Thymic Selection and Thymic Major Histocompatibility Complex Class II Expression Are Abnormal in Mice Undergoing Graft-versus-Host Reactions

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Summary

The graft-vs.-host reaction (GVHR) results in damage to the epithelial and lymphoid compartments of the thymus and thus in abnormal maturation and function of thymocytes in mice undergoing GVHR. In this report, the effects of GVHR on thymic T cell receptor (TCR) expression and usage have been investigated. GVHR was induced in unirradiated F_1 hybrid mice by the intravenous transfer of parental lymphoid cells. Expression of the CD3/TCR complex on thymocyte subsets defined by CD4 and CD8 was studied by three-color flow cytometry. The level of CD3/TCR was decreased on $CD4^+CD8^-$, but not $CD4^-CD8^+$, mature thymocytes. The lack of upregulation of CD3/TCR on CD4 single-positive thymocytes, but not on their $CD8^+$ counterparts, suggested an abnormality of class II major histocompatibility complex (MHC) expression in the thymuses of mice undergoing GVHR. Immunofluorescence staining of thymic frozen sections revealed that MHC class II expression was dramatically decreased in GVH-reactive mice. GVHR-induced changes in positive and negative selection were evaluated by determining the incidence of specific Vβ TCR segment usage in the thymus. In normal mice, thymocyte usage of any given Vβ segment was highly consistent between individuals of the same strain and age; however, a marked divergence in the incidence of TCR $V\beta6^{hi}$ and $V\beta8^{hi}$ cells between GVH-reactive littermate mice was observed, suggesting that thymic positive selection had become disregulated in these animals. Furthermore, negative selection was defective; the incidence of phenotypically self-reactive $V\beta6^{hi}$ T cells was significantly greater in the thymuses of GVH-reactive mice bearing the endogenous superantigen Mls-1^a than in untreated controls. Thus, mice undergoing GVHR showed defective TCR upregulation on $CD4^+CD8^-$ thymocytes and changes in TCR usage reflecting aberrant thymic selection, in conjunction with decreased expression of MHC class II. Most abnormalities of TCR expression and usage on $CD4^+$ thymocytes observed in GVH-reactive mice were analogous to those of class II knockout mice.

The graft-vs.-host reaction (GVHR)[1] induced by injection of parental lymphoid cells into unirradiated F_1 hybrid recipients consists of an acute syndrome of immunosuppression, epithelial cell lesions, and cachexia, often leading to death. Survivors of the acute reaction develop chronic GVHR, a multisystem disease characterized by autoimmune features and persistent immunosuppression (1–3). These manifestations of chronic GVHR represent T cell functional abnormalities possibly resulting from defective education within the GVHR-dysplatic thymus (4, 5). Indeed, the thymus sustains severe histopathological damage during acute GVHR, including injury to medullary epithelial cells, effacement of the corticomedullary junction, progressive disappearance of Hassal's corpuscles (6), and cortisone-dependent effects such as death of the immature $CD4^+8^+$ thymocytes (4). In addition, the immune activation that occurs early in GVHR results in the release of cytokines such as IFN (7), which primes macrophages (8) and modulates MHC antigens in peripheral tissues, and may similarly affect the thymus. Thus, the thymic microenvironment is markedly altered by GVHR; furthermore, histological abnormalities persist well into the chronic phase of the reaction (9).

In normal animals, the thymus provides the microenvironment for T cell maturation and selection. Pre-T cells enter the thymus as $CD4^-8^-3^-$ precursors; as they mature, they become double-positive $CD4^+8^+$ immature, cortisone-sensitive thymocytes and acquire low levels of CD3/TCR ($CD3^{lo}$); they then lose expression of one of the two accessory molecules and become mature, cortisone-resistant single-

[1] *Abbreviation used in this paper:* GVHR, graft-vs.-host reaction.

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positive $CD4^+8^-$ or $CD4^-8^+$ T cells with peripheral levels of CD3/TCR expression ($CD3^{hi}$). The transition from $CD3^{lo}$ to $CD3^{hi}$, an upregulation of approximately an order of magnitude, requires the thymic microenvironment and is thought to be a consequence of positive selection (10).

Thymic stromal components positively select T cells able to interact at low affinity with self-MHC molecules, while bone marrow–derived elements mediate the deletion of potentially autoreactive cells (11–14). These intrathymic events are believed to be primarily responsible for the self-restriction and self-tolerance of the peripheral T cell repertoire (15). Antibody-mediated blocking of the interactions between thymic MHC molecules and the CD4 or CD8 accessory molecules on immature T cells has resulted in defective thymic selection (16–18), as has the absence of these molecules in transgenic mice with deletion mutations (19–24). Thus, GVHR-induced alterations of the thymic stroma, whether by direct injury or by cytokine-triggered modulation of cell surface molecules, are likely to affect T cell maturation and selection.

To investigate this hypothesis, the level of CD3/TCR expression and the incidence of representative positively or negatively selected TCRs were studied during acute and chronic GVHR. The effects of GVHR on positive selection were monitored by following the upregulation of CD3 expression on thymocytes and by changes in the incidences of specific TCR Vβ segment usage in the thymus. Before selection, the incidence of each Vβ segment is germline controlled; however, the final incidence of each Vβ among mature thymocytes is determined by thymic selection processes (25). Thus, in unmanipulated age- and sex-matched mice, the incidence of any given TCR Vβ segment among mature ($CD3/TCR^{hi}$) thymocytes is highly consistent and results from positive selection pressures. We therefore chose to use deviations in normal incidences before antigen-driven expansion in the periphery as an indicator of abnormal positive selection. Negative selection results in the intrathymic deletion of potentially autoreactive clones; in mice bearing the endogenous retrovirus encoding the Mls-1^a antigen, T cells bearing Mls-1^a-reactive Vβ segments such as Vβ6 are deleted (26, 27). Thus, we induced GVHR in a strain combination in which both the donor and recipient were Mls-1^{a+}, and monitored the incidence of normally deleted $V\beta6^{hi}$ T cells in the thymuses of GVH-reactive mice in order to detect defective negative selection. Our results demonstrate aberrant positive and negative selection concomitant with decreased thymic MHC class II expression during GVHR. Our findings demonstrated remarkable similarities between the $CD4^+$ thymocytes in GVH-reactive mice and class II knockout mice; this suggested that the abnormal level and distribution of thymic class II during GVHR could explain the impaired thymic maturation and defective peripheral function of the class II–restricted T cells observed in GVH-reactive mice.

Materials and Methods

Animals. Mice of the inbred strains CBA/J (H-2^k, Mls-1^a), A (H-2^a, Mls-1^b), and C57BL/6 (B6) (H-2^b, Mls-1^b), and the F_1 hybrids B6 × CBA/J, A × CBA/Ca (H-$2^{a/k}$, Mls-$1^{b/b}$), and B6 × A ($B6AF_1$) were used. All mice were bred and maintained in our animal colony.

GVHR Induction. GVHRs were induced as described previously (4). Briefly, pooled parental spleen and lymph nodes were made into single-cell suspensions and injected intravenously into unirradiated F_1 hybrid recipients (28). Cell dose varied from 2–6 × 10^7 viable donor lymphoid cells depending on the strain combination and the required severity of the GVHR.

Direct Plaque-forming Cell (PFC) Response to SRBC. Mice were immunized intravenously with 5 × 10^8 SRBC in 0.3 ml saline. 4 d later the direct splenic PFC response was assessed by the method of Cunningham and Szenberg (29) as modified in this laboratory (30).

Mitogen Assays. T cell proliferative responses to Con A and B cell responses to LPS were evaluated by [^{3}H]thymidine incorporation as described previously (31). Briefly, 5 × 10^5 spleen cells were cultured for 48 h in triplicate with or without mitogen, then for a further 16 h with 1 μCi/well [^{3}H]thymidine. DNA was harvested with an automated cell harvester and [^{3}H]thymidine incorporation was quantitated on a beta counter (LKB Instruments, Turku, Finland). Data are expressed as: net cpm = cpm of mitogen stimulated culture − cpm of unstimulated culture. The counts in unstimulated cultures (background) did not exceed 6,000 cpm for normal mice, and 2,500 cpm for GVH-reactive mice.

Flow Cytometry Analysis. Single-cell suspensions of thymocytes were prepared by gentle tamping through 50-mesh stainless steel screens. Suspensions were washed, made up to 2 × 10^7 cells/ml in RPMI 1640 supplemented with 10% FCS, and incubated for 1 h at 37°C. Aliquots of thymocytes (10^6 cells per sample) were incubated with primary antibodies at 4°C for 20 min, then washed and incubated at 4°C for 20 min with secondary reagents if necessary. Labeled thymocytes were resuspended in 1 ml RPMI and analyzed by flow cytometry. For each sample, 12,000–25,000 events were acquired on a FACScan® flow cytometer (Becton Dickinson & Co., Mountain View, CA) with light scatter gating to exclude erythrocytes and debris, and propidium iodide exclusion of dead cells whenever possible (for one- and two-color analyses). Data were analyzed with Consort 30 or FACScan® Research Software. TCR and CD3 labeling produced two positive peaks, of "hi" and "lo" intensity fluorescence, corresponding to immature and mature thymocytes (10, 32, 33). Analysis gates were set to assess the TCR^{hi} or $CD3^{hi}$ populations.

Reagents for Flow Cytometry. Culture supernatants from hybridomas KJ16–133 (anti-Vβ8.1 + 8.2 antibody, kindly provided by Dr. P. Marrack, University of Colorado Health Sciences Centre, Denver, CO [34]) and 44-22-1 (anti-Vβ6, a gift from Dr. G. Prudhomme, McGill University, Montreal, Canada, originally obtained from Dr. H. Hengartner, University of Zürich, Zürich, Switzerland [35]) were diluted 1:2 in PBS and used as a source of primary antibodies in conjunction with goat anti-rat FITC (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) as the second-step reagent. Anti-CD3-FITC (Boehringer Mannheim Canada Ltd., Laval, Canada) was used as single-step reagent, either alone or for three-color analysis with anti-CD4-PE, and anti-CD8-biotin followed by streptavidin-allophycocyanin (Becton Dickinson & Co.). Reagents obtained commercially were used at the recommended dilutions.

Immunofluorescence Labeling of Frozen Sections. Thymuses were removed, rinsed in HBSS, and immediately embedded in Tissue-Tek O.C.T. compound (Miles Inc. Diagnostics Division, Elkhart, IN) on dry ice. The tissue blocks were cut into 12-μm sections on a Histostat microtome (American Optical, Buffalo, NY) at −20°C and the sections were mounted on gelatin-coated slides.

The sections were incubated with normal goat serum (Cedarlane Laboratories, Hornby, Ontario, Canada) for 1 h at room temperature to block nonspecific binding, washed, then incubated overnight at 4°C with hybridoma P7/7 supernatant (anti-MHC class II specificity, kindly provided by Dr. T. Owens, McGill University). The slides were washed, incubated for 1 h with goat anti-rat FITC (Kirkegaard & Perry Laboratories, Inc.), washed again, and mounted in Gelvatol (glycerol and polyvinyl alcohol in Tris-HCl buffer). Background staining was assessed by substituting PBS alone for P7/7 supernatant during the overnight incubation.

Results

CD3 Expression Is Altered in the Thymus during GVHR. CD3 expression was examined in the thymuses of mice 25 d after induction of GVHR. $B6AF_1$ mice injected with 5×10^7 B6 parental lymphoid cells developed an intense, lethal reaction as shown by complete suppression of splenic mitogen responses (Fig. 1, legend) and 70% GVHR-induced mortality (the remaining 30% GVH-reactive mice were killed on day 25). Thymocytes were analyzed by flow cytometry for expression of CD3 on subsets defined by the CD4 and CD8 accessory molecules (Fig. 1). The distribution and levels of CD3 on the most immature subset, the $CD4^-8^-$ thymocytes, were unchanged in mice undergoing GVHR. In untreated mice, the majority of $CD4^+8^+$ thymocytes expressed low levels of CD3 after an incubation at 37°C; this cortisone-sensitive $CD3^{lo}$ $CD4^+8^+$ subset was largely ablated by the high levels of circulating glucocorticoids that we and others have observed during severe GVHR (36; and K. E. You-Ten and W. S. Lapp, manuscript in preparation). A small subpopulation of the $CD4^+8^+$ thymocytes, representing the most mature cells of this subset, expressed high levels of CD3; only this small subpopulation of $CD3^{hi}$, $CD4^+8^+$ cortisone-resistant cells persisted during GVHR. In normal mice, the most mature thymic subsets lose expression of one of the two accessory molecules and expressed peripheral levels of CD3. However, $CD4^-8^+$ GVH-reactive thymocytes showed higher than normal mean levels of CD3, while the $CD4^+8^-$ thymocytes had lower levels of CD3 expression, suggesting a selective failure of CD3 upregulation on $CD4^+8^-$ thymocytes in mice undergoing GVHR (Fig. 1).

Thymic TCR Usage Is Altered during Severe GVHR. Potential alterations in positive selection were addressed by analyzing the incidence of TCRs that are normally positively selected, as inferred by their high proportional representation among mature thymocytes and peripheral T cells. In this study, only mature thymocytes were considered in order to examine the postselection repertoire while avoiding bias due to peripheral clonal expansion in response to antigenic stimulation.

In the $B6AF_1$ mice described above, the incidence of $V\beta8^{hi}$ thymocytes was analyzed by flow cytometry (Fig. 2). Severe GVHR induces high glucocorticoid production, resulting in the disappearance of the cortisone-sensitive $CD4^+8^+$ thymocytes and consequent thymic involution and increased incidence of mature thymocytes (4, 36; and K. E. You-Ten and W. S. Lapp, manuscript in preparation); to correct for this effect, results were expressed in terms of the ratio of $V\beta8^{hi}$ thymocytes to total mature thymocytes, as deter-

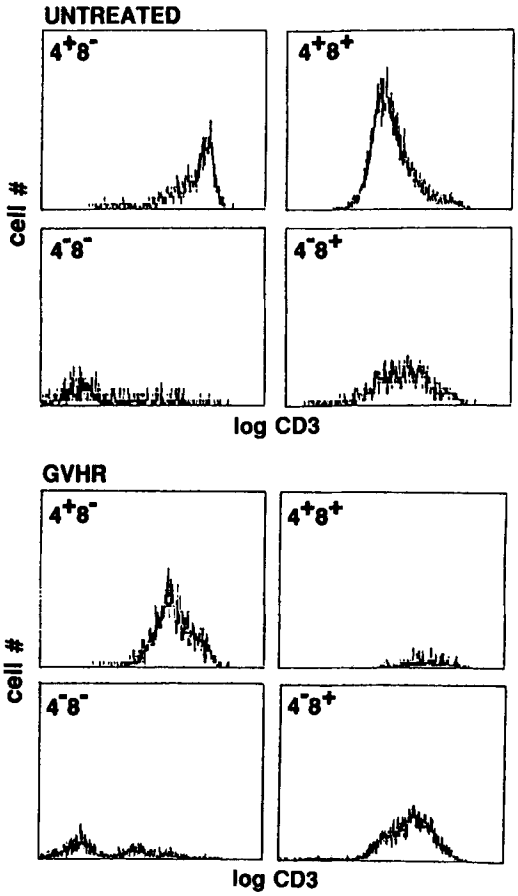

Figure 1. CD3 expression on thymocytes is affected by GVHR. Thymocytes were labeled with anti-CD4, anti-CD8, and anti-CD3 antibodies and analyzed by three-color flow cytometry. The histograms represent log-CD3 fluorescence of gated CD4/CD8 populations of thymocytes from untreated and GVH-reactive $B6AF_1$ mice 25 d after transfer of 5×10^7 B6 lymphoid cells. GVHR was confirmed by immunosuppression assessed by mitogen responses to ConA and LPS. Net responses to ConA and LPS stimulation, respectively, were 70,578 ± 11,403 and 52,980 ± 7,702 cpm in normal mice, and 592 ± 2,684 and −7,488 ± 486 cpm in GVH-reactive mice.

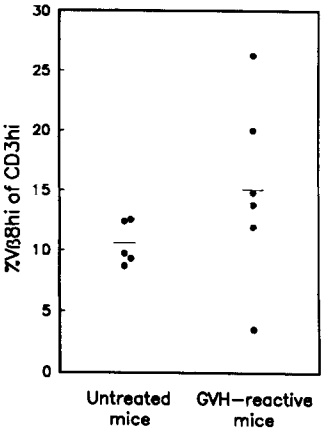

Figure 2. The incidence of TCR $V\beta8$ usage by mature thymocytes becomes inconsistent during severe GVHR. The incidence of $V\beta8^{hi}$ cells as a percentage of total $CD3^{hi}$ thymocytes was assessed by flow cytometry and is shown for individual $B6AF_1$ animals. GVH-reactive mice were those described in Fig. 1.

mined by $CD3^{hi}$ expression. The incidence of $V\beta8^{hi}$ thymocytes varied by an order of magnitude among GVH-reactive mice, whereas it was highly consistent among the untreated age- and sex-matched control mice. Results indicate that the range of $V\beta8$ incidence in $B6AF_1$ GVH-reactive mice spanned from 3% to >25% of the mature thymocytes, a 10-fold variability. Both increases and decreases in $V\beta8^{hi}$ incidence with respect to normal were observed in GVH-reactive animals, suggesting that the normally tightly regulated thymic selection processes were affected stochastically by the GVHR. The high incidence of $V\beta8$ cells was unlikely donor infiltration of mature cells into the host thymus since studies using a DNA probe to detect donor cells in similar reactions never revealed high chimerism this early in GVHR (B. Wisse, K. E. You-Ten, and W. S. Lapp, manuscript in preparation).

Altered incidences of $V\beta$ usage were also detected in the reciprocal GVHR, induced by injecting 2×10^7 A strain cells into $B6AF_1$ recipients to produce a severe lethal reaction (Fig. 3). All GVH-reactive mice were completely immunosuppressed, confirming GVHR induction, at the time of assay (Fig. 4). As above, results were expressed as a ratio of $V\beta6^{hi}$ or $V\beta8^{hi}$ to $CD3^{hi}$ thymocytes to normalize specific receptor usage; the increased variability in incidence among the mice undergoing GVHR was again remarkable when compared with the consistency of usage in the untreated control mice. The incidences of mature thymocytes expressing $V\beta6$ and $V\beta8$ were examined in the same mice; although both increased in variability within the GVH-reactive group, these changes were not coordinated within a given thymus: an increase in the incidence of $V\beta6^{hi}$ did not correlate consistently with a concomitant increase or decrease in $V\beta8^{hi}$ incidence, again suggesting a stochastic variation in receptor usage during GVHR.

Thymic TCR Usage Is Altered during Nonlethal GVHR. We chose to study a nonlethal GVHR model to determine if our findings were reproducible in a less severe GVHR. A × CBA/Ca F_1 hybrid recipients of 5×10^7 A strain lymphoid cells developed a chronic GVHR with complete immunosuppression (Table 1) but no mortality. Results were expressed directly as percentages of total thymocytes rather than as ratios of mature cells since the relatively mild GVHR did not result in cortisone-dependent thymic atrophy. As early as 14 d after GVHR induction, changes were detected in the incidence of $V\beta6^{hi}$ and $V\beta8^{hi}$ thymocytes (Table 2). Again, the variability in thymic TCR incidences was increased during GVHR, as demonstrated by the high standard deviations, the large ranges, and the significantly greater mean dispersion of specific $V\beta$ incidences in the GVHR groups.

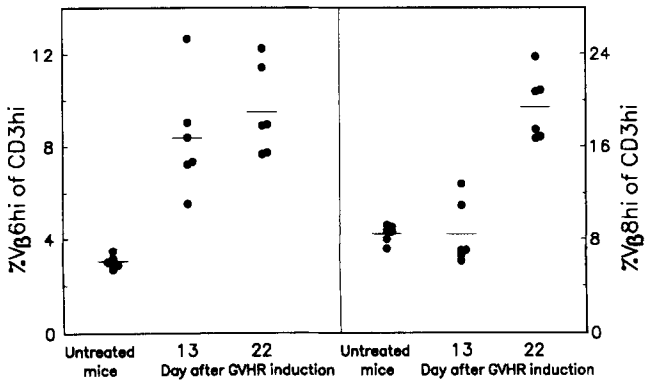

Figure 3. The incidence of TCR $V\beta$ usage by mature thymocytes becomes inconsistent early in GVHR. The incidence of $V\beta6^{hi}$ and $V\beta8^{hi}$ cells as a percentage of total $CD3^{hi}$ thymocytes is shown for individual $B6AF_1$ hybrid mice 13 and 22 d after the induction of GVHR by the injection of 2×10^7 A strain lymphoid cells. GVHR was confirmed by suppressed mitogen responses (see Fig. 4).

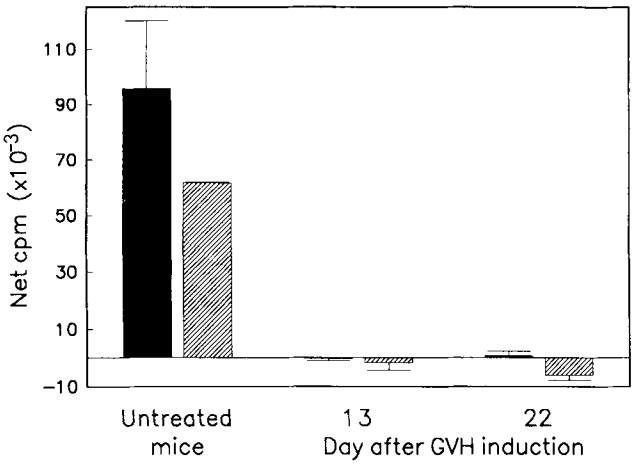

Figure 4. GVH-reactive mice are immunosuppressed. Net responses of splenocytes to the T cell mitogen Con A (*filled bars*) and the B cell mitogen LPS (*hatched bars*) are shown. GVH-reactive mice were those described in Fig. 3.

Negative Selection Is Defective in CBA/J × B6 ($Mls^{a/b}$) Mice Undergoing GVHR. A potential defect in negative selection in CBA/J × B6 mice undergoing GVHR was detected by examining their thymuses for the presence of T cells bearing phenotypically autoreactive receptors, which would normally be deleted. Both CBA/J (donor) and CBA/J × B6 F_1 (recipient) mice were Mls-1^{a+} and thus delete T cells expressing $V\beta6$, 7, 8.1, or 9, resulting in a very low incidence (<0.2% of thymocytes) of these cells in the thymuses of normal young adult mice (26, 27, 37–39). Mls-1^a strain donors were used so that the graft used to induce GVHR would not contain $V\beta6^+$ cells; thus, the presence of cells expressing $V\beta6$ at high levels ($V\beta6^{hi}$) in the recipient thymus could not be attributed to donor cell infiltration. In addition, in this system any donor-derived cells able to mediate negative selection would also delete $V\beta6^+$ thymocytes in the event of eventual stromal chimerism. Finally, this model was chosen to avoid irradiation, which can cause GVHR-independent thymic damage, thus complicating interpretation of the results (40).

As shown in Fig. 5 and Table 3, the incidence of potentially self-reactive thymocytes expressing $V\beta6$ at high levels was significantly increased by 21 d after induction of GVHR. In most GVH-reactive mice, a distinct $V\beta6^{hi}$ population was

Table 1. *Immunosuppression of GVH-reactive Mice Assayed by Their PFC Response to SRBC*

Strain combination	Mean PFC/10^6 splenocytes ± SD			
		Day after GVHR induction		
	Normal F_1	14	21	28
A → A × CBA/Ca	1,323 ± 195	0 ± 0	2 ± 3	0 ± 0
CBA/J → CBA/J × B6	1,158 ± 228	0 ± 0	5 ± 4	0 ± 0

clearly visible by flow cytometry 3 wk after induction of GVHR (Fig. 5). The increased incidence of Vβ6^{hi} thymocytes was maintained during the acute phase of the reaction, and was observed to undergo a further increase during the chronic phase, 55 d after induction of GVHR (Table 3). As in the A × CBA/Ca F_1 recipients, the GVHR induced by 5 × 10^7 cells in this strain combination produced complete immunosuppression (Table 1) but was not lethal and relatively mild, resulting in minimal cortisone-dependent thymic involution that resolved after the acute phase. Thus, the increased incidence of Vβ6^{hi} cells did not represent merely a decrease in the percentage of immature thymocytes. Furthermore, the increased incidence of potentially autoreactive cells was mirrored by the absolute number of Vβ6^{hi} thymocytes, which was as much as sixfold greater in chronic GVH-reactive animals than in normal age-matched control mice.

Thymic MHC Class II Expression Is Decreased during GVHR. Class II expression was examined in the thymuses

Table 2. *Variability of the Incidence of TCR Vβ Usage by Mature Thymocytes in GVH-reactive AxCBA/Ca Mice*

Group	Vβ6^{hi}		Vβ8^{hi}	
	Mean ± SD (range)	Mean dispersion*	Mean ± SD (range)	Mean dispersion*
Untreated mice	1.58 ± 0.11 (1.40–1.60)	0.09	2.51 ± 0.44 (2.09–3.10)	0.35
Day 14 GVH-reactive mice	2.59 ± 0.88‡ (1.58–3.65)	0.66§	1.56 ± 1.32‖ (0.05–2.51)	1.01§
Day 21 GVH-reactive mice	2.62 ± 0.64§ (2.14–3.56)	0.47§	4.22 ± 0.72§ (3.55–5.24)	0.51‖
Day 28 GVH-reactive mice	3.20 ± 0.90§ (2.30–4.10)	0.60‡	5.80 ± 1.42§ (4.70–5.30)	1.07§
GVH-reactive mice¶	2.77 ± 0.77§ (1.58–4.10)	0.64§	3.90 ± 2.02‖ (0.05–7.40)	1.47**

The incidence of Vβ6^{hi} and Vβ8^{hi} cells among total thymocytes is shown for untreated and GVH-reactive A × CBA/Ca F_1 hybrid mice. GVHR was induced by the injection of 5 × 10^7 A strain lymphoid cells, and confirmed by suppressed PFC responses (Table 1).

* Mean dispersion (or mean deviation), $\Sigma|\bar{x} - x|/N$, is a measure of the variability of the data.

‡ $p < 0.01$ compared with untreated mice by the student's t test.

§ $p < 0.005$.

‖ $p < 0.1$.

¶ Statistics are shown for all the GVH-reactive animals considered together.

** $p < 0.025$.

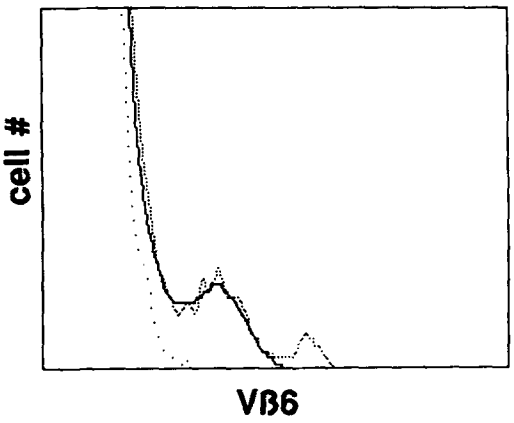

Figure 5. Negative selection is defective during GVHR. Vβ6^{hi} expression in Mls-1^{a+} CBA/J × B6 F_1 mice was determined by flow cytometry in untreated (———) and GVH-reactive (----) mice 21 d after GVHR induction by the transfer of 5 × 10^7 CBA/J (Mls-1^{a+}) lymphoid cells. Background staining (·····) represents labeling with the second antibody alone. The peaks represent TCR Vβ6$^-$, Vβ6^{lo}, and Vβ6^{hi} expression displayed on a logarithmic scale.

of A × CBA/Ca mice injected with 5 × 10^7 lymphoid cells from A strain parental donors, and B6AF$_1$ mice injected with 2 × 10^7 A strain cells or 6 × 10^7 B6 strain cells. At these cell doses, the animals were completely immunosuppressed at the time of class II labeling, between 13 and 15 d after GVHR induction. In every strain combination examined, all of the GVH-reactive mice showed greatly diminished class II staining (Fig. 6). In normal mice, there was a clear histologic demarcation between the thymic cortex and medulla, with a corresponding clear-cut difference in class II expression, with the medullary staining appearing more intense (Fig. 6 *A*). During severe GVHR, the cortex atrophies due to the cortisone-mediated disappearance of the CD4$^+$8$^+$ thymocytes, and the cortico-medullary junction disappears (1). At low magnification, class II staining in the thymic medulla was dramatically decreased, and appeared no brighter than normal cortical staining; at higher power, small areas of normal brightness could be seen interspersed with areas devoid of any detectable label (Fig. 6 *B*). Preliminary time course studies indicated that 1 wk after GVH induction, no change in class II expression was apparent; by 2 wk after cell transfer, the GVH-reactive thymus was largely devoid of class II; and after 3 wk, some sparse but bright class II staining appeared at the periphery of the thymus. This labeling probably corresponded to class II expressed on macrophages infiltrating the thymus at this stage of GVHR. Thus, both the distribution and intensity of class II expression were altered in the GVH-reactive thymus.

Discussion

In this report, the effect of GVHR-induced thymic injury on positive and negative selection was examined. We used nonirradiated F_1 hybrid recipients of parental lymphoid cells to dissect out the consequences of GVHR on thymic maturation and education of T cells. T cell maturation and positive selection appeared dramatically altered in GVH-reactive mice, as indicated by lack of upregulation of CD3 on CD4$^+$8$^-$ thymocytes and variability of up to an order of

Table 3. *Defective Negative Selection in GVH-reactive B6xCBA/J (Mls-1^{a+}) F_1 Hybrid Mice*

	Percent Vβ6^{hi}	
Group	Mean ± SD	Range
Untreated mice	0.09 ± 0.05	0.03–0.17
Day 14 GVH-reactive mice	0.04 ± 0.01	0.03–0.04
Day 21 GVH-reactive mice	0.30 ± 0.10*	0.20–0.40*
Day 28 GVH-reactive mice	0.31 ± 0.08*	0.21–0.41
Day 55 GVH-reactive mice	0.43 ± 0.14*	0.29–0.61*

The incidence of Vβ6^{hi} cells among total thymocytes is shown for untreated and GVH-reactive B6 × CBA/J (Mls-1^{a+}) F_1 hybrid mice. A representative FACS® profile of Vβ6 expression in the thymus of a day 21 GVH-reactive mouse is shown in Fig. 5. GVHR was induced by the injection of 5 × 10^7 CBA/J (Mls-1^{a+}) lymphoid cells and confirmed by suppressed PFC responses (Table 1).
* $p < 0.001$ compared with untreated mice by the student's t test.

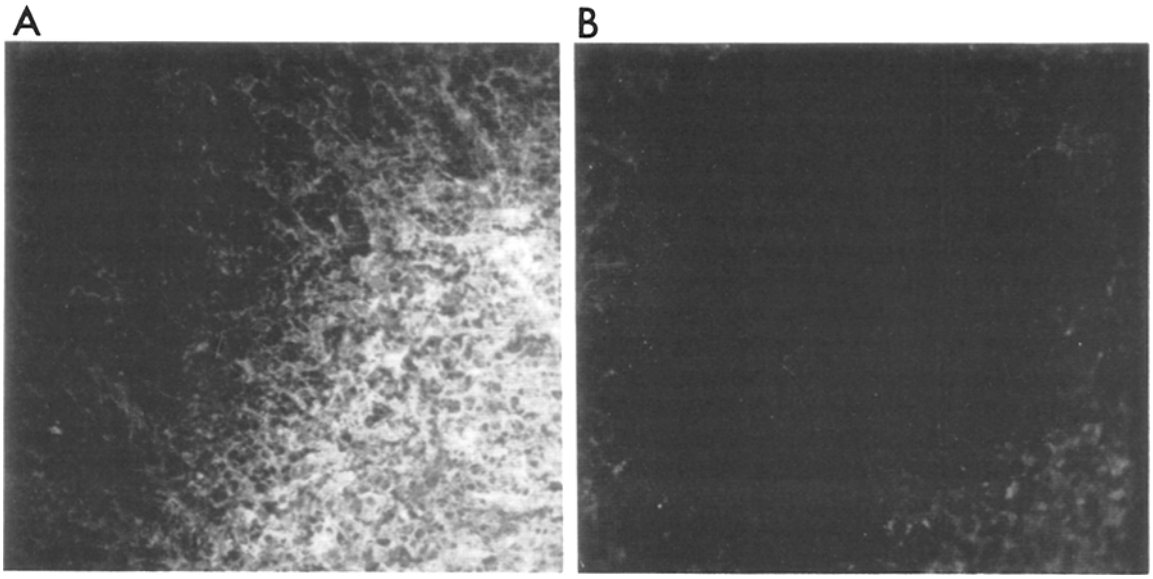

Figure 6. Immunohistochemical staining for thymic class II MHC was dramatically decreased in GVH-reactive mice. $B6AF_1$ mice were studied 13 d after the induction of GVHR by the injection of 6×10^7 B6 lymphoid cells. At high magnification (×250), a well-defined cortico-medullary junction was seen in normal mice, with intense class II expression in the medulla and dimmer staining in the cortex (*A*). In GVH-reactive mice, no thymic cortico-medullary junction was detectable; throughout the thymus of GVH-reactive mice, faint class II staining was observed interspersed with areas entirely devoid of class II (*B*).

magnitude in the incidence of specific Vβ segments on mature thymocytes. Concomitantly, the appearance of phenotypically self-reactive T cells in the GVH-damaged thymuses demonstrated impaired thymic deletion processes. The defects in selection may have resulted from the profound changes in the level and pattern of thymic MHC class II expression observed during GVHR.

We report here that thymic class II MHC expression is dramatically decreased during GVHR. Class II–bearing tissues, including lymphoid organs and epithelia such as gut, lung, liver, salivary glands, and skin, are targets of GVHR-induced lesions (1, 41–46). We have previously reported that medullary epithelial cells are injured during GVHR (6); the loss of the intense staining for class II in the thymic medulla may be due to the destruction of class II–bearing stroma. This seems especially likely since viable thymic epithelial cells would be expected to upregulate their class II expression in response to the IFN-γ production induced early during GVHR (7, 47). However, the relative contributions of epithelial cell damage versus class II MHC downregulation have not yet been examined. The net result of the decreased cell surface class II expression appeared to be a defect in class II–mediated selection events.

It is interesting that most of the abnormalities of $CD4^+$ cell maturation and function reported in class II knockout mice were also observed in GVH-reactive animals. GVH-reactive animals (4, and reported herein) and class II knockout mice (21) both demonstrated decreased expression of CD3 on class II–restricted, $CD4^+8^-$ thymocytes; cortisone sensitivity of $CD4^+8^-$ thymocytes; abnormal variability in thymocyte Vβ incidence between mice; paucity of $CD4^+$ cells in the thymus and the periphery; and peripheral immunosuppression. Thus, the class II–dependent acquisition of functions and phenotype by $CD4^+$ thymocytes appears to be as flawed during GVHR as in the complete absence of class II expression. It seems, then, that the class II molecules remaining in the thymus during GVHR were expressed on cell types inefficient at mediating selection; or that the class II antigens were themselves altered and unable to deliver complete or correct selection signals. Reduced affinity of the TCR/MHC interaction has been shown to result in inefficient positive selection and functionally defective self-restriction in the periphery in mice transgenic for allelic variants of class II (48). During GVHR, the affinity of the class II/TCR interaction may be affected by the presentation of different, GVHR-induced peptides; acute phase proteins and endogenous viral antigens induced by the high cytokine expression during GVHR (49) likely give rise to peptides not usually presented within the thymus, potentially displacing endogenous peptides that normally play a role in positive selection (50).

In transgenic mice with class II expression restricted to specific regions of the thymus (51; ΔX and ΔY mice), positive selection was defective in mice without cortical class II despite its normal medullary expression (52). We have observed defective positive selection despite low levels of class II expression; this supports the hypothesis that the class II antigens remaining in the GVHR-injured thymus are unable to deliver normal selection signals. In fact, we have previously reported that the cortico-medullary junction, normally

clearly demarcated histologically, is lost during GVHR (1); this may be in part a reflection of loss of a certain characteristic cortical cell able to mediate positive selection.

Our findings greatly resemble those reported in cyclosporine A (CsA)-treated mice. CsA treatment in conjunction with thymic irradiation also triggers GVHR-like thymic histopathology, disappearance of medullary class II expression (53), and the appearance of autoreactive (Mls-reactive) T cells (54). CsA treatment in human recipients of autologous bone marrow transplants has resulted in a syndrome dubbed "syngeneic GVHR," consisting of a GVHR-like syndrome with autoimmune features (55–57). Our present results suggest that the primary etiology of the T cell selection defect in allogeneic (as well as syngeneic) GVHR may be the abnormally reduced expression of class II MHC in the thymus.

Defective positive selection has also been described in TCR transgenic mice lacking the appropriate MHC haplotype (58). In these mice, there is an abnormal accumulation of $CD3^{hi}$ $CD4^-8^-$ cells; we have not observed an analogous phenomenon during GVHR. Thus, the changes seen in GVHR more closely resemble those that occur in the absence of class II expression than in the absence of an appropriately restricted TCR/MHC class I interaction.

Negative selection of TCR $V\beta6^+$ cells in response to $Mls\text{-}1^a$ was found to be defective in GVHR. The incidence of $V\beta6^{hi}$ thymocytes in GVH-reactive mice, however, did not reach the frequency observed in nondeleting strains; this may reflect a leakiness in the negative selection process rather than a complete failure of deletion. The latter hypothesis conforms to a view of thymic selection in which developing T cells associate with a group of stromal cells and receive most of their selection signals in a spatially restricted microenvironment: those maturing on undamaged stroma are normally tolerized and restricted, while thymocytes interacting with injured epithelia or infiltrating APCs may receive aberrant signals. This hypothesis correlates with the previously described focal nature of GVHR-induced histopathological thymic lesions (59, 60), and the patchy pattern of class II loss and stochastic disruption in $V\beta$ incidences reported here.

Defective positive and negative selection observed in mice undergoing GVHR could provide a basis for the symptoms characteristic of clinical chronic GVH disease. As in murine allogeneic GVHR, human GVHR results in thymic dysplasia (60, 61), and therefore may also induce the production of aberrantly self-restricted and self-tolerized T cells. We report here that the incidence of specific $V\beta$ segments was dramatically altered during GVHR; these disproportional decreases or increases may result in a limited repertoire, and reflect defective thymic class II–mediated signaling. In fact, selective defects in $CD4^+$ T cell self-restricted responses and IL-2 production have been detected in the periphery of GVH-reactive mice (5, 62, 63). In turn, these functional defects contribute to the immunosuppression and susceptibility to bacterial and viral pathogens that complicate GVHR. The autoimmunity that occurs clinically in spite of the profound immunosuppression may be triggered by the self-reactive T cells generated during GVHR. Although the phenotypically self-reactive cells arising during the GVHR were not examined for functional autoreactivity in this study, by analogy with CsA-induced autoreactivity, T cells educated in the GVHR-dysplastic thymus may express autoimmune activity in irradiated hosts (54).

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