Induction of Peripheral Tolerance to Class I Major Histocompatibility Complex (MHC) Alloantigens in Adult Mice: Transfused Class I MHC-Incompatible Splenocytes Veto Clonal Responses of Antigen-Reactive Lyt-2$^+$ T Cells

By Klaus Heeg and Hermann Wagner

From the Institute of Medical Microbiology and Hygiene, Technical University of Munich, D-8000 Munich, Federal Republic of Germany

Summary

The efficacy and the mode of action of pretransplant transfusion with class I major histocompatibility complex (MHC)-disparate splenocytes in establishing a state of peripheral tolerance in adult mice is analyzed. Adult mice injected intravenously with a critical number of $\sim 5 \times 10^7$ allogenic splenocytes accept skin grafts and develop chimerism in the peripheral lymphatic tissues, but not in thymus and bone marrow. In parallel, a split tolerance evolves: the frequency of class I MHC-reactive Lyt-2$^+$ cytotoxic T lymphocyte precursor (CTL-p)- and interleukin 2 (IL-2)-producing T cells falls off in the peripheral lymphoid tissue, but remains unaltered intrathymically. In particular, high affinity CTL-p become clonally undetectable. In vivo generation of tolerant cells is cyclosporin A resistant, but dependent on recipient L3T4$^+$ T cells. Loss of Lyt-2$^+$ CTL-p- and IL-2-producing T cell precursors is not due to active suppression, but is caused by clonal anergy. Donor-derived chimeric cells positively selected 7 d after intravenous transfusion exhibit in vitro the hallmarks of veto cells, i.e., paralyze CTL-p reactive to donor-type class I MHC alloantigens. We conclude that the peripheral (split) tolerance induced in vivo by pretransplant transfusion operates because donor-type cells develop in vivo efficiently into "veto cells," which in turn induce a state of clonal anergy within antigen-reactive Lyt-2$^+$ T lymphocytes.

A prime aim in transplantation immunology is to define gentle methods able to convert immune reactivity to transplantation antigens into a state of immune unresponsiveness. This conversion is successful in neonates: introduction of foreign antigens into a developing immune system prevents the system from responding further on to these antigens (1, 2). Recent evidence indicates that both in natural tolerance and in experimentally induced neonatal unresponsiveness maturing antigen-reactive thymocytes become either clonally deleted (3–8) or at least clonally silenced (9–11).

Induction of unresponsiveness in a mature peripheral T cell pool meets difficulties: receptor occupancy by antigens primarily induces sensitization rather than tolerization (12–14). Therefore, most strategies attempt first to reduce the pool of immunocompetent peripheral T lymphocytes, and thereafter to induce unresponsiveness by exposing the regenerating (neonatal) immune system to antigen. In the first step, rather invasive techniques are used, such as whole body (15, 16) or total lymphoid irradiation (17), systemic application of antiproliferative drugs (18, 19), or a combination of either of these methods.

Evaluation of pretransplant transfusion effects have indicated that intravenous confrontation with allogeneic cells may induce specific immunosuppression rather than sensitization (20–28). Independently, Miller, Bevan, and associates (29–36) pioneered the veto cell concept. Accordingly, unlinked to the specificity of their own antigen receptor, veto cells paralyze in vitro the response of T cells reacting to antigens displayed by the veto cells (29–36). Interestingly, upon intravenous transfusion of allogeneic lymphocytes (23–28) or even MHC-transfected recipient cells (37), recipient mice develop a state of specific unresponsiveness as if the transfused lymphocytes were veto cells.

Since intravenous cell transfusion represents a gentle method, and, in addition, bears the promise to induce unresponsiveness in peripheral mature T cells, we have analyzed the efficacy of this approach in vitro and in vivo. The main findings described here are that: (*a*) injected mice develop a long lasting chimerism that is paralleled by an unresponsiveness to skin

[1] *Abbreviations used in this paper:* CsA, cyclosporin A; CTL-p, CTL precursor; f, frequency; IL-2 TL-p, IL-2-producing T lymphocyte precursor; LD, limiting dilution; 95% CL, 95% confidence limit; *p*, probability of single hit kinetics.

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allografts; (*b*) injected mice display a state of split tolerance (i.e., peripheral but not intrathymic CTL precursors (CTL-p)[1] bearing high affinity tolerogen-reactive TCRs are clonally silenced); (*c*) L3T4$^+$ recipient T cells are necessary for tolerance induction; and (*d*) donor-derived lymphocytes isolated from chimeric recipients display in vitro effective veto functions.

Materials and Methods

Mice. Breeding pairs of C57Bl/6 (B6) ($H2K^bIA^bIE^bD^b$), B6.C.$H2^{bm1}$ (bm1) ($H2K^{bm1}IA^bIE^bD^b$), B6.C.$H2^{bm12}$ (bm12) ($H2K^bIA^{bm12}IE^bD^b$) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. B10.BR ($H2K^kIA^kIE^kD^k$) and B10.A ($H2K^kIA^kIE^kD^d$) mice were purchased from OLAC, Blackthorn, UK. CB17 ($H2K^dIA^dIE^dD^d$) mice were bred in the animal facility of the Ulm University. The mice were used at an age of 8–12 wk.

Reagents. Human rIL-2 was a kind gift from Eurocetus, Amsterdam, The Netherlands (batch no. LP-370B). Con A was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Cyclosporine A (CsA) was generously provided by Dr. Borel, Sandoz AG, Basel, Switzerland. CsA (1 mg) was dissolved in 100 μl C_2H_5OH and 20 μl Tween 80 (Sigma Chemical Co., Munich, FRG). A stock solution (1 mg/ml) was prepared by adding 880 μl PBS. Mice received 20 mg/kg body weight daily intraperitoneally. Selected rabbit low-tox complement was purchased from Cederlane, Hornby, Canada. Rat IgG was purchased from Sigma Chemical Co. PE-coupled streptavidin was from Becton Dickinson & Co., Heidelberg, FRG.

mAbs. FITC-conjugated anti-Lyt-2 antibodies and biotin-coupled anti-L3T4 and anti-Thy-1.2 antibodies were obtained from Becton Dickinson & Co., and used for cell sorter analyses. Hybridomas GK-1.5 (anti-L3T4, a kind gift from Dr. Fitch, University of Chicago), 53-6.72 (anti-Lyt-2; American Type Culture Collection, Rockville, MD), HO-13-4 (anti-Thy-1.2; American Type Culture Collection), and 34-4-20S (anti-$H2D^d$; American Type Culture Collection) were grown as ascites. mAbs were purified from ascites fluid using standard methods (38). Anti-D^d antibodies were coupled with FITC to a FITC/protein ratio of 2 using the method of Goding (39).

Cell Staining and Cytofluorometry. For analyses, 2–5 × 10^5 cells were incubated with the respective mAb for 30 min at 4°C. Thereafter, the cells were washed twice with PBS, and, when necessary, a second incubation with PE-coupled streptavidin was performed. Subsequently, the cells were fixed with paraformaldehyde. 1–3 × 10^4 cells were analyzed with an Epics V cell sorter (Coulter Immunology, Hialeah, FL). Cells were gated on forward angle light scatter, and the green (FITC) and red (PE) fluorescence was recorded. For cell sorting, 10^7 cells were stained and sorted according their fluorescence intensity. The purity of the sorted population exceeded 98% when reanalyzed.

Complement Lysis. Splenocytes (10^7/ml) were incubated for 30 min on ice with the respective antibody. After washing twice with PBS, the cells were incubated at 37°C with a 1:10 dilution of rabbit low-tox complement (Cederlane) for 30 min. This procedure was repeated twice. Cell sorter analyses revealed that the efficacy of complement lysis was >98%.

Cell Preparation. Thymi and spleens were removed aseptically and then teased. The cell suspension was freed from erythrocytes by a brief incubation in NH_4Cl. Spleen cells were always passed over a nylon wool column.

Limiting Dilution Cultures. Limiting dilution (LD) cultures were set up as detailed elsewhere (40). Briefly, replicates (n = 16) of graded numbers of responder cells were cultured together with 3 × 10^5 irradiated (12 Gy) stimulator cells in 200 μl of medium. Culture medium (Click/RPMI; Biochrom, Berlin, FRG) was supplemented with 10% heat-inactivated FCS (Biochrom), 10 mM Hepes buffer, 2 mM glutamine, 5 × 10^{-5} M mercaptoethanol, 1 μg/ml indomethacin, and antibiotics. Note that LD cultures for the estimation of CTL-p frequencies were supplemented with 20 U/ml rIL-2, while in LD cultures for the determination of IL-2 producer T cells, 10% WEHI-3 and 10% P388-D1 supernatant (41) was added.

Assay for IL-2 Activity and Cytotoxicity. IL-2 activity in the supernatant of LD cultures was assayed after restimulation with 2 × 10^5 irradiated (100 Gy) (41) stimulator cells using a colorimetric assay (41, 42). The ^{51}Cr release assay was performed as described (40).

Statistical Analyses. In LD analyses, all cultures generating cytotoxicity or containing IL-2 exceeding the mean values plus three times the SD of cultures containing only stimulator cells were considered positive. Calculation of frequencies (f), 95% confidence limits of the frequencies (95% CL), and probabilities of single hit kinetics (p) were performed as described (40).

Skin Grafting. Mice were anesthetized with pentobarbital (0.6 mg/10 g mouse weight). Tail skin grafts (5 × 5 mm) were then transplanted onto the left chest wall according to the method of Brent et al. (43). Skin grafts were scored for viability three times a week.

Results

Transfusion with MHC Class I–Disparate Splenocytes Alters T Cell Reactivity. Upon intravenous injection of spleen cells from bm1 mice into adult B6 recipients, the in vivo and in vitro reactivity of recipient mice changes dramatically. The majority of B6 recipients receiving 5 × 10^7 allogeneic bm1 spleen cells tolerated bm1 skin grafts for >4 wk. As detailed in Fig. 1 *A*, the cell number injected was critical for the induction of unresponsiveness in vivo. In vitro, the frequency of anti-bm1-reactive CTL-p in the recipient spleen dropped from 1:513 (control) to 1:10,500 within 8 d (Fig. 1 *B*). This frequency reduction compares well with the loss of antigen-reactive CTL-p observed in neonatally induced tolerance to class I MHC antigens (9–11). Reduction of anti-bm1-reactive CTL-p occurred rapidly within 24 h, peaked at day 10–20, lasted for at least 4 wk (Fig. 1 *C*), and was antigen specific, since the frequency of third party–reactive CTL-p remained unaltered (data not shown). Fig. 1 *D* further details that the selective reduction of anti-bm1-reactive T cells was not confined to CTL-p, but also included anti-bm-1-reactive IL-2-producing T cells. Using cell sorter–purified Lyt-2$^+$ T cells from recipient mice, we found that it is this subset that developed specific unresponsiveness (vide infra). Essentially similar results were obtained in the B10.A → B10.BR combination (H-$2D^d$ difference).

Not Thymic, but Peripheral CTL-p Become Affected. Next, the frequency of anti-bm1-reactive purified thymic Lyt-2$^+$ L3T4$^-$ T cells was compared with that of peripheral T cells 7 d after intravenous injection of 5 × 10^7 bm1 spleen cells into B6 recipients. While in peripheral T cells, the frequency of anti-bm1-reactive CTL-p was reduced to ~18% of the con-

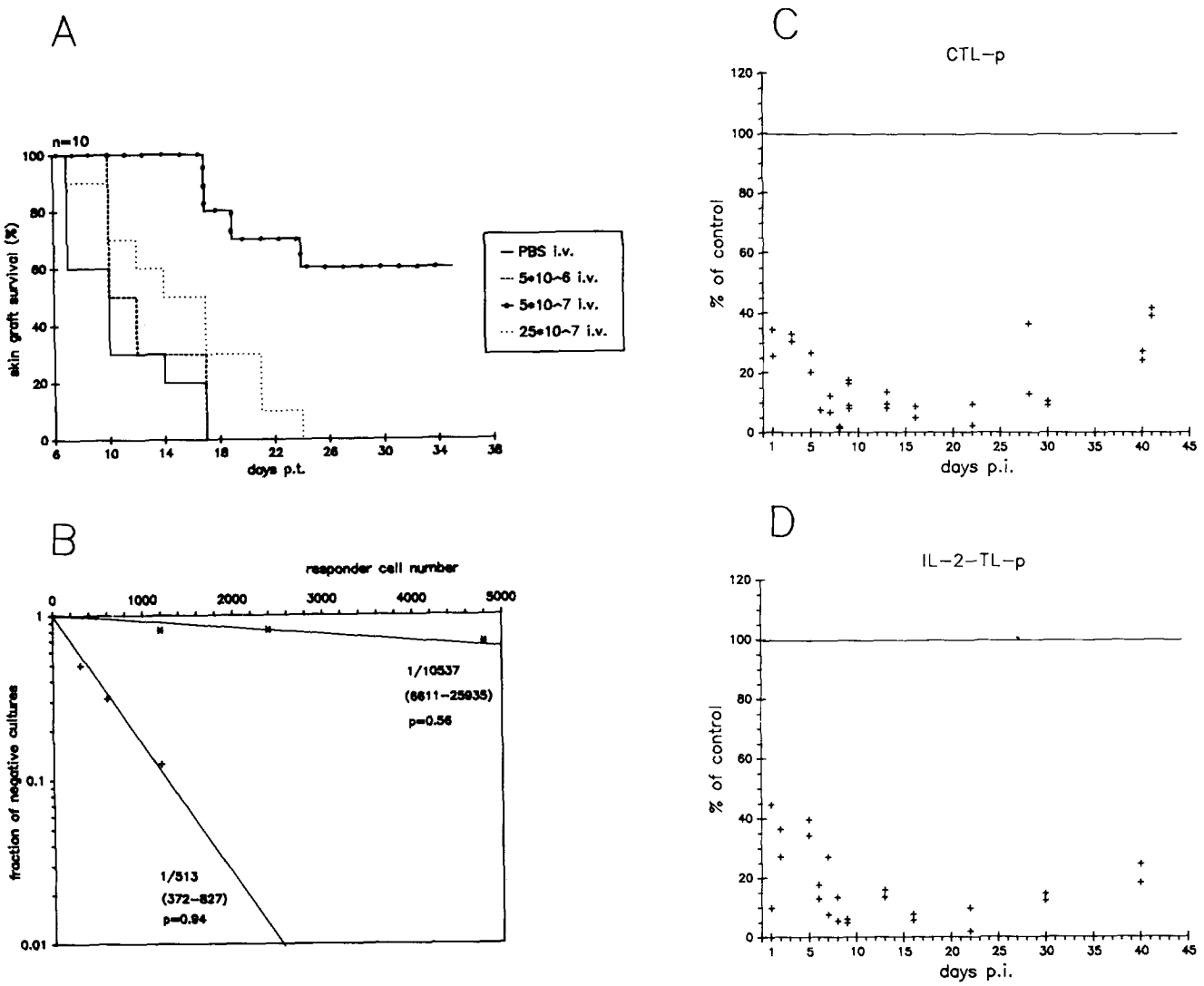


Figure 1. (*A*) Skin graft survival after injection of allogeneic spleen cells. B6 mice (n = 10 per group) were injected intravenously with the indicated numbers of allogeneic (bm1) spleen cells. 1 d later, the mice were grafted with bm1 tail skin grafts. Starting at day 7, the grafts were inspected three times a week. (*B*) Limiting dilution analysis of splenocytes from tolerant mice. B6 mice were injected intravenously with 5 × 10^7 bm1 spleen cells. 8 d later, nylon wool–nonadherent splenic cells were prepared and incubated with bm1 stimulator cells under limiting dilution conditions. After 7 d, cells of individual cultures were tested for lytic activity against bm1 blast target cells. The fraction of negative replicate cultures is plotted against the number of responder cells seeded. $1/n$ = frequency, (n-n) = 95% confidence limit of the frequency, p = probability of single hit kinetics). (*C* and *D*) Kinetics of frequency reduction. B6 mice were injected with 5 × 10^7 bm1 spleen cells. After different time points, the frequency of bm1-reactive CTL-p (*C*) or IL-2 TL-p (*D*) of splenic T cells was assessed in LD analyses. The frequency reduction is displayed as a fraction of the frequency of normal control mice.

trol (Fig. 2 *A*), the number of thymic CTL-p remained unaffected (Fig. 2 *B*).

We also analyzed whether the few remaining anti-bm1-reactive CTL-p in the peripheral T cell pool of tolerant B6 mice could be scored as high affinity or low affinity CTL-p. To this, LD cultures were set up and clonally developing CTL colonies (probability for clonality >80%) were assayed for lytic activity towards bm1 targets in the presence or absence of blocking anti-Lyt-2 mAb (44). As detailed in Fig. 3 *A*, 7 of 30 (23%) colonies from splenic T cells of normal B6 mice were able to lyse bm1 targets in the presence of Lyt-2 mAb, and thus have to be scored as T cells bearing high affinity TCRs (44). On the other hand, no high affinity anti-bm1-reactive CTL-p were found in the spleen of tolerant B6 mice. (Fig. 3 *B*).

In Vivo Tolerance Induction of Lyt-2$^+$ T Cells Is L3T4$^+$ T Cell Dependent, but CsA Resistant. In vivo injection of L3T4-, Lyt-2-, or even Thy-1.2-depleted splenic bm1 cells into B6 recipients was almost equally effective in causing a selective loss of anti-bm1-reactive CTL-p (Fig. 4). On the other hand, recipient L3T4$^+$ T cells were necessary for in vivo induction of tolerance. Thus, B6 mice injected intraperitoneally with 2 mg of anti-L3T4 mAb to deplete their L3T4$^+$ T cells (45–47) retained their ability to reject bm1 skin grafts

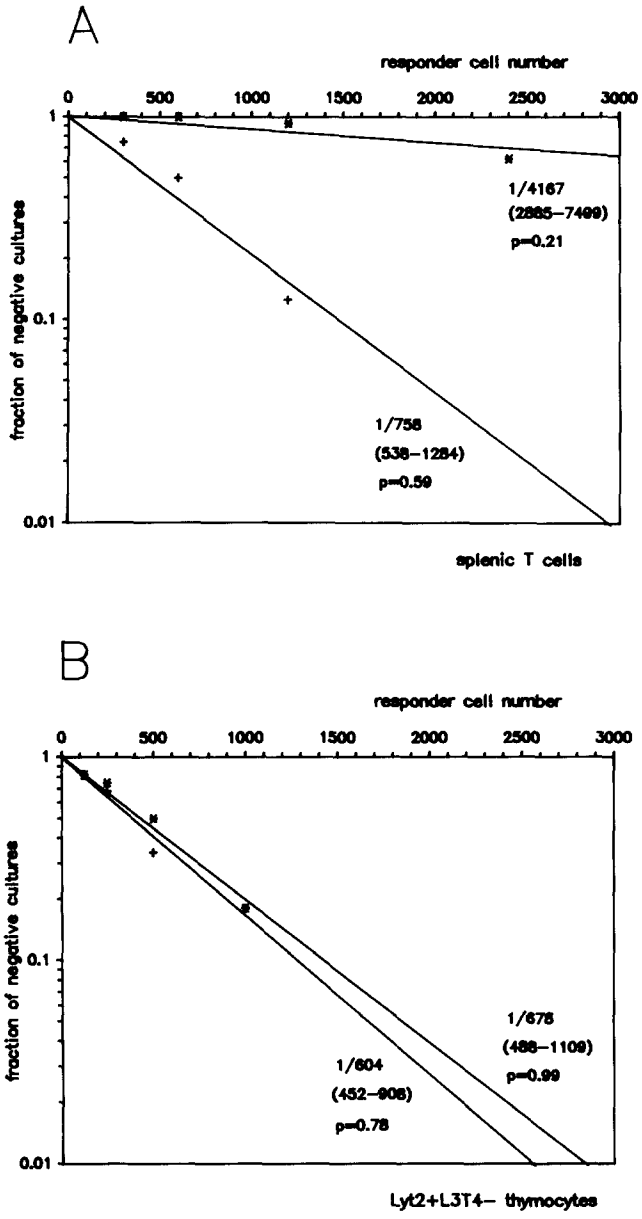


Figure 2. Frequency analysis of splenic T and mature thymocytes in tolerant mice. Splenic T cells (*A*) and cell sorter-purified Lyt-2$^+$L3T4$^-$ mature thymic T cells (*B*) from normal (+) and tolerized (*) (7 d after intravenous injection of bm1 spleen cells) B6 mice were stimulated under LD conditions with bm1 stimulator cells. After 7 d, the LD cultures were tested for lytic activity against bm1 blast target cells. The fraction of negative cultures is plotted against the number of responder cells seeded.

(Fig. 5), and the frequency of anti-bm1-reactive CTL-p was altered only marginally (Table 1). These results cannot be explained by the assumption that donor L3T4$^+$ T cells in the inoculum were critical for tolerance induction, since L3T4$^+$ T cell-depleted splenocytes are effective in inducing CTL-p reduction (Fig. 4). Thus, these results imply that recipient L3T4$^+$ T cells are essential for tolerance induction.

CsA inhibits not only lymphokine transcription and secretion in T cells (48), but also TCR-mediated signals downstream from IL-2R expression during primary activation of resting T cells (40, 49, 50). Since induction of tolerance is antigen specific at the clonal level, and thus likely to be channeled via the TCR/T3 complex, we anticipated that CsA would interfere with tolerance induction in the model system studied here. However, the results given in Table 2 clearly demonstrate that the mechanism of tolerance induction by transfusion with class I MHC-incompatible spleen cells in adult mice is CsA resistant.

Both Donor and Recipient T Cells Are Clonally Silenced, and Donor Lymphocytes Veto the Reactivity towards Their Own Class I MHC Antigens. Using an FITC-labeled H-2D^d mAb, we analyzed the state of chimerism in the B10.A → B10.BR mouse combination. While in bone marrow or thymus no donor cells were found (Table 3), significant numbers (3–9%) of D^d-positive B10.A donor cells were detected for up to 20 d in the spleen of recipient B10.BR mice (Table 3, Fig. 6). About 50–60% of these donor cells expressed the Thy-1.2 T cell marker (Table 3).

Upon depletion, by cell sorting, of donor cells from the spleen of tolerant mice, the recipient B10.BR T cells remained unresponsive towards donor-type stimulator cells (Table 4). Thus, unresponsiveness of recipient T cells is not due to active suppression by donor T cells, but represents a state of unresponsiveness due to either functional or clonal deletion. This type of result also extends to donor-derived B10.A lymphocytes from tolerized mice. As detailed in Table 5, B10.A cells positively selected from chimeric (tolerant) mice contained ~10-fold reduced frequencies of antirecipient (D^k)-reactive CTL-p.

Even though donor lymphocytes were found to be tolerant, i.e., clonally depleted for antirecipient (anti-D^k)-reactive CTL-p, the very same cells efficiently suppressed the primary activation of CTL-p with reactivity to donor class I MHC antigens; i.e., expressed veto cell function. For example, when B10.A (donor) cells that were positively selected from spleens of B10.BR (recipient) mice 7 d after intravenous injection of B10.A spleen cells were subsequently mixed at a ratio of 1:1 with normal B10.BR splenic responder cells, and thereafter cocultured at limiting dilution with 3×10^5 irradiated B10.A stimulator cells, a biphasic LD curve is obtained. At high cell input (>1,000 veto cells), the frequency of anti-B10.A-reactive CTL-p is effectively reduced (Fig. 7 *A*; Table 6, biphasic curve II), while at low cell numbers (<1,000 cells), the frequency remained unaltered (Fig. 7 *A*, biphasic curve I). Thus, as few as 1,000 veto cells are sufficient to block in LD cultures the primary activation of naive B10.BR CTL-p reactive to MHC antigens also expressed on veto cells. This suppression was antigen specific, since the reactivity to third-party antigens was not affected (Fig. 7 *B*; Table 6). Donor-derived B10.A cells from chimeric mice appear to be enriched for veto function, because B10.A cells from normal mice failed to suppress anti-B10.A responses in vitro (Table 6).

Discussion

The results described here will be discussed in the context of pretransplant transfusion effects (20–28), the veto cell

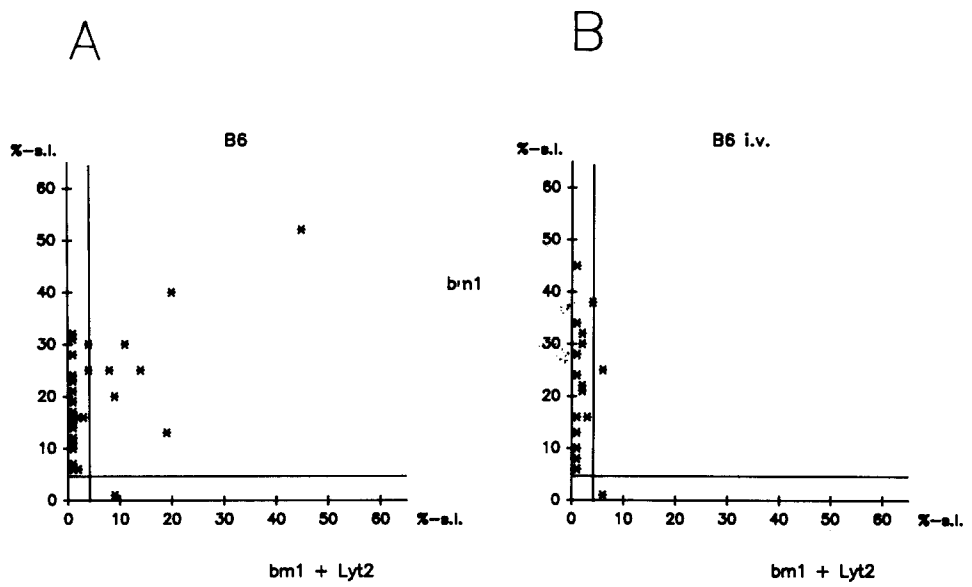


Figure 3. High affinity CTL become tolerized. A LD analysis of control (*A*) and tolerized (day 7) (*B*) splenic T cells was set up. After 7 d, the LD cultures were split and tested against bm1 blast target cells in the presence or absence of blocking anti-Lyt-2 antibodies. The lytic reactivity of individual colonies displaying a high probability (>80%) of clonality is plotted. The lines represent the statistical cut-off points for lysis.

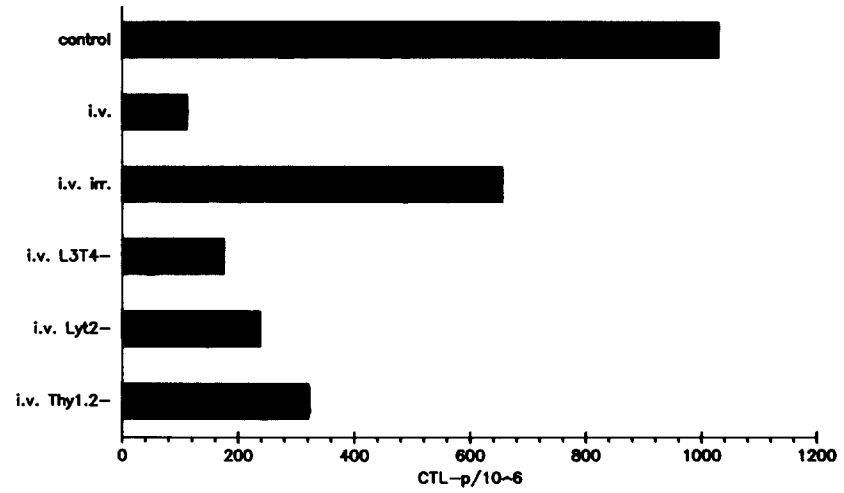


Figure 4. L3T4$^-$, Lyt-2$^-$, and Thy-1.2$^-$ splenic bm1 cells induce selective reduction of CTL-p. B6 mice were injected with 5 × 10^7 bm1 splenocytes or splenocytes depleted for Thy-1.2$^+$, Lyt-2$^+$, or L3T4$^+$ cells, or irradiated bm1 splenocytes. After 7 d, the number of bm1-reactive CTL-p was enumerated.

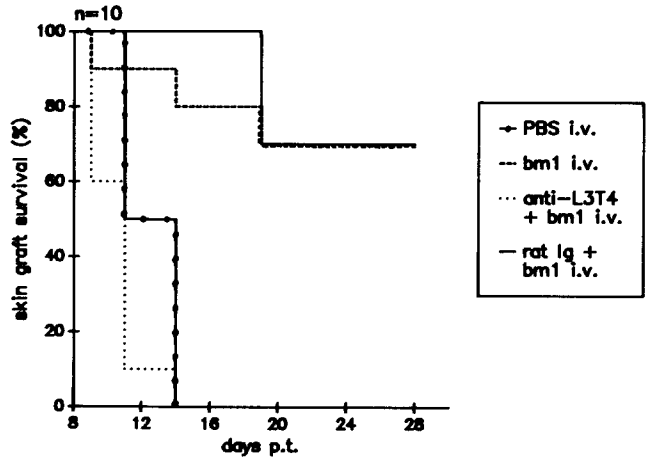


Figure 5. L3T4$^+$ T cell depletion prevents tolerance induction. B6 mice were first injected intraperitoneally with 2 mg anti-L3T4 antibodies or 2 mg rat Ig. 2 d later, the mice received intravenously 5 × 10^7 bm1 spleen cells and were subsequently grafted with bm1 skin. Anti-L3T4 and rat Ig injection was continued twice a week. Starting at day 7, grafts were inspected three times a week.

phenomenon first described by Miller and associates (29–36), and the concept of peripheral tolerance as opposed to tolerance induced by intrathymic clonal deletion of antigen-reactive T lymphocytes (1–11). We show here that after intravenous injection of a critical number of allogeneic class I MHC-incompatible donor lymphocytes, recipient mice accept donor type skin grafts for >30 d. This state of unresponsiveness is paralleled by a state of chimerism in peripheral lymphatic tissues, but not in bone marrow or thymus. This split chimerism correlates with a state of split tolerance. Thus, the frequency of anti-donor-reactive CTL-p as well as IL-2-producing T cells falls off dramatically in the peripheral lymphatic organs, reaching, after 4–5 d, 5–10% of control values. Yet, in the thymus, the frequency of donor-reactive CTL-p remains unaltered. Both anti-donor-reactive recipient CTL-p as well as anti-recipient-reactive donor CTL-p are clonally silenced. Interestingly, it is the subset of CTL-p bearing high affinity TCRs that becomes functionally deleted. In vitro, donor-type lymphocytes, positively selected from tolerant chimeric mice, bear the hallmarks of veto cells, in that they

Table 1. *L3T4⁺ T Cell Depletion Prevents the Induction of Tolerance to Class I MHC Alloantigens*

Anti-L3T4 injection	Rat Ig injection	Anti-bm1 tolerization	CTL-p 1/f	CTL-p 95% CL	CTL-p *p*
No	No	No	681	429–1,103	0.55
No	No	Yes	12,884	7,780–37,464	0.83
Yes	No	No	488	352–795	0.99
Yes	No	Yes	1,641	1,182–2,681	0.98
No	Yes	No	381	275–681	0.95
No	Yes	Yes	9,045	5,561–24,224	0.40

B6 mice were injected intraperitoneally with 2 mg anti-L3T4 antibodies, 2 mg rat Ig, or PBS twice a week. As indicated, some mice were further injected intravenously with 5×10^7 bm1 spleen cells. The frequency of bm1-reactive CTL-p of splenic T cells was determined 7 d later.

Table 2. *Effect of CsA during the In Vivo Induction of Tolerance*

CsA	Percent of control frequency: B10.A (tolerogen)	Percent of control frequency: bm12 (third party)
–	16	87
–	23	109
+	7	85
+	20	97

B10.BR mice were injected intravenously with 5×10^7 B10.A splenocytes. Some mice ($n = 2$) received daily 20 mg/kg body weight CsA intraperitoneally. After 7 d, the number of B10.A (tolerogen)- or bm12 (third-party)-reactive CTL-p was determined. The percentages of the frequencies of control animals ($n = 3$) are given.

Table 3. *Donor Cell Chimerism in Tolerant Mice*

Tissue	Percent staining cells: Control mice	Percent staining cells: Intravenously injected mice
Spleen	0.5	9.2 (Thy-1.2⁺, 65.3%)
Thymus	0.4	0.4
Mesent. lymph node	0.2	8.4 (Thy-1.2⁺, 46.9%)
Bone marrow	0.4	0.6

B10.BR mice were injected with B10.A spleen cells. After 7 d, the splenocytes, thymocytes, lymph node cells, and bone marrow cells were prepared and stained with a monoclonal FITC-conjugated anti-D^d antibody and biotin-coupled anti-Thy-1.2 antibodies. After further incubation with streptavidin-PE, the cells were analyzed for red and green fluorescence.

Table 4. *Frequency Analysis of Donor Cell-Depleted Host Lymphocytes*

Intravenous tolerization (day 0)	Donor cell depletion (day 7)	B10.A (tolerogen) 1/F	B10.A (tolerogen) 95% CL	B10.A (tolerogen) *p*	bm12 (third party) 1/F	bm12 (third party) 95% CL	bm12 (third party) *p*
No	No	2,979	2,085–4,982	0.91	1,327	965–2,098	0.99
Yes	No	>74,435			1,289	888–2,345	0.64
Yes	Yes	36,381	17,017–74,421	0.94	765	505–1,582	0.15

B10.BR mice were injected with B10.A spleen cells. After 7 d, splenocytes were depleted by cell sorting of donor-derived cells, and, subsequently, the frequency of CTL-p was determined with reactivity to either the tolerogen (B10.A cells) or third-party cells (bm12).

specifically block primary activation of CTL-p reactive towards the veto cells' own class I MHC alloantigens. Since in vivo the donor (veto) cells do not affect the repertoire of maturing thymocytes, we conclude that transfusion of adult mice with class I MHC-incompatible lymphocytes leads to a state of peripheral (split) tolerance and thus allows chimerism to be maintained. Why do donor and recipient T cells not reject each other? Peripheral tolerance appears not to be due

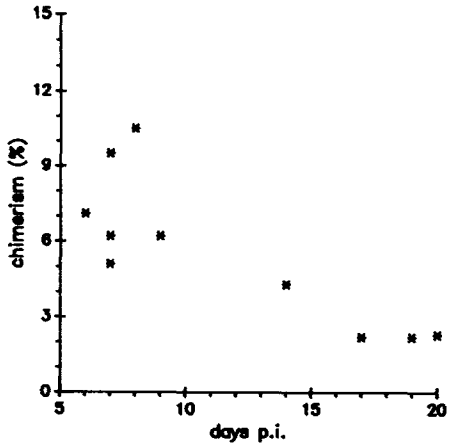
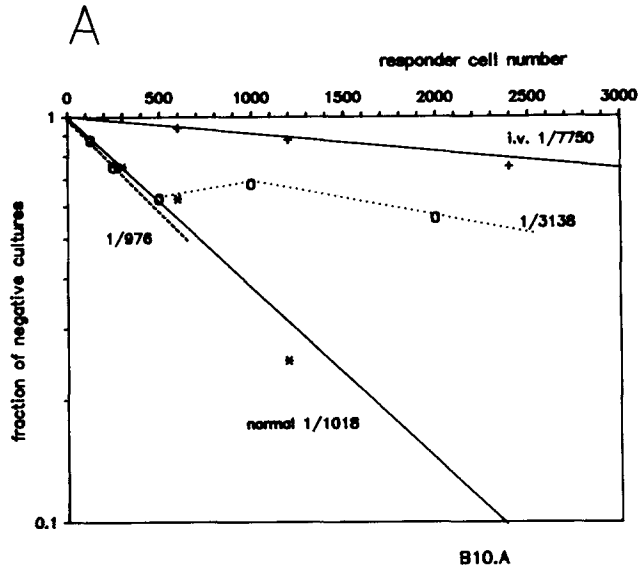


Figure 6. Donor cell chimerism in tolerized mice. B10.BR mice were injected with 5 × 10^7 B10.A splenocytes. After different time points, spleen cells from tolerized mice were stained with an FITC-coupled anti-D^d antibody and analyzed with an Epics V cell sorter. Staining of control B10.BR spleen cells was <0.5%.

Table 5. *Frequency Analysis of Donor-Derived Lymphocytes from Chimeric (Tolerant) Mice*

Responder cells	Stimulator cells	CTL-p 1/F	CTL-p 95% CL	CTL-p *p*
B10.A (control)	B10.BR	762	545–1,265	0.94
Donor-derived B10.A	B10.BR	8,843	5,454–18,688	0.99

B10.BR mice were tolerized by intravenous injèction of B10.A spleen cells. After 7 d, donor-derived cells were positively selected by cell sorting, and subsequently, the frequency of antirecipient (B10.BR)-reactive CTL-p was enumerated and compared with that of B10.A cells of control mice.



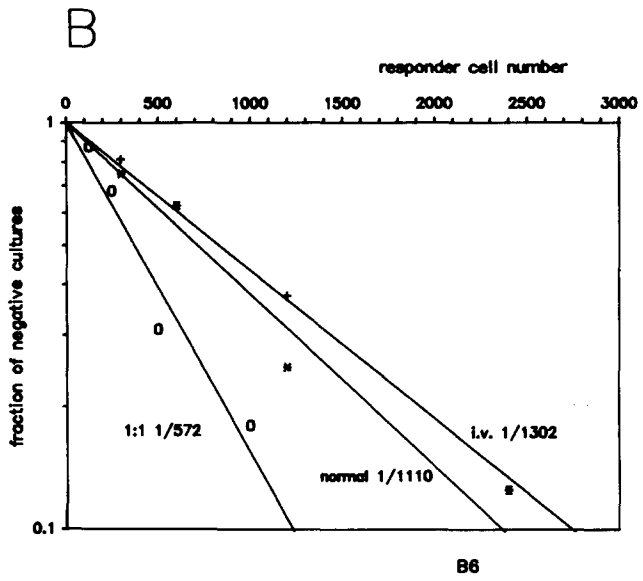


Figure 7. Donor-derived cells specifically suppress antidonor responses. B10.BR mice were injected intravenously with B10.A spleen cells. After 7 d, the frequency of splenic T cells from control and tolerized mice against the tolerogen (B10.A) and third-party MHC antigens (B6) was tested. A fraction of tolerized spleen cells was further stained with anti D^d antibodies, and donor-derived cells were positively selected with a cell sorter. These cells were mixed 1:1 with splenic T cells from normal nontolerized B10.BR mice and, subsequently, a LD analysis was performed. (*A*) (*) Control mouse, f = 1:1,018 (1:711–1,796), p = 0.87; (+) tolerized mouse f = 1:7,750 (1:5,128–1:15,846) p = 0.99; (O) 1:1 mixture of donor-derived cells and control B10.BR cells. (----) Linear part I of the biphasic LD analysis; frequency estimate, 1:976; (·····) linear part II of the biphasic LD analysis; frequency estimate, 1:3,138. (*B*) (*) Control mouse, f = 1:1,110 (1:815–1:1,739), p = 0.97; (+) tolerized mouse f = 1:1,302 (1:950–1:2,071), p = 0.99; (O) 1:1 mixture f = 1:572 (1:416–1:915), p = 0.96.

to active suppression, but due to clonal paralysis of antigen-reactive Lyt-2^+ CTL-p- and IL-2-producing Lyt-2^+ T cells. Since donor lymphocytes exhibit clonal anergy to recipient class I MHC antigens, yet express in vitro remarkably efficient veto functions, we believe that the reciprocal peripheral tolerance in the adult chimeric mice is caused, and maintained, by veto activity of donor and recipient cells.

Deletion of recipient L3T4$^+$ T cells before pretransplant transfusion abrogates the ability of the recipient to be tolerized (Fig. 5; Table 1). Although establishment of tolerance to class I MHC antigens by pretransplant transfusion meets difficulties if the transfused allogeneic lymphocytes in addition are class II MHC incompatible (26, 27, unpublished data), a problem that is overcome by systemic application of anti-L3T4 mAb (27), our data, in addition, imply that recipient L3T4$^+$ cells are essential for the in vivo development of veto

Table 6. *Donor (B10.A)-Derived Cells, but Not Normal B10.A Cells, Specifically Suppress Anti-B10.A Responses In Vitro*

Responder cells	B10.A (tolerogen)			bm12 (third party)		
	1/F	95% CL	*p*	1/F	95% CL	*p*
B10.BR	1,682	1,174–2,966	0.94	948	687–1,529	0.59
B10.BR + B10.A (1:1)	1,833	1,327–2,963	0.38	362	254–575	0.79
B10.BR + donor-derived B10.A cells (1:1)						
biphasic part I	1,619			899	619–1,639	0.85
biphasic part II	6,953					
B10.BR tolerized	7,662	4,946–17,000	0.97	704	508–1,149	0.79

B10.BR mice were injected with B10.A spleen cells. After 7 d, donor-derived cells were positively selected via cell sorting. Splenic T cells from B10.BR mice (line 1) were admixed with spleen cells from B10.A mice (line 2) or B10.A donor-derived cells (line 3) at a ratio of 1:1. These cell populations and splenocytes from tolerized mice (line 4) were subjected to a LD analysis. Mixing of donor-derived B10.A cells to normal B10.BR splenic responder cells resulted in a biphasic LD curve when the reactivity against B10.A stimulator cells was analyzed. Biphasic parts I and II give the frequency estimates of the linear parts of the respective segment of the LD analysis.

cell-dependent peripheral tolerance. Recipient L3T4$^+$ T cells might interact with MHC class II–expressing donor veto cells directly, or might support the activation of veto functions by actively participating in the in vivo response to class I MHC antigens via lymphokine secretion. Assuming the latter, why then is the generation of veto cell activity resistant to the effects of cyclosporine A known to block not only lymphokine transcription and secretion, but also primary activation of resting T cells? In ongoing experiments, we are analyzing whether systemic application of defined recombinant lymphokines such as IL-2 can restore the ability of L3T4$^+$ T cell–deficient adult mice to become tolerized by pretransplant transfusion.

We show that pretransplant transfusion results in a state of split tolerance, i.e., high affinity TCRs bearing peripheral, but not thymic, CTL-p become functionally deleted. This contrasts to neonatally induced tolerance, in which thymic and bone marrow chimerism develops (9). Since in the adult mouse chimerism induced by pretransplant transfusion fades with time, an effect that is paralleled by the reappearance of tolerogen-reactive CTL-p and IL-2-producing T lymphocyte precursor (IL-2 TL-p) (Fig. 1, *C* and *D*), we presently investigate conditions to induce a stable, long-lasting chimerism by bone marrow grafting as well.

In the model system of neonatal tolerance, removal of donor-derived cells revealed the existence of anti–donor-reactive T cells (10, 11, 51). No evidence for active suppression was obtained in the model system detailed here (Table 4). Yet, unlike naive donor type cells, donor type cells selected 7 d after transfusion efficiently vetoed primary CTL responses in vitro (Fig. 7; Table 6), as if they were enriched for veto cells. Obviously, these cells are the candidates for the cells that adoptively transferred tolerance in vivo, as recently reported by Martin and Miller (28).

Since there is evidence that in vitro veto cells do not clonally delete antigen-reactive T cells, but rather induce paralysis that can be overcome within time (unpublished data), we anticipate the continuous existence of clonally silenced CTL-p in adult mice transfused with class I MHC-incompatible splenic cells. The availability of TCR-transgenic mice (7, 8) will allow us to experimentally approach this question. There is already evidence that neonatal tolerization for reactivity to Mls antigens phenotypically abolishes anti-Mls reactivity in vitro and in vivo without clonally deleting the respective Vβ-bearing lymphocytes (52).

Even though there are numerous indications that pretransplant transfusion can induce specific immunosuppression rather than sensitization (20–28), the results presented here provide compelling evidence that pretransplant transfusion-induced peripheral tolerance to class I MHC antigens is associated with the in vivo activation and selection of remarkably efficient veto cells. Opposing thymic deletion of antigen-reactive T cells as a basis for negative selection of maturing T lymphocytes (4–8), veto cells operating in peripheral tolerance clonally appear to silence mature Lyt-2$^+$ T cells. If so, then the in vivo induction and maintenance of tolerance to MHC class I antigens by veto cells might represent a paradigm for natural peripheral tolerance to self-antigens not present in the thymus. Accordingly, veto cells control the reactivity towards endogenously produced proteins and peptides, and might thus be further involved in immunoregulatory networks. On the other hand, pretransplant transfusion represents a gentle procedure to "open the window" of unresponsiveness in adult mice for the establishment of conditions for inducing a stable chimerism that then would imply an indefinitive state of transplantation tolerance against foreign MHC alloantigens.

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Address correspondence to Klaus Heeg, Institute of Medical Microbiology and Hygiene, Technical University of Munich, Ismaninger Str. 22, D-8000 Munich, FRG.

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References

1. Nossal, G.J.V. 1983. Cellular mechanisms of immunologic tolerance. *Annu. Rev. Immunol.* 1:33.
2. Streilein, J.W. 1979. Neonatal tolerance: towards an immunological definition of self. *Immunol. Rev.* 46:125.
3. Kappler, J.W., U. Staerz, J. White, and P. Marrack. 1988. Self-tolerance eliminates T cells specific for Mls-modified products of the major histocompatibility complex. *Nature (Lond.).* 332:35
4. MacDonald, H.R., R. Schneider, K. Lees, R.C. Howe, H. Acha-Orbea, R.M. Festenstein, R.M. Zinkernagel, and H. Hengartner. 1988. T-cell receptor Vβ use predicts reactivity and tolerance to Mls^a-encoded antigens. *Nature (Lond.).* 332:40.
5. White, J., A. Herman, A.M. Pullen, R. Kubo, J.W. Kappler, and P. Marrack. 1989. The Vβ-specific superantigen Staphylococcal enterotoxin B: stimulation of mature T cells and clonal deletion in neonatal mice. *Cell.* 56:27.
6. MacDonald, H.R., T. Pedrazzini, R. Schneider, J.A. Louis, R.M. Zinkernagel, and H. Hengartner. 1988. Intrathymic elimination of Mls^a-reactive (Vβ6$^+$) cells during neonatal tolerance induction to Mls^a-encoded antigens. *J. Exp. Med.* 167:2005.
7. Kisielow, P., H. Blüthmann, U.D. Staerz, M. Steinmetz, and H. von Boehmer. 1988. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4$^+$8$^+$ thymocytes. *Nature (Lond.).* 333:742.
8. Sha, W.C., C.A. Nelson, R.D. Newberry, D. Kranz, J.H. Russell, and D.Y. Loh. 1988. Positive and negative selection of an antigen receptor on T cells in transgenic mice. *Nature (Lond.).* 336:73.
9. Nossal, G.J.V., and B.L. Pike. 1981. Functional clonal deletion in immunological tolerance to major histocompatibility complex antigens. *Proc. Natl. Acad. Sci. USA.* 78:3844.
10. Stockinger, B. 1984. Cytotoxic T-cell precursor revealed in neonatally tolerant mice. *Proc. Natl. Acad. Sci. USA.* 81:220.
11. Heeg, K., and H. Wagner. 1985. Analysis of immunological tolerance to major histocompatibility complex antigens. I. High frequencies of tolerogen-specific cytotoxic T lymphocyte precursors in mice neonatally tolerized to class I major histocompatibility complex antigens. *Eur. J. Immunol.* 15:25.
12. Lafferty, K.J., L. Andrus, and S.J. Prowse. 1980. Role of lymphokine and antigen in the control of specific T cell responses. *Immunol. Rev.* 51:279.
13. MacDonald, H.B. 1986. T-cell activation. *Annu. Rev. Cell Biol.* 2:231.
14. Mueller, D.L., M.K. Jenkins, and R.H. Schwartz. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu. Rev. Immunol.* 7:445.
15. Ildstad, S.T., and D.H. Sachs. 1984. Reconstitution with syngeneic plus allogeneic or xenogeneic bone marrow leads to specific acceptance of skin allografts or xenografts. *Nature (Lond.).* 307:168.
16. Auchincloss, H., and D. Sachs. 1983. Mechanisms of tolerance in murine bone marrow chimeras: I. Non-specific suppression of alloreactivity by spleen cells from early but not late chimeras. *Transplantation (Baltimore).* 36:436.
17. Slavin, S., S. Strober, Z. Fuks, and S.J. Kaplan. 1977. Induction of specific transplantation tolerance using fractionated total lymphoid irradiation in adult mice: long term survival of allogeneic bone marrow and skin grafts. *J. Exp. Med.* 146:34.
18. Mayumi, H., K. Himeno, T. Shin, and K. Nomoto. 1985. Drug-induced tolerance to allografts in mice. IV. Mechanisms and kinetics of cyclophosphamide-induced tolerance. *Transplantation (Baltimore).* 39:209.
19. Many, A., and R.S. Schwartz. 1970. On the mechanism of immunological tolerance in cyclophosphamide-treated mice. *Clin. Exp. Immunol.* 6:87.
20. Van der Kwast, T.H., A.J.J. Binanchi, H. Bril, and R. Benner. 1981. Suppression of antigraft immunity by preimmunization. I. Kinetic aspects and specificity. *Transplantation (Baltimore).* 31:79.
21. Faustman, D., P. Lacy, J. Davie, and V. Hauptfeld. 1982. Prevention of allograft rejection by immunization with donor-blood depleted of Ia-bearing cells. *Science (Wash. DC).* 217:157.
22. Sigal, D.P., D. Ludwin, and M.A. Blajchman. 1985. Blood transfusion and renal transplantation. *Br. J. Haematol.* 61:595.
23. Rammensee, H.G., P.J. Fink, and M.J. Bevan. 1984. Functional clonal deletion of class I specific cytotoxic T lymphocytes by veto cells that express antigen. *J. Immunol.* 133:2390.
24. Rammensee, H.G., and M.J. Bevan. 1987. Mutual tolerization of histoincompatible lymphocytes. *Eur. J. Immunol.* 17:893.
25. Sano, S., T. Suda, J.H. Qian, S. Sato, R. Ikegami, T. Hamaoka, and H. Fujiwara. 1987. Abrogation of the capacity of delayed-type hypersensitivity responses to alloantigens by intravenous injection of neuraminidase-treated allogeneic cells. *J. Immunol.* 139:3652.
26. Kast, W.M., E. van Twuyver, R.J.D. Mooijaart, M. Verveld, A.G.A. Kamphuis, C.J.M. Melief, and L.P. de Waal. 1988. Mechanism of skin allograft enhancement across an H-2 class I mutant difference. Evidence for involvement of veto cells. *Eur. J. Immunol.* 18:2105.
27. Rammensee, H.G., and D. Hügin. 1989. Masking of veto function in vivo by activated CD4$^+$ T lymphocytes. *Eur. J. Immunol.* 19:643.
28. Martin, D.R., and R.G. Miller. 1989. In vivo administration of histoincompatible lymphocytes leads to rapid functional de-

letion of cytotoxic T lymphocyte precursors. *J. Exp. Med.* 170:679.
29. Miller, R.G., and H. Derry. 1979. A cell population in nu/nu spleen cells can prevent generation of cytotoxic lymphocytes by normal spleen cells against self antigens of the nu/nu spleen. *J. Immunol.* 122:1502.
30. Miller, R.G. 1980. An immunological suppressor cell inactivating cytotoxic T-lymphocyte precursor cells recognizing it. *Nature (Lond.).* 287:544.
31. Muraoka, S., D.L. Ehman, and R.G. Miller. 1984. Irreversible inactivation of activated cytotoxic T lymphocyte precursor cells by "anti-self" suppressor cells present in murine bone marrow T cell colonies. *Eur. J. Immunol.* 14:1010.
32. Fink, P.J., H.G. Rammensee, J.D. Benedetto, U.D. Staerz, L. Lefrancois, and M.J. Bevan. 1984. Studies on the mechanism of suppression of primary cytotoxic responses by cloned cytotoxic T lymphocytes. *J. Immunol.* 133:1769.
33. Fink, P.J., H.G. Rammensee, and M.J. Bevan. 1984. Cloned cytolytic cells can suppress primary cytotoxic responses directed against them. *J. Immunol.* 133:1775.
34. Rammensee, H.G., P.J. Fink, and M.J. Bevan. 1985. The veto concept: an economic system for maintaining self-tolerance of cytolytic T lymphocytes. *Transplant. Proc.* 17:689.
35. Miller, R.G. 1986. The veto phenomenon and T cell regulation. *Immunol. Today.* 7:112.
36. Fink, P.J., R.P. Shimonkevitz, and M.J. Bevan. 1988. Veto cells. *Annu. Rev. Immunol.* 6:115.
37. Madson, J.C., R.A. Superina, K.J. Wood, and P.J. Morris. 1988. Immunological unresponsiveness induced by recipient cells transfected with donor MHC genes. *Nature (Lond.).* 332:161.
38. Goding, J.W. 1986. Monoclonal Antibodies: Principles and Practice. 2nd ed. Academic Press, London. 122–125.
39. Goding, J.W. 1986. Monoclonal Antibodies: Principles and Practice. 2nd ed. Academic Press, London. 255–266.
40. Heeg, K., S. Gillis, and H. Wagner. 1988. IL-4 bypasses the immune suppressive effect of Cyclosporin A during the in vitro induction of murine cytotoxic T lymphocytes. *J. Immunol.* 141:2330.
41. Heeg, K., C. Steeg, H. Schmitt, and H. Wagner. 1987. Frequency analysis of class I MHC-reactive Lyt2$^+$ and class II MHC-reactive L3T4$^+$ IL2-secreting T lymphocytes. *J. Immunol.* 138:4121.
42. Heeg, K., J. Reimann, D. Kabelitz, C. Hardt, and H. Wagner. 1985. A rapid colorimetric assay for the determination of IL2-producing helper T cell frequencies. *J. Immunol. Methods.* 77:237.
43. Brent, L., J.A. Hansen, P.J. Kilshaw, and A.V. Thomas. 1973. Specific unresponsiveness to skin allografts in mice. I. Properties of tissue extracts and their synergistic effect with antilymphocytic serum. *Transplantation (Baltimore).* 15:160.
44. MacDonald, H.R., N. Thiernesse, and J.C. Cerottini. 1981. Inhibition of T-cell mediated cytolysis by monoclonal antibodies directed against Lyt2: heterogeneity of inhibition at the clonal level. *J. Immunol.* 126:1671.
45. Cobbold, S., M. Martin, and H. Waldmann. 1986. Monoclonal antibodies for the prevention of graft-versus-host disease and marrow graft rejection. *Transplantation (Baltimore).* 42:239.
46. Seaman, W.E., and D. Wofsy. 1988. Selective manipulation of the immune response in vivo by monoclonal antibodies. *Annu. Rev. Med.* 39:231.
47. Heeg, K., C. Steeg, and H. Wagner. 1988. L3T4$^+$ T cell independent reactivity of Lyt2$^+$ T cells in vivo. *Cell. Immunol.* 111:148.
48. Krönke, M., W.J. Leonhard, J. Depper, S.K. Arya, F. Wang-Staal, R.C. Gallo, T.A. Waldmann, and W.C. Greene. 1984. Cyclosporin A inhibits T cell growth factor gene expression at the level of mRNA transcription. *Proc. Natl. Acad. Sci. USA.* 81:5214.
49. Hess, A.D. 1985. Effect of interleukin 2 on the immunesuppressive action of cyclosporin. *Transplantation (Baltimore).* 39:62.
50. Heeg, K., K. Deusch, W. Solbach, D. Bunjes, and H. Wagner. 1984. Frequency analysis of cyclosporin-sensitive cytotoxic T lymphocyte precursors. *Transplantation (Baltimore).* 38:532.
51. Stockinger, B., H. Darjes, and P.H. Krammer. 1986. Adsorption on B cell hybridomas removes suppressor cells from spleen cells of neonatally tolerized mice. *Eur. J. Immunol.* 16:301.
52. Rammensee, H.G., R. Kroschewski, and B. Frangoulis. 1989. Clonal anergy induced in mature Vβ6$^+$ T lymphocytes on immunizing Mls-1^b mice with Mls-1^a expressing cells. *Nature (Lond.).* 339:541.