CD4 Expression Is Differentially Required for Deletion of MLS-1a-reactive T Cells

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Summary

Clonal deletion of thymocytes expressing potentially self-reactive T cell receptors (TCRs) occurs during thymocyte ontogeny. Mice deficient for CD4 expression provide a unique model system to study the contribution of the CD4 molecule in negative selection of T cells reactive against the major histocompatibility complex class II-associated retroviral self-superantigen, Mls-1a. In the presence of Mls-1a determinants, mature CD8+ T cells expressing Vβ6, 8.1, and 9 were deleted in CD4-deficient mice, thus demonstrating that TCR affinity for Mls-1a is sufficient for deletion and that a signal through CD4 was not required. However, in instances where the TCR affinity for Mls-1a is low, as in the case of Vβ7+ T cells, CD4 expression was required for clonal deletion. These results demonstrate that for Mls-1a-mediated clonal deletion of T cells, the requirement for the accessory or coreceptor function of CD4 depends on the affinity of the TCR.

One mechanism by which self-tolerance is maintained is through clonal deletion of T cells expressing self-reactive TCRs during thymocyte ontogeny (1). The nature of the tolerizing signal delivered to the developing thymocyte, however, has not yet been characterized. In addition, the contribution of other cell surface molecules, for instance, CD4, to tolerance induction is still controversial.

Recently, it has been demonstrated that Mls-1a determinants are encoded by an endogenous mouse mammary tumor virus, Mtv-7, integrated on chromosome 1 (2, 3). T cells expressing Vβ6, 7, 8.1, and 9 are deleted during thymic differentiation in Mls-1a strains of mice (4). Mls-1a stimulation in vitro of T cells or clonal deletion of thymocytes requires the presence of MHC class II molecules, and there is a hierarchy for MHC class II presentation of Mls-1a; namely, H-2k, H-2d > H-2b > H-2d, with I-E molecules being better presenters than I-A molecules (4).

Mice rendered CD4 deficient by gene targeting (5) provide a unique system in which to study the requirement for CD4 in negative selection of CD8+ T cells mediated by self-superantigens. TCR Vβ expression was examined in CD4-deficient mice (CD4−/−) bred into Mls-1a and Mlsb backgrounds. Vβ6+, 8.1+, and 9+ T cells were deleted in CD4−/− Mls-1a mice in I-Ek haplotypes. However, Vβ7+ T cells were not deleted in CD4−/− Mls-1a mice.

Materials and Methods

Mice and Mtv-7 Analysis. B10.BR, BALB.K, CBA/J, and DBA/2 strains of mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice homozygous for the CD4 mutation (CD4−/−) have been described previously (5). The breeding strategy to obtain CD4−/− Mls-1a mice was as follows: CD4 heterozygous (CD4+/-) F1 mice were obtained from crosses between female DBA/2 (H-2d, Mls-1a), CBA/J (H-2b, Mls-1a), or B10.BR (H-2b, Mlsb), and male CD4−/− I-E+ animals. The CD4−/− F1 progeny from each pair were mated, and pups from the F2 generation were typed for CD4 expression and H-2 haplotype by immunofluorescence. Mice were typed for Mls-1a by Southern blot analysis for Mtv-7 integration, which is determined by the presence of an 11.7-kb band in blots of EcoRI-digested genomic DNA probed with an MMTV env probe (6, 7, and data not shown).

In Vitro Culture. Spleen cell responders (2 x 10^6) from mice aged 6–12 wk were cocultured with 5 x 10^5 thymocytes or 5 x 10^6 anti-Thy-1 and complement-depleted and irradiated (1,000 rad) splenic stimulator cells in 2 ml of IMDM supplemented with 10% FCS, 2-ME, antibiotics, and 10% ratspleen Con A supernatant as a source of IL-2. In experiment 2, responder cells (2 x 10^6) were stimulated with 2.5 µg/ml Con A (Pharmacia, Uppsala, Sweden) in medium lacking IL-2. On day 3, responding T cell blasts were isolated on a Ficoll density gradient and cultured for another 2 d in the presence of IL-2, after which the cells were harvested and stained for CD8 and TCR Vβ expression.

Immunofluorescence. Blood samples (200 µl) were collected in heparinized capillary tubes, washed once in immunofluorescence staining buffer (PBS, 4% FCS, 0.1% NaN3), and incubated with the following anti-H-2 culture supernatants obtained from American Type Culture Collection (Rockville, MD): B8-24 (anti-H-2k) (TIB 139), 34-2-12 (anti-H-2d) (HB87), and 11-4-1 (anti-H-2b) (TIB 95), followed by FITC-conjugated goat anti–mouse antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL). For CD4 analysis, blood samples were stained with PE-conjugated anti-CD4 (Becton Dickinson & Co., Mountain View, CA). For TCR
VO analysis, the following rat mAb cell culture supernatants were used: 44-22-1 (anti-Vβ6) (8), TR31 (anti-Vβ7) (9), KJ16 (anti-Vβ8.1, 8.2) (10), and B20.6 (anti-Vβ2) (11). The following mouse mAbs were used: F23.2 (anti-Vβ8.2) (12) and MR10-2 (anti-Vβ9) (13). Single cell suspensions of thymocytes and mesenteric lymph nodes from 6-8 wk-old mice were resuspended in staining buffer and incubated with appropriate anti-TCR VO culture supernatants, washed, and labeled with PE-conjugated goat anti-rat Ig or FITC-conjugated goat anti-mouse Ig (Southern Biotechnology Associates, Inc.). Lymph node samples were incubated with 1 µg of rat IgG (Sigma Chemical Co., St. Louis, MO) to block remaining anti-rat Ig sites, and cells were then double stained with anti-CD8 (Becton Dickinson & Co.) followed by Streptavidin-RED613 (Gibco Laboratories, Grand Island, NY). T cell blasts were stained as described for lymph node samples. Samples were analyzed using a FACS® (Becton Dickinson & Co.). The percentage of Vβ8.1+ T cells was calculated by subtracting the percentage of Vβ8.2+ cells from the percentage of Vβ8.1− Vβ8.2+ cells. Mean values of TCR VO expression were statistically compared between Mls-1+ and Mls-1− mice within the same H-2 haplotype using an unpaired Student’s t test. Probability values, p < 0.05, were taken to be significantly different.

Results and Discussion

To assess the requirement for CD4 expression in the Mls-1a-mediated deletion of CD8+ T cells bearing Vβ6, 7, 8.1, and 9, the CD4 mutation was bred into different Mls and H-2 backgrounds, as described in Materials and Methods. The presence of Mls-1a was determined by Southern blot analysis for Mtv-7 integration (data not shown). We first determined whether the lack of CD4 expression during ontogeny would adversely affect the development of Mls-1-reactive T cells in CD4 deficient (CD4−/−) Mls-1a (Mtv-7−) mice. As previously reported, CD4−/− mice have a large peripheral TCR α/β+ CD8− T cell population and a smaller TCR α/β+ CD4− CD8− population (5). CD8+ peripheral T cells expressing Vβ6, 7, 8.1, and 9 were present in the lymph nodes of CD4−/− Mls-1b H-2k or H-2d mice at levels similar to CD4+− Mls-1b littermates or BALB.K (Mls-1b, H-2k) mice (Table 1 and Fig. 1).

After in vitro Mls-1a stimulation, CD8+ T cell blasts from CD4−/− Mls-1b mice were enriched for Vβ6 and 8.1 expression, as were CD8+ T cell blasts from control BALB.K mice (Table 2). CD8+ T cells expressing Vβ7+ TCRs were poorly enriched in anti-Mls-1 cultures from both the CD4−/− Mls-1b or control BALB.K mice, which is in agreement with previous data showing that Vβ7+ T cells are the last Mls-1a reactive in vitro (14). Vβ9+ T cells were also not significantly enriched in these cultures, and this may be due to the strain combination used in this experiment. Therefore, the lack of CD4 expression during thymic ontogeny in CD4−/− Mls-1b mice did not affect the development of T cells expressing Mls-1a-reactive Vβs or in vitro reactivity against Mls-1a determinants.

Spleen cells from CD4−/− Mls-1b H-2a or H-2d mice were tolerant against Mls-1a as judged by low cell recovery and lack of enrichment for Vβ6, 7, 8.1, or 9 CD8+ T cells after a 5-d in vitro Mls-1a stimulation (Table 2, and data not shown). Lymph node cells from CD4−/− Mls-1a− mice were analyzed for expression of Mls-1a-associated Vβs in order to determine if clonal deletion was the mechanism of tolerance against Mls-1a (Table 1, and Fig. 1). CD8+ Vβ6+ T cells were significantly reduced in CD4−/− Mls-1a H-2d and H-2k mice when compared with their CD4+− Mls-1a littermates (p < 0.05). Vβ6+ T cells were also not present in the CD4−/− CD8− compartment of CD4−/− Mls-1a− mice (data not shown). CD8+ T cells bearing Vβ8.1 and 9 were also reduced in CD4−/− Mls-1a H-2d mice to the same extent as in CD4−/− Mls-1a H-2d littermates and CBA/J (H-2k, Mls-1a) control mice. These results demonstrate that in the absence of CD4 expression, CD8+ T cells bearing Vβ6, 8.1, and 9 can be deleted by Mls-1a determinants.

| Mouse* | Mls | H-2 | I-E | Percent of CD8+ lymph node cells expressing: |
|--------|-----|-----|-----|---------------------------------------------|
|        | Vβ6 | Vβ7 | Vβ8.1 | Vβ9 | Vβ2 |
| BALB.K | b k  | 11.6 ± 1.0 | 3.2 ± 0.5 | 15.8 ± 1.7 | 2.1 ± 0.3 | 6.7 ± 2.0 |
| CBA/J  | a k  | 0.1 ± 0.0 | 0.9 ± 0.5 | 5.5 ± 1.2 | 0.2 ± 0.1 | 16.5 ± 1.2 |
| CD4−/− | b d  | 12.1 ± 0.3 | 4.0 ± 1.8 | 11.4 ± 3.3 | 3.1 ± 0.9 | 7.7 ± 1.4 |
| CD4−/− | a d  | 0.6 ± 0.1 | 1.3 ± 0.3 | 3.3 ± 0.5 | 0.6 ± 0.3 | 8.7 ± 0.7 |
| CD4−/− | b d  | 10.6 ± 0.7 | 5.0 ± 1.6 | 9.2 ± 1.6 | 2.0 ± 0.1 | 6.3 ± 1.1 |
| CD4−/− | a d  | 1.4 ± 0.2 | 2.9 ± 0.5 | 5.5 ± 0.1 | 0.9 ± 0.1 | 6.6 ± 0.5 |
| CD4−/− | b k  | 8.9 ± 2.0 | 4.5 ± 0.6 | ND | ND | 7.0 ± 1.0 |
| CD4−/− | a k  | 1.4 ± 0.2 | 3.2 ± 0.4 | ND | ND | 9.9 ± 1.0 |

Table 1. TCR Vβ Expression on CD8+ Lymph Node T Cells from Control and CD4−/− Mice

Table 2. TCR Vβ Expression on CD8+ Lymph Node T Cells from Control and CD4−/− Mice

- Mice were typed for CD4, H-2, and Mls-1a as described in Materials and Methods. Mesenteric lymph node cell suspensions from mice aged 6-8 wk were stained with appropriate anti-TCR Vβ mAbs and anti-CD8, and analyzed by flow cytometry. Results are expressed as mean percent of Vβ+ CD8+ T cells ± SEM. Underlined values are not significantly different from one another.
- At least three mice per group were analyzed.
- Staining for Vβ2, which is not Mls-1a reactive, is also included.
T cells expressing Vβ7 were not significantly reduced in the lymph nodes of CD4⁻/⁻ Mls⁻¹ H-2d or H-2k mice as compared with CD4⁺/⁺ Mls⁺ H-2d or H-2k littermates, or CBA/J mice, thus demonstrating that CD4 expression is required for deletion of Vβ7-bearing T cells (Table 1, and data not shown). Although mice heterozygous for the CD4 mutation (CD4⁺/⁻) have a slightly reduced intensity of surface CD4 staining (5), Vβ7⁺ T cells were deleted in CD4⁻/⁻ Mls⁻¹ H-2d mice to the same extent as in CBA/J mice (Table 1). Therefore, a reduction in CD4 surface intensity on T cells does not adversely affect deletion of Vβ7⁺ T cells.

We next investigated whether deletion of Vβ6⁺ T cells could be detected in the thymus of CD4⁻/⁻ Mls⁻¹ mice. In Mls⁻¹ mouse strains, T cells expressing Mls⁻¹-reactive Vβ6 are present among the TCR⁺ immature thymocyte population, but are markedly reduced in the mature TCR⁺ thymocyte population (1). As summarized in Table 3, the Vβ6⁺ population of thymocytes was present in CD4⁻/⁻ Mls⁻¹ and in control BALB.K (Mls⁺, H-2d) mice. However,

Table 2. TCR Vβ Expression on CD8⁺ T Cell Blasts after In Vitro Mls⁻¹ Stimulation

| Exp. | Responder | Stimulator | Vβ6 | Vβ8.1 | Vβ8.2 | Vβ7 | Vβ9 |
|------|-----------|------------|-----|-------|-------|-----|-----|
| 1    | BALB.K    | BALB.K     | 7.1 | NT    | NT    | NT  | NT  |
|      | H-2d, Mls | CBA/J      | 43.0| 14.6  | 3.3   | NT  | NT  |
|      | CD4⁻/⁻    | BALB.K     | 7.7 | 9.7   | 14.1  | NT  | NT  |
|      | H-2d, Mls | CBA/J      | 41.5| 17.0  | 7.6   | NT  | NT  |
|      | CD4⁻/⁻    | BALB.K     | 2.9 | NT    | NT    | NT  | NT  |
|      | H-2d, Mls | CBA/J      | 2.1 | 5.6   | 14.4  | NT  | NT  |
| 2    | BALB.K    | Con A      | 13.1| 18.4  | 16.2  | 10.2| 3.3 |
|      |           | BALB.K     | 16.5| NT    | NT    | NT  | NT  |
|      |           | CBA/J      | 66.0| 17.2  | 3.7   | 15.0| 2.4 |
|      | CD4⁻/⁻    | Con A      | 12.4| 11.4  | 19.2  | 5.0 | 3.1 |
|      | H-2d, Mls | BALB.K     | 10.8| 10.9  | 17.0  | 6.8 | 3.5 |
|      | CBA/J     | 64.0       | 11.1| 13.3  | 2.8   | 1.5 |
|      | CD4⁻/⁻    | Con A      | 1.1 | 0.3   | 27.4  | 4.0 | 1.3 |
|      | H-2d, Mls | BALB.K     | 1.4 | 3.8   | 20.3  | 3.9 | NT  |
|      | CBA/J     | 0.9        | 7.9 | 16.0  | 2.8   | 1.3 |

Spleen cell responders (2 x 10⁶) from mice aged 6-12 wk were stimulated as described (Materials and Methods) and double stained with anti-Vβ mAb and CD8.
* NT, not tested.
† CBA/J stimulators are H-2d, Mls⁻¹.
Single cell suspensions of thymocytes from 6-8-wk-old mice were stained for V06, V07, and V32 expression. Samples were analyzed on a FACScan® and the percentage of thymocytes with high-intensity TCR Vβ expression was determined. Results are expressed as mean percent of Vβ high thymocytes ± SEM, and the data are compiled from at least three animals per group. It should be noted that the percentages of TCR α/β high thymocytes were similar in all mice tested.

In vitro and in vivo studies have shown that Vβ7+ T cells are the least reactive of all Mls-1-reactive Vβs described to date (14). In addition, the degree of reduction of Vβ7+ T cells in CD4+ Mls-1+ mouse strains is not as drastic as that of Vβ6+ T cells (9). The data demonstrating that CD4 expression is required for deletion of Vβ7-expressing T cells are consistent with these previous findings and supports a model in which Vβ7 has low affinity for Mls-1a.

Reactivity against MHC class II-associated self-superantigens is more readily demonstrated with CD4+ T cells expressing appropriate Vβs, however, both mature CD4+ and CD8+ T cells expressing these Vβs are deleted during thymic development (15). A contribution of CD4 in deletion mediated by MHC class II-associated self-superantigens was inferred from studies in which anti-CD4 treatment was able to prevent the deletion of CD8+ T cells bearing self-superantigen-reactive Vβs (16, 17). Therefore, it was postulated that CD8+ T cells expressing self-superantigen-reactive Vβs were targeted for deletion as a result of CD4 expression at the double-positive stage of thymocyte development. However, it has been subsequently demonstrated that CD8+ T cells from Mls1 mice are enriched for Vβ6, 8.1, and 9 expression after in vitro and in vivo Mls-1 stimulation (18-20), and that the in vitro anti-Mls-1 α response of CD8+ Vβ6+ T cells is MHC class II dependent (21). Our results now demonstrate that CD4 expression is not absolutely required for Mls-1-reacted clonal deletion of CD8+ Vβ6+ T cells. However, the avidity required for clonal deletion of Mls-1-reactive TCRs may be attained through the interaction of CD8 with MHC class I in the thymus. To address this, experiments on clonal deletion in CD4/8-deficient mice are currently underway.

In summary, CD8+ T cells expressing Vβ6, 8.1, and 9 TCRs are deleted from the periphery of CD4-/- Mls-1+ mice. Analysis of Vβ expression on thymocytes has revealed that deletion of Vβ6+ T cells can be detected in the thymus of CD4-/- Mls-1+ mice. Thus, deletion of T cells with high affinity for Mls-1 determinants can occur in the absence of a signal through CD4. Vβ7+ T cells were not deleted from the periphery or the thymus in CD4-/- Mls-1+ mice. Therefore, there is a differential requirement for CD4 expression in clonal deletion such that CD4 expression is required for deletion when TCR affinity is reduced. There is evidence that T cell recognition of superantigens is different from recognition of nominal peptide antigen in the groove of MHC class II (22, 23). Whether or not the finding that CD4 expression is differentially required for Mls-1 self-superantigen-mediated deletion of T cells can be extended to deletion of T cells mediated by self-peptides presented in the groove of MHC class II requires further study.
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