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Circumvention of natural killer cell and T-cell mediated allogeneic target killing with tacrolimus (FK506) in small bowel transplantation related graft-vs-host disease

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Abstract The role of simultaneous donor-specific transfusion of unprocessed cellular bone marrow (BM) together with solid organ transplantation, a postulated concept to achieve long-term graft acceptance, was investigated in an experimental setting of semiallogeneic transplantation of parental small bowel (SBTx) to F1 hybrids. The established graft-vs-host (GvH) model revealed that simultaneous transfer of SB/BM substantially enhanced GvH-mediated immune responses in recipient target organs, e.g. skin, gut, and liver. In comparison to isolated SBTx, animal survival decreased from 16.1(± 0.9) to 10.1(± 0.8) days after additional BM transfusion, $P < 0.001$. Severe tissue injury of GvH-susceptible target organs in the setting of simultaneous SB and BMTx was associated with significant changes in recruitment and tissue distribution of NKR-P1$^+$ cells during the GvH-related proliferative immune response. Tacrolimus effectively suppressed these initial events and prevented recipient animals from clinically and histologically observed damage caused by GvH disease.

Key words Small bowel transplantation · Tacrolimus · Microchimerism · Natural killer cells · NKR-P1 receptor

Introduction

After the causal relationship of hematopoietic chimerism to drug-free immunological tolerance had been established by Billingham, Brent, and Medawar [1], their findings were implicated in further experimental [2] and clinical settings [3]. The idea to transplant cells of bone marrow or lymphatic origin and solid organs from the same donor simultaneously was based on the assumption of augmented mutual recognition by two-way migration of "passenger leucocytes" exchanged between graft and host. Besides, the concept was based on the detection of donor-derived cells in tissues of human recipients with functioning kidney [4] and liver [5] grafts, 10–30 years posttransplantation. The chimeric state in these patients had developed, irrespective of the various immunosuppressive protocols applied, while some patients had been off all immunosuppressive medication. In contrast, there is now a large body of evidence from clinical and experimental data which outline the inherent difficulty in separating cause from effect in the framework of cellular chimerism [6–8]. The study of Bushell et al. [7] indicated that the short-term persistence and presentation of donor-derived antigen, rather than the development of microchimerism, is the critical factor in the induction of operational tolerance.

So far not focused on as a relevant subject of discussion in the context of combined bone marrow plus solid organ transplantation is a non-adaptive killing mechanism also designated "allogeneic lymphocyte cytotoxicity" (ALC). ALC is mediated by natural killer (NK) cells (NKR-P1$^+$, CD3$^-$ cells) and characterized as allospecific lysis of lymphocytes and cells of hematopoietic origin. After allogeneic challenge of an MHC-disparate host with either one of these cells, host NK cells eliminate the non-self target cells within 6 to 12 h and with-

out the need for a prior antigen-specific sensitization step [9, 10]. Activation of the host NK cell population is associated with increased release of important cytokines, such as γ-IFN, TNF-α, and IL-2 [11, 12]. Early NK cell production of IFN-γ is necessary to promote MHC class II antigen processing and presentation by macrophages and the development of Th1 T cells [13]. This experimental study was designed to investigate the impact of coincidentally administered donor bone marrow (BM) in a graft-vs-host (GvH) model of small bowel transplantation (SBTx) on: (1) the clinical course and histological grade of graft-vs-host disease (GvHD) in lymphatic and non-lymphatic targets, (2) the migration and tissue distribution of NK cells, (3) the immunoregulatory role of NK cells on T-cell proliferation during the initial recipient immune response, and (4) the T-cell-mediated immune responses of F1 recipients under immunsuppressive treatment with tacrolimus.

Material and methods

Animal groups

A GvH model was established by heterotopic transplantation of male DA[Rt1^{aaavl}] parental small bowel on F1 hybrids, a crossing between male DA and female LEW[Rt1^{l}] inbred rats weighing between 160 and 250 g. Animals were raised and maintained under conventional animal facilities and screened routinely for common rat pathogens. Heterotopic SBTx was performed as previously reported [14]. Bone marrow cells were obtained from femurs, tibiae, and humeri of sacrified donor strain (DA) rats. After flushing the bones through a 100 μg Nytex filter with a sterile needle and Hank's balanced salt solution (HBSS) cells were washed three times and counted. T-cells were not depleted. After suspension of 5×10^7 bone marrow cells in 1 ml of HBSS, the transplant was infused into the penile vein of the F1 recipient rat. The following experimental groups ($n = 6$) were included into this study: (1) DA(SB)> F1, no treatment (NT), (2) DA(SB + 5×10^7 BM cells) > F1, (3) DA(SB) > F1, FK 506, 1 mg/kg body weight, day 0–14, i.m., and (4) F1(SB) > F1, NT.

Histology

Tissue specimens were harvested on postoperative days (POD) 3, 7, 10, and 14 after HSBTx. Note, however, that in group II only two animals lived for 12 days and one animal for 14 days. These animals were pooled for histological comparison at POD 14. Tissue specimens were sliced into small pieces, either snap-frozen in liquid nitrogen and stored at –80 °C or subjected to 10 % buffered formaldehyde, embedded in paraffin, cut into 4 μm sections, and stained with hematoxylin and eosin. Immunohistological analysis was performed using an indirect APAAP-staining technique. Histological evaluation of the tissue specimens was performed by two observers who had not been informed of the experimental group by grading six different sections of each specimen. Hereby, the number of positive staining cells were counted in serial tissue sections using the photographic view finder of a Zeiss Photomicroscope at 630× magnification under oil immersion (area = 0.030 mm^2) and were expressed as numbers of positive cells per unit tissue area. Conventional criteria were used to diagnose four different grades of GvHD: (1) grade 0 (none), (2) grade I (moderate); (3) grade II (acute), and (4) grade III (severe), as outlined in Tables 1, 2.

Table 1 Degree of graft-vs-host disease according to clinical and autoptic findings following heterotopic small bowel transplantation. For appropriate classification, each animal had to demonstrate at least three of the listed items for grades I, II, or III. Animals which demonstrated two features of two different columns were assigned to the appropriate grade of GvH according to the histological pattern of tissue lesions (*MLN* mesenteric lymph nodes)

	Grade I	Grade II	Grade III
Weight loss	0–10 %	10–20 %	> 20 %
Appearance	Redness of eyes paws, snout, diarrhea	Piloerection	Listlessness, hunched posture
Spleen index	1–2	2–5	> 5
Volume of MLN	1–1.5	1.5–3	> 3

Preparation of splenocytes and mesenteric lymph nodes

Donor and recipient mesenteric lymph nodes and recipient spleens were excised, minced, and pressed gently through a 60-gauge-mesh stainless steal screen into HBSS. Lymph node lymphocytes and splenocytes were obtained by centrifugation on Ficoll-Hypaque gradients (density = 1.077) at 300 × g for 20 min at room temperature. After collection of the mononuclear cells from the gradient interface they were resuspended in RPMI-FBS, washed three times, and immediately used in the experiments described.

Mixed Lymphocyte Reaction

Freshly isolated spleen cells from control and experimental rats of groups 1 to 4 were cultured in triplicate in 0.2 ml complete medium at a concentration of 1×10^5 cells/well in the presence or absence of phytohemagglutinin (PHA) in 96-well flat-bottomed microplates together with 3×10^5 irradiated (2000 rad) stimulator cells. After 96 h of incubation at 37 °C in 5 % CO_2/95 % air, cells were pulsed with 1 μCi of [^{3}H]thymidine and subsequently harvested for measurement of radioactivity in a liquid-scintillation spectrometer 18 h later.

Statistics

Animal survival rates were calculated according to Kaplan-Meier life-table analysis using the statistical program package of Astute Module 1, Statistics Add-in for Microsoft Excel, University of Leeds, UK. Differences in survival between the various groups were assessed using the generalized Savage (Mantel-Cox) log-rank test. Deaths secondary to technical complications (with functioning grafts) were censored. The F-test was used to test for equality of the variances of the two samples to be compared. Depending on the results of the F-test, Student's *t*-test for equal or unequal variances was used for further analysis.

Table 2 Degree of graft-vs-host disease according to histological findings following heterotopic small bowel transplantation. Each animal was classified histologically according to histological evaluation of eight different specimen, small bowels *(SB)*, mesenteric lymph nodes *(MLN)*, and peyer patches *(PP)* of donor and recipient origin and skin and liver of the host. Grade I, II, or III was diagnosed, if in four of the eight analyzed specimen the designated histological features were present

	Grade I	Grade II	Grade III
SB mucosa	Reduction of villus height, mononuclear cellular infiltrate of lamina propria	Crypt necrosis, clubbing of villi, sloughing of villi, apoptotic cells 2–4/6 crypts	Necrotizing enteritis, epithelial replacement with granulation tissue, apoptotic cells > 4/6 crypts
MLN, PP, spleen	Appearance of histocytoid cells, plasma cells, immunoblasts	Immunoplastic proliferation, circumscribed lymphoid depletion	Loss of follicular architecture, stromal fibrosis, necrosis, depletion of lymphocytes
Skin	Spongiosis and vacuolation of upper dermis	Cell necrosis (apoptosis), lymphocytic infiltration	Polymophonuclear cell infiltration
Liver	Mild portal tract infiltration with mononuclear cells (< 10)	Enhanced infiltration of portal fields with mononuclear cells (10–20)	Liver parenchyma and portal fields are infiltrated, bile duct lesions, endothelialitis of central veins

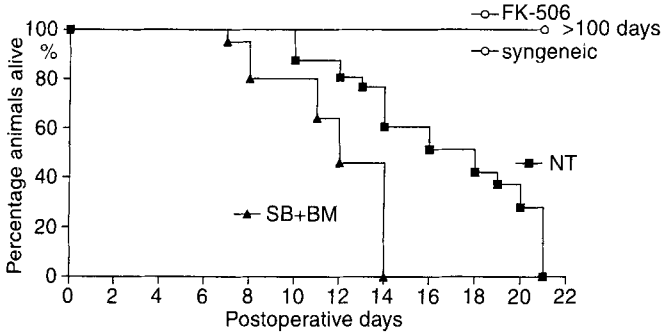

Fig. 1 Survival after DA-parental SBTx of untreated (NT; $n = 24$), DA small bowel plus bone marrow transplanted (SB + BM; $n = 9$), tacrolimus (FK506; $n = 10$) immunosuppressed, and F1 small bowel transplanted F1 recipient animals (syngeneic; $n = 10$) is depicted. Simultaneous transplantation of parental DA small bowel and 5×10^7 bone marrow cells significantly decreased the mean survival of 16.1 (± 0.9) days in group I to 10.1 (± 0.8) days, *$P > 0.001$. Median survival was determined by the method of Kaplan and Meier.* Univariate comparisons of survival was calculated with the log-rank test

Results

Simultaneous transplantation of unprocessed DA-parental BM plus SB into F1 recipients severely impaired the hosts immune response to cope with GvH-related donor immunogenicity. As Fig. 1 illustrates, mean survival of F1 hybrids was significantly reduced to 10.1 (± 0.8) days in comparison to 16.1 (± 0.9) days after isolated SBTx. Individual survival of the four different investigated groups is summarized in Table 3. Three F1 animals which received combined DA BM/SB were immunsuppressed with FK 506 according to the protocol of group III. Similar to F1 hybrids of groups III and IV, these animals did not suffer from GvHD and showed long-term graft acceptance > 100 days (data not shown). Beside the observed decrease in life expectancy, animals of group II presented with significantly higher grades of GvHD and a more rapid onset and clinical progression of GvHD including severe diarrhea, weight loss, hunched posture, and massive enlargement of the spleen and MLNs, as catagorized in Tables 1, 2. In conjuction with BMTx, non-lymphatic target tissues, such as recipient gut, skin, and liver, were profoundly impaired as GvHD progressed. Skin lesions included vast damage to epidermis and upper dermis with concomitant infiltration of mononuclear and polymorphonuclear cells. Liver injury related to the donor-derived cytotoxic immune attack showed characteristic features of mononuclear cell infiltration of portal fields, bile duct lesions, and, in some cases, substantial damage to vessel endothelium. Table 4 summarizes the most important data on clinical and histological changes gathered from animals which succumbed to GvHD.

Immunohistochemical analysis of NKR-P1$^+$ cells by use of 3.2.3-mAb in various splenic compartments at POD 3 and 10 was performed to investigate cell frequency, distribution, and migration pattern of this cellular lymphocyte subset. Autographic studies from [^{3}H]-uridine-labeled T- and B cells have traced these lymphocytes for a 24 h period after i. v. injection and revealed that allogeneic lymphocytes had been destroyed within the T-cell areas of both the lymph nodes and the spleen white pulp at 3 h after injection. Fragments of the destroyed cells could be detected inside large non-lymphoid cells of the lymph node paracortex and spleen periarteriolar lymphocyte sheath (PALS) and not in the marginal zone and red pulp of the spleen, where NK cells normally reside [15, 16]. Therefore, our interest was focused on the distribution pattern of NKR-P1$^+$ cells within various, functionally distinguishable, splenic compartments in the four different experimental settings as summarized in Fig. 2 A, B. At POD 14, groups I and II differed significantly from groups III and IV in

Table 3 Survival after small bowel transplantation from DA to F1 (DA × LEW) rats

Group	Donor	Recipient	Treatment	Number	Survival (days × number)	Mean ± SEM
I	DA	F1	SB	24	10 × 4, 12 × 2, 13 × 1, 14 × 4, 16 × 2, 18 × 2, 19 × 1, 20 × 2, 21 × 6	16.1 ± 0.9
II	DA	F1[a]	SB + BM	9	7 × 1, 8 × 3, 11 × 2, 12 × 2, 14 × 1	10.1 ± 0.8[b]
III	DA	F1[c]	SB + FK506	10	> 100 × 10	> 100
IV	F1	F1	SB	10	> 100 × 10	> 100

[a] F1 recipients received DA-parental small bowel and 5×10^7 unprocessed bone marrow cells, i.v.
[b] $P > 0.001$ for group I vs group II (according to Mantel-Cox log-rank test)
[c] F1 recipients received DA-parental small bowel and were immunosuppressed with 1 mg FK506/kg body weight, i.m. from day 0 to day 14

Table 4 Effect of simultaneous SB/BMTx on the grade and clinical course of GvHD

Experimental group *n* (%)	Clinical and histological grade of GvHD				First signs of GvHD (day ± SD)	Duration of GvHD (days ± SD)
	None	I	II	III		
No treatment ($n = 24$)	5 (20.8)[a]	6 (25)	6 (25)	7 (29.2)	10.2 (± 1.2)	7.3 (± 2.4)
DA(SB + BM) ($n = 9$)	None	1 (11)	2 (22)	6 (67)	5.7 (± 0.7)[b]	3.75 (± 0.7)[c]

[a] These 5 animals without signs of GvHD suffered and died from peritonitis and sepsis due to a paradoxical immune response with substantial injury of their parental small bowel grafts and mesenteric lymph nodes
[b] Combined SB + BMTx treated animals suffered from more severe and progressive GvHD with significant earlier appearance of GvHD symptoms as compared to host animals without additional transfer of DA-parental bone marrow cells ($P < 0.001$)
[c] The enhanced severity of GvHD in F1 hosts after simulaneous SB + BMTx was also mirrored in a shorter clinical course of GvHD until death in these animals as compared to untreated animals ($P < 0.0001$)

NK cell density, both in the area around the central artery of germinal follicles and in the area of the PALS. There is indirect evidence that in the BM/SB group high numbers of these NK cells are of F1 origin since flow cytometric analysis showed that 35 % of the gated F1 splenocytes were OX-3$^+$, a LEW MHC class II antigen specific marker which does not cross-react with DA epitopes. Among the OX-3$^+$ cells, 20 % were NKR-P1$^+$/OX-3$^+$ and 9 % NKR-P1$^+$/OX-3$^-$, in contrast to group I animals were no NKR-R1$^+$/OX-3$^+$ double-positive cells were detected among the 18.6 % OX-3$^+$ lymphocytes, data not shown. Altogether, NKR-P1$^+$ cells comprised 15.9 % (± 2.4) of gated splenocytes in group II in contrast to 12.3 % (± 9.9) NK cells in group I. This clearly indicates that additional transfer of donor-specific bone marrow increases the percentage of F1 NK cells and stimulates the upregulation of MHC class II molecules on the surface of these cells. The large amount of NK cells entering the spleen at POD 14 via the central artery (Fig. 2 A), basically homed to the red pulp medullary areas in the spleen. Conversely, at POD 3, high numbers of NK cells were recognized within the marginal zone in all allogeneic groups (I–III) and apparently differed from syngeneic animals where NK cells basically resided in the red pulp. The fact that high densities of NK cells were found in those areas where recirculating lymphocytes normally pass during the initial "proliferative" phase of GvHD (marginal zones of lymph nodes and spleen) emphasize findings of others [15] that lymphocyte killing on one side and cell fragmentation and antigen presentation on the other side might not be spatially and temporally coinciding events. Immunosuppressive treatment of F1 recipients efficiently inhibited NK cell activity and proliferation, as mirrored in Fig. 2 A, B which demonstrate that the migration pattern and distribution within the spleen of FK-treated rats is very similar to syngeneically transplanted animals.

In addition to these findings, T-cell-mediated proliferation responses of freshly harvested F1 splenocytes to F1 syngeneic responder cells was investigated using one-way mixed lymphocyte cultures. Figure 3 gives evidence that combined SB/BMTx led to increased anti-F1 responses, as early as 3 days after transplantation of DA-parental grafts. These results are consistent with the early onset and rapid progression of GvHD in this group, as described above. The increasing percentage of DA immunocompetent cells among recipient splenocytes, hence, considerably enhanced the T-cell response against host stimulator cells at POD 10. Tacrolimus treatment significantly decreased anti-host-directed T-cell activity after DA-parental SBTx. However, whereas

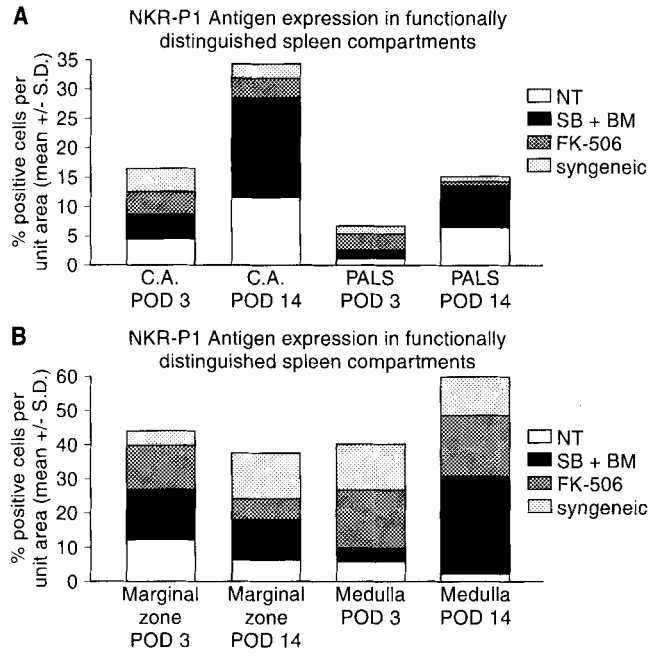

Fig. 2 A, B Four, anatomically and functionally distinguishable compartments within the spleen of F1 recipients were analyzed and evaluated independently by two observers without knowledge of the experimental groups. Each column presents the mean of six different areas of 0.030 mm^2 for each designated structural unit, using a Zeiss photomicroscope at 630 × magnification under oil immersion. Values of six animals per group have been averaged and are expressed as numbers of positive cells per unit tissue. (*C.A.* Central artery of a germinal follicle, *PALS* periarteriolar lymphocyte sheath)

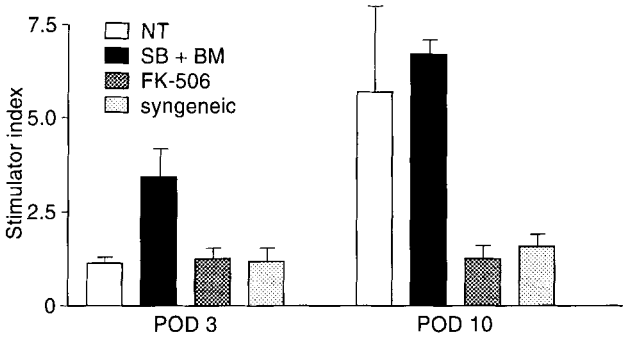

Fig. 3 [^{3}H]Thymidine incorporation assays were performed. One-way MLR was performed with freshly isolated F1 splenocytes used as responder cells and co-cultured with DA or F1 irridiated (2000 rad) stimulator cells. Varying numbers of stimulator cells were mixed in triplicate with F1 responder cells. Columns represent the mean of 6–10 experiments consisting of two pooled rats per experiment. Stimulator index was calculated as E/C, where E = cpm of [^{3}H]thymidine-treated responder cells cocultured with irridiated stimulator cells and C = cpm of [^{3}H]thymidine-treated responder cells not cocultered with stimulator cells. Notice the increased anti-F1 proliferation response in group II animals at POD 3 and 10

splenocytes from F1 recipients of the syngeneic group were still able to proliferate in response to third party stimulator cells, this response was suppressed in FK 506 treated animals (not shown).

Discussion

Coutinho [17] and Cohen [18] have outlined the importance of sustained lymphocyte activity in complex communicating networks as a prerequisite to acquiring transplantation tolerance at a postthymic peripheral level. According to their views, the immunological step for "self"-recognition is linked more to activation of "connected" lymphocytes than to suppression of alloreactive clones. In this framework of operating immune networks, successful transfer of network components, defined by the donor, is required to assure donor-specific tolerance to the graft. According to Starzl et al. [5] this might be achieved by augmentation of cell chimerism in conjunction with non-specific immunosuppression. He speculates that unspecific immunosuppressive drugs exert a "permissive" effect, allowing for mutual recognition, activation and, finally, non-reactivity of engaging donor and recipient derived immunocompetent cells. In this context, the herein presented study explored the significance of increased graft immunogenicity by additional donor-specific BMTx on non-adaptive and cell-mediated immune response patterns in a GvH model of SBTx. NK cells, by virtue of their ability to elicit ALC and various cytokines, function as regulators of myeloid precursor cells and of extramedullary myelopoiesis in the spleen [19]. In fact, we were able to demonstrate that additional transfer of donor-derived BM significantly increased the percentage and density of NKR-P1$^+$ cells at sites of antigen recognition (marginal zones, PALS) in close vicinity to interdigitating dendritic cells, concomitant with MHC class II antigen upregulation on NK cells, at POD 10 and later. Besides, BMTx stimulated "homing" mechanisms of NK cells to the medullary areas of recipient spleens via the central follicle arteries. The strategic position of NK cells close to or within the vascular network implicates a high statistical probalility of encountering donor-specific allografted BM and stem cells, which both have been demonstrated to be susceptible to NK cell killing [20]. Our own observations conclusively showed that the initial host NK cell activity following an allogeneic challenge strictly correlated with the degree of GvHD, with F1 host survival, and with anti-parental T-cell responses (submitted for publication). Conversely, BMTx coincided with increased levels of anti-host T-cell activity, suppressed under FK 506 treatment. Whether activation of host activity by BMTx is related to augmented long-term chimerism cannot be answered with this study. However, higher initial lysis of alloantigeneic cells gen-

erated by host NK cells led to high levels of processed alloantigen, a crucial factor in the induction of operational tolerance [7].

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