Tolerogen-Producing Cells in Allogeneic Bone Marrow Chimeras Established with Spontaneously Leukemia-Prone Mice

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Using SL/Kh mice and AKR/J mice, which are animal models for spontaneous pre-B-cell leukemia and thymic lymphoma, respectively, we studied the protective influence of allogeneic bone marrow transplantation (BMT) and the induction of tolerance to Mls-1^a, a host antigen. When BM cells from allogeneic C57BL/6 mice were used to reconstitute self-tolerance SL/Kh mice, these [B6→SL] chimeric mice survived for a longer time than non-treated SL or [SL→SL] syngeneic chimeras. These findings are compatible with results previously obtained for [B6→AKR] chimeras. In [B10. D2→SL] and [B10. D2→AKR] chimeras, Vβ6$^+$ T-cells reactive to Mls-1^a were eliminated 5 weeks after BMT. On the other hand, minor graft versus host reaction (GVHR) abrogated the clonal elimination of Vβ6$^+$ T-cells in both [B10. D2→SL] and [B10. D2→AKR] chimeras. The cause of this abrogation was attributed to the early disappearance of Mls-1^a-producing host T-cells in the GVHR chimeras. The cells responsible for the Mls-1^a production were revealed to be mainly CD8$^+$ CD44$^+$ T-cells, by *in vitro* mixed lymphocyte reaction (MLR) and *in vivo* tolerance induction. The present findings indicate that host CD8$^+$ CD44$^+$ T-cells constitute the major source of Mls-1^a antigens in the [Mls-1^b→Mls-1^a] BM chimera system.

Key words allogeneic bone marrow chimera, minor lymphocyte stimulatory antigen 1^a, negative selection, leukemia-prone mice

INTRODUCTION

T-cells undergo both negative and positive selections in the thymus[1]. To elucidate the selection mechanism, especially negative selection, the minor lymphocyte stimulatory (Mls)-1^a antigen (Ag) system, an intrinsic super-antigen, has been widely employed. T-cell reactivity to Mls-1^a Ag correlates with the expression of certain T-cell Ag receptor (TCR)-Vb regions[2,3]. The expression of *Mtv-7*, an endogenous mammary tumor virus gene which determines the Mls-1^a phenotype, results in the deletion among developing thymocytes, of T-cells that express Vβ6, Vβ7, Vβ8.1, or Vβ9[4-6]. Using [Mls-1^b→Mls-1^a] bone marrow (BM) chimeras, we demonstrated that Mls-1^a-reactive T-cells are eliminated from the developing thymocyte population that is derived from the donor BM[7,8]. Furthermore, the presence of thymic stromal cells derived from the donor BM has been shown to be the primary requirement for the effective deletion of Mls-1^a-reactive thymocytes[7,9,10]. We have reported that activated CD8$^+$ and CD4$^+$ T-cells both produce Mls-1^a Ag *in vitro*, although only CD8$^+$ T-cells, not CD4$^+$ T-cells, can produce Mls-1^a Ag under non-stimulated conditions[11]. Taking into account thatclonal deletion is a major mechanism for inducing and maintaining self-tolerance, it is important to determine the source of the relevant tolerogen in this chimera system, paying particular attention to the role of T-cells. In the present study, we first analyzed the protective influence of allogeneic bone marrow transplantation (BMT) in SL/Kh mice, an animal model for pre-B-cell leukemia. We then analyzed which cell components in lethally irradiated recipient mice provides Mls-1^a Ag and ultimately contribute to clonal elimination of the Mls-1^a-reactive T-cell repertoire in the BM chimeric mice. We

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show here that the residual radio-resistant recipient T-cells are the cells responsible for this intrathymic clonal elimination.

MATERIAL AND *METHODS*

Mice

AKR/J (AKR) (H-2^k, Mls-1^a, Thy1.1) mice were obtained from the Jackson Laboratory (Bar Harbor, ME). C57BL/6 (B6) (H-2^b, Mls-1^b, Thy1.2), B10. BR/SgSnSlc (BR) (H-2^k, Mls-1^b, Thy1.2), and B10. D2 (D2) (H-2^d, Mls-1^b, Thy1.2) mice were obtained from JAPAN SLC Co. (Hamamatsu, Japan). SL/Kh (SL) (K^s, A^q, E^-, Dq) and (AKR×BR) F_1 mice were bred and maintained in our animal facility at Hokkaido University.

Bone marrow transplantation

Eight-week-old female AKR or SL mice were subjected to either 10 or 11 Gy X-ray irradiation. Twenty four hours later, these mice were treated to achieve hematopoietic and immunologic reconstitution with 2×10^7 BM cells taken from 8-week-old B6, D2 or syngeneic mice. Prior to BMT, BM cells were treated *in vitro* with anti-Thy1.2 (F7D5, Olac, Bicester, UK) monoclonal antibody (mAb) plus selected rabbit complement (C)[12]. To induce subclinical GVHR, BM cells treated with anti-Thy1.2 mAb alone were injected intravenously into the recipient mice[13–15]. As reported earlier[15], these GVHR chimeras scarcely showed overt signs of GVHR (ruffled fur, hunched back, significant loss of weight), but clonal elimination of self-reactive T-cells (i. e. against recipient's Ag) was abrogated. Chimeras prepared by injecting T-cell-depleted BM cells alone will be referred to as [donor→recipient] chimeras or control chimeras. Chimeras which were given BM cells pretreated with anti-Thy1.2 mAb alone will be referred to as GVHR [donor→recipient] chimeras. In some experiments, 2×10^6AKR T-cell subsets obtained from untreated (AKR×BR) F_1 mice were introduced intravenously into [BR→BR] syngeneic BM chimeras one week after BMT. [BR→BR] chimera mice were prepared as described above.

Cell purification

Spleen cells were passed over nylon wool columns and the purified T-cells were treated with either anti-CD4 mAb or anti-CD8 mAb and selected rabbit C. These cells were further purified by M-450 Dynabeads (Dynal Inc., Norway) coated with anti-rat immunoglobulin G (IgG) and anti-mouse IgG antibodies (Ab). The purified cell fraction showed more than 99% relevant cells. The $CD4^+$and $CD8^+$ T-cell fractions were then treated with anti-CD44 mAb and stained cells were sorted using the FACStar system as described elsewhere[16].

Flow cytometry

Three-color FACS analyses were carried out as previously described[17,18]. Thymocytes from chimeric mice were treated *in vitro* before analysis with the following primary mAbs: anti-Thy1.1 (T11D7e. Olac), anti-CD3ε(2C11), anti-Vβ6 (44-22-1) or anti-Vβ8.1, 2, 3 (F23.1). Biotinylated anti-mouse and anti-rat IgG secondary Ab (Cappel, West Chester, PA, USA) were also used, followed by TANDEM-streptavidin (Southern Biotech., Birmingham, AL, USA) to treat the thymocytes. After blockingbinding sites of these secondary Ab, phycoerythrin (PE)-anti-CD4 and fluorescein isothiocyanate (FITC)-anti-CD8 (Becton-Dickinson, Mountain View, CA) were reacted with the thymocytes. When the population of host-derived T-cells was analyzed, cells were first incubated with biotinylated anti-mouse IgG followed by PE-streptavidin. Then, anti-Thy1.1 or anti-Thy1.2 mAb and FITC-anti mouse IgG were added. Stained cells were analyzed with a FACScan system (Becton-Dickinson).

RNA preparation and PCR analysis of MTV-7

Total cellular RNA was extracted from spleen cells using the guanidinium isothiocyanate method[19]. Reverse-transcription (RT) was performed, and the Mls-1^a-specific sequence contained within the ORF of the 3'-LTR of MTV-7 cDNA was amplified[20] using the following Mls-1^a-specific primers: 5'-primer GTCAAAGAACAGGTGCAAGGAC and 3'-primer AAGGGATCGAAGCCAACGCG. Theβ-Actin cDNA was amplified for control (5-primer TGGAATCCTGTGGCATCCATGAAAC and 3'-primer

TAAAACGCAGCTCAGTAACAGTCCG).

Mixed lymphocyte reaction (MLR)

MLR was performed as described elsewhere[11,16]. BR T-cells were stimulated with AKR or (AKR×BR) F_1 T-cell subsets in the presence of mitomycin-treated BR spleen cells as antigen-presenting cells (APC). In some experiments, Vβ 8.2− T-cells of BR mice were used.

Statistical analyses

Statistical analyses were carried out using Student's t test. P values of less than 0.05 were considered significant.

RESULTS

Effect of allogeneic BMT on leukemogenesis in SL mice

The SL mouse, which develops pre- B-cell leukemia, was established in Japan[21,22]. It has been reported that allogeneic BMT prevents leukemogenesis in spontaneous and radiation-induced models[13,22,23]. Thus, we attempted to determine whether allogeneic BMT also prevents leukemogenesis in the SL mouse, a pre-B-cell leukemia model.

Fig. 1A shows that all non-treated SL mice died of leukemia within 36 weeks. No difference was observed between male and female mice. SL mice irradiated with a dose of 10 Gy and received syngeneic SL BM cells showed a survival pattern similar to non-treated SL mice (Fig. 1B). How-

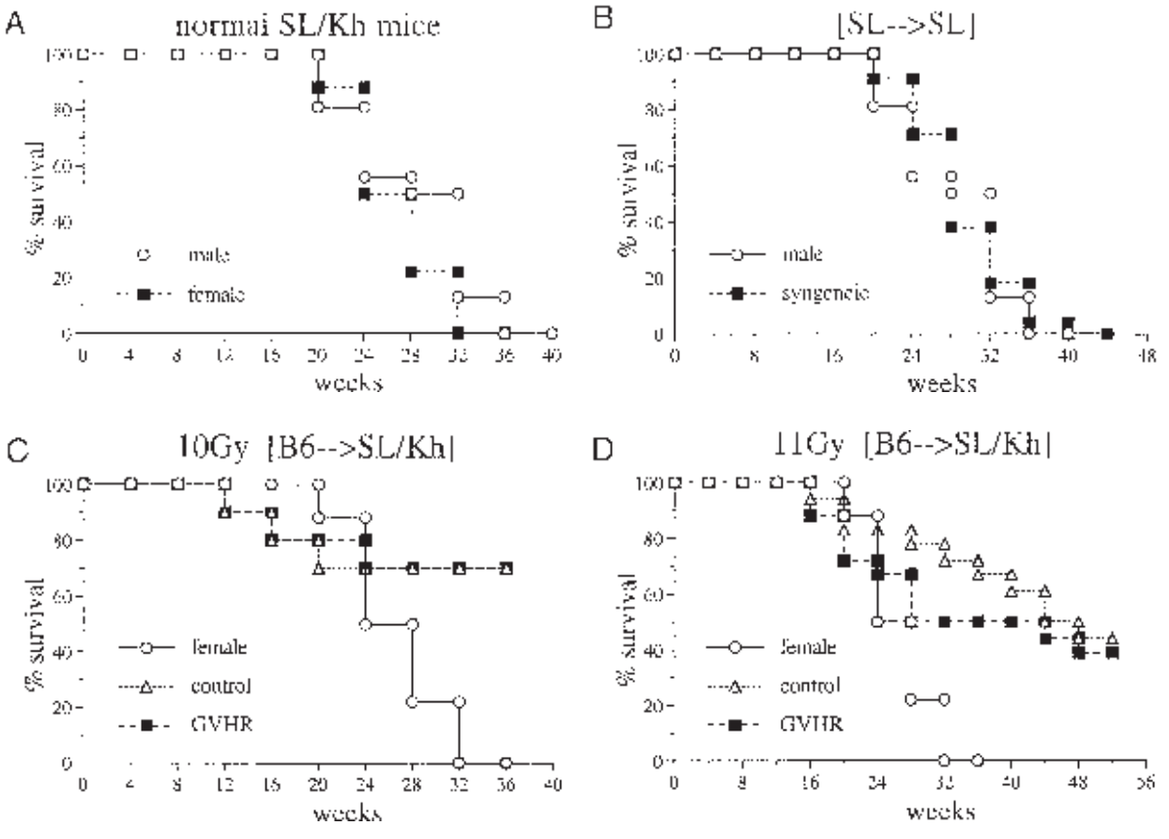

Fig. 1. Survival of [B6→SL] after BMT. SL recipient mice were lethally irradiated (B, C : 10 Gy, D : 11 Gy) and their self tolerance reconstituted with 2×10^7 BM cells. A. Non-treated male (○) and female (■) mice. B. Syngeneic [SL→SL] male (○) and female (■) chimeras. C. SL mice irradiated with a dose of 10 Gy, whose self tolerance was reconstituted with B6 BM cells pretreated with anti-Thy1.2 plus C (control△) or B6 BM cells pretreated with anti-Thy1.2 alone (GVHR■). D. SL mice irradiated with a dose of 11 Gy, whose self tolerance was reconstituted with B6 BM cells pretreated with anti-Thy1.2 plus C (control△) or B6 BM cells pretreated with anti-Thy1.2 alone (GVHR■). Survival curves of non-treated female mice (○) are also illustrated in C and D for comparison.

ever, when allogeneic (B6) BM cells were transplanted to SL mice irradiated with doses of 10 Gy or 11 Gy, approximately 70% or 50% of the mice, respectively, survived more than 36 weeks after BMT (Fig. C, D). No difference in the survival curve was detected between the [B6→SL] (control) and the GVHR [B6→SL] chimeras.

Elimination of Vβ6⁺ T-cells in the [D2→SL] and the [D2→AKR] chimeras

We reported that Vβ6⁺ T-cells reactive to Mls-1ᵃ plus MHC class II were eliminated in the thymus and the spleen of [B10. AQR→AKR] and [B10. BR→AKR] chimeras as the result of negative selection[7,25]. The elimination of Vβ6⁺ T-cells, however, was abrogated in GVHR chimeras[12,15,26]. Since the SL background partially containsAKR genes, it was assumed that SL mice expressed Mls-1ᵃ. Thus, we used RT-PCR to examine whether Mls-1ᵃ messages were present in SL cells or not. Fig. 2 shows that the SL mouse is an Mls-1ᵃ-positive strain. We next analyzed Vβ6⁺ T-cells in [D2→SL] chimeras. We also reported that MHC class II molecules, especially H-2E must be present on the surface of BM-derived cells for the elimination of Mls-1ᵃ-reactive T-cells. Thus, instead of B6 (H-2E⁻), D2 mice (H-2E⁺) were used as donors of BMT in this experiment. Table 1 shows that Vβ6⁺ T-cells are eliminated from both the thymus and the lymph nodes (LN) of [D2→SL] chimeras as well as from the thymus of [D2→AKR] chimeras. By contrast, in GVHR [D2→SL] chimeras, significant proportions of Vβ6⁺ T-cells were detected in the thymus and LN. This finding is consistent with the results obtained with the [D2→AKR] chimeras (Table 1, and Refs. 12, 25).

Perhaps as was shown in [B10. AQR→AKR] chimeras[12,15,16,25], minor GVHR eradicated the radio-resistant recipient cells that otherwise might have supplied Mls-1ᵃ molecules. Indeed, we found residual SL T-cells (20%) in the LN of [D2→SL] chimeras, but only less than 2% in GVHR [D2→SL] chimeras 5 weeks after BMT. No B-cells of the recipient type were seen in either the control or GVHR chimeras. These findings are consistent with our previous reports with AKR chimeras[11,15,25]. A variation in the proportion of Vβ8⁺ T-cells appeared to result from partial elimination of Vβ8.1⁺ T-cells that also react with Mls-1ᵃ[12]. These findings taken together demonstrate that the major population supplying Mls-1ᵃ Ag is the T lineage cells of SL recipient mice.

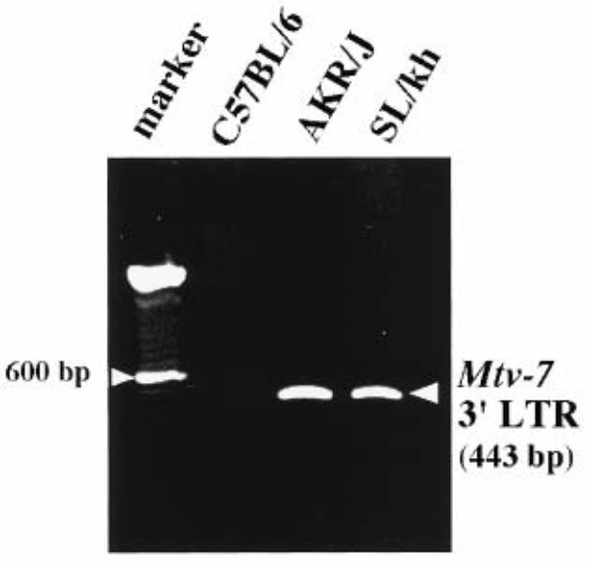

Fig. 2. Identification of Mls-1 type of SL mice. Total RNA was isolated from spleen cells and reverse transcription PCR was performed with the primers specific for the 3'-LTR of *MTV-7*. Note the *MTV-7* bands (443bp) in SL as well as in AKR/J cells.

Table 1. Proportions of Vβ6⁺ and Vβ8⁺ cells in the thymus and the LN from allogeneic chimeras

	CD4⁺8⁻			CD4⁻8⁺		
	CD3	Vb6/CD3	Vb8/CD3	CD3	Vb6/CD3	Vb8/CD3
	%	%	%	%	%	%
[D2→SL]						
thymus	93	0	33	50	1	19
LN	83	0	25	80	0	12
GVHR [D2→SL]						
thymus	98	4	36	86	10	27
LN	72	9	43	52	6	56
[D2→AKR]						
thymus	96	2	21	64	1	25
GVHR [D2→AKR]						
thymus	97	7	21	81	12	29

*Pooled cells from 2 to 3 mice per group were analyzed. Representative data from 3 independent experiments are shown.

Mls-1ᵃ producing T-cell subsets

Prior findings suggested that radio-resistant recipient T-cells produce Mls-1ᵃ. Using modified MLR[11]. We next analyzed T-cell subsets that are

responsible for the production of Mls-1[a]. Since the SL mouse is from an H-2 recombinant and their use as stimulators in MLR presented a number of complications, we used AKR or (AKR×BR) F_1 cell as stimulators. Whole T-cells or a population of T-cell depleted of Vβ8.2$^+$ cells from BR mice were cultured with syngeneic APC in the presence of the T-cell subsets from AKR mice. Table 2 shows that the proportion of Vβ6$^+$ T-cells increased significantly among blast cells of BR responders, in the presence of CD8$^+$ T-cells from AKR mice. No appreciable increase of Vβ6$^+$ T-cells was induced with AKR CD4$^+$ T-cells. Thus, the major population that produces Mls-1 Ag is the CD8$^+$ T-cell fraction. Similar results were obtained with (AKR×BR) F1 stimulators (data not shown).

Next, AKR T-cells were further fractionated and analyzed in MLR. To activate only stimulator AKR T-cells, in this particular experiment, Vβ8.2$^-$ T-cells of BR mice were used as responders. Table 2 shows that CD44$^+$ memory-type T-cells of AKR mice vigorously stimulate BR T-cells as compared to CD44$^-$ native T-cells. Furthermore, when the stimulatory cells were activated with immobilized anti-Vβ8.2 mAb, the proportions of responding Vβ6$^+$ T-cells were markedly increased. In this condition, even the CD4$^+$ CD44$^-$ stimulators generated considerable MLR. These findings are essentially compatible with our previous reports[11,16].

Table 2. Proportions of Vβ6$^+$ cells in BR blasts

Stimulator	Activation with anti-Vβ8.2	Vβ6	Vβ8.2
-a		13.5±0.2%	15.5±1.1%
CD4$^+$a	−	14.1±1.2	15.3±1.2
CD4$^+$CD44$^+$b	−	24.1±0.4**	ND
CD4$^+$CD44$^+$b	+	30.5±0.1**	ND
CD4$^+$CD44$^-$b	−	14.2±0.5	ND
CD4$^+$CD44$^-$b	+	21.2±1.1**	ND
CD8$^+$a	−	27.0±1.2*	10.5±0.7
CD8$^+$CD44$^+$b	−	34.1±0.1**	ND
CD8$^+$CD44$^+$b	+	62.0±1.2**	ND
CD8$^+$CD44$^-$b	−	22.5±1.9**	ND
CD8$^+$CD44$^-$b	+	22.6±1.5**	ND

a : BR T-cells were stimulated with T-cell subsets from AKR mice in the presence of BR APC. Each number represents the mean proportion of Vβ6$^+$/CD3$^+$ or Vβ8$^+$/CD3$^+$±SD of triplicate cultures. A representative result from three separate experiments is shown. *Significantly higher than control without stimulators ($p<0.001$).

b : Proportions of Vβ6$^+$/CD3$^+$ were calculated in the responder Vβ8.2$^-$ blast cells from BR mice after coculture with stimulator T-cell subsets from AKR mice in the presence of BR APC. **Significantly higher than control value without stimulators (12.5±0.1%) ($p<0.01$).

Clonal elimination of Vβ6$^+$ T-cells in [BR→BR] syngeneic chimeras after administration of (AKR×BR) F_1 T-cell subsets

We next intravenously administrated subsets of T-cells from (AKR×BR) F_1 mice into [BR→BR] chimeras, one week after BMT. Six weeks after administration of (AKR×BR) F_1 T-cells, proportions of Vβ6$^+$ T-cells in the thymus and LN were analyzed by flowcytometry. Table 3 shows that administration of CD8$^+$ CD44$^+$ T-cells resulted in significant decreases of Vβ6$^+$ T-cells in both the thymus and the LN of [BR→BR] miceas if CD8$^+$ CD44$^+$ T-cells were present in the [BR→BR] thymus and LN. We reported that CD8$^+$ CD44$^+$ AKR T-cells, which had been inoculated into [BR→BR] mice one week after BMT, were present in the thymus even at later stages[16].

Inoculation of CD8$^+$ CD44$^-$ T-cells of (AKR×BR) F_1 mice reduced the proportion of Vβ6$^+$ T-cells only in the LN of chimeras. Administration of CD4$^+$ T-cells subsets showed no reduction of Vβ6$^+$ T-cells at all. These findings are consistent with the results obtained with

Table 3. Proportion of Vβ6$^+$ T-cells in the thymus and the LN of [BR→BR] syngenic chimeras

T-cell subsets administered	Thymus LN			
	CD4$^+$8$^-$	CD4$^-$8$^+$	CD4$^+$	CD8$^+$
-[a]	7.8±0.5	13.1±0.8	7.5±0.3	12.3±0.4
CD4$^-$ CD44$^+$	7.8±0.2	13.3±0.3	6.9±0.3	11.5±0.4
CD4$^+$ CD44$^-$	7.5±0.1	13.5±0.1	7.5±0.4	12.5±0.5
CD8$^+$ CD44$^+$	3.0±0.3*	2.8±1.5*	2.0±0.1*	2.9±1.0*
CD8$^+$ CD44$^-$	7.3±0.1	12.0±0.1	5.0±0.1*	7.1±0.0*

(AKR×BR) F_1 T-cell subsets (2×10^6) were injected intravenously into [BR→BR] syngenic chimeras 1 week after BMT. Six weeks after reconstitution, FACS analyses were carried out.

Data show mean±SD of 3 mice per each group.

[a]No F1 T-cells were injected (control).

*Significantly lower than control value ($p<0.01$).

MLR and our previous study in which the T-cell repertoire was analyzed at later periods after BMT[16]. The present results indicate again that $CD8^+$ $CD44^+$ T-cells are the most potent Mls -1^a-producing cells.

DISCUSSION

BMT is one of the most promising therapies for many hematopoietic and immunodeficiency diseases that can not otherwise be treated effectively[27,28]. In the present study, we showed that allogeneic BMT prevented leukemogenesis in SL mice, an animal model for spontaneous pre-B-cell leukemia, as was shown in AKR/J mice, an animal model for spontaneous thymic lymphoma[13,24]. It has been reported that GVHR may exert beneficial influences on the reconstitution of recipient hematopoietic and lymphoid tissues by donor-derived cells (graft enhancement)[29,30]. In addition, GVHR may be associated with graft versus leukemia (GVL) effect[31]. However, we could not detect any appreciable influences of minor GVHR on the survival of SL chimeras in the conditions we tested. Similar survival curves were observed in the control and the GVHR [B6→SL] chimeras.

We have reported that minor GVHR resulted in the abrogation of negative selection of T-cells reactive to recipient Ag[12,15,25,26]. In the SL chimera system, we also found that minor GVHR led to the failure of clonal elimination of Mls-1^a-reactive T-cells. At first, the abrogation of the negative selection was attributed to the lack of Mls-1^a Ag-producing cells in GVHR [Mls-1^b→Mls-1^a] chimeras. However, we found recently that GVHR resulted in the failure of clonal elimination of T-cells reactive to donor Ag[32].

Thus, itmay be concluded that the GVHR also induces functional changes to the thymus. Similar observation was reported by Desbarats and Lapp[33].

We reported that the acute GVHR induced in AKR recipients shifted the T-cell responses to the Th2 dominant state[34]. This early Th2 shift appeared to be associated with the subsequent T-cell responsiveness, since T-cells recovered from acute GVHR showed the Th2 dominant state. Thus, T-cells from such chimeras prominently producedIL-4 but not IFN-γupon stimulation. In addition, these T-cells exhibited significant MLR but not cytotoxic T-lymphocyte responses to the recipient Ag (split tolerance)[35]. Although we did not analyze T-cell responsiveness in [B6→SL] chimeras, a similar functional state appeared to be generated in these GVHR chimeras., since significant proportions of Mls-1^a-reactive T-cells were detected in GVHR [D2→SL] mice but not in control [D2→SL] chimeras. We first expected that these Mls-1^a-reactive T-cells might be exerting the GVL effect. However, as described above, no difference was observed in the survival rate between [B6→SL] and GVHR [B6→SL] chimeras. It is possible that these Mls-1^a-reactive T-cells induced neither harmful GVHR responses nor beneficial GVL responses in GVHR [B6→SL] chimeras. It seems important to elucidate the basic mechanism underlying the immunological alteration induced by GVHR in further studies.

In the present study, we demonstrated that the major population of Mls-1^a-producing cells of the recipients were $CD8^+$ $CD44^+$ T-cells, although these findings were obtained with the AKR chimera system but not with the SL chimera system. These findings are essentially compatible with our previous reports[11,16,25]. In our previous studies[11,16], we demonstrated that $CD8^+$ $CD44^+$ T-cells expressed larger amounts of *MTV-7* mRNA than $CD8^+$ $CD44^-$ T-cells. Since cells of donor mice cannot produce Mls-1^a, it is clear that Mls-1^a Ag derived from radio-resistant recipient cells (mainly $CD8^+$ $CD44^+$) are transferred to and presented on the surface of donor MHC class II^+ cells in the thymus (cross-presentation). We and others have reported that these Mls-1^a Ag plus MHC class II on the surface of donor BM-derived cells (APC) eliminate the Mls-1^a-reactive T-cells between 2 and 3 weeks after BMT in the thymic medulla[7,9,10].

Although we analyzed here T-cell reactivities to a superantigen, GVHR appears to be induced by various allogeneic protein Ags including MHC Ag. It is now understood that the major GVHR is not induced by the recipient MHC Ag alone but by complexes of MHC and peptide Ag bound in the Ag-binding groove of the MHC[36,37]. These recipient peptides with a specific motif for binding to the MHC[38] appeared to be derived from cellular components of the recipient. Thus, identification of the peptide Ag involved in the rather complex GVHR is essential to explain the influence of the GVHR on the development of the recipient immune system,

especially a state tolerant to the recipient Ag. The determination of the peptide Ag also leads to elucidation of the tissue injury induced by T-cells specific for the peptide Ag.

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