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Paradoxical early upregulation of intragraft Th1 cytokines is associated with graft acceptance following donor-specific blood transfusion

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Abstract Donor-specific blood transcfusion (DSBT) promotes graft acceptance in certain experimental and clinical situations. However, the exact nature of the operating mechanisms, and in particular the role of Th1/Th2 cytokines, remains controversial. We recently described a fully mismatched rat combination [RA rat (RT1p) to PVG rat (RT1c)] in which a single pre-transplant (Tx) DSBT induces donor-specific tolerance following heart transplantation (Htx). In order to delineate better the role of Th1 and Th2 cytokines in the development of tolerance versus rejection, we studied the intragraft mRNA for Th1 and Th2 cytokines at different time points post-Tx in rejecting and DSBT-tolerized heart grafts. We performed reverse transcriptase–polymerase chain reaction (RT–PCR) to examine intragraft Th1/Th2 cytokines profile. In contrast to findings from previous data, we detected an early (post-Tx day 2) Th1 cytokine upregulation. Th1 downregulation/Th2 immune deviation was observed only at a later period (post-Tx day 30). Consistent with this Th1 early upregulation, a dense mononuclear cellular infiltrate was seen in tolerized grafts, which was equivalent to that seen in rejecting grafts. Despite in vivo unresponsiveness, peripheral lymphocyte derived from tolerant animals proliferated against donor-matched antigens to the same degree as against those of a third party until post-Tx day 30. Altogether, these observations (dense graft cellular infiltrate, early post-Tx Th1 cytokine production, early preserved cellular proliferative responses), suggest that the development of tolerance is preceded by an active Th1 cytokine-mediated immune response. The mechanisms by which an early Th1 response promotes tolerance need to be further studied.

Keywords Cytokine · Tolerance · Donor-specific blood transfusion

Introduction

Tolerance can be induced by various immunomodulatory strategies. In certain models, induction of tolerance is accompanied by a Th1 downregulation/Th2 immune deviation, but data have been conflicting [20]. Donor-specific blood transfusion (DSBT) is a well-established immunomodulatory strategy to promote graft acceptance, clinically and experimentally [3, 15]. However, the nature of the operating mechanisms, in particular the role of the Th1/Th2 cytokines balance, remains controversial. We recently developed a rat model in which a single pre-transplant (Tx) DSBT induces donor-specific tolerance after heart transplanta-

tion (Htx). In order to delineate better the mechanisms underlying DSBT-induced tolerance in that model, we analyzed intragraft mRNA cytokine profiles at different time points, early and late post-Tx, in DSBT-tolerized grafts and in rejecting grafts. In addition, at the same time points, we looked at the degree of mononuclear cell infiltration in DSBT-tolerized grafts and in rejecting grafts. Finally, we examined the peripheral mixed lymphocyte culture (MLC) responses in tolerant recipients.

Materials and methods

Transplant surgical model

Fully mismatched male rats RA (RT1p–RT1Au:B/Dl), weighing 150~200 g, and male PVG rats (RT1c–RT1Ac: B/Dc), weighing 200~250 g were used as donors and recipients, respectively. The heart graft was implanted in the recipient abdomen by standard microsurgical technique [11]. The heartbeat was monitored daily through the abdominal wall by manual palpation. Rejection was defined as complete cessation of heart beat.

Immunomodulation for tolerance induction

A transfusion of 1.5 ml of heparinized donor-specific blood was given to the recipient via the dorsal penile vein on pre-Tx day 12 [9].

Confirmation of donor-specific tolerance

To confirm donor-specific tolerance, we implanted a donor-matched or third-party secondary heart graft in the right cervix on post-Tx day 100 as previously described [8].

Experimental transplant groups

1. Syngeneic control group: RA to RA syngeneic Htx was carried out ($n=6$).
2. Allo-Htx rejecting control group: RA to PVG allo-Htx was carried out with no DSBT ($n=6$).
3. DSBT-tolerant group: RA to PVG allo-Htx was carried out; DSBT was given 12 days before Tx ($n=6$).

Histological analysis for mononuclear cell infiltrate in the graft

The heart grafts were harvested from the DSBT-tolerant group and the syngeneic control group on post-Tx days 2, 5, 9, 30, and 90. Heart grafts from the Allo-Htx rejecting control group were harvested on post-Tx days 2, 5 and 9. The horizontal section of the mid-portion of the specimen was fixed with 10% formalin and embedded in paraffin. Sections 4-μm thick were prepared and stained with hematoxylin eosin (HE). In each group and at each time point, five grafts were analyzed. To quantify the degree of mononuclear cell infiltration, we used a scoring system. Endocardial and perivascular infiltrates were scored as follows: 0, no infiltrate; 1, mild infiltrate; 2, moderate infiltrate; 3, severe infiltrate. Both scores were added, and a 0-to-6 score was reported for each sample.

Real-time reverse transcriptase–polymerase chain reaction

Total RNA was extracted from the heart graft with Trizol (Life Technologies, Gaithersburg, Md.). A constant amount of 1 μg of target RNA was reverse transcribed using 100 U Superscript 2 RT (Life Technologies) at 42 °C for 80 °min in the presence of 5 μmol/l oligo$(dT)_{16}$. Real-time quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) was performed for IFN-gamma, IL-2, IL-10, IL-4 and hypoxanthine phosphoribosyl transferase (HPRT). Briefly, PCR reactions were performed with the ABI Prism 7700 Sequence Detector (Perkin Elmer/Applied Biosystem, Foster City, Calif.).

The system uses the 5 nuclease activity of the Tag polymerase to cleave a non-extendable dual-labeled fluorogenic probe. Fluorescent emission was measured continuously during the PCR reaction. Therefore, PCR amplification and detection were done in a single step. PCR amplifications were performed in a total volume of 25 μl containing 0.5 μl cDNA sample; 1×buffer A (50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, 10 mmol/l EDTA, 60 nmol/l passive reference 1); 200 μmol/l dNTPS; 3–9 mmol/l $MgCl_2$; 100–200 nmol/l of each primer; and 0.625 U AmpliTag-Gold. Each reaction was performed in triplicate wells under the following conditions: 2 min at 50 °C and 10 min at 94 °C, followed by a total of 40 or 45 two-temperature cycles (15 s at 94 °C and 1 min at 60 °C).

For the generation of standard curves, plasmid clones containing a partial cDNA sequence of the target DNA were constructed by the cloning of the corresponding PCR fragments into pGEM Teasy plasmid vector (Promega, Madison, Wis.). The exact identity of the cloned fragments was confirmed by sequence analysis (Pharmacia, Uppsala, Sweden). Serial dilutions from the resulting plasmid clones were used as standard curve, each containing a known amount of template copy number. We derived normalized values by dividing the measured fluorescent emission increase for the transcript of interest by the mean HPRT value for the individual sample. Table 1 shows primer and probe sequences for each cytokine as well as for HPRT [13]. In the syngeneic control group and the DSBT-tolerant group, RNA was extracted from the heart graft on post-Tx days 2, 5, 9, 30, and 90. In the allo-Htx rejecting control group, RNA was extracted in the same manner on post-Tx days 2, 5, and 9. At each point in each group, five grafts were examined.

Mixed lymphocyte reaction

Recipient animals in the DSBT-treated tolerant group were killed at the time of Htx, on post-Tx day 30, or on post-Tx day 90. At each time point, three animals were examined. Mixed lymphocyte reaction (MLR) was performed as described previously. Recipient peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by density gradient centrifugation over Percoll (Pharmacia, Uppsala, Sweden). Single-cell suspensions of the spleen were prepared for the stimulator by the gentle compression of the minced spleen on nylon mesh. The medium used for culture was RPMI with 10% Fetal calf serum (FCS) and 2-mercaptoethanol. Cells were seeded at a concentration of 5×10^5 cells/well in 96-well microtiter plates in replicates of 4. The cells from one animal were divided into four wells per plate; the mean value of counts per min (cpm) for those four wells was used for calculation. Per well, 5×10^5 cells

Table 1 Primer and probe sequences for the cytokines and HPRT. Forward (*FW*) and reverse (*RV*) primers are always located in different exons. Fluorogenic probes (*TP*) are FAM-labeled at the 5 end and TAMRA-labeledat the 3 end. All cDNA sequences were obtained from the Genbank database

Name		Sequence	Amplicon length (bp)
IFN-gamma	IFN-gamma-TP	CCG TCC TTT TGC CAG TTC CTC CAG	299
	IFN-gamma-FW	CCA TGA GTG CTA CAC GCC G	
	IFN-gamma-RV	CAG GTG CGA TTCGAT GAC AC	
IL-2	IL-2-TP	CCC AAG CAG GCC ACA GAA TTG AAA C	271
	IL-2-FW	TGT CCT CCT TGT CAA CAG CG	
	IL-2-RV	TCC AGC GTC TTC CAA GTG AA	
IL-10	IL-10-TP	CTG CGA CGC TGT CAT CGA TTT CTC CC	194
	IL-10-FW	GGT TGC CAA GCC TTG TCA GA	
	IL-10-RV	TTC ACC TGC TCC ACT GCC TT	
IL-4	IL-4-TP	CAA CAA GGA ACA CCA CGG AGA ACG AG	208
	IL-4-FW	TCC ACG GAT GTA ACG ACA GC	
	IL-4-RV	TTG TTC TTC AAG CAG GGA GGT	
HPRT	HPRT-TP	TGA GAG ATC ATC TCC ACC AAT AAC TTT TAT GTC CC	127
	HPRT-FW	TTA TCA GAC TGA AGA GCT ACT GTA ATG ATC	
	HPRT-RV	TTA CCA GTG TCA ATT ATA TCT TCA ACA ATC	

were irradiated (3,000 rad), and stimulator lymphocytes were added to the culture for 96 h. After 72 h of incubation, 50 µl of ^{3}H-thymidine uptake was added per well at a concentration of 10 U Ci/ml. The cells were incubated for a further 12 h, and the ^{3}H-thymidine uptake was determined by standard techniques [14].

Statistical analysis

Fisher's protected least significant difference (PLSD) test and the *t*-test were performed by StatView-J4.02 (Macintosh). $P < 0.05$ was regarded as significant.

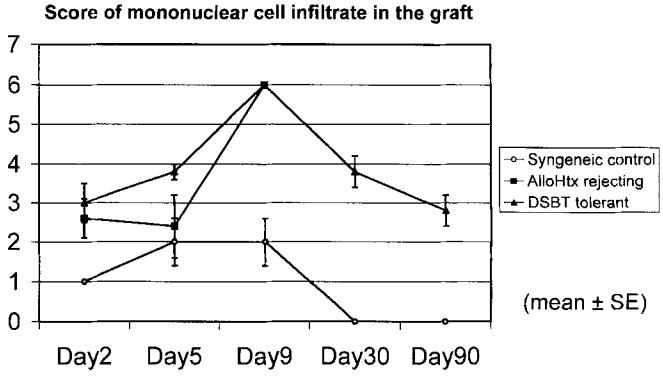

Fig. 1 Scoring for mononuclear cell infiltrate in the graft on post-Tx days 2, 5, 9, 30 and 90

Results

In vivo findings

Primary and secondary graft survival

Syngeneic Htx survived indefinitely (> 140 days). Allo-Htx rejecting controls succumbed to rejection on post-Tx day 9 ± 0.6. However, DSBT prolonged primary graft survival indefinitely (> 140 days) and induced tolerance, as assessed by the survival of the second donor-specific but not the third-party graft. The donor-specific second graft ($n = 5$) survived for more than 40 days, while third party grafts ($n = 1$) (WKAKH) were rejected in 7 days.

Mononuclear cell infiltrate in the graft

Mononuclear cell infiltrate in the graft is shown in Fig. 1. In syngeneic control rats, a transient infiltration was seen on post-Tx days 2–9 but had completely disappeared by day 90. In both the allo-Htx-rejecting and the DSBT-tolerant groups, a substantial infiltration was seen as early as day 2 post-Tx. The extent of that infiltration increased from day 2 to day 9 in both the allo-Htx-rejecting and the DSBT-tolerant groups. On post-Tx day 5, the DSBT tolerant group showed a more dense infiltration than the allo-Htx group, but this difference did not reach significance (DSBT-tolerant group and allo-Htx-rejecting group: score 3.8 ± 0.4 and 2.4 ± 1.9, respectively; $P = 0.1$). In the DSBT-tolerant group, the graft infiltrate declined until post-Tx day 90, but remained higher than in syngeneic controls (DSBT-tolerant group and syngeneic: score 2.8 ± 0.8 and 0 ± 0, respectively; $P < 0.05$).

Intragraft cytokines

Figure 2 shows intragraft cytokines on post-Tx day 2. The DSBT-tolerant group displayed an increased IFN-gamma production compared with allo-Htx-rejecting and syngeneic controls ($P < 0.005$). IFN-gamma production in the DSBT-tolerant group was ten-times

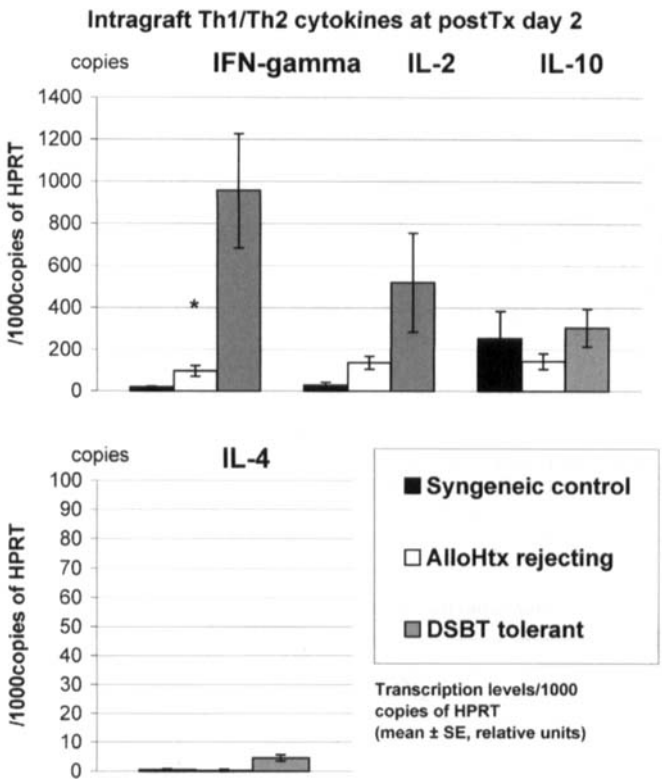

Fig. 2 Normalized transcription levels of T cell-derived intragraft Th1/Th2 cytokines from syngeneic control, Allo-Htx-rejecting group and DSBT-tolerant group on post-Tx day 2

higher than in allo-Htx-rejecting controls. Similarly to IFN-gamma, IL-2 was augmented in the DSBT-tolerant group, but the difference did not reach significance. In contrast, IL 4 and IL 10 did not differ among the groups.

Figure 3 shows the kinetics of each cytokine from post-Tx day 2 to post-Tx day 90 in allo-Htx and in tolerant rats. On post-Tx day 5, all cytokines tested showed higher peaks in allo-Htx controls than in tolerant groups. Both IFN-gamma and IL-10 were significantly suppressed in the DSBT-tolerant group, compared with allo-Htx-rejecting controls (for IFN-gamma, $P < 0.005$; for IL-10, $P < 0.001$). Similarly, IL-2 and IL-4 were lower in the DSBT-tolerant group than in allo-Htx-rejecting controls, although this difference did not reach significance (for IL-2, $P = 0.1$; for IL-4, $P = 0.6$). In the DSBT-tolerant group, IFN-gamma and IL-2 reached their highest values on post-Tx days 9 and 5, respectively, before gradually declining until post-Tx day 90. In contrast, IL-4 and IL-10 in the DSBT-tolerant group continued to rise and reached their highest values by post-Tx day 30, before showing an abrupt decline by post-Tx day 90. To determine the evolution of the Th1/Th2 cytokine balance, we measured the IFN-gamma/IL-10 ratio at different time points in the rejecting and tolerized groups (Fig. 4). The IFN-gamma/IL-10 ratio was highest on post-Tx day 2 in the DSBT-tolerant group. It declined thereafter and reached its lowest value by post-Tx day 30. The ratio in rejecting animals was the lowest at day 2 but then slightly increased until day 9. A Similar tendency was observed in the ratios of IFN-gamma/IL-4, IL-2/IL-10, and IL-2/IL-4 (data not shown). In syngeneic controls, Th1 and Th2 mRNA cytokines remained poorly produced at all time points tested.

Mixed lymphocyte reaction

Proliferative responses were examined for donor and third-party antigens. As shown in Fig. 5, vigorous anti-donor MLR responses were maintained from the time of Tx until post-Tx day 30. These responses were equivalent to the MLR responses against third-party antigen. Hypo-responsiveness was observed on post-Tx day 90.

Discussion

The efficacy of DSBT has long been described in both experimental and clinical situations [3, 15]. However, the operating mechanisms are not elucidated yet. Various mechanisms have been hypothesized: a specific development of suppressor cells, anti-idiotypic antibodies, a non-specific macrophage-mediated immunosuppression, development of regulatory cells, and microchimerism [7, 17, 18]. A Th2 cytokine immune deviation was involved in the development of tolerance following various immunomodulatory strategies – including DSBT –but the exact role of the Th1/Th2 cytokine unbalance in DSBT-induced tolerance remains controversial [1, 4, 5]. Our study was not consistent with previous studies suggesting that a Th2 immune deviation solely is pivotal in the development of tolerance. In contrast, we found an early (day 2) Th1 cytokine upregulation in grafts destined to become tolerized. In addition, IL-10 was more expressed in rejecting grafts at day 5 than in tolerized grafts. However, a Th2 profile (IL-10 and in particular IL-4) was observed, but only at a later period (day 30) in the tolerized grafts.

In addition to this intriguing early intragraft Th1 upregulation, we noted that DSBT-tolerized grafts do contain a pronounced mononuclear cellular infiltrate that is equivalent in intensity to that present with rejecting grafts. This infiltrate was present as early as day 2 post-Tx and reached its maximum value by day 9, before gradually decreasing. This shows that an active immune response is taking place in the graft and probably precedes the development of tolerance, a phenomenon that has already been documented in certain transplant models [2, 10]. The question arises as to the nature of that "protective" infiltrate and the operating

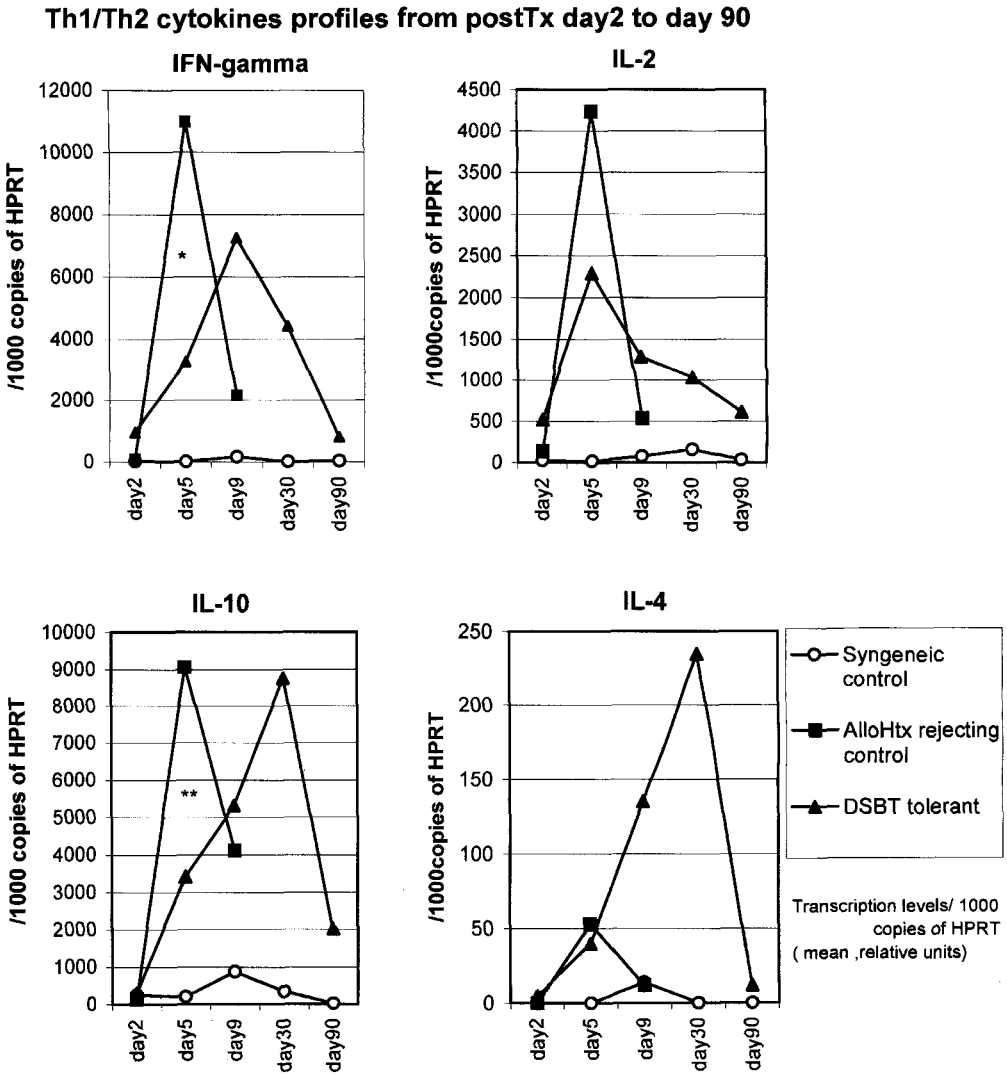

Fig. 3 Intragraft Th1/Th2 cytokine profiles from post-Tx days 2 to 90

mechanisms. First, graft-infiltrating cells may undergo apoptosis in certain situations where lymphocytes are abnormally or exceedingly stimulated. This phenomenon has been particularly well documented in certain liver transplant models in rodents [16]. In those models, tolerance is accompanied by an intragraft Th1 cytokines upregulation [2, 16]. Th1 cytokines, which are necessary for lymphocyte proliferation, are paradoxically known to cause lymphocyte apoptosis [2]. Thus, despite a severe infiltration, the liver graft escapes from rejection via a Th1-dependent mechanism referred to as "activation-induced cell death". Activation-induced cell death has also been shown to operate in a model where tolerance is induced by leukocyte infusion [19]. We are currently investigating whether activation-induced cell death intervenes in our model.

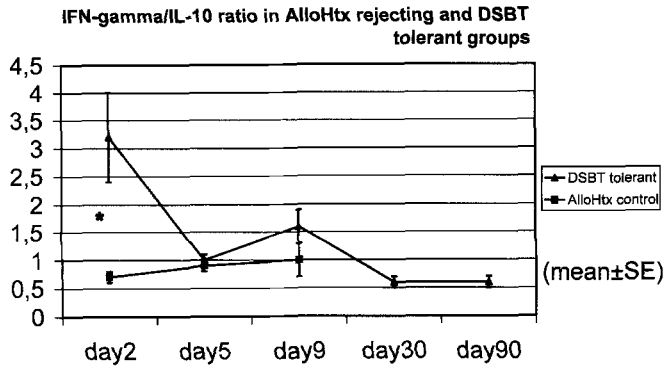

Fig. 4 Evolution of intragraft Th1/Th2 cytokine balance in AlloHtx-rejecting and DSBT-tolerant groups represented by the IFN-gamma to IL-10 ratio at each time point

An alternative possibility is that the infiltrating cells in tolerized grafts represent, at least in part, a population of regulatory cells. Regulatory cells have been shown to mediate tolerance in some models. Several studies have demonstrated the ability of T cells to transfer tolerance from tolerant to naïve animals, a phenomenon termed

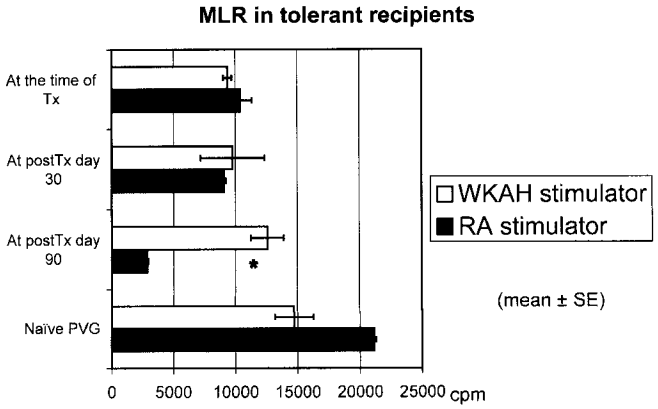

Fig. 5 MLR in tolerant recipients at the time of Tx, on post-Tx days 30 and 90

infectious tolerance [12, 21]. In these studies, the source of transferred T cells was the spleen. More recently, however, in a model of regulatory T cell-mediated tolerance, it was shown through techniques of T cell receptor gene polymorphism, that T cell clonal expansion preferentially took place within accepted grafts themselves rather than in the spleen or the draining lymph nodes [22]. These authors hypothesized that regulatory T cells originate and expand at the graft site early on and migrate toward the peripheral lymphoid organs thereafter. If this hypothesis is true, it is not surprising to find an early transient intragraft Th1 cytokine upregulation that may contribute to expansion of regulatory cells. In fact, an early Th1 cytokine upregulation was observed in a previous study showing the role of regulatory cells in DSBT-induced tolerance. One graph in that work clearly shows a very early Th1 cytokine upregulation in the graft, although this fact was not discussed in that paper [6]. Further studies are in progress to determine the phenotype of both infiltrating cells and circulating cells, and particularly the role of CD4+ CD25+ cells in DSBT-induced tolerance.

Finally, we found preserved MLR responses in vitro, against donor-matched antigen, until post-Tx day 30. Preserved peripheral proliferative responses contrast with the observed in vivo unresponsiveness, a phenomenon previously referred to as split tolerance. This observation indirectly supports the hypothesis that the induction phase of tolerance is probably taking place in the graft itself. However, by day 90, hypo-responsiveness is also present peripherally.

That an active Th1 cytokine-dependent immune process may be necessary for the induction of tolerance has important implications for the judicious use of immunosuppression. In fact, it has been shown that certain immunosuppressive agents – particularly steroid and calcineurin inhibitors – though effective at blocking rejection, may equally block tolerance and trigger rejection under certain circumstances [2, 9, 10]. For example, we showed earlier in an analogous DSBT tolerance model that administration of steroids at the time of DSBT or at transplantation triggers rejection [9].

In conclusion, we report a paradoxical, early intragraft Th1 upregulation in a model of DSBT-induced tolerance. This state of tolerance was associated with the presence of a dense mononuclear infiltration within the graft and with a preserved peripheral proliferative response at early post-Tx. We hypothesize that an intragraft early Th1 cytokine upregulation is a prerequisite for the development of tolerance. That the development of tolerance is preceded by a dynamic/active Th1 response may have important clinical implications for the use of immunosuppressive drugs that influence Th1 cytokine production. Further studies using IFN-γ neutralizing antibodies will be needed to determine whether this early Th1 cytokine upregulation is a simple marker of tolerance or a prerequisite for the development of tolerance.

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