Characterization of the Ligand(s) Responsible for Negative Selection of V\(\beta\)11- and V\(\beta\)12-Expressing T Cells: Effects of a New Mls Determinant

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Summary

During T cell development, events occur that result in the generation of a T cell population capable of recognizing foreign antigens in association with self major histocompatibility complex (MHC) gene products. However, selective events also occur during thymic education that result in the deletion of T cells expressing \(\alpha/\beta\) T cell receptors with high affinity for self determinants alone, i.e., potentially self-reactive T cells. Both MHC- and non-MHC-encoded self antigens appear to play critical roles in this negative selection of self-reactive T cells. We recently observed that T cells expressing V\(\beta\)5, V\(\beta\)11, V\(\beta\)12, or V\(\beta\)16 products are deleted in most strains of H-2\(^k\) type, but not in congenic H-2\(^b\) strains. In contrast, the H-2\(^k\) strain C58/J deleted V\(\beta\)5\(^+\) and V\(\beta\)16\(^+\) T cells, but failed to delete T cells expressing V\(\beta\)11 or V\(\beta\)12. Based upon this observation, in the present study we have analyzed the genetic regulation of the ligands responsible for deletion of V\(\beta\)11- and V\(\beta\)12-expressing T cells, and have tested the possibility that these ligands can function as strong alloantigens analogous to the known minor lymphocyte stimulatory (Mls)- and MHC-encoded antigens. Two major findings have resulted from these studies. First, the ligands recognized by V\(\beta\)11\(^+\) and V\(\beta\)12\(^+\) T cells were regulated by both MHC- and multiple non-MHC-encoded genes. Correlation between expression of these two V\(\beta\)s in backcross animals suggested that shared, though not necessarily identical, ligands mediated deletion of V\(\beta\)11- and V\(\beta\)12-expressing T cells. Second, the ligand for deletion of V\(\beta\)11- and V\(\beta\)12-expressing T cells functions as a newly defined Mls alloantigen that stimulates primary proliferative responses in T cell populations from mice that express V\(\beta\)11\(^+\) and V\(\beta\)12\(^+\) T cells.

The process of T cell development results in the matura-
tion of a heterogeneous T cell population capable of recognizing foreign antigens in association with self MHC gene products. Selective events also occur during thymic education that result in the deletion of potentially autoreactive T cells expressing TCR-\(\alpha/\beta\) with high affinity for self determinants alone. The self ligands involved in the negative selection of self-reactive T cells appear to involve both MHC- and non-MHC-encoded antigens. For example, non-MHC-encoded minor lymphocyte stimulatory (Mls)\(^1\) antigens in association with appropriate MHC products are capable of eliciting deletion of essentially all T cells expressing certain V\(\beta\) products, regardless of the other TCR \(\alpha\) or \(\beta\) chain products expressed. Mls\(^a\) and Mls\(^b\) have been defined as alloantigens capable of inducing strong proliferative responses by MHC-identical T cells. T cells expressing V\(\beta\)6, V\(\beta\)8.1, or V\(\beta\)9, all of which are strongly associated with T cell specificity for Mls\(^a\) determinants, are deleted in Mls\(^a\)-positive mouse strains (1–5), and T cells expressing V\(\beta\)3 are similarly absent in Mls\(^a\)-positive strains of the appropriate MHC haplotype (6–8). In other instances, expression of specific MHC products, in the absence of identified Mls antigens, results in analogous negative selection of T cells expressing other V\(\beta\) gene products (4, 9–14). Evidence suggests that these latter instances of negative selection are not due to recognition of self MHC alone, but also reflect recognition of as yet unidentified self antigens in association with self MHC products (10, 15–17).

The fact that essentially all T cells expressing a given V\(\beta\) product are deleted in animals expressing a specific self determinant suggests that this determinant can be recognized by effectively all T cells expressing that V\(\beta\). Therefore, mice that do express that V\(\beta\), because they do not express the self ligand responsible for its deletion, should maintain a high proportion of T cells capable of recognizing this ligand as an alloan-

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\(^{1}\) Abbreviation used in this paper: Mls, minor lymphocyte stimulatory.
tigen. This is clearly the case for Mls+ and Mls− determinants that are capable of inducing T cell responses at extremely high precursor frequencies in Mls+ and Mls−-negative strains. In other instances, however, the ability of determinants to mediate Vβ-specific negative selection has not been so clearly related to their ability to act as alloantigens in the stimulation of mature T cells expressing appropriate Vβ products (15).

We recently observed that T cells expressing Vβ5, Vβ11, Vβ12, or Vβ16 products are deleted in most strains of H-2k type, but not in congenic H-2b strains (4). In contrast, the H-2k strain C58/J deleted Vβ5+ and Vβ16+ T cells, but failed to delete T cells expressing Vβ11 or Vβ12. Based upon this observation, in the present study we have analyzed the genetic regulation of the ligand responsible for deletion of Vβ11- and Vβ12-expressing T cells, and have tested the possibility that this ligand can function as a strong alloantigen analogous to the known Mls- and MHC-encoded antigens. Two major findings have resulted from these studies. First, the ligands recognized by Vβ11+ and Vβ12+ cells were regulated by both MHC- and multiple non-MHC-encoded genes. Correlation between expression of these two Vβs in backcross animals suggested that shared, though not necessarily identical, ligands mediate deletion of Vβ11- and Vβ12-expressing T cells. Second, the ligand for deletion of Vβ11- and Vβ12-expressing T cells functions as a newly defined Mls alloantigen that stimulates primary proliferative responses in T cell populations from mice that express Vβ11+ and Vβ12+ T cells.

Materials and Methods

Mice. (B10 x C58/J)F1, (B10.A x C58/J)F1, and (B10.A x C58/J)F1 x C58/J backcross mice were bred at BioQual (Rockville, MD). CBA/Ca and C57BL/6NCR were obtained from BioQuail (Rockville, MD). CBA/Ca and C58/J were obtained from The Jackson Laboratory (Bar Harbor, ME). All other mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Northern Blot Analysis. Techniques utilized for preparation of RNA from T cells and Northern blot analysis of TCR Vβ expression have been previously described (4). Vβ-specific probes were generously provided by Dr. D. Loh (Washington University, St. Louis, MO). Vβ expression for each strain was standardized to Cβ expression, and values were expressed in relation to those obtained for C58/J, or in some cases, C57BL/10 (B10).

Flow Cytometry. Spleen cells were enriched for T cells by passing over rabbit anti-mouse Ig-coated plates. 10^6 T-enriched spleen cells were stained at previously described (18). The antibody specific for Vβ11, RR3-15, was obtained from Dr. O. Kanagawa (Washington University) (15).

Culture Medium. Medium for culturing cells was RPMI 1640, supplemented with 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM Hapes, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 2 mM l-glutamine, and 5 x 10^-5 M 2-ME.

T Cell Proliferation Assays. Responder T cells were isolated from spleens by enrichment over rabbit anti-mouse Ig-coated plates. Stimulator spleen cells were treated with 50 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, MO) at a concentration of 1-5 x 10^6/ml for 20 min at 37°C. 3 x 10^6 T cells were cultured with 6 x 10^5 or with titrated numbers of mitomycin-treated stimulators for 72 h at 37°C in a 5% CO2-humidified air atmosphere. Proliferation was assessed by [3H]Tdr incorporation during a subsequent 18-h pulse (1 μCi/well; New England Nuclear, Boston, MA).

Results

Expression of Vβ11 and 12 in C58/J. In a previous study (4), we demonstrated by Northern blot analysis that expression of specific MHC haplotypes (H-2d-k) generally resulted in decreased T cell expression of Vβ5, Vβ11, Vβ12, and Vβ16. However, it was also noted that C58/J, despite its H-2k haplotype, did not exhibit decreased expression of Vβ11 or Vβ12 (Table 1). High levels of Vβ11 expression in C58/J were also confirmed by flow cytometry (Fig. 1); expression was equivalent in CD4+ and CD8+ T cell subsets (data not shown). This did not represent a generalized defect in negative selection in this strain, since C58/J (H-2k, Mls+â) mice did show the expected deletions in Vβ16 (Table 1), as well as in Vβ3, Vβ5, Vβ6, Vβ7, and Vβ9 (4). Further studies were therefore undertaken to analyze the genetic basis for negative selection of Vβ11- and Vβ12-expressing T cells.

Table 1. Expression of Vβ11 and Vβ12 in C58/J

| Strain | H-2 | Vβ11 | Vβ12 | Vβ16 |
|--------|-----|------|------|------|
| B10    | b   | 1.00 | 1.00 | 1.00 |
| B10.BR | k   | 0.16 | 0.16 | 0.37 |
| C3H/HeJ| k   | 0.09 | 0.09 | 0.09 |
| AKR/J  | k   | 0.29 | 0.06 | 0.28 |
| CBA/J  | k   | 0.31 | 0.02 | 0.17 |
| BALB.K | k   | 0.08 | 0.03 | 0.11 |
| C58/J  | k   | 1.38 | 1.04 | 0.11 |

Values are expressed as the means of densitometric readings of two to four individual filters.
of interest that Vβ11 and Vβ12 depletion was also observed in (B10 × C58/J)F1 mice. B10 mice fail to delete Vβ11+ and Vβ12+ T cells due to the failure to express an appropriate MHC product, since the MHC congenic strains B10.A and B10.Br delete efficiently. The observed deletion of Vβ11- and Vβ12-expressing cells in (B10 × C58/J)F1 mice therefore suggested that the MHC (H-2) expressed by C58/J is competent to mediate Vβ11 and Vβ12 deletion, but that a non-MHC gene product is also required for deletion, and that C58/J fails to express this non-MHC product, while all strains of the B10 background do express the appropriate product.

**Backcross Analysis of Vβ11 and Vβ12 Expression.** The role of MHC and non-MHC gene products in negative selection of Vβ11+ and Vβ12+ T cells was formally assessed by backcross analysis. Evaluation of (B10.A × C58/J) × C58/J backcross animals demonstrated that the lack of negative selection in C58/J is not attributable to an MHC polymorphism (Table 2). The mAb 34-2-12, which detects Dk but not Dk, was used to determine whether backcross animals did or did not express the MHC type of B10.A origin. In two of the eight animals analyzed (nos. 1 and 2), decreased expression of Vβ11 and Vβ12 occurred in the absence of the B10.A haplotype, indicating that the C58/J MHC gene products are competent to support negative selection, and that the absence of negative selection in C58/J is due to polymorphism in non-MHC-encoded products.

It also appeared from these results that the self ligand(s) mediating deletion of Vβ11+ T cells are linked to those mediating deletion of T cells expressing Vβ12. Table 2 illustrates that out of the 8 (B10.A × C58/J)F1 × C58/J backcross animals analyzed, only one expressed high levels of Vβ11, and the same animal also expressed high levels of Vβ12. This observation was extended by an analysis of 34 (CBA/Ca × C58/J)F1 × C58/J backcross animals in which it was again noted that the animals expressing the highest levels of Vβ11 also expressed the highest levels of Vβ12. Statistical analysis by Pearson correlation shows a strong correlation (r = 0.67; p < .0001) between Vβ11 and Vβ12 expression in these backcross animals.

Analysis of backcross data also allows an estimation of the number of genes involved in decreased expression of Vβ11 and Vβ12. Fig. 3 summarizes flow cytometry data collected from the 42 backcross animals analyzed. Some backcross mice expressed levels of Vβ11 comparable with C58/J (nondeletion); others expressed Vβ11 comparable with (CBA/Ca × C58)F1 (deletion); and others expressed intermediate levels (partial deletion). It was notable that only 12 of the 42 backcross mice (28%) fell within 2 SD of the mean expression of Vβ11 in C58/J (nondeletion), whereas 10 of 42 (23%) expressed levels of Vβ11 within 2 SD of the mean expression by the F1 (complete deletion), and the remaining 48% expressed intermediates levels. These results are statistically incompatible with the expected proportion of nondeleting mice (50%) if deletion were determined by a single segregating gene ($\chi^2 = 7.72; p < 0.01$), and thus indicate that deletion is determined by two or more independently segregating genes.

**Figure 1.** Expression of Vβ11 in F1 mice. 10^6 T cells were stained with RR3-15 (anti-Vβ11) culture supernatant and goat anti-rat FITC followed by Thy-1.2-biotin and Texas red-avidin. Percentages indicate the number of Thy-1.2+ cells that are Vβ11+. The percentage of cells staining with an irrelevant rat antibody has been subtracted.

**Figure 2.** Expression of Vβ11 and Vβ12 mRNA in F1 mice. A Northern blot of RNA isolated from Con A-activated T cells was hybridized sequentially with Vβ11-, Vβ12-, and Cβ-specific probes. Densitometric analysis of relative Vβ11 and Vβ12 mRNA expression for each strain was expressed as a percent of total β chain message, then expressed relative to C58/J. Values are the means of densitometric readings on two to six individual filters.
Table 2. Backcross Analysis of Vβ11 and Vβ12 Expression

| Animals                        | Relative Vβ expression |
|--------------------------------|------------------------|
|                                | H-2^2 Vβ11 Vβ12        |
| Strain:                        |                        |
| C58/J                          | - 1.00 1.00            |
| B10.A                          | + 0.01 0.14            |
| B10.A x C58/J                  | + 0.02 0.10            |
| Backcross animals:             |                        |
| (B10.A x C58/J) x C58/J        | 1 - 0.02 0.20          |
| 2 - 0.18 0.23                  |
| 3 + 0.18 0.29                  |
| 4 + 0.03 0.18                  |
| 5 + 0.09 0.21                  |
| 6 + 0.06 0.18                  |
| 7 + 0.04 0.13                  |
| 8 - 1.03 2.18                  |

Values are expressed as the means of densitometric readings on two individual filters.

Discussion

It had been noted in a previous study (4) that the numbers of T cells expressing Vβs 5, 11, 12, and 16 were generally decreased in H-2^d, H-2^k, or H-2^k strains of mice. However, it was noted that the inbred strain C58/J, also H-2^k, failed to delete Vβ11- and Vβ12-expressing T cells. The same strain showed that expected deletions of other Vβs, demonstrating that this was not a generalized defect in negative selection. Results of the present study demonstrated that the failure...
of C58/J to delete $\text{V}11^+$ and $\text{V}12^+$ T cells is due to the failure of this strain to express the self ligand that is necessary for these deletions. Moreover, these studies resulted in two major findings. First, it was shown that the ligand(s) for $\text{V}11$ and $\text{V}12$ deletion consist of both MHC and non-MHC gene products, and that more than one non-MHC-linked gene can contribute to this ligand. Furthermore, it was demonstrated that the ligand that mediates $\text{V}11$ and $\text{V}12$ deletion functions as a novel Mls antigen that elicits proliferative T cell responses in $\text{V}11^+$ and $\text{V}12^+$ expressing T cell populations. Non-MHC influences on $\text{V}11$ expression have previously been suggested in several studies. It has been noted that some mouse strains, for example, on the A background, show less dramatic decreases in expression of $\text{V}11$ than MHC congenic strains with differing non-MHC backgrounds (10, 15). The effect of non-MHC gene products on $\text{V}11$ expression was also noted in the BXD recombinant inbred strains (15). The results of the present study are consistent with these findings. The results presented here further indicate that more than one non-MHC gene product can be involved in successful deletion of $\text{V}11^+$ and $\text{V}12^+$ expressing T cells. Moreover, the finding of intermediate levels of $\text{V}B$ expression (partial deletion) by backcross analysis suggests that expression of only one or a subset of these non-MHC gene products is insufficient for complete deletion to occur. The effect of multiple non-MHC gene products on deletion may be an additive effect on a single T cell population. Alternatively, different non-MHC products may be the ligands for distinct subset of $\text{V}11^+$ or $\text{V}12^+$ T cells. Expression of one of these determinants would then result in deletion of only one of the subsets, and only when all non-MHC ligands were expressed could complete deletion of $\text{V}11$ or $\text{V}12$ occur.

Although $\text{V}11$ and $\text{V}12$ expression were highly correlated in C58/J backcross animals, the ligands recognized by $\text{V}11^+$ and $\text{V}12^+$ T cells may not be identical. As mentioned above, the A background has been demonstrated to be inefficient in the deletion of $\text{V}11^+$ T cells (4, 10, 15) yet these same animals efficiently delete $\text{V}12^+$ T cells (4). Backcross analysis in the present study revealed that while there was a strong overall correlation between $\text{V}11$ and $\text{V}12$ expression, there does exist a small group of animals that essentially totally deleted $\text{V}12^+$ T cells but still expressed some $\text{V}11$ mRNA (data not shown). Given the finding that two or more non-MHC gene products contribute to deletion of $\text{V}11$ or $\text{V}12$, this observation could be interpreted as the existence of multiple non-MHC ligands necessary for $\text{V}11$ or $\text{V}12$ deletion, only some of which influence both $\text{V}11$ and $\text{V}12$. The ligands for $\text{V}11$ and $\text{V}12$ would thus be overlapping but not necessarily identical sets.

Despite decreased expression of $\text{V}11$ in I-E$^+$ strains of mice, it has been difficult to demonstrate reactivity of $\text{V}11^+$ T cells to EoE in vitro (15). In contrast, specific recognition of EoE by $\text{V}11^+$ T cells has been reported in vivo (20). It is therefore interesting that, in the present study, expression of $\text{V}11^+$ T cells in backcross animals correlated with the ability of these T cells to proliferate in vitro. Several explanations could account for the previous failure to observe such reactivity in $\text{V}11^+$ T cells. First, the proliferative responses of C58/J, as well as backcross animals to H-2-matched CBA/Ca stimulators, could be due predominantly to $\text{V}12^+$ T cells. However, preliminary experiments analyzing C58/J anti-CBA/Ca T cell lines suggest that expansion of $\text{V}11^+$ T cells does occur. If both $\text{V}11^+$ and $\text{V}12^+$ T cells are responding, the inability of others to detect this response may be due to differential expression of the appropriate stimulatory antigen in various strains. For instance, responses to B10.BR were very weak as compared with responses to CBA/Ca or other H-2$^k$ strains, despite the fact that each of these strains deletes $\text{V}11^+$ and $\text{V}12^+$ expressing T cells equally well. Previous analysis of the responses of $\text{V}11^+$ T cells to EoE in vitro used stimulators from the B10 background (15). Thus, some ligands that are capable of mediating $\text{V}11$ deletion may not function effectively as stimulatory alloantigens in vitro. Alternatively, quantitative requirements of ligand expression for T cell deletion in vivo may differ from the requirements for in vitro stimulation. Either of these two possibilities could explain the presence of some backcross animals with intermediate levels of $\text{V}11^+$ T cells that do not respond to in vitro challenge, as shown in Table 4.

This study has demonstrated the effect of a non-MHC-encoded determinant on the expressed $\text{V}B$ repertoire. By conventional criteria, this determinant appears to define a novel Mls antigen. Mls$^a$ and Mls$^b$ determinants were initially defined as alloantigens capable of inducing strong proliferative responses by MHC-identical T cells; recognition of these determinants was subsequently found to be mediated by specific $\text{V}B$ products (reviewed in reference 21). Similar to Mls$^a$ and Mls$^b$, the determinant(s) characterized in the present study is capable of causing proliferation of T cells to H-2-matched stimulators (19, and this study), as well as causing deletion of T cells expressing $\text{V}11$ or $\text{V}12$. C58/J is the only identified H-2$^k$, H-2$^b$, or H-2$^d$ strain known to lack this determinant, as demonstrated by the lack of $\text{V}11$ and $\text{V}12$ deletion. Therefore, the influence of MHC haplotypes other than H-2$^k$ on the stimulatory capacity of this determinant has been demonstrated.

| $\text{V}B$11 expression* | Mean† | $p$ value§ |
|--------------------------|-------|---------|
| High (>5.5%)             | 38.0 ± 9.7 | <0.01 |
| Intermediate (2.5-5.5%)  | 4.2 ± 2.2  | <0.01 |
| Low (<2.5%)              | 2.5 ± 1.4  | <0.01 |

* $\text{V}B$11 expression was determined by flow cytometry on individual (CBA/Ca x C58/J) x C58/J backcross animals.
† Mean of responses (± SEM) of individual animals to mitomycin-treated CBA/Ca stimulators, expressed as a percent of the response to control MHC-allogeneic BALB/c stimulators.
§ Significance of difference between the high $\text{V}B$11 group and the intermediate or low group as assessed by student’s $t$ test.
been reported, based upon analysis of Mls strains, that non-
or in association with other self antigens (4). It has recently been unclear whether the influence was a result of MHC alone expression of V\(\beta\)5, V\(\beta\)11, V\(\beta\)12, and V\(\beta\)16, although it was thought that the expression of non-MHC haplotypes resulted in decreased expression of V\(\beta\)3, V\(\beta\)6, V\(\beta\)8.1, and V\(\beta\)9 are decreased in Mls\(^+\) strains of the correct MHC haplotype (1-5). While the ligand for V\(\beta\)7 has not been identified, it does appear to be influenced by MHC as well as non-MHC self antigens (4). In our previous study, it was reported that expression of specific MHC haplotypes resulted in decreased expression of V\(\beta\)5, V\(\beta\)11, V\(\beta\)12, and V\(\beta\)16, although it was unclear whether the influence was a result of MHC alone or in association with other self antigens (4). It has recently been reported, based upon analysis of RI strains, that non-MHC genes influence V\(\beta\)5.2 (17). Results in the present report strongly support previous suggestions that V\(\beta\)11 expression is influenced by non-MHC genes, and demonstrated in addition that V\(\beta\)12 expression is affected by a highly correlated set of non-MHC products. Out of the 10 V\(\beta\)s shown to be affected by negative selection, eight have now been shown to be deleted not by recognition of MHC alone, but by some interaction of non-MHC gene products with self MHC.

The mechanism by which non-MHC gene products interact with self MHC determinants to mediate negative selection of T cells is unclear. The most conventional model for such interaction would involve TCR-mediated recognition of a ligand that consists of processed non-MHC peptide antigens presented in the groove of self MHC molecules (1). Alternatively, as has been suggested for recognition of stimulatory Mls antigens, tolerizing self ligands may not be processed self peptides, but rather a unique set of self molecules capable of interacting directly with external domains of both MHC products and the TCR V\(\beta\) (22). The findings in this study suggest that, whatever its mechanism of action, this class of Mls antigens may be more extensive than previously thought. The observed pattern of Mls influence on V\(\beta\) selection may not therefore be an unusual phenomenon, but rather, may be characteristic of a class of antigens that mediate an important role in T cell repertoire selection.

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