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Immunological suppression by human CD8$^+$ T cells is receptor dependent and HLA-DQ restricted

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ABSTRACT **Mechanisms of specific immunologic unresponsiveness or tolerance and their regulation by the major histocompatibility complex remain central issues in immunology. Recent findings that potentially reactive anti-self T cells are not completely clonally deleted in the thymus and that specific immunological unresponsiveness can be acquired in certain infectious diseases, such as leprosy, suggest that peripheral unresponsiveness can be developed and maintained in adults. Human antigen-specific T suppressor cells represent one mechanism of peripheral tolerance. Clones of CD8$^+$ T suppressor cells have been derived from blood or lesions of patients with lepromatous leprosy who are selectively unable to mount cellular immunity to *Mycobacterium leprae*. Using a panel of *M. leprae*-specific CD4$^+$ and CD8$^+$ T-cell clones of differing major histocompatibility complex class II haplotypes, suppression *in vitro* was found to be restricted by HLA-DQ and not by HLA-DR and inhibited by antibodies to HLA-DQ. In addition, antigen-induced suppression could be inhibited by antibodies specific to appropriate polymorphic T-cell receptor β chains of the CD8$^+$ clones. The results establish that activation of specific T suppressor cells is dependant on their polymorphic T-cell receptors and suggest that HLA-DQ serves as the preferred restricting element for suppression.**

A number of mechanisms have been invoked to explain specific immunological unresponsiveness to self and foreign antigens. They include (*i*) clonal deletion of antigen-specific B or T cells (1–4), (*ii*) clonal anergy of T cells (5–8), (*iii*) "veto" (9), and (*iv*) T-cell-mediated suppression (10). Although clonal deletion of specific T cells in the thymus appears to be the major developmental mechanism for generating self-tolerance neonatally, there is increasing evidence that unresponsiveness can be maintained in adult individuals despite the existence of antigen-reactive T-cell clonotypes in the periphery. It is in this context that leprosy is of fundamental immunological interest, because it is a chronic human infectious disease in which a significant proportion of patients, those with the lepromatous form of the disease, fail to develop detectable cell-mediated immunity to antigens of *Mycobacterium leprae*. In the majority of lepromatous patients, the unresponsiveness is not irreversible, since it has been possible to overcome the unresponsiveness in the majority of the lepromatous patients using immunotherapeutic vaccines (11–13). Leprosy therefore provides a relatively unique model in humans for studying peripheral tolerance. Several laboratories have observed antigen-induced suppression of CD4$^+$ T-cell responses to antigens *in vitro* mediated by CD8$^+$ T cells from lepromatous patients (14, 15). The phenotype of such T suppressor (Ts) cells obtained in our laboratory from biopsies of skin lesions or from blood of these patients is found to be CD8$^+$, CD4$^-$, CD3$^+$, HLA-DR$^+$, FcR$^+$, CD28$^-$, TCR $\alpha\beta^+$, differing from cytotoxic T lymphocytes by their failure to express CD28.

It is the current immunological paradigm that cytotoxic CD8$^+$ T cells recognize antigen in association with class I molecules of the major histocompatibility complex (MHC) and that CD4$^+$ T cells are restricted by MHC class II molecules. Nevertheless, in studies from several laboratories CD8$^+$ Ts cells and clones were unexpectedly, but almost invariably, able to suppress CD4$^+$ cells and clones in a MHC class II restricted fashion (14). Though susceptibility to clinical leprosy is not known to be genetically linked to the HLA locus, an association between HLA type and the form of disease has been reported (16). The present work seeks to clarify more precisely the MHC class II restriction on suppression and the recognition of antigen in that context by the T-cell receptor (TCR) of the suppressor cells.

MATERIALS AND METHODS

Monoclonal Antibodies. Antibodies HU11 (anti-DQ1) and HU13 (anti-DQ3) were kind gifts of T. Sasazuki (Kyushu University, Kyushu, Japan), SPVL3 was a gift from H. Spits (DNAX, Palo Alto, CA), and anti-DR4 antibody was a gift from F. Ward (Duke University). Murine monoclonal antibodies to the human TCR variable (V) regions (Diversi-T-TCR screening panel) were purchased from T Cell Sciences (Cambridge, MA) and included the following seven antibodies: βV5(a), βV5(b), βV8(a), βV12(a), βV6(a), αV2(a), and $\alpha\beta$V(a). Monoclonal antibodies for cell surface receptors CD3, CD4, and CD8 were bought from Coulter Clone.

Assay for Suppressor Activity. The procedures for obtaining *M. leprae*-specific CD8 suppressor and CD4 helper clones from blood and lesions of leprosy patients have been described in earlier reports (17). Suppressive activity of the CD8$^+$ clones was measured by inhibition of [^{3}H]thymidine incorporation of antigen-reactive CD4 clones. CD4 clones (1×10^4) and γ-irradiated peripheral blood cells (1×10^5) as antigen-presenting cells (APCs) in the presence or absence of 1×10^4 Ts clones, were stimulated with 10 μg of *M. leprae* antigen per ml in 96-well plates. The cells were cultured at 37°C in Iscove's medium containing 10% human AB serum in a total volume of 200 μl. Proliferation of the CD4$^+$ clone was measured at day 3 by [^{3}H]thymidine incorporation. Percent suppression was calculated as [100 − (cpm from antigen-containing cultures)/(cpm from antigen-containing cultures + Ts cells) × 100].

MHC Haplotype of CD4 and CD8 clones and APCs. Peripheral blood mononuclear cells or Epstein–Barr virus-transformed B-lymphoblastoid cell lines from lepromatous patients and lepromin-positive contacts of lepromatous patients were HLA typed at The Children's Hospital Blood Bank (Los Angeles). To investigate the role of HLA-DQ in Ts-mediated suppression a panel of Ts and T helper (Th) clones was established from donors of different HLA haplotypes. APCs from rare donors with recombinant haplotypes

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Abbreviations: MHC, major histocompatibility complex; Ts, T suppressor; Th, T helper; TCR, T-cell receptor; APC, antigen-presenting cell; V, variable.

were very kindly provided by C. Hurley (Georgetown University Medical Center) and D. Eckels (The Blood Center of Southeastern Wisconsin).

RESULTS

$CD8^+$ Ts Clones Are HLA-DQ Restricted. The availability of $CD8^+$ Ts clones, CD4 T-cell clones, and APCs from donors of different haplotypes made it possible to explore the MHC restriction on suppression. In extensive studies by Ottenhoff *et al.* (18) and all of our previous work, antigen-specific $CD4^+$ clones recognized *M. leprae* antigens almost exclusively in association with HLA-DR. Our previous studies using HLA typed Ts clones indicated that their suppressive activity appeared to be MHC class II restricted (14). Despite the fact that DR and DQ genes are in strong linkage disequilibrium, we sought MHC class II matched and mismatched combinations of $CD4^+$, $CD8^+$, and APCs that would permit us to identify the restricting element on T-cell suppression by our $CD8^+$ clones. Matching of APC, $CD8^+$, and $CD4^+$ clones at DR and DQ (Fig. 1, *A–C*, line 1) resulted in significant suppression of proliferative responses of specific $CD4^+$ clones to antigens of *M. leprae*. In Fig. 1*A*, line 1, the APCs (HLA-A24/30, B56, Cw3/w6) and Ts cells (HLA-A23/32, B-, Cw2) are totally mismatched at class I, yet suppression is produced, confirming our earlier observations that the $CD8^+$ Ts cells are not class I restricted. No suppression was observed when the Ts cells were mismatched at DR and DQ with APCs (Fig. 1 *A–C*, line 2). In two combinations of cells matched at DQ but mismatched at DR (Fig. 1 *A* and *B*, line 3), suppression was still observed. Since the β and α chains of the DQ molecule are polymorphic it is important to note that DR4,DQ3- and DR5,DQ3-positive haplotypes express DQ molecules with similar β chains but different α chains (19). In contrast, suppression was not observed (Fig. 1 *B* and *C*, line 4) using APCs from rare donors that were matched with the Ts cells at DR and mismatched at DQ. Although HLA-DR5 is usually linked to DQ3 and HLA-DR7 to DQ2, there are uncommon recombinational events in which DR5 is associated with DQ1 and DR7 with DQ3. The haplotype of the Ts cells in Fig. 1*B*, line 4, is DR5,DQ3 and that of the APCs is DR5,DQ1; similarly, in Fig. 1*C*, line 4, the Ts cell is DR7,DQ2 and the APC cell is DR7,DQ3. The DR5,DQ1 haplotype differs from the DR5,DQ3 haplotype in DQ α and β chains. However the only significant class II difference between the lines expressing DR7,DQ2 and DR7,DQ3 is in the DQβ chains (20). These data suggest that, in these cases, the critical restricting element on $CD8^+$ suppressor cells resides on the DQβ molecule.

The haplotype combination in Fig. 1*C* allowed us to discriminate whether the critical DQ restricting element for antigen presentation to the Ts cells was provided by APC'c or the $CD4^+$ responding cells. When suppression by the DR7,DQ2 Ts clone was measured in the presence of either DQ matched or mismatched $CD4^+$ cells, suppression was not observed when the Ts clones and $CD4^+$ were mismatched at DQ (Fig. 1*C*, line 3). Even when the $CD4^+$ clone in this group was matched at DR and DQ with the $CD8^+$ clone, suppression was not observed (Fig. 1*C*, line 4). This combination establishes that the Ts cells recognize antigen in association with DQ molecules expressed on APCs.

Anti-DQ Antibody Blocks Ts-Mediated Suppression. The importance of HLA-DQ in regulating Ts activity of the $CD8^+$ clones *in vitro* was confirmed by blocking experiments using haplotype-specific HLA-DQ antibodies. *M. leprae*-specific proliferation of $CD4^+$ Th clones used in this study was blocked by anti HLA-DR antibodies, confirming that antigen presentation to the $CD4^+$ clones studied was HLA-DR restricted (data not shown). The effect of polymorphic anti-DQ3 and DQ1 antibodies on proliferation of $CD4^+$ clones in the presence of $CD8^+$ Ts clones (Fig. 2) indicated that anti-DQ3 antibodies significantly inhibited the suppressive activity of the $CD8^+$ clones on $CD4^+$ *M. leprae*-responsive clones. Under the same conditions anti-DQ1 antibody did not block suppression. Allele-specific anti-DR4 antibody also did not block suppression by Ts clone LL14#7 (DR4/w6, DQ1/3) of a DR5-restricted $CD4^+$ clone. Anti-MHC class I antibody did not inhibit suppression by Ts clone LLi#13 and LL14#7. As reported earlier in the case of class II restricted $CD8^+$ cytotoxic T lymphocytes (21), the suppression was inhibited by anti-$CD8^+$ antibodies.

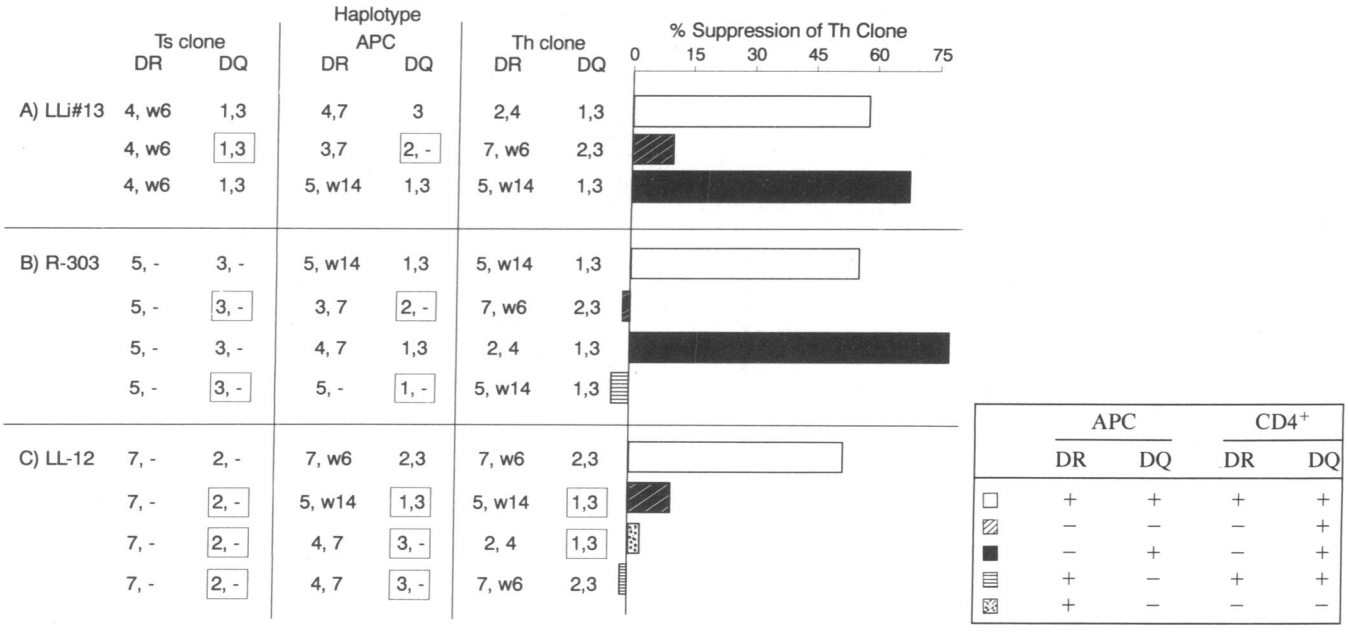

FIG. 1. Restriction of T cell suppression by HLA-DQ. Six $CD8^+$ suppressor clones from six different lepromatous leprosy patients and five $CD4^+$ antigen-responsive clones from four different donors were generated as described (14). They were mixed in different combinations to provide mismatching for MHC class II DR or DQ antigens, and data from three such combinations [LLi#13 (*A*), R-303 (*B*), and LL-12 (*C*)] are presented. A summary of the MHC compatibility with Ts is given in *Inset*.

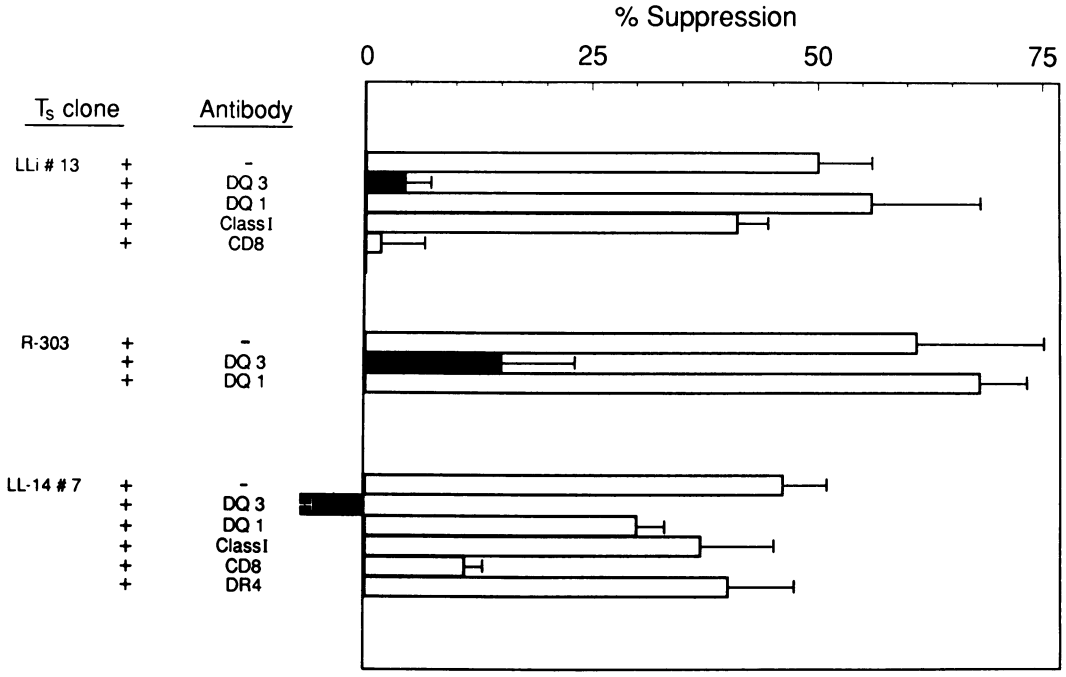

FIG. 2. Inhibition of suppression by anti-DQ antibodies. *M. leprae*-specific $CD4^+$ cells (1×10^4) were cultured with feeder cells (1×10^5), 10 μg of *M. leprae* antigen per ml, and $CD8^+$ cells (1×10^4) in the presence or absence of anti-DQ monoclonal antibody. Antibodies HU11 (DQ1) and HU13 (DQ3) were used at 1:300 dilution of the ascites fluid, anti-CD8 was used at 2.5 μg/ml, anti-class I (W6/32) was used at 1:1000 dilution of the ascites fluid, and anti-DR4 was used at 1:10 dilution of the supernatant. The antibodies were added at the start of culture. Proliferation was measured at day 3 by [^{3}H]thymidine incorporation. The data are presented as mean ± SE of three experiments.

Ts Clones Express Functional TCR. All $CD8^+$ Ts clones we have examined expressed surface TCR α and β chains (22). Using monoclonal antibodies directed at specific TCR V regions expressed on individual $CD8^+$ Ts clones but not expressed on the responding $CD4^+$ clones, it was possible to establish that these represent functional receptors. Two of our Ts clones expressed TCR Vβ8 and one clone expressed Vβ5, neither of which was present on $CD4^+$ Th clones they suppressed. Monoclonal antibodies specific for these TCR Vβ chain epitopes in each case abrogated the suppression (Fig. 3), formally demonstrating that the TCRαβ receptor on the Ts clones, in fact, mediated the DQ-restricted recognition

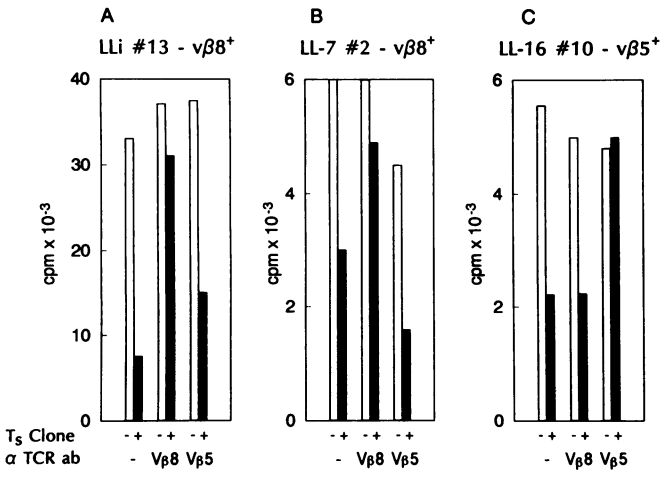

FIG. 3. Inhibition of suppression by anti-Vβ antibodies specific for Ts cell receptor. Suppression by $CD8^+$ Ts clones expressing Vβ5 or Vβ8 clonotypes were assayed in the presence of anti-clonotypic TCR antibodies. $CD4^+$ (1×10^4) cells were admixed with feeder cells, *M. leprae* antigen, and Ts cells in the presence or absence of anti-TCR antibodies (2 μg/ml), and proliferation was measured at day 3.

of antigen. The anti-TCR antibodies acted by blocking subsequent antigen recognition and not by killing the Ts cells—e.g., by antigen-dependent cell-mediated cytotoxicity (data not shown). Further, the expression of more than one V region by the Ts clones (Table 1) is suggestive of the presence of multiple epitopes on *M. leprae* capable of inducing Ts cells.

DISCUSSION

Few areas of immunology are more controversial than that of the nature and function of Ts cells. For many years cogent reasons existed for general skepticism regarding Ts cells. (*i*) Most studies of suppression had been phenomenological and carried out on murine T-T hybridomas rather than well-characterized Ts cell clones. (*ii*) Studies of the receptors of putative antigen-specific murine T-T hybrids found that most either lacked the gene for the TCR β chain or had failed to productively rearrange them and could thus not make a functional receptor (23). (*iii*) Although the genetic restriction on function of Ts cells and hybridomas in the mouse mapped to the I-J region within the MHC, studies of the DNA sequence of that region failed to provide any evidence for a unique open reading frame capable of encoding the I-J restriction element (24). (*iv*) A number of antigen-specific suppressor factors have been described in the mouse, yet there was virtually no consistent molecular characterization of these factors. (*v*) Finally, strong experimental support for alternative models of tolerance have recently been developed. Suffice it to say that recent studies have addressed many of these concerns. All human primary Ts clones and the majority of murine Ts clones and some hybridomas studied do express rearranged TCRαβ (25), although it had not previously been demonstrated that they functioned in antigen recognition. There appears further to be a developing consensus that soluble suppressor factors derive, at least in part, from the TCR α chain.

Table 1. V-region diversity of Ts clones

Ts clone	HLA type		TCR	V-region family
	DR	DQ		
LLi#13	4, w6	1, 3	Vβ8	Vα2(a)
R-303	5	3	—	—
LL-16#10	4, w6	1, 3	Vβ5(a)	—
LL-14#7	4, w6	1, 3	—	Vα2(a)
LL-7#2	—	—	Vβ8	—
J.G.#5	4	3	—	—

The Ts clones were analyzed for their TCR V-region usage with a panel of murine monoclonal antibodies to the human TCR V regions supplied by T Cell Sciences (Diversi-T-TCR screening panel). Ts cells were analyzed by flow cytometry with each of the seven anti-TCR antibodies followed by fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin. A — signifies that the cells were not typeable.

Though clonal deletion has been established as the dominant mechanism of neonatally induced self-tolerance, from studies in transgenic mice it has become evident that not all cells of the clonotype are deleted. Failure of the residual 0.2–15% of the original clonotype-bearing cells to expand in response to the transgene-encoded antigens indicates the need for mechanisms to maintain unresponsiveness. In the case of unresponsiveness in infectious diseases such as lepromatous leprosy, it is unlikely that the T-cell clones to the multiplicity of antigens of a bacterial pathogen could all be deleted neonatally, long prior to infection. As noted above, a majority of such patients can be immunized therapeutically to clinically upgrade their immune responses to *M. leprae* and, in some cases, cure their infection (11). Lymphocytes from a third of lepromatous patients studied, although unresponsive to intact *M. leprae*, have been reported to be able to respond to electrophoretic fractions of *M. leprae* extracts (26). These findings are most consistent with the interpretation that suppression is one mechanism of peripheral tolerance in leprosy.

Human MHC class II antigens, comprising the three subclasses, HLA-DR, -DQ, and -DP, are expressed constitutively in a limited number of cell types known to be involved in antigen presentation to $CD4^+$ T cells and can be induced in several other cell types by lymphokines. The questions why subclasses of the MHC class II evolved in virtually all mammalian species and whether they differ functionally in their regulation of immune responses remain problematic. In the present experiments, recognition of antigens of *M. leprae* leading to suppression *in vitro* was shown to occur only when the APC and the $CD8^+$ cells shared HLA-DQ haplotypes. Individuals matched for DQ and mismatched for DR and MHC class I suppressed; mismatches at DQ failed to suppress irrespective of matches for other MHC loci. Finally, the suppression was inhibited by specific anti-DQ antibodies. The restriction of Ts activity was found to be determined by the interaction of the $CD8^+$ clones and APC, and there was no necessity for the Ts and $CD4^+$ cells to share DQ. These results using six independent $CD8^+$ Ts clones establish that HLA-DQ compatibility is a sufficient condition for inducing suppression and support the view that DQ may be the preferred restriction element for T-cell suppression.

There are a number of precedents for specialization of MHC loci for unresponsiveness or immunosuppression. In the mouse, Lyt-2 T cells specific for lactic dehydrogenase (27) and the F protein of liver (28) and myeloma protein (29) were induced in mice when the antigens were presented in the context of I-E molecules. Several mouse strains lacking I-E tend to have a high incidence of autoimmune disease, which can be corrected by introduction of a transgene expressing the I-E molecule. In humans, too, there are examples of unresponsiveness associated with MHC class II subsets, particularly HLA-DQ, some of which may be mediated by Ts cells. Van Rood *et al.* (30) showed association between DQ and low responsiveness to tuberculin purified protein derivative. In the mixed lymphocyte reaction system DQ molecules on activated $CD8^+$ T cells act as major regulatory molecules that could be blocked by anti-DQ antibody (31). In important family segregation studies, Kikuchi *et al.* (32) found an association between T-cell unresponsiveness to various antigens and HLA-DQ antigens, and in the case of a patient with schistosomiasis, antigen unresponsiveness *in vitro* was dominant or epistatic to responsiveness (33). Finally, in two autoimmune diseases in which self-tolerance is not maintained, insulin-dependent diabetes (34) and pemphigus vulgaris (35), a strong association between mutant DQ allele and disease has been observed.

Ts cells, since they are MHC class II restricted $CD8^+$, constitute a notable exception to the general paradigm that antigen recognition by $CD4^+$ cells is MHC class II restricted and that antigen recognition by $CD8^+$ cells is MHC class I restricted. Again there is precedent for antigen recognition by $CD8^+$ cells being MHC class II restricted—e.g., some alloreactive cytotoxic $CD8^+$ cells (21). Further, T cells from $I\text{-}E^+$ mice depleted Vβ11 T cells of $CD4^+$ and $CD8^+$ subsets, establishing that some $CD8^+$ cells had to have had I-E reactivity (36). In those and in our experiments in which suppression was inhibited by anti-CD8 antibodies, it is not clear what the ligand for the CD8 molecule on MHC class II reactive T cells is or whether they function not as accessory molecules but in signal transduction only. Nevertheless, although there is no obvious reason for negative selection of such cells in the thymus, it remains quite puzzling how such cells can be positively selected to recognize antigen in the context of MHC class II antigens.

The precise mechanism of T-cell suppression(s) in this system is still unclear. We have previously provided evidence that $CD8^+$ Ts clones do not effect suppression by (*i*) killing the responding $CD4^+$ cells, (*ii*) killing the APCs, or (*iii*) a veto mechanism or presentation of antigen to $CD4^+$ cells in the absence of costimulatory signal (37). Our results are most consistent with the view that these Ts clones function by blocking production of a second signal by APCs or by negating appropriately delivered stimulatory signals to $CD4^+$ cells and inducing a state of anergy. One model for suppressor function, consistent with functions of other T-cell subsets, would hold that Ts cells are activated by specific antigens in an HLA-DQ-specific fashion and exert their biological activity on $CD4^+$ cells in an antigen-independent manner. It will clearly be of interest to learn how presentation of antigen by HLA-DR and -DQ lead preferentially to recognition by different T-cell subsets. Of particular interest will be the question whether deficiencies in mechanisms of peripheral tolerance are associated with susceptibility to human autoimmune diseases, particularly those with genetic associations to HLA-DQ (38, 39). Finally, because there exist obvious clinical needs for specific immunological modulation of immune responses in adults, understanding suppression and clonal anergy remain relevant as well as challenging immunological problems.

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