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Tolerance, mixed chimerism and protection against graft-versus-host disease after total lymphoid irradiation

Elizabeth H. Field[1,2] **and Samuel Strober**[3*]

[1]*Department of Veterans Affairs Medical Center, Iowa City, IA 52246, USA*
[2]*Department of Internal Medicine, University of Iowa College of Medicine, Iowa City, IA 52242, USA*
[3]*Division of Rheumatology and Immunology, Department of Medicine, Stanford University School of Medicine, Stanford, CA 94305, USA*

Total lymphoid irradiation (TLI), originally developed as a non-myeloablative treatment for Hodgkin's disease, has been adapted for the induction of immune tolerance to organ allografts in rodents, dogs and non-human primates. Moreover, pretransplantation TLI has been used in prospective studies to demonstrate the feasibility of the induction of tolerance to cadaveric kidney allografts in humans. Two types of tolerance, chimeric and non-chimeric, develop after TLI treatment of hosts depending on whether donor bone marrow cells are transplanted along with the organ allograft. An advantageous feature of TLI for combined marrow and organ transplantation is the protection against graft-versus-host disease (GVHD) and facilitation of chimerism afforded by the predominance of $CD4^+NK1.1^+$-like T cells in the irradiated host lymphoid tissues. Recently, a completely post-transplantation TLI regimen has been developed resulting in stable mixed chimerism and tolerance that is enhanced by a brief course of cyclosporine. The post-transplantation protocol is suitable for clinical cadaveric kidney transplantation. This review summarizes the evolution of TLI protocols for eventual application to human clinical transplantation and discusses the mechanisms involved in the induction of mixed chimerism and protection from GVHD.

Keywords: tolerance; chimerism; total lymphoid irradiation; graft-versus-host disease; natural killer T cells

1. TOTAL LYMPHOID IRRADIATION: A NON-MYELOABLATIVE REGIMEN THAT FACILITATES ACCEPTANCE OF BONE MARROW AND ORGAN ALLOGRAFTS IN LABORATORY ANIMALS AND HUMANS

After Medawar and his colleagues demonstrated permanent and specific transplantation tolerance across the major histocompatibility complex (MHC) barriers in neonatal mice (Medawar 1953; Billingham & Brent 1956), many investigators attempted to reproduce these results in adult laboratory animals for eventual application to organ transplantation in humans. Total lymphoid irradiation (TLI), used to treat Hodgkin's disease and non-Hodgkin's lymphoma (Kaplan 1972; Hoppe *et al.* 1987), was the first non-myeloablative host-conditioning regimen shown to induce tolerance and stable mixed chimerism following MHC-mismatched bone marrow (BM) transplantation in a variety of adult laboratory animals including mice, rats and dogs (for reviews see Strober *et al.* 1979; Hoppe & Strober 1987; Waer & Strober 1990). High-level chimeras permanently accepted organ allografts such as skin and heart transplants derived from the marrow donors but rejected third-party transplants. Subsequently, a wide variety of non-myeloablative host-conditioning regimens have been successfully developed to produce mixed chimerism in adult laboratory animals (Sharabi & Sachs 1989; Sykes *et al.* 1997; Colson *et al.* 1995; McSweeney & Storb 1999; Wekerle *et al.* 2000), and many of these are discussed in this review.

The use of non-myeloablative host-conditioning regimens to induce chimerism after human leucocyte antigen (HLA)-identical BM or peripheral blood stem cell transplantation has been recently adapted to human protocols (Giralt *et al.* 1997; Khouri *et al.* 1998; Slavin *et al.* 1998; Childs 1999). However, the high incidence of severe graft-versus-host disease (GVHD), even in the setting of mixed chimerism (Giralt *et al.* 1997; Khouri *et al.* 1998; Slavin *et al.* 1998; Childs *et al.* 1999), limits the widespread use of the non-myeloablative approach in clinical transplantation. In this regard, TLI may prove to be an advantageous non-myeloablative-conditioning regimen. TLI treatment in rodents, especially when combined with the potent T-cell depletive agent anti-thymocyte globulin (ATG), results in mixed chimerism after BM transplantation and protects against the development of GVHD, even when the BM transplant is heavily contaminated with donor peripheral blood T cells (Lan *et al.* 2001). In contrast, recipients that are conditioned with non-myeloablative whole-body irradiation (WBI) develop lethal GVHD after BM cell infusions that contain donor peripheral blood T cells.

The non-myeloablative TLI regimen selectively irradiates the thymus and the peripheral lymphoid tissues,

*Author for correspondence (sstrober@stanford.edu).

including the spleen and the axillary, cervical, hilar, aortic and inguinal lymph nodes, while shielding non-lymphoid tissues in the head, chest, abdomen and pelvis (Kaplan 1972; Strober *et al.* 1979). The radiation is given in small doses (80–250 cGy) once a day and delivered consecutively over a few weeks. The fractionated radiation results in transient quantitative and qualitative changes in the peripheral lymphoid system. For example, TLI markedly depletes mature T and B cells from the lymphoid organs (Strober *et al.* 1979; Hoppe & Strober 1987; Waer & Strober 1990), but expands the proportion of $CD4^+NK1.1^+$-like T cells in mice (see § 4(a)). Exposure of animals to foreign antigens shortly after the completion of TLI, when most spleen T cells are $CD4^+NK1.1^+$-like T cells, results in tolerance to the antigen. In rodents, the T- and B-cell compartment gradually recovers over the next four to eight weeks, as does the ability to mount normal immune responses to alloantigens and other protein antigens (Strober *et al.* 1979; Hoppe & Strober 1987; Waer & Strober 1990).

Patients given TLI treatment for Hodgkin's disease also develop marked peripheral blood T- and B-cell lymphopenia immediately following the completion of TLI, but both lymphocyte number and function gradually return towards normal by 18–24 months (Strober *et al.* 1979; Hoppe & Strober 1987; Waer & Strober 1990). TLI has been administered safely to thousands of patients with Hodgkin's disease (Kaplan 1972; Hoppe *et al.* 1987), and the risk of secondary haemological and lymphoid malignancies in long-term follow-up studies has been remarkably low (Coleman *et al.* 1977).

Pretransplantation TLI (total dose of 800–1800 cGy) without BM transplantation has been used successfully to induce tolerance to heart and kidney allografts in baboons and dogs (Strober *et al.* 1984; Myburgh *et al.* 1984). Based on these studies, pretransplantation TLI (total dose of *ca.* 2000 cGy) combined with post-transplantation ATG was used successfully without BM transplantation in a prospective study to induce tolerance in humans to HLA-mismatched cadaveric kidney allografts, such that immunosuppressive drugs could be discontinued for at least 12 years without rejection of the transplant in some patients (Strober *et al.* 1989, 2000). Despite its success in the induction of tolerance in large outbred laboratory animals, and in humans, the TLI-conditioning regimen without BM transplantation has not been applied extensively to human organ transplantation, because only a small proportion of patients developed tolerance. However, one major reason to consider using TLI combined with ATG and BM transplantation is that this combination used post-transplantation along with a brief course of cyclosporine reliably induces a state of stable mixed chimerism and organ allograft tolerance without concomitant GVHD (Lan *et al.* 2000).

2. ORGAN ALLOGRAFT TOLERANCE IN SMALL AND LARGE LABORATORY ANIMALS AFTER PRETRANSPLANTATION TLI

(a) *Chimeric tolerance after pretransplantation TLI in rodents*

Slavin *et al.* (1978*a*) first showed that, after TLI treatment (17 fractions × 200 cGy) and intravenous infusion of C57BL/Ka ($H\text{-}2^b$) donor BM cells, the majority of recipient BALB/c ($H\text{-}2^d$) mice developed stable mixed chimerism (Slavin *et al.* 1977). When 30×10^6 C57BL/Ka BM cells were infused into recipient BALB/c mice one day after completion of TLI, 90% of the recipients became mixed chimeras with 50–90% of donor-type lymphocytes and donor red blood cells in the peripheral blood. None of the chimeric mice developed evidence of GVHD during a subsequent observation period of more than 200 days (Slavin *et al.* 1977, 1978*a*; Waer *et al.* 1982).

The ability to successfully induce mixed chimerism depended on the timing of the BM infusion and the TLI regimen. When BM infusion was delayed seven days after a standard dose of 3400 cGy TLI (17 fractions × 200 cGy), only 40% of mice became chimeric, and no mice developed chimerism when BM infusion was delayed 21 days (Gottlieb *et al.* 1979). Lowering the total TLI dose to 1800, 900 or 450 cGy (in 18 fractions) prevented the development of chimerism (Gottlieb *et al.* 1979). Compacting the fractionation programme, such that animals received TLI treatments on multiple occasions per day, also decreased the incidence of chimerism (Waer *et al.* 1982).

When C57BL/Ka skin grafts were transplanted to TLI-treated BALB/c recipients at the same time as donor BM infusion, the grafts survived more than 250 days. However, chimeric mice rejected third-party, C3H ($H\text{-}2^k$) skin grafts within two to three weeks (Slavin *et al.* 1978*a*). TLI-treated BALB/c mice that received skin grafts but no BM infusion showed prolonged survival of C57BL/Ka skin grafts, but the mean survival time was limited to 50 days (Slavin *et al.* 1978*a*). Therefore, the establishment of mixed chimerism was required for the establishment of long-term donor-specific tolerance.

Chimeric donor cells present in the TLI-conditioned recipients were tolerant to host alloantigens (Gottlieb *et al.* 1980). Donor-type (C57BL/Ka) spleen cells were isolated from C57BL/Ka→BALB/c chimeras more than 100 days after BM transplantation and 25×10^6 viable donor-type cells were injected intravenously into sublethally irradiated BALB/c or third-party (C3H) hosts. Injection of the chimeric donor-type spleen cells into irradiated third-party hosts resulted in the development of a severe wasting syndrome. Only 2 out of the 12 of these third-party mice survived more than 15 days, and none survived more than 30 days. In contrast, 17 out of 20 BALB/c hosts survived in apparent good health for more than 60 days after injection of chimeric donor-type spleen cells (Gottlieb *et al.* 1980). Thus, the cells of donor origin in the chimeras lost their reactivity to the tissues of the host strain, even when removed from the chimeric environment, but retained the ability to mediate GVHD against a third-party strain.

A TLI regimen similar to that used in mice was adapted to adult Lewis rats to establish chimerism and transplantation tolerance in ($RT1^l$) rats (Slavin *et al.* 1978*b*). In order to achieve uniform chimerism after a standard dose of TLI (3400 cGy) a large dose of BM cells (300×10^6) from ACI ($RT1^a$) donors was required, and the entire pelvis had to be included in the irradiation fields. The ACI→Lewis mixed chimeras contained about 50% donor-type lymphocytes in the peripheral blood and showed no evidence of GVHD (Slavin *et al.* 1978*b*).

ACI skin grafts placed simultaneously with the infusion of BM cells survived for at least six months with full hair growth. Chimeric recipients that had long-term ACI skin grafts rejected third-party skin grafts within three weeks (Slavin *et al.* 1978*b*). Thus, specific transplantation tolerance was achieved in both mice and rats after TLI.

To evaluate the effect of TLI on the survival of vascularized organ allografts, Lewis rats were given TLI, and ACI or BN hearts were directly anastomosed to the abdominal aorta and inferior vena cava (Slavin *et al.* 1978*b*). Graft function was determined by direct palpation or by electrocardiography. The heart transplants survived no more than ten days in untreated recipients, but four out of five animals given TLI and both heart and BM transplants maintained functioning heart grafts for at least 300 days.

(b) *Adaptation of pretransplantation TLI to large outbred animals*

The adaptation of the rodent protocols for TLI and BM transplantation to dogs and non-human primates required further modification of the radiation regimen. The wide fields and high total TLI doses given simultaneously above and below the diaphragm, which were used in rodents, produced considerable toxicity in large animals (Slavin *et al.* 1979). Therefore, either the radiation fields were narrowed, or the radiation was delivered in separate fractions to the lymphoid tissue above and below the diaphragm, or the total radiation dose was reduced from 3400 cGy (used in mice and rats) to between 800 and 1800 cGy. In addition, it was frequently difficult to recover enough BM cells from a single large animal to replicate the dose of BM cells used to achieve uniform chimerism in the rodent models (*ca.* 1×10^9 cells kg^{-1} host body weight).

These necessary changes in the TLI regimen decreased its efficacy of achieving mixed chimerism and tolerance. For example, successful BM engraftment occurred in only two out of five dogs given high doses of TLI (2800–3500 cGy), delivered separately to fields above and below the diaphragm, and infused with *ca.* 0.5×10^9 cells kg^{-1} donor BM cells (Slavin *et al.* 1979). The incidence of mixed chimerism improved when dogs were treated with a lower total TLI dose of 1800 cGy TLI, delivered to a combined above- and below-diaphragm field (Gottlieb *et al.* 1980). All of the 12 dogs treated this way achieved stable mixed chimerism when examined 2–11 months later. However, in a subsequent study, a group of dogs given 1800 cGy pretransplantation TLI, BM cell infusion (*ca.* 0.5×10^9 cells kg^{-1} body weight), and a heterotopic heart allograft, from the same donor on day 0, all rejected their allografts by 272 days (Strober *et al.* 1984).

Others have encountered similar limitations of the narrow field and low-dose TLI regimen. Myburgh *et al.* (1981) used both short-course and maintenance courses of TLI before combined BM cell infusion and kidney transplantation in baboons. In a total group of 28 animals treated with various schedules, only nine were alive with functioning grafts at 82–350 days. Howard *et al.* (1981) delivered 2400 cGy of TLI through narrow fields to four beagle dogs that were splenectomized prior to TLI. Following TLI the animals simultaneously received an allogeneic kidney transplant and a small dose of donor BM cells. Two out of the four animals showed no rejection of their kidney grafts, but chimerism could not be demonstrated.

Thus, using narrow fields, lower total doses of radiation, or lower doses of donor BM cells did not produce reliable tolerance to transplanted organ allografts as in the earlier rodent studies. This was probably because the narrow field and lower dose of radiation allowed a larger number of host peripheral T cells to remain, and mediate rejection of the combined organ and marrow allografts. Also, the low number of BM cells injected was probably insufficient to overcome this host T-cell barrier to both allografts. Strategies for depleting these host T cells in preparation for non-myeloablative haemopoietic progenitor cell transplantation have been developed recently, as well as techniques for harvesting larger numbers of donor progenitors using recombinant cytokines (Giralt *et al.* 1997; Khouri *et al.* 1998; Slavin *et al.* 1998; Childs *et al.* 1999).

(c) *Non-chimeric tolerance after pretransplantation TLI*

Extensive studies of the effect of TLI without BM infusion on renal and hepatic allografts in primates were done in a large series of baboons (Myburgh *et al.* 1984). Of the many regimens investigated, a wide TLI-field regimen (with shielding of only the skull, lungs and major parts of the legs) with a small total dose (eight fractions $\times$ 100 cGy, given twice weekly), resulted in permanent renal allograft survival in 60% of the animals. Extending the shielding, removing the lung shields, or increasing the total radiation dose either led to unacceptable side-effects or decreased the incidence of tolerance. The latter studies demonstrate that an appropriate TLI-conditioning regimen can lead to permanent allograft survival in a large percentage of outbred non-human primates without the need of concomitant BM infusion or immunosuppressive agents.

Strober *et al.* (1984) examined heart allograft survival in outbred dogs given TLI alone or in combination with ATG. Table 1 shows that untreated control mongrel recipients rejected unmatched heart allografts within five to seven days. TLI treatment of recipients before transplantation (total dose of 1800 cGy) prolonged graft survival, but all animals rejected the grafts by 28 days. Treatment of the recipients with ATG alone (4 mg kg^{-1}) intramuscularly on days 0, 2, 4, 6, 8 and 10 after heart transplantation also prolonged graft survival, but all animals rejected the grafts by 33 days (table 1). However, five out of the eight recipients given TLI, a heart allograft, and ATG showed prolonged survival, and three of the animals maintained functioning grafts for at least 360, 494 and 495 days (table 1).

The last three dogs were examined for evidence of donor-specific tolerance. Transplantation of third-party hearts was performed between 200 and 270 days after placement of the initial allograft. The dogs rejected the third-party heart allografts within two weeks, and biopsies of the rejected third-party hearts showed a typical picture of diffuse mononuclear cell infiltrates, myonecrosis and patchy infarction. Subsequent biopsies of the initial allograft showed normal heart tissue or minimal patchy mononuclear cell infiltrate without abnormalities of

Table 1. *Allograft survival in dogs treated with TLI and ATG*

treatment	heart allograft survival (days)
none	5, 5, 6, 6, 7
TLI alone	6, 6, 8, 8, 9, 28
ATG alone	15, 16, 21, 28, 31, 33
TLI + ATG	45, 81, 89, 139, 200, > 360[a], > 494[a], > 495[a]

[a] Rejected third-party allograft.

muscle cells. Thus, three out of eight dogs given TLI and ATG showed specific tolerance to the initial allograft (Strober *et al.* 1984).

3. TOLERANCE IN HOSTS GIVEN POST-TRANSPLANTATION TLI AND ATG

(a) *Adaptation to clinical cadaveric organ transplantation*

Because one cannot easily predict the timing of cadaveric renal transplantation surgery for a given recipient, one needs to adapt a completely post-transplantation TLI-conditioning regimen for use in cadaveric transplantation. Studies in the adult Lewis rat showed that hosts given a non-myeloabative post-transplantation regimen of TLI (ten doses of 240 cGy) plus ATG (five doses) after ACI heart transplantation developed donor-specific tolerance, if the animals also received an intravenous infusion of donor blood or BM cells (Woodley *et al.* 1993; Zeng *et al.* 1996; Hayamizu *et al.* 1998*a*,*b*, 1999). Lewis rats given TLI and ATG without donor cell infusions showed prolonged graft survival (median of 53 days), but all grafts were eventually rejected (Woodley *et al.* 1993; Zeng *et al.* 1996). Infusions of donor whole blood, peripheral blood mononuclear cells (PBMCs), granulocyte-colony stimulating factor (G-CSF)-mobilized PBMC, or BM cells after the completion of TLI, all improved the mean survival of ACI heart grafts to more than 150 days (Woodley *et al.* 1993; Zeng *et al.* 1996; Hayamizu *et al.* 1999). However, when the Lewis hosts bearing long-term ACI heart grafts (more than 150 days) were analysed for evidence of chimerism, mixed chimerism was only detected in the Lewis hosts that received ACI BM cells (Hayamizu *et al.* 1999).

Histological examination of long-term surviving heart grafts from the Lewis hosts that received infusions of normal PBMCs showed evidence of chronic rejection. In comparison, heart grafts from Lewis hosts that received infusions of donor BM cells showed little or no evidence of chronic rejection. Heart grafts from Lewis hosts that received infusion of G-CSF-mobilized PBMCs showed variable histological profiles; some grafts had substantially less chronic rejection than those given normal PBMCs, and some grafts showed no chronic rejection (Hayamizu *et al.* 1999).

Thomas, *et al.* (1992) reported that the post-transplantation regimen of 600 cGy TLI (six doses of 100 cGy) in combination with six injections of ATG and a single intravenous infusion of selected $CD3^{-}DR^{dim}CD8^{+}$ donor BM cells induced tolerance in 60% of rhesus monkey kidney transplant recipients. These recipients received no maintenance immunosuppression after the two-week induction regimen, and none had rejection episodes requiring treatment with immunosuppressive drugs. The recipients were observed for at least one year, and the serum creatinine levels in tolerant recipients were normal. Specific unresponsiveness to donor cells was observed in the mixed lymphocyte reaction (MLR), and no serum anti-donor antibodies were detected during the observation period.

Table 2. *Percentage of $NK1.1^{+}$ T cells in the spleen of C57BL/6 mice treated with TLI and ATS*

(Percentage of cells determined by flow cytometry from pools of C57BL/6 mice.)

	percentage of cells in spleen	
treatment	all TCR^{+} T cells	$NK1.1^{+}$ T cells amongst all T cells
untreated control	33.2	1.3
WBI (single dose of 450 cGy)	8.3	4.5
TLI (2 doses, 480 cGy)	25.6	4.3
TLI (8 doses, 1920 cGy)	13.4	22.0
TLI (17 doses, 4080 cGy)	9.4	65.9
TLI (17 doses + 3 doses of anti-thermocyte serum)	3.6	92.4

(b) *Effect of cyclosporine on chimerism and tolerance after post-transplantation TLI*

It would be advantageous if the non-myeloablative regimens developed for combined organ and BM transplantation were compatible with a brief course of immunosuppressive drugs in current use, such as cyclosporine, because safe clinical protocols would involve tapering and discontinuing these drugs while monitoring the state of tolerance (see §6). Therefore, cyclosporine was added to a post-transplantation TLI-conditioning regimen in a rat cardiac allograft model. Lewis rat hosts were transplanted with ACI hearts and given TLI, ATG, and ACI blood transfusions without cyclosporine. Grafts were accepted for more than 100 days in seven out of nine cases. However, the addition of a 60-day course of cyclosporine (days 15–75) to the latter protocol prevented long-term graft acceptance, and all grafts were rejected between 3 and 39 days after the discontinuation of cyclosporine (Lan *et al.* 2000). Thus, cyclosporine interfered with the induction of tolerance when given in conjunction with TLI, ATG and donor blood transfusion.

In contrast to the above result, cyclosporine facilitated long-term graft acceptance in Lewis hosts given TLI, ATG and donor BM infusion. Whereas the majority of hosts given the treatment protocol without cyclosporine rejected their grafts, all hosts that received the 60-day course of cyclosporine showed prolonged graft survival without rejection. Flow cytometry studies to detect the presence of donor cells showed that cyclosporine facilitated the development of mixed chimerism. Only a minority of hosts that did not receive cyclosporine achieved detectable donor T- or B-cell chimerism in the blood, but all hosts that received cyclosporine achieved detectable T- and B-cell chimerism (Lan *et al.* 2000).

Table 3. *Alterations in spleen T-cell subsets and patterns of cytokine secretion following TLI treatment*

(Spleen cells were harvested from BALB/c mice at 2–28 days after the completion of TLI, pooled and stimulated with anti-CD3ϵ for 48 h. Supernatants were collected and the level of cytokine determined by enzyme-linked immunosorbent assay. $CD4^+$ and $CD8^+$ cells were determined by flow cytometry. Values are means ± s.e. Abbreviation: n.d., not done.)

	cytokine (pg ml^{-1})				
sample	IL-4	IFN-γ	IL-10	$CD4^+$ (%)	$CD8^+$ (%)
TLI + 2 days post-TLI	291 ± 27	23 ± 10	17 ± 3	14.2 ± 2.8	1.2 ± 0.3
TLI + 5 days post-TLI	n.d.	n.d.	n.d.	6.6 ± 2.1	1.0 ± 0.3
TLI + 8 days post-TLI	90 ± 37	321 ± 15	47 ± 13	3.1 ± 0.5	0.8 ± 0.3
TLI + 14 days post-TLI	n.d.	n.d.	n.d.	3.8 ± 0.5	1.0 ± 0.3
TLI + 28 days post-TLI	78 ± 10	1550 ± 366	74 ± 16	7.9 ± 0.6	3.5 ± 0.5
untreated control	186 ± 35	1438 ± 423	114 ± 36	23.7 ± 1.7	10.3 ± 1.0

4. ALTERATIONS IN T-CELL SUBSETS AND PATTERNS OF CYTOKINE SECRETION AFTER TLI

(a) *Predominance of $CD4^+NK1.1^+$ T cells, $CD4^-CD8^-$ (double negative) T cells and 'natural suppressor cells' after TLI*

During and immediately after TLI there are marked alterations in the proportions of T-cell subsets in the peripheral lymphoid compartment. The subsets of residual T cells in the spleen of C57BL/6 mice given TLI with or without anti-thymocyte serum (ATS) were examined for the presence of $NK1.1^-$ or $NK1.1^+$ T-cell receptor (TCR) $\alpha\beta^+$ T cells. As the dose of TLI increased, the absolute number of $NK1.1^-$ T cells decreased, while the absolute number of $NK1.1^+$ T cells remained unchanged, resulting in a dramatic increase in the percentage of $NK1.1^+$ T cells amongst all T cells (table 2). Addition of ATS further increased the percentage of $NK1.1^+$ T cells to more than 90% of all residual splenic T cells. About 80% of the $NK1.1^+$ T-cell population were $CD4^+$ T cells, and the remainder were $CD4^-CD8^-$ T cells (Lan *et al.* 2001). It is noteworthy that the peculiar alteration in the T-cell phenotype appears to be unique to standard TLI treatment, because a lower dose of TLI or a non-myeloablative dose of WBI (450 cGy) does not cause similar phenotypic changes (table 2).

While the expansion of $CD4^+NK1.1^+$ T cell is best seen in NK1.1-expressing mouse strains, an expanded $CD4^+NK1.1^+$-like T-cell population was also detected in TLI-treated BALB/c mice. About 60% of splenic $CD4^+$ T cells from TLI-treated BALB/c mice coexpressed the $CD4^{lo}$, $CD3^{lo}$, interleukin 2 receptor (IL-2R) β^+, $DX5^+$, and $CD44^{hi}$ phenotype (Lan *et al.* 2001), all characteristic features of $NK1.1^+$-like T cells (Arase *et al.* 1993; Emoto *et al.* 1995; MacDonald 1995).

(b) *Alterations in patterns of cytokine secretion after TLI*

The cytokine milieu of the peripheral lymphoid environment is also quite different in TLI-treated mice. Immediately after the completion of TLI, spleen cells from TLI-treated mice produced significantly higher levels of IL-4 upon anti-CD3 engagement, but significantly lower amounts of IL-2, interferon-γ (IFN-γ) (Field & Rouse 1995) and IL-10, compared with spleen cells from untreated control mice (table 3). Sorting experiments demonstrated that the $CD4^+$ T cells (Field *et al.*, unpublished data) or the $NK1.1^+$ T cells (Lan *et al.* 2001) obtained from the spleen after TLI were the predominant T-cell source of IL-4. It is therefore likely that most of the IL-4 is coming from $CD4^+$NK1.1-like T cells. Indeed, $CD4^+$NK1.1-like T cells secrete a variety of lymphokines, including IL-4, IL-5, IFN-γ, IL-10 and IL-2 (Bendelac & Schwartz 1991; Bendelac *et al.* 1992), and the cells may produce more IL-4 in relation to the other cytokines (Arase *et al.* 1993). Therefore, during the immediate post-TLI period, when mice are most susceptible to tolerance induction, the animals produce the highest amount of IL-4 and the peripheral T-cell compartment is composed of mainly $CD4^+$NK1.1-like T cells with few, if any, $CD8^+$ cells (Field *et al.*, unpublished data). Whether these $CD4^+NK1.1^+$-like T cells migrate to peripheral tissue sites in the post-TLI period and/or give rise to $CD4^-CD8^-NK1.1^+$-like T cells through the down-regulation of surface CD4 remains unknown. $CD4^-CD8^-$ T cell clones that have been generated from TLI-treated mice and propagated *in vitro* prevent lethal GVHD *in vivo* (Strober *et al.* 1987).

5. MECHANISMS OF PROTECTION AGAINST GVHD AND DEVELOPMENT OF MIXED CHIMERISM AFTER ALLOGENEIC BM TRANSPLANTATION IN HOSTS CONDITIONED WITH TLI

(a) *Allogeneic peripheral T cells and BM T cells in the induction of GVHD*

In humans, even HLA-matched siblings that have only minor histocompatibility antigen mismatches can develop severe GVHD, and anti-GVHD prophylactic drugs are commonly used (Weisdorf *et al.* 1991; Sullivan 1994; Ferrara *et al.* 1996). The incidence and severity of GVHD is markedly reduced when recipients are given BM transplants that have been stringently depleted of donor T cells (Burnett *et al.* 1988; Martin & Kernan 1990).

In contrast to humans, GVHD in mice after BM transplantation across major MHC barriers is usually mild, even when the BM T cells are not depleted and prophylactic drugs are not used (Palathumpat *et al.* 1992*a*, 1995). In fact, one frequently needs to add back $\alpha\beta^+$ peripheral T cells to the BM inoculum in order to produce a murine

model of severe GVHD. This suggests that peripheral T cells cause GVHD in mice, rather than the resident BM T cells. The make-up of BM T-cell subsets differs from that of peripheral T cells. BM T cells contain an unusually high proportion of NK1.1$^+$ T cells and CD4$^-$CD8$^-$ T cells (Sykes 1990; Makino *et al.* 1995; Bendelac *et al.* 1997; Zeng *et al.* 1997). Both of these subsets have been reported to ameliorate autoimmune diseases and GVHD (Gombert *et al.* 1996; Baxter *et al.* 1997; Hammond *et al.* 1998).

Zeng *et al.* (1999) directly compared the ability of highly purified CD4 and CD8 T cells obtained from the peripheral blood or BM of C57BL/6 mice to induce acute GVHD in lethally irradiated BALB/c hosts co-injected with stringently T-cell depleted C57BL/6 BM cells. GVHD severity was judged by mortality and by histopathological changes in the skin and large intestines. The peripheral blood T cells were at least 30-fold more potent inducers of GVHD than the BM T cells on a per-cell basis; BM T cells produced no significant mortality when co-injected in the dose range tested. Flow cytometric analysis showed that about 30% of the BM T cells were NK1.1$^+$ T cells, but only 1% of the blood T cells were NK1.1$^+$ T cells (Zeng *et al.* 1999). Sorted BM T cells were only able to induce GVHD if the NK1.1$^+$ T cells were removed or if the BM T cells were obtained from IL-4$^{-/-}$ donors. Sorted BM NK1.1$^+$ T cells from wild-type mice secreted high levels of both IL-4 and IFN-γ and, when co-injected, suppressed the development of GVHD induced by NK1.1$^-$ BM T cells. In contrast, sorted BM NK1.1$^+$ T cells from IL-4$^{-/-}$ mice secreted high levels of IFN-γ without IL-4 and exacerbated GVHD induced by NK1.1$^-$ BM T cells (Zeng *et al.* 1999).

Therefore, peripheral blood or BM NK1.1$^-$ $\alpha\beta^+$ T cells induce and NK1.1$^+$ $\alpha\beta^+$ T cells potently suppress acute lethal GVHD in lethally irradiated hosts, and the suppression of GVHD by BM NK1.1$^+$ $\alpha\beta^+$ T cells requires IL-4. The results agree with and extend previous studies which show that CD4$^-$CD8$^-$ T cells ('natural suppressor' cells) from the BM inhibit GVHD (Palathumpat *et al.* 1992*b*; Sykes *et al.* 1990; Strober *et al.* 1996).

(b) *Protection against GVHD after TLI: role of NK1.1$^+$ T cells and IL-4*

Protection from GVHD was noted in recipients that were conditioned with combined TLI and ATS (Lan *et al.* 2001), a non-myeloablative regimen that maximizes the proportion of NK1.1$^+$ $\alpha\beta^+$ T cells in the spleen (table 2). Control adult BALB/c mice given a single dose of WBI (800 cGy), intravenous infusion of C57BL/6 BM cells (3×10^6), and 5×10^6 C57BL/6 PBMC all died of severe GVHD during a 100-day observation period. The addition of three intraperitoneal injections of ATS on days -12, -10 and -8 prior to the cell infusion did not improve survival. BALB/c hosts given a TLI-conditioning regimen (17×240 cGy) and intravenous infusion of C57BL/6 BM and PBMC developed less severe GVHD, and only 60% of the hosts died. However, the addition of ATS during the first week of TLI (days -12, -10 and -8) protected further against the development of GVHD following infusion of BM and PBMC, and all mice survived. Staining for C57BL/6 (H-2K^b) donor cells in the blood of BALB/c hosts given TLI, ATS, and an injection of donor marrow and PBMC showed that all hosts that survived without GVHD became mixed chimeras (Lan *et al.* 2001).

Because sorted NK1.1$^+$ BM T cells from C57BL/6 mice protected against GVHD (Zeng *et al.* 1999), and TLI-treatment plus ATS increased the proportion of splenic NK1.1$^+$ $\alpha\beta^+$ T cells (table 2), we examined whether sorted splenic TCR$\alpha\beta^+$ T cells from TLI-treated C57BL/6 mice protected against GVHD. GVHD was induced by infusing splenic T cells from untreated C57BL/6 mice into lethally irradiated BALB/c hosts. Co-injection of splenic T cells from TLI-treated donors decreased the incidence of GVHD and significantly improved survival of the BALB/c hosts (Lan *et al.* 2001). In a similar model of GVHD, King *et al.* (1981) previously showed that co-injection of splenic T cells from TLI-treated BALB/c mice also decreased the incidence of GVHD. Thus, spleen T cells from either TLI-treated donors or TLI-treated hosts are able to protect against GVHD.

We speculated that CD4$^+$NK1.1$^+$-like T cells, which predominate in the TLI-treated mice, function to suppress development of GVHD through an IL-4-dependent pathway. To test this, wild-type and IL-4$^{-/-}$ BALB/c mice were conditioned with TLI and given BM transplants from IL-4$^{-/-}$ C57BL/6 donors. As previously noted, BM T cells from IL-4$^{-/-}$ C57BL/6 donors produced lethal GVHD when infused into lethally irradiated BALB/c recipients (Zeng *et al.* 1999). Survival of TLI-treated wild-type hosts was significantly improved as compared with TLI-treated IL-4$^{-/-}$ hosts (Lan *et al.* 2001). The experimental results indicate that the mechanism by which TLI-treated mice protect against GVHD depends on IL-4.

(c) *Immunoredirection and the role of IL-4 in the establishment of mixed chimerism*

Both Th1 CD4$^+$ and TCl CD8$^+$ cells play a role in graft rejection (Fowler *et al.* 1994, 1996; Krenger *et al.* 1995), and the development of tolerance often depends on blocking the generation of these critical effector subsets (Chen *et al.* 1996; Gao *et al.* 1996). In order to identify the mechanisms by which TLI-treated mice become tolerant of donor cells, haploidentical spleen cells from CAF_1 strain mice (BALB/c $\times$ A/J) were injected into the peritoneal cavity of TLI-treated or control BALB/c mice, to establish a model of mixed chimerism. In the $F_1\rightarrow$parental strain combination, the CAF_1 cells do not generate GVHD against the BALB/c host, and they are normally rejected by immunocompetent BALB/c cells. Five to seven weeks after injection the presence of donor cells was determined in the spleen of the hosts using fluorescence-activated cell sorting (FACS) analysis. As expected, control BALB/c hosts rapidly rejected CAF_1 donor spleen cells as none of the mice showed evidence of mixed chimerism. When CAF_1 cells were injected between two and seven days after the completion of TLI, 70–80% of TLI-treated BALB/c hosts became mixed chimeras. However, none of the TLI-treated hosts became mixed chimeras if the injection of the CAF_1 cells was delayed for 14 or 28 days (Field *et al.* 1997).

The immune responses of the chimeric and non-chimeric mice were examined in the MLR assay. TLI-treated hosts that developed mixed chimerism

Table 4. *Clinical characteristics of renal transplant recipient after 176 months*

(High-resolution DRB1 typings were performed directly on genomic DNA by the sequence-specific oligonucleotide probe–enzyme-linked immunosorbent assay method.)

sex/age (years)	F/68
graft survival (months)	176
period without drugs (months)	145
latest serum creatinine (mg dl^{-1})	1.4
absolute CD4$^+$ T cells (ml^{-1})	1228
absolute CD8$^+$ T cells (ml^{-1})	818
recipient HLA type	A2, A11; B27, B44; DR4 (DRB1 0401), DR5 (DRB1 1103)
donor HLA type	A28, A30; B14, B17; DR2 (DRB1 1503), DR8 (DRB1 0804)

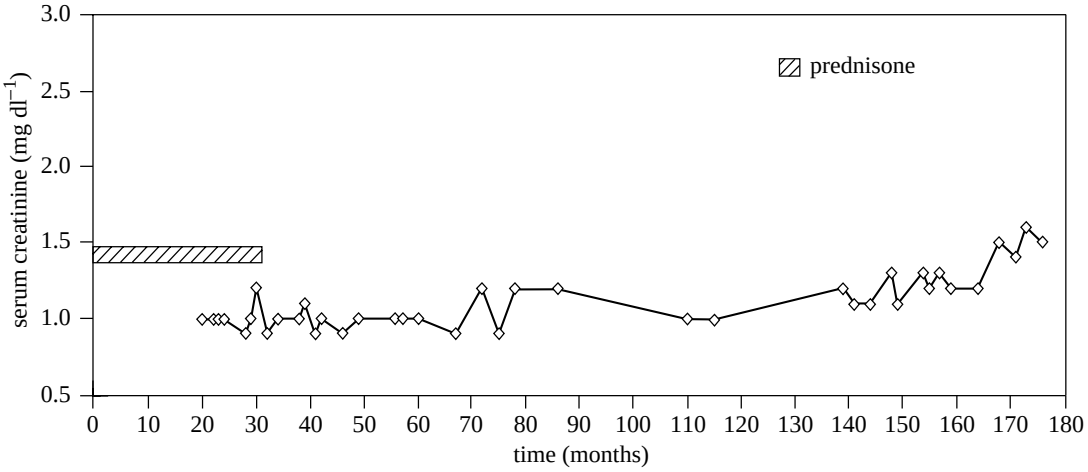

Figure 1. Serum creatinine concentrations during the post-transplantation period. The bar shows the time interval in which immunosuppressive drug therapy (prednisone) was administered. Values were obtained from a single out-patient clinic used after 20 months post-transplantation.

produced more IL-4 and less IFN-γ in response to fully allogeneic A/J stimulator cells as compared with either non-chimeric TLI-treated hosts or non-chimeric control hosts. In contrast, the cytokine response to third-party (SJL) stimulator cells was the same among the three groups (Field *et al.* 1997).

To identify the source of the IL-4 and IFN-γ, the CD4$^+$ and CD8$^+$ T cells were sorted by FACS and the frequency of IL-4-producing Th2 CD4$^+$, IFN-γ-producing Th1 CD4$^+$, and IFN-γ-producing TCl CD8$^+$ T cells was determined using an ELISPOT MLR assay and A/J stimulator cells (Field & Rouse 1995). TLI-treated hosts that developed mixed chimerism contained a higher frequency of Th2 CD4$^+$ T cells, lower numbers of Th1 CD4$^+$ T cells and almost no TCl CD8$^+$ T cells reactive against A/J compared with non-chimeric TLI-treated hosts or non-chimeric control hosts (Field *et al.*, unpublished data). Thus, the development of mixed chimerism, indicating the presence of host-versus-graft tolerance, was associated with immunoredirection; that is, the enhanced generation of IL-4-producing Th2 CD4$^+$ T cells and the inhibited generation of TCl CD8$^+$ T cells. In contrast, rejection of the donor cells leading to a non-chimeric state was associated with preferential development of IFN-γ-producing Th1 CD4$^+$ and TCl CD8$^+$ T cells.

As noted above, TLI-treated mice contained the highest proportion of IL-4-producing CD4$^+$NK1.1$^+$-like T cells immediately after the completion of TLI. Because IL-4 can drive the maturation of Th2 CD4$^+$ T cells (O'Garra & Murphy 1994) and inhibit the maturation of TCl CD8$^+$ T cells (Croft *et al.* 1994), we examined the role of IL-4 in the development of tolerance. TLI-conditioned BALB/c hosts were treated with either anti-IL-4 or irrelevant monoclonal antibody (mAb) beginning at the time of injection of CAF_1 spleen cells. Treatment with anti-IL-4, but not irrelevant mAb, decreased the incidence and the level of mixed chimerism (Field *et al.*, unpublished data). The results indicate that the establishment of mixed chimerism depended on IL-4.

6. HUMAN TOLERANCE AFTER TLI

Twenty-eight cadaveric renal transplant recipients were given 1800 cGy TLI pretransplantation, rabbit ATG post-transplantation, and low-dose prednisone as the sole maintenance immunosuppressive therapy (Saper *et al.* 1988). Graft survival of the group was similar to patients treated with standard cyclosporine-based immunosuppression (Saper *et al.* 1988). Eleven of the TLI-treated transplant recipients had no rejection episodes in the first year after transplantation; 9 out of 11 showed specific unresponsiveness to donor antigens in the MLR. Three recipients were subsequently completely withdrawn from all maintenance immunosuppressive drugs, and the

patients showed donor-specific unresponsiveness to donor antigens in the MLR and cell-mediated lympholysis (CML) assays while off immunosuppression (Strober *et al.* 1989). Therefore, these three patients met the criteria for tolerance: normal graft function; no immunosuppressive drug therapy; and specific unresponsiveness to donor antigens by *in vitro* assays.

One of these patients was recently studied (Strober *et al.* 2000). The 68-year-old recipient had renal transplantation surgery one day after the completion of TLI in August 1984, and received a six-antigen-mismatched cadaveric donor kidney (table 4). The patient received six injections of rabbit ATG intramuscularly on alternate days starting on the day of surgery. Maintenance immunosuppressive therapy was started with prednisone alone at 15 mg day^{-1}, and was tapered to 10 mg day^{-1} five months after transplantation. Fourteen months after transplantation, gradual tapering of the prednisone was undertaken, based on a lack of rejection episodes, a stable creatinine level, and the continued presence of donor-specific unresponsiveness in both the MLR and the CML assays. Prednisone was completely discontinued 31 months after transplantation. No rejection episodes occurred during an observation of 176 months thereafter (figure 1), and the patient had no hospitalizations for infection. Thus, the recipient has remained healthy and operationally tolerant to the cadaveric allograft during the period after drug discontinuation. The results demonstrate that it is feasible to achieve long-term graft survival in humans based on immune tolerance.

7. FUTURE DIRECTIONS

Despite the successful use of immunosuppressive drugs to prevent organ allograft rejection in humans, half of all current cadaveric kidney transplant recipients will reject their grafts within 20 years, and half of those patients who experience an acute rejection episode in the first year post-transplantation will lose their grafts within ten years (Hariharan *et al.* 2000). Therefore, the important goal of achieving transplantation tolerance remains. The development of true tolerance would eliminate the life-long side-effects and financial costs of the immunosuppressive drugs and reduce the risk of graft failure from acute or chronic rejection. The goal of our future research will be to adapt to human cadaveric kidney transplantation the protocol of organ transplantation followed by post-transplantation non-myeloablative TLI, ATG and a donor BM or haemopoietic progenitor infusion, which has been successful in achieving tolerance in rats. The critical advantages of this protocol are (i) the completely post-transplantation conditioning procedures, (ii) the development of tolerance based on stable mixed chimerism, (iii) the protection against GVHD afforded by the regimen, and (iv) the compatibility with currently used immunosuppressive drugs, such as cyclosporine.

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