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Requirement of a higher degree of chimerism for skin allograft tolerance in cyclophosphamide-induced tolerance

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Abstract By using a cyclophosphamide (CP)-induced tolerance system, we previously raised the possibility that the degree of chimerism might determine the induction of heart and skin allograft tolerance. When C3H (H-2^k; Thy1.2, Mls-1^b) mice were intravenously primed with 1×10^8 spleen cells (SCs) from H-2 matched AKR (H-2^k; Thy1.1, Mls-1^a) mice and then treated intraperitoneally with 200 mg/kg CP, the survival of AKR skin grafts was permanently prolonged in a tolerogen-specific fashion. After this treatment, a minimal degree of mixed chimerism and the clonal destruction of Mls-1^a-reactive $CD4^+V\beta6^+$ T cells in the periphery were observed. When AKR SCs and 100 mg/kg CP were used for conditioning, the survival of the AKR skin grafts was mildly prolonged. The clonal destruction of $CD4^+V\beta6^+$ T cells in the periphery was induced and a minimal degree of mixed chimerism was detectable. The degree of mixed chimerism induced with AKR SCs and 200 mg/kg CP was significantly higher than that with AKR SCs and 100 mg/kg CP during the observation. On the other hand, neither skin allograft prolongation nor permanent mixed chimerism could be induced when C3H mice were treated with AKR SCs and 50 mg/kg CP. In order to increase the degree of mixed chimerism, we injected 1×10^8 tolerant AKR SCs on day 3 into the recipient C3H mice that had been treated with AKR SCs on day 0 and with 100 mg/kg CP on day 2. The reason that we used tolerant SCs was that untreated AKR SCs caused graft-versus-host disease in most of the recipients. Tolerant AKR SCs were harvested from AKR mice that had been treated with C3H SCs and 200 mg/kg CP 2 weeks earlier, and did not contain regulatory cells. By adoptive transfer, the degree of chimerism was stably and significantly increased in all recipients, and AKR skin graft tolerance was induced in half of the recipients. T-cell-depleted bone marrow cells (BMCs) from untreated AKR mice induced skin allograft tolerance in 83% of recipients. Thus, the present study strongly confirmed the hypothesis that a higher degree of chimerism is required for the induction of skin allograft tolerance in CP-induced tolerance.

Keywords Chimerism · Clonal destruction · Adoptive transfer · Skin allograft tolerance · Cyclophosphamide

Introduction

Various protocols to induce immunological tolerance against allogeneic antigens have been reported in murine systems [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12]. The induction and persistence of tolerance in these systems have been typically shown to depend on the establishment of a mixed chimeric state in which donor-derived cells are detected to some degree in the recipients [4, 5, 8, 11, 13]. Recent reports, however, demonstrated that the presence of microchimerism does not always mean the establishment of tolerance [14, 15]. Thus, the role of mixed chimerism in transplantation tolerance seems to remain controversial.

By using a cyclophosphamide (CP)-induced tolerance system, we have recently elucidated the possible role of chimerism in skin and heart allograft tolerance [16]. When C3H (H-2^k; Thy-1.2, Mls-1^b) mice were intravenously (i.v.) primed with 1×10^8spleen cells (SCs) from H-2 matched AKR (H-2^k; Thy-1.1, Mls-1^a) mice on day 0 and then treated intraperitoneally (i.p.) with 200 mg/kg CP on day 2, survival of both AKR skin and heart grafts were permanently prolonged in a tolerogen-specific fashion. After this treatment, a minimal degree of mixed chimerism was detectable. When AKR SCs and 100 mg/kg CP were used for conditioning, AKR heart grafts were permanently accepted, but survival of the AKR skin grafts was only mildly prolonged. Mixed chimerism was detectable, but the degree of mixed chimerism induced with SCs and 100 mg/kg CP was significantly lower than that with SCs and 200 mg/kg CP during the observation. Thus, we have suggested that a higher degree of mixed chimerism is required for the induction of skin allograft tolerance.

The aim of the present study was to further investigate the role of mixed chimerism for the induction of skin allograft tolerance. To enhance the degree of mixed chimerism, injection of 1×10^8 SCs from tolerant AKR mice on day 3 was added to conditioning with AKR SCs and various doses of CP. The reason that we used tolerant SCs was that untreated AKR SCs caused graft-versus-host disease (GVHD) in most of the recipients. The additional treatment with tolerant donor SCs significantly enhanced the degree of mixed chimerism, and donor skin grafts were accepted over 100 days in half of the recipient C3H mice. When 2×10^7 T cell-depleted bone marrow cells (BMCs) from untreated AKR mice were used instead of tolerant SCs, most of the recipient C3H mice accepted donor skin grafts. These manipulations support our proposed hypothesis.

Materials and methods

Animals

Inbred female C3H/HeNSLc (C3H; H-2^k, Mls-1^b, Thy1.2), AKR/N (AKR; H-2^k, Mls-1^a, Thy1.1) and B10.BR SgSnSlc (B10.BR; H-2^k, Mls-1^b) mice were obtained from Japan SLC (Hamamatsu, Shizuoka, Japan). Recipients were used at 12–16 weeks of age.

Cell preparation

The mice were killed by decapitation. The spleens were collected and kept on ice in RPMI 1640 medium (GIBCO, Grand Island, N.Y., USA) supplemented with antibiotics (100 μg/ml penicillin G and 100 μg/ml streptomycin). The spleen was disrupted in the medium by our pressing spleen fragments between two glass slides. Cell suspensions were filtered through cotton gauze and washed three times with RPMI medium. Viable nucleated cells were counted and adjusted, usually to 2×10^8/ml.

Bone marrow cell preparation and T-cell depletion

The bone marrow in the femoral and tibial bones was flushed out with a 5-ml syringe with 26-G needle (Terumo, Tokyo, Japan). Cell suspensions were washed two times with RPMI medium. T cell-depletion was performed as described, by using anti-Thy-1.1 monoclonal antibodies (mAbs) (Meiji, Tokyo, Japan) and complement (Low-Tox-M Rabbit Complement, Cedarlane, Ontario, Canada) [17]. Viable nucleated cells were counted by a standard trypan blue dye exclusion method and adjusted to 4×10^7/ml.

Conditioning of CP-induced tolerance

An aliquot of 0.5 ml, containing 1×10^8 SCs from AKR mice, was injected into the tail vein of recipient C3H mice. Two days later, CP (Endoxan, Shionogi, Osaka, Japan) dissolved in PBS at a concentration of 20 mg/ml was injected i.p. in a dose of 50, 100 or 200 mg/kg. The day of the injection of AKR SCs is day 0 throughout this report.

Skin grafting

Using the procedure we have previously reported [17], we performed skin grafting. Briefly, a square full-thickness skin graft (1 cm^2) was prepared on the right lateral thoracic wall of the recipient mouse. The graft was fixed to the graft bed with eight interrupted sutures of 5-0 silk thread and was covered with protective tape. The first inspection was carried out on the 7^{th} day, followed by daily inspection for 3 weeks. The graft was considered as rejected at the time of complete sloughing or when a dry scar was formed. Survival was expressed as median survival time.

Flow cytometry for chimerism and T cell receptor expression

Phenotyping was performed at various times after injection of SCs. Recipients were tail-bled and white blood cells (WBCs) were prepared by hypotonic shock. Staining with both donor-specific and recipient-specific mAbs was performed on each recipient, and control mouse cells were incubated with fluorescein-isothiocyanate (FITC)-conjugated anti-Thy1.1 (PharMingen, San Diego, Calif., USA) or Thy1.2 (PharMingen) monoclonal antibody (mAb) for 30 min at 4°C and then washed twice. To block non-specific FcγR binding of labeled antibodies, we added 10 µl undiluted culture supernatant of 2.4G2 (rat anti-mouse FcγR mAb; [18]) to the first incubation.

For analysis of T cell receptor (TCR) expression, we used WBCs. WBCs for the assay of clonal destruction were labeled with FITC-conjugated anti-Vβ6 or Vβ8.1/8.2 mAb (PharMingen) and phycoerythrin (PE)-conjugated anti-CD4 mAb (PharMingen) for 30 min at 4°C.

All data were analyzed with a FACScan (Becton Dickinson, Sunnyvale, Calif., USA). We excluded dead cells by gating out low forward scatter high propidium iodide-retaining cells.

Statistics

The statistical significance of the data was determined by the Mann–Whitney U-test when the data seemed non-parametric. When the data seemed parametric, however, Student's *t*-test was used. A P value of less than 0.05 was considered to be statistically significant.

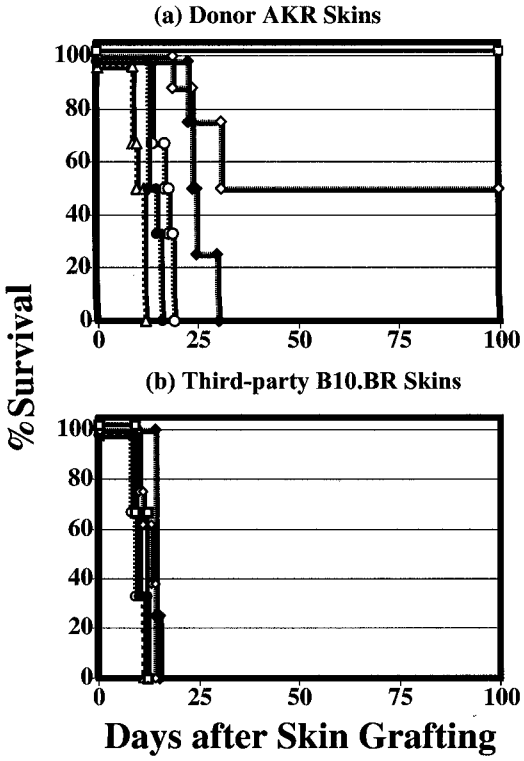

Fig. 1a, b Skin allograft survival in the recipient C3H mice treated with AKR SCs and CP or treated with SCs, CP and tolerant AKR SCs on day 3. Recipient mice were grafted with skin graft from donor AKR (**a**) or third party B10.BR (**b**) mice 4 weeks after treatment. B10.BR skin grafts were rejected within 14 days after grafting in all groups. △ untreated C3H mice ($n=6$; 10 days), ♦ C3H mice treated with SCs/100 mg/kg CP ($n=8$; 23.5 days), • C3H mice treated with SCs/50 mg/kg CP ($n=6$; 13.5 days), □ C3H mice treated with SCs/200 mg/kg CP/tolerant SCs ($n=6$; > 100 days), ◇ SCs/100 mg/kg CP/tolerant SCs ($n=8$; 65 days), ○ C3H mice treated with SCs/50 mg/kg CP/tolerant SCs ($n=6$; 16.5 days)

Results

Skin allograft tolerance in the H-2 matched AKR (H-2^k) → C3H (H-2^k) combination by use of 1×10^8 AKR SCs followed by 50, 100 or 200 mg/kg CP

When C3H (H-2^k) mice were grafted with H-2 matched AKR skin (H-2^k), allografts were rejected within 14 days (Fig. 1a; $n=5$; median 10 days). All AKR skin allografts survived for over 100 days in recipient C3H mice treated with AKR SCs followed by 200 mg/kg CP ($n=6$; median > 100 days). By contrast, AKR skin grafts were rejected within 50 days when C3H mice were treated with AKR SCs followed by 100 mg/kg ($n=8$; median 23.5 days). Survival of AKR skin grafts was hardly prolonged in recipient C3H mice treated with AKR SCs and 50 mg/kg ($n=6$; median 13.5 days). This skin allograft prolongation was tolerogen-specific because third-party skin B10.BR (H-2^k) allografts were rejected in a normal fashion (Fig. 1b). AKR heart grafts were specifically accepted in C3H mice treated with AKR SCs and 200 or 100 mg/kg CP as previously reported [16]. In C3H mice treated with AKR SCs and 50 mg/kg CP, however, AKR hearts were chronically rejected, as seen in the untreated C3H mice (data not shown).

Prolongation of skin allograft survival by additional injection with tolerant donor SCs on day 3

In our previous study [16], we showed the possibility that the degree of chimerism strongly influences skin and heart graft tolerance in the AKR into C3H combination, i.e., the requirement of higher chimerism for skin allograft tolerance. In order to confirm this, we examined whether the injection of 1×10^8 SCs on day 3 from AKR mice made tolerant of C3H antigens can induce the prolongation of donor skin graft survival in C3H mice treated with AKR SCs and 100 mg/kg CP. The tolerant

SCs were harvested from AKR mice that had been treated with 1×10^8 SCs from untreated C3H mice followed by 200 mg/kg CP 2 weeks earlier and were demonstrated to have no regulatory cells [19]. Skin grafting was performed on day 28.

As shown in Fig. 1a, AKR skin graft survival was prolonged for more than 100 days in recipient C3H ($n=8$) mice treated with AKR SCs on day 0 and 100 mg/kg CP on day 2 followed by injection of tolerant AKR SCs on day 3 (median 65; $P<0.05$ compared with group with AKR SCs and 100 mg/kg CP). By contrast, survival of AKR skin grafts was mildly prolonged in the C3H mice treated with AKR SCs, 50 mg/kg CP and tolerant AKR SCs ($n=6$; median 16.5 days). This skin graft prolongation was donor antigen-specific because third-party B10.BR (H-2^k) allografts were rejected in a normal fashion (Fig. 1b). Furthermore, a second set of skin grafts from donor AKR mice survived for more than 100 days in a tolerogen-specific fashion in the four C3H mice treated with AKR SCs, 100 mg/kg CP, and tolerant AKR SCs, and accepting AKR skin grafts, indicating the induction of skin graft tolerance (data not shown).

A chimeric analysis in WBCs of the recipient C3H mice treated with AKR SCs plus 200, 100 or 50 mg/kg CP

As we reported previously, a minimal degree of mixed chimerism was detected in the C3H mice made tolerant of AKR skin allografts [16]. A mixed chimeric state induced with AKR SCs (Thy1.1) and 200, 100 or 50 mg/kg CP was examined with FITC-conjugated anti-Thy1.1 and Thy1.2 mAbs. WBCs were obtained from the recipient C3H (Thy1.2) mice at 2, 12 and 40 weeks after tolerance induction (Table 1).

In C3H mice treated with AKR SCs and 200 mg/kg CP (group 7), 2.1–2.4% of Thy1.1$^+$ cells were clearly detectable in the recipient WBCs during observation. In contrast, a lower degree of chimerism was detected at 2 weeks (mean ± SD = 1.4 ± 0.3; $P<0.01$ compared with group 7), and seemed still detectable at 40 weeks in the C3H mice treated with AKR SCs and 100 mg/kg CP (group 5). The degree of chimerism treated with AKR SCs and 50 mg/kg CP appeared detectable at 2 weeks and ended by 12 weeks (group 3).

We further elucidate whether injection of 1×10^8 tolerant SCs induced higher level of mixed chimerism (groups 4, 6 and 8). The injection of tolerant donor SCs on day 3 increased the degree of mixed chimerism in C3H mice treated with AKR SCs and 100 or 200 mg/kg CP (groups 6, and 8) during the observation. There is statistical significance between groups 5 and 6 ($P<0.01$), and groups 7 and 8 ($P<0.01$). In C3H mice treated with AKR SCs, 50 mg/kg CP, and tolerant SCs, mixed chimerism was clearly detectable at 2 weeks but seemed to end by 12 weeks after treatments (group 4).

Table 1 Chimerism in the recipient C3H mice treated with AKR SCs, CP and tolerant AKR SCs

Group	Recipient	Treatment[a]			Code of mice	Chimeric analysis (% positive cells ± SD)					AKR skin graft survival
		SCs	CP (mg/kg)	Tolerant AKR SCs		Thy1.1			Thy1.2		
		(day 0)	(day 2)	(day 3)		2 Weeks	12 Weeks	40 Weeks	2 Weeks	12 Weeks	40 Weeks	days
1	C3H	(−)	(−)	(−)	11–16 ($n=6$)	0	0	0	22.4 ± 12.3	18.6 ± 8.2	17.3 ± 6.9	8~11
2	AKR	(−)	(−)	(−)	21–26 ($n=6$)	18.6 ± 5.5	15.7 ± 5.6	15.2 ± 4.3	0	0	0	Not tested
3	C3H	AKR	50	(−)	31–36 ($n=6$)	0.5 ± 0.2	0.1 ± 0.1	ND	21.0 ± 2.5	21.9 ± 5.1	ND	13~15
4	C3H	AKR	50	(+)	41–46 ($n=6$)	1.6 ± 0.6[b]	0.2 ± 0.1	0.2 ± 0.1	31.8 ± 6.2	21.7 ± 5.0	23.1 ± 6.4	13~18
5	C3H	AKR	100	(−)	51–56 ($n=8$)	1.4 ± 0.3[b]	1.0 ± 0.3[b]	0.5 ± 0.2	22.9 ± 4.9	15.6 ± 4.9	15.1 ± 5.3	22~29
6	C3H	AKR	100	(+)	61–68 ($n=8$)	2.7 ± 0.3[c]	2.1 ± 1.0[c]	1.4 ± 0.7[c]	25.7 ± 3.5	18.1 ± 2.5	20.4 ± 3.3	18~ > 100
					61	2.7	1.2	0.6	26.8	21.9	17.3	18
					62	3.4	2.0	2.0	28.3	20.4	20.2	> 100
					63	2.4	1.3	0.9	21.2	16.3	20.8	30
					64	2.6	1.2	0.7	30.9	16.0	27.7	23
					65	2.9	3.4	2.1	28.0	17.8	18.8	> 100
					66	2.6	3.9	2.3	21.5	14.2	19.5	> 100
					67	2.3	1.9	1.8	22.7	18.7	17.6	> 100
					68	2.5	1.1	0.9	25.9	19.4	21.1	30
7	C3H	AKR	200	(−)	71–76 ($n=6$)	2.1 ± 0.3[c]	2.4 ± 0.6[c]	2.4 ± 0.8[c]	20.3 ± 5.6	15.0 ± 5.2	14.4 ± 6.1	> 100×6
8	C3H	AKR	200	(+)	81–86 ($n=6$)	4.3 ± 1.2[d]	4.9 ± 2.4[d]	4.6 ± 0.7[d]	21.2 ± 2.8	13.4 ± 2.7	12.2 ± 5.0	> 100×6

[a] The recipient mice were primed intravenously with 1×10^8 viable AKR SCs on day 0 and then given 50, 100, or 200 mg/kg CP on day 2. Some groups were injected with 1×10^8 SCs on day 3 from AKR mice made tolerant of C3H antigens

[b] $P<0.01$ compared with group 3

[c] $P<0.05$ compared with group 5

[d] $P<0.05$ compared with group 7

Strong correlations between the degree of mixed chimerism and AKR skin graft prolongation

We further analyzed the relationship between the degree of mixed chimerism and AKR skin graft prolongation in C3H mice treated with AKR SCs, 100 mg/kg CP, and tolerant AKR SCs (group 6; Table 1). Four of eight recipient C3H mice showing over 1.8% Thy1.1$^+$ T cells accepted donor AKR skin grafts for more than 100 days. On the other hand, the other recipients showing fewer than 0.9% Thy1.1$^+$ T cells rejected AKR skins within 30 days post-grafting.

Skin allograft survival and chimeric analysis in the recipients treated with AKR SCs, CP and untreated AKR SCs

To examine whether the untreated SCs were sufficient to induce longer graft prolongation and higher level of mixed chimerism, on day 3 we injected 1×10^8 SCs from untreated AKR mice into the recipients treated with SCs on day 0 and various doses of CP on day 2. The results of skin graft survival and degree of chimerism are shown in Table 2. In recipients treated with AKR SCs, 200 mg/kg CP, and untreated AKR SCs (group 8; $n=6$), all died within 49 days. All recipients treated with AKR SCs, 200 mg/kg CP, and untreated AKR SCs showed splenomegaly and wasting syndrome (loss of hair, rough skin, body weight loss, diarrhea), indicating that all recipients died of GVHD. On the other hand, skin graft prolongation was not observed in five of six recipients treated with AKR SCs, 100 mg/kg CP, and untreated AKR SCs (group 6; median 18.5 days), compared with recipients treated with AKR SCs and 100 mg/kg CP (MST ± SD in group 5 vs 6:24.8 ± 3.3 days vs 30.7 ± 34.4 days; $P>0.05$). Four of six recipients seemed to have died of GVHD. Although all recipients could not be tail-bled, the injection of untreated AKR SCs on day 3 could not induce higher level of chimerism. In the recipients treated with AKR SCs, 50 mg/kg CP, and untreated AKR SCs ($n=6$), neither skin graft prolongation nor higher level of chimerism was observed (group 4).

Analysis of clonal destructions in WBCs of the recipient C3H mice treated with AKR SCs plus 200, 100 or 50 mg/kg CP

Analysis of clonal destruction was performed in the donor-reactive T cells of C3H (Mls-1^b; Vβ6$^+$) mice treated with AKR (Mls-1^a; Vβ6$^-$) SCs and 200, 100 or 50 mg/kg CP. As reported previously [20, 21], the induction mechanism of CP-induced tolerance is the clonal destruction of antigen-stimulated and proliferating T cells by the anti-mitotic drug CP. We examined the expression of Mls-1^a-reactive CD4$^+$Vβ6$^+$ in the C3H mice treated with AKR SCs and various doses of CP. The WBCs from the recipient C3H mice were stained with FITC-conjugated anti-Vβ6 mAb and PE-conjugated anti-CD4 mAb (Table 3).

In WBCs of untreated C3H mice, CD4$^+$Vβ6$^+$ T cells were detected (group 1), whereas they were hardly detected in WBCs of the untreated AKR mice (group 2). In all C3H mice treated with AKR SCs and 200 mg/kg CP (group 7) or AKR SCs, 200 mg/kg CP and tolerant SCs (group 8), Vβ6$^+$CD4$^+$ T cells were significantly reduced at both 5 and 15 weeks, compared with untreated C3H mice. Similar results were obtained in the WBCs of C3H mice treated with AKR SCs and 100 mg/kg CP (group 5) or AKR SCs, 100 mg/kg CP and tolerant SCs (group 6). The disappearance of T cells in WBCs was specific for Vβ6$^+$ T cells because the percentage of the Vβ8.1/8.2$^+$ T cells was not significantly altered. This disappearance of Vβ6$^+$CD4$^+$ T cells has been explained as being a result of destruction of antigen-stimulated and proliferating T cells [20, 21]. On the other hand, Vβ6$^+$CD4$^+$ T cells were reduced to 0.3 or 0.4% at 5 weeks but were significantly restored to 0.9 or 0.8% at 15 weeks in WBCs of C3H mice treated with AKR SCs and 50 mg/kg CP (group 3) or AKR SCs, 50 mg/kg CP and tolerant SCs (group 4), respectively.

Prolongation of skin allograft survival by additional injection with T-cell-depleted donor bone marrow cells on day 3

In order to evaluate further the efficacy of donor cell injection to prolong skin graft prolongation, on day 3 we administered 2×10^7 T cell-depleted bone marrow cells (BMCs) from untreated AKR mice instead of tolerant SCs. Survival of AKR skin grafts was markedly prolonged in C3H mice treated with AKR SCs, 100 mg/kg CP, and T cell-depleted AKR BMCs, and grafts were accepted for more than 100 days in 83% of these recipients (Fig. 2a; $n=6$; median >100 days). This skin allograft prolongation was tolerogen-specific because third party B10.BR (H-2^k) skin allografts were rejected in a normal fashion (Fig. 2b). When 2×10^7 non T cell-depleted BMCs from untreated AKR mice were injected on day 3, survival of donor skin grafts was not prolonged at all (Fig. 2a).

Furthermore, transplantation tolerance was induced by conditioning with AKR SCs, 100 mg/kg CP and donor BMCs because second-set skin grafts were accepted for over 100 days in a donor-specific manner (data not shown).

Table 2 Chimerism in the recipient C3H mice treated with AKR SCs, CP and untreated AKR SCs (*ND*not done—results could not be obtained because recipients became too weak to be tail-bled)

Group	Recipient	Treatment[a]			Code of mice	Chimeric analysis (% positive cells ± SD)				AKR skin graft survival[b] (days)	Recipient survival[c] (on day X)
		SCs (day 0)	CP (mg/kg) (day 2)	Untreated AKR SCs (day 3)		Thy1.1		Thy1.2			
						2 Weeks	12 Weeks	2 Weeks	12 Weeks		
1	C3H	(−)	(−)	(−)	101–106 ($n=6$)	0	0	20.1 ± 11.8	22.3 ± 9.4	7∼11	> 100×6
2	AKR	(−)	(−)	(−)	111–116 ($n=6$)	18.9 ± 4.5	17.2 ± 8.2	0	0	Not tested	> 100×6
3	C3H	AKR	50	(−)	121–126 ($n=6$)	0.7 ± 0.4	0.2 ± 0.1	23.3 ± 4.5	22.5 ± 3.9	13∼15	> 100×6
4	C3H	AKR	50	(+)	131–136 ($n=6$)	1.3 ± 0.8	0.4 ± 0.0[d]	12.4 ± 3.3	20.2 ± 2.0	13∼18	> 100×6
5	C3H	AKR	100	(−)	141–146 ($n=6$)	1.6 ± 0.4[d]	0.9 ± 0.2[d]	20.6 ± 4.4	18.7 ± 4.8	22∼29	> 100×6
6	C3H	AKR	100	(+)	151–156 ($n=6$)	1.5 ± 0.4[e]	0.6 ± 0.2[e]	17.6 ± 4.8[e]	19.6 ± 0.0[e]	14∼ > 100	38∼ > 100
					151	1.4	ND	22.3	ND	23[f]	51[f]
					152	ND	ND	ND	ND	14[f]	42[f]
					153	1.1	0.46	12.7	19.6	23	> 100
					154	1.9	0.69	17.8	19.6	> 100	> 100
					155	ND	ND	ND	ND	10[f]	38[f]
					156	ND	ND	ND	ND	14[f]	42[f]
7	C3H	AKR	200	(−)	161–166 ($n=6$)	2.4 ± 0.7[g]	2.9 ± 0.9[g]	19.6 ± 3.2	16.4 ± 4.1	> 100×6	> 100×6
8	C3H	AKR	200	(+)	171–176 ($n=6$)	2.2 ± 1.1	ND	1.1 ± 0.5	ND	7∼21	35∼49
					171	3.5	ND	1.4	ND	21[f]	49[f]
					172	1.2	ND	0.5	ND	18[f]	46[f]
					173	ND	ND	ND	ND	15[f]	43[f]
					174	ND	ND	ND	ND	7[f]	35[f]
					175	2.0	ND	1.5	ND	21[f]	49[f]
					176	ND	ND	ND	ND	7[f]	35[f]

[a]The recipient mice were primed intravenously with 1×10^8 viable AKR SCs on day 0 and then given 50, 100, or 200 mg/kg CP on day 2. Some groups were injected with 1×10^8 SCs on day 3 from untreated AKR mice
[b]Skin grafting was performed on day 28
[c]Recipient mice died on day X
[d]$P < 0.01$ compared with group 3
[e]Mean ± SD was calculated in three recipients at 2 weeks and two recipients at 12 weeks. No statistical significance compared with group 5
[f]Skin graft still survived when the recipient died
[g]$P < 0.05$ compared with group 5

Table 3 Clonal destruction of $CD4^+V\beta6^+$ T cells in the WBCs of the C3H mice treated with AKR SCs, CP and tolerant AKR SCs

Group	Recipient	Treatment[a]			Analysis of TCR expression (% positive cells ± SD)					
		SCs	CP	Tolerant AKR SCs	$CD4^+V\beta6^+$		$CD4^+V\beta8.1/8.2^+$		$CD4^+V\beta6^+/CD4^+$	
		(day 0)	(day 2)	(day 3)	5 Weeks	15 Weeks	5 Weeks	15 Weeks	5 Weeks	15 Weeks
1	C3H	(−)	(−)	(−)	3.6±2.2	3.0±1.2	4.8±3.0	3.5±1.1	14.3±3.1	12.9±0.6
2	AKR	(−)	(−)	(−)	0	0	2.8±1.5	1.9±0.2	0	0
3	C3H	AKR	50	(−)	0.4±0.2[b,c]	0.9±0.3[b,c]	3.2±0.5	2.9±0.4	3.9±0.7[b]	3.8±0.9[b]
4	C3H	AKR	50	(+)	0.3±0.1[b,c]	0.8±0.3[b,c]	1.1±0.4	2.1±1.1	5.0±2.2[b]	6.3±1.8[b]
5	C3H	AKR	100	(−)	0.4±0.1[b]	0.1±0.0[b]	3.8±1.3	2.4±0.6	1.6±0.5[b]	0.3±0.1[b]
6	C3H	AKR	100	(+)	0.3±0.1[b]	0.2±0.2[b]	2.0±0.8	3.0±0.7	2.0±0.4[b]	1.0±0.5[b]
7	C3H	AKR	200	(−)	0.2±0.1[b]	0.1±0.0[b]	3.8±1.6	3.8±1.5	0.8±0.3[b]	0.2±0.1[b]
8	C3H	AKR	200	(+)	0.2±0.1[b]	0.1±0.1[b]	2.0±0.6	2.0±0.6	1.6±0.4[b]	0.7±0.2[b]

[a]The recipient mice were primed intravenously with 1×10^8 viable AKR SCs on day 0 and then given 50, 100, or 200 mg/kg CP on day 2. Some groups were injected with 1×10^8 SCs on day 3 from AKR mice made tolerant of C3H antigens

[b]$P<0.01$ compared with group 1

[c]$P<0.01$ compared with data at 5 weeks

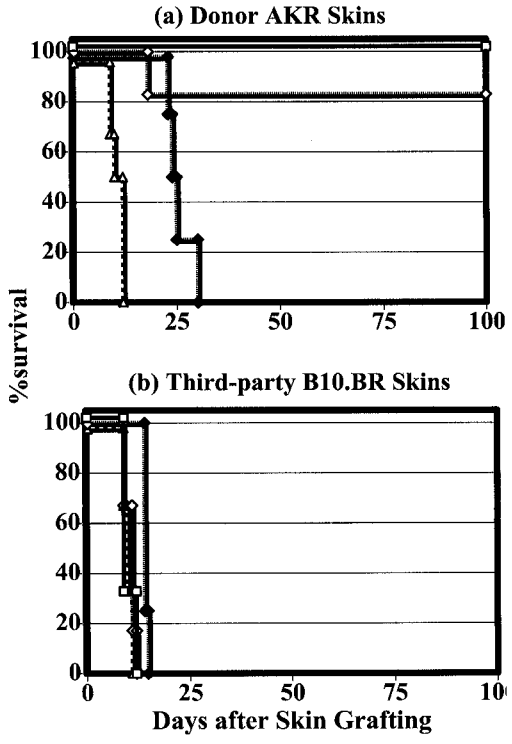

Fig. 2a, b Skin allograft survival in recipient C3H mice treated with AKR SCs, CP, and donor AKR T cell-depleted BMCs. Recipient mice were grafted with skin graft from donor AKR (**a**) or third party B10.BR (**b**) mice 4 weeks after treatments. B10.BR skin grafts were rejected within 14 days after grafting in all groups. △ untreated C3H mice ($n=6$; 10 days), □ C3H mice treated with SCs/200 mg/kg CP ($n=6$; >100 days), ◇ C3H mice treated with SCs/100 mg/kg CP/T cell-depleted BMCs ($n=6$; >100 days), ♦ C3H mice treated with SCs/100 mg/kg CP/non-T cell-depleted BMCs ($n=6$; 26 days), • C3H mice treated with SCs/100 mg/kg CP ($n=8$; 23.5 days)

Discussion

By using the H-2 matched murine combination and mAbs against T cell markers (Thy1.1 and Thy1.2) and TCR Vβ6, we demonstrated the sequential mechanisms of CP-induced tolerance [19]. These mechanisms are: (1) clonal destruction of antigen-stimulated and then proliferating T cells by CP; (2) establishment of stable mixed chimerism; (3) intrathymic clonal deletion; (4) regulatory mechanisms at the late stage of tolerance. These four conditions are achieved by SCs and 200 mg/kg CP alone without any other supportive treatments in most H-2 matched mouse combinations.

By analyzing recipient C3H mice treated with AKR SCs and 200 or 100 mg/kg CP, we previously reported the possibility that the degree of chimerism may determine the induction of heart and skin allograft tolerance [16]. In order to increase the degree of chimerism, we set up adoptive transfer experiments with the injection of 1×10^8 tolerant SCs or T cell-depleted BMCs on day 3 from donor mice. By adoptive transfer, the degree of chimerism was stably and significantly increased (groups 6 and 8; Table 1), and skin allograft tolerance could be induced in 50% and 83% of recipients treated with AKR SCs and 100 mg/CP followed by tolerant SCs and donor BMCs, respectively. In recipients treated with AKR SCs and 50 mg/kg CP, adoptive transfer with tolerant donor SCs could not induce stable mixed chimerism (group 4; Table 1) or prolongation of donor skin graft survival (Fig. 1a), or could not maintain the clonal destruction (group 4; Table 3). These results support the hypothesis described above.

As a source to enhance mixed chimerism (Table 1), tolerant AKR SCs were used for the following reasons. Firstly, mixed chimerism in AKR and C3H combination is detectable by means of T cell alleles, i.e., Thy1.1 vs. Thy1.2. SCs are more suitable than BMCs to detect

chimerism objectively because SCs include T cells much more than BMCs. By using tolerant SCs, we could show the strong correlation between the level of mixed chimerism and skin allograft tolerance (group 6; Table 1). Secondly, the injection of untreated AKR SCs on day 3 caused GVHD in recipients treated with SCs and 200 or 100 mg/kg CP (Table 2). Furthermore, injection of untreated AKR SCs on day 3 induced neither skin graft prolongation nor stable mixed chimerism. Thirdly, tolerant SCs from C3H mice that had been treated with AKR SCs and 200 mg/kg CP 2 weeks earlier had no regulatory cells [17, 19].

In the following experiment (Fig. 2), we selected donor BMCs for the source of adoptive transfer. When T cell-depleted BMCs were used, skin graft prolongation was enhanced, but the degree of mixed chimerism (T cell level) was not (data not shown). This discrepancy may be explained the following way. In order for T cell-depleted BMCs to be detected, the injected BMCs have to migrate into recipient thymus and differentiate, and are finally supplied as T cells in the periphery. In CP-induced tolerance systems, however, a minimal degree of mixed thymic chimerism is detectable [17, 19, 20], suggesting that CP has a minimal effect on ablating the thymus. Thus, T cell chimerism, which is the only method to estimate chimerism in AKR into C3H combination, may not be correlated with WBC chimerism.

Non-T-cell-depleted BMCs were not effective in the enhancement of graft prolongation, and we could not show any reason why this was so, although T cells included in unmodified BMCs may be activated and change the very fine balance between graft-versus-host reaction and host-versus-graft reaction [22, 23, 24]. On the other hand, donor-specific transfusion has been known to induce transplantation tolerance or graft prolongation [25]. Although injection of AKR SCs or BMCs alone could not enhance graft prolongation in C3H mice in our previous studies ([17, 19]; Y. Tomita, unpublished data), skin graft survival might have been enhanced by the injection of tolerant SCs or T cell-depleted BMCs on day 3 in the present study.

The induction of allogeneic mixed chimerism as an approach to transplantation tolerance has been reviewed and discussed [4, 5, 11, 13, 16, 17, 19, 26, 27]. Taniguchi et al. [28] hypothesized the presence of the threshold of mixed chimerism for the induction of skin allograft tolerance. By using only I-E antigen-disparate and Ly-5 congenic murine combination and mAbs against anti-Ly-5.1 and Ly-5.2 mAb and I-E-reactive Vβ11 mAb, they measured the survival of donor skin grafts, peripheral chimerism, and the peripheral deletion of Vβ11$^+$ T cells. They clearly indicated a strong correlation among skin graft prolongation, the degree of mixed chimerism, and the deletion of Vβ11$^+$ T cells. However, their study has the following weak points. Firstly, I-E antigens alone do not act as transplantation antigens [29]. Secondly, they showed the data of chimerism and deletion of Vβ11$^+$ T cells only once. They did not observe whether low degree of chimerism was stable, increasing or reducing. Recent reports (Tomita et al., Transplantation Biology Research Center of Massachusetts General Hospital) indicated the importance of stable chimerism and T cell-chimerism in the maintenance of long-term skin allograft tolerance in irradiation-induced tolerance [30]. In some recipients showing an eventual decrease in mixed chimerism, donor skin grafts were rejected, or I-E-reactive Vβ11$^+$ T cells were not deleted in the periphery (Y. Tomita, unpublished data). Prigozhina et al. showed that lower levels of mixed chimerism cannot induce long-term skin graft prolongation and that addition of T cell-depleted donor BMCs resulted in skin allograft tolerance [31]. Thus, our previous and present studies confirm the importance of stable chimerism, the presence of the threshold of mixed chimerism to induce allograft tolerance and the requirement of a higher degree of mixed chimerism to induce skin graft tolerance than heart graft tolerance [16].

Clonal deletion as a mechanism does not occur in the recipient thymus, and thus it takes some time for donor-reactive T cells to be deleted clonally after SC–CP treatment [19]. These results suggest that T cells generated in the recipient thymus by the establishment of thymic chimerism may become the effectors to reject subsequent skin allografts. Thymic T cells actually break down the tolerance induction to MHC alloantigens in a non-myeloablative regimen conditioned with anti-CD4 and CD8 mAb and 3-Gy whole-body irradiation without thymic irradiation [30]. By contrast, Zamoyska et al. [32] reported that tolerance is dominant to H-2 matched alloantigens. They showed that recipient nude mice accepted both donor skin grafts when nude B6 (H-2^b) mice were grafted with two thymuses from Balb/b (H-2^b) and C3H.SW (H-2^b) mice. Thus, this CP-induced tolerance system may induce and maintain skin allograft tolerance to H-2 matched alloantigens without thymic ablation.

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