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In vivo depletion of NKR-P1 positive cells in the recipient prior to small bowel transplantation enhances graft-versus-host disease (GvHD) in the rat

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Abstract Recent evidence for major histocompatibility complex (MHC) class I antigen-directed recognition mechanisms of natural killer cells (NKs) have revived interests in investigating non-adaptive immune responses in the framework of solid organ transplantation. A semiallogeneic rat model of heterotopic small bowel transplantation (HSBTx) from male DA parental to male F1 hybrid rats (DA × LEW) was established to investigate the role of host NKs to attenuate graft-versus-host (GvH)-mediated immunosuppression and tissue injury. By use of anti-NKR-P1 monoclonal antibody (mAb) 3.2.3, host NKs were depleted effectively in vivo after triple intraperitoneal injection prior to HSBTx. In contrast to non-depleted animals, an initial lack of NK activity in F1 hosts significantly decreased the mean survival ($P < 0.01$) and substantially enhanced graft-versus-host disease (GvHD)-related damage to lymphoid and non-lymphoid target organs. These findings emphasize the important immunoregulatory role of host NKs during the early onset of GvHD.

Key words Graft-versus-host disease (GvHD) · Allogeneic lymphocyte cytotoxicity · Natural killer cells · Small bowel transplantation · Major histocompatibility complex (MHC)

Introduction

Natural killer cells (NKs) are endowed with clonally distributed surface receptors which recognize major histocompatibility complex (MHC) class I alleles on potential targets such as tumor cells, virally infected cells, and allogeneic lymphocytes [1–5]. Two genetically distinct NK cell receptor-like molecules have been cloned and categorized and were designated as belonging to the NKR-P1 and Ly-49 murine gene families [6, 7]. Functionally, both receptor molecules were found to deliver opposing signals, with activation of NK cells by NKR-P1 engagement and suppression of NK-mediated killing after encounter of Ly-49 molecules with specific MHC class I epitopes on target cells [6, 8]. In the context of allograft rejection, it is important to note that rapid elimination of MHC-disparate lymphocytes in unsensitized recipient animals, i.e., allogeneic lymphocyte cytotoxicity (ALC), was shown to be conferred by NKs and thus reveals an important mechanism of the non-adaptive immune response to recognize and defeat allogeneic target cells [9, 10]. Independent studies in the mouse have described elimination of parental target cells by F1 hybrids, aligning ALC with "hybrid resistance" against parental allogeneic bone marrow cell grafts [11, 12]. The large amount of lymphatic tissues together with the high frequency of immunocompetent cells transferred in small bowel transplantation bears a potential risk of inducing graft-versus-host disease (GvHD) in the recipient organism. It was a major objective of this experimental study to investigate the immunoregulatory role of the host's initial NK activity as a first line barrier defense directed against donor-specific lymphocytes invading recipient tissues after heterotopic small bowel transplantation (HSBTx). By use of the highly specific monoclonal antibody (mAb) 3.2.3, which

engages with the lectin-binding protein NKR-P1 on rat NK cells [13], in vivo depletion of the host NK population can efficiently be achieved [14]. The lack of initial host NK activity, in conjunction with donor-imposed graft-versus-host (GvH)-related immunosuppression and subsequent tissue injuries in the framework of semiallogeneic HSBTx are described and analyzed immunofunctionally.

Materials and methods

Animals and transplant procedures

Male DA (RT1.^{aaav1}) and male F1 hybrids, a crossing between male DA and female LEW (RT1.^{1}) inbred rats, weighing 160–250 g were raised and maintained at the Institute of Immunology, University of Kiel, Germany and kept under conventional animal facilities. Animals were routinely screened for common rat pathogens and given water and rat chow ad libitum. The experimental protocol was approved by the Ministry of Nature, Environment and Country Development, Schleswig-Holstein, Germany. HSBTx was performed as described previously [15].

Animal groups and experimental points

HSBTx was performed between male DA and male F1 hybrid rats in the following groups: (1) DA > F1, no other treatment (NT), $n = 24$; (2) DA > F1, pretreated with ascites mAb 3.2.3, 50 µl intraperitoneally (i.p.), at days − 2, − 1, and 0, $n = 10$; and (3) F1 > F1, syngeneic control, $n = 10$. Intestine graft survival was considered synonymous with recipient death or sacrifice before then because of a moribund state. In addition, six animals in each group were sacrificed selectively at postoperative days (POD) 3, 7, 10, and 14 and analyzed histologically. Flow cytometric analysis, cytotoxicity assays, and MLRs were performed from animals sacrificed selectively at POD 3 and 10 after HSBTx. Control experiments included triple injection of an irrelevant isotype-matched mAb prior to HSBTx of F1 hosts and in vivo depletion with mAb 3.2.3 and sham operation of F1 recipients (data not shown).

Histology

Conventional criteria were used to diagnose GvHD and rejection by use of paraffin-embedded tissue specimens, cut at 4 µm, and stained with hematoxylin and eosin (H&E).

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 steel screen into Hank's balanced salt solution (HBSS). Lymph node lymphocytes and splenocytes were obtained by centrifugation on Ficoll-Hypaque gradients (density = 1.077 g/ml) 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 3 times, and immediately used in the experiments described.

Flow cytometric analysis

Cell populations of recipient spleens and peripheral blood leukocytes were prepared for fluorescence-activated cell sorter (FACS) analysis. Directly conjugated antibodies were matched with an irrelevant, non-reactive isotype-matched control with the same fluorescent tag. Monoclonal antibodies used in cell sorting experiments were all directly labeled. Cells (1×10^6/ml) were preincubated for 10 min with saturating concentrations of anti-Fcγ receptor to block unspecific Fc receptor binding. Incubation with fluorescein isothiocyanate (FITC)-coupled monoclonal antibodies for 30 min followed. After a double washing step in FACS medium, cells were incubated with PE-labeled monoclonal antibodies at saturating concentrations if double staining was performed, washed twice, and mounted in phosphate-buffered saline (PBS) for cytofluorometric analysis. Staining was assessed using a FACStar/Plus flow cytometer (Becton Dickinson, Mountain View, Calif.).

Pretreatment of F1 animals with mAb 3.2.3 (anti-NKR-P1)

Ascites fluid was produced in Balb/c mice bearing the hybridoma 3.2.3 as a source of anti-NKR-P1 mAb. Aliquots of 50 µl of ascites fluid in 0.5 ml HBSS were used on 3 consecutive days for suppression of NK activity in recipient rats, as determined by Yac-1 target cell lysis. mAb 3.2.3 contained 10 mg/ml protein, of which 30% was analyzed to be monoclonal IgG1.

Target cell preparation

To assess target cell lysis in cell-mediated cytotoxicity assays, the rat NK-sensitive Moloney virus-induced lymphoma cell line YAC-1 of A/Sn mouse origin was used. This cell line was maintained in suspension culture in RPMI 1640 and contained 10% (v/v) fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (Sigma, Darmstadt, Germany). Target cells were labeled by incubating 1×10^6 cells in 0.2 ml medium with 200 µCi $Na_2{}^{51}CrO_4$ for 60 min at 37 °C for 1 h.

Cell-mediated cytotoxicity assays

Variable numbers of effector cells were added at five different effector to target cell ratios ranging from 50:1 to 3.125:1 to 10^4 labeled target cells in U-bottomed, 96-well plates in a final volume of 0.2 ml complete medium. The plates were centrifuged at 40 *g* for 3 min and then incubated at 37 °C in a humidified chamber containing 5% CO_2. After 4 h the plates were centrifuged at 400 *g* for 5 min, and 0.1 ml of supernatant was collected and radioactivity estimated in an autogamma counter. Spontaneous ^{51}Cr release was determined by incubation of target cells with medium alone. The percentage of specific lysis was calculated as 100 (E − S/T − S) where E is the experimental ^{51}Cr release, S is the spontaneous release, and T is the total incorporated counts.

Mixed lymphocyte reaction

Responder spleen cells (1×10^5) from control, NK-depleted or non-depleted rats were cultured in triplicate in 0.2 ml complete medium together with 3×10^5 irradiated stimulator cells (2000 rad) in the presence or absence of phytohemagglutinin (PHA) in 96-well flat-bottomed microplates. After 96 h of incubation of 37 °C in 5% CO_2/95% air, cells were pulsed with 1 µCi of

Table 1 Effect of monoclonal antibody *(mAb)* 3.2.3 pretreatment on progression and extent of clinical graft-versus-host disease (GvHD)

	DA (SB) > F1 (no treatment)	DA (SB) > F1 (mAb 3.2.3 pretreatment)	Level of significance (*P* values)
First signs of GvHD (days)	10.2 (± 1.2)	7.2 (± 1.8)[a]	< 0.001
Mean duration of GvHD (days)	7.3 (± 2.4)	4.3 (± 0.8)[b]	< 0.0001

[a] Suppression of host natural killer cell *(NK)* activity with mAb 3.2.3 enhanced the development of GvHD in recipient animals with significant earlier onset

[b] Aggrevated GvHD resulted in significant reduction of the mean time period between the first signs of GvHD until death in NK-depleted animals

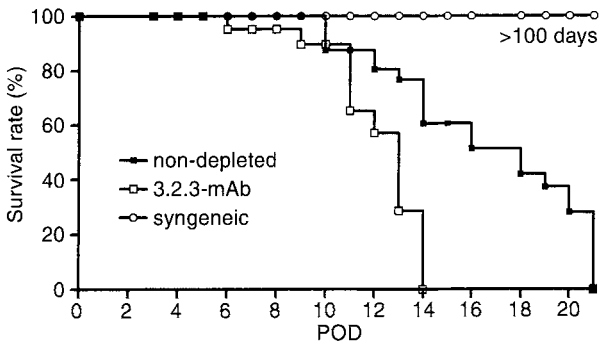

Fig. 1 Survival of monoclonal antibody (mAb) 3.2.3-treated *(3.2.3-mAb)*, $n = 10$; non-depleted, $n = 24$; and syngeneic F1 recipient animals, $n = 10$, after semiallogeneic heterotopic small bowel transplantation *(HSBTx)* of parental small bowel from DA rats is presented. mAb 3.2.3 pretreatment of F1 hybrid rats included triple intraperitoneal injection of 50 µl ascites of the highly specific anti-NKR-P1 monoclonal antibody 3.2.3 on days − 2, − 1, and 0 prior to transplantation. Kaplan-Meier curves were generated and statistical analysis with the Savage (Mantel-Cox) log-rank test showed a significant difference in survival ($P < 0.01$) between natural killer cell (NK) depleted and non-depleted animals

[^{3}H]thymidine, 18 h before harvesting for measurement of radioactivity in a scintillation counter.

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. Difference in survival between the various groups was 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.

Results

For depletion of host NKs, F1 hybrid rats received a triple i.p. injection of mAb 3.2.3 prior to HSBTx, as outlined above. F1 recipients lacking NKs suffered from GvH-mediated manifestations far earlier and to a greater extent. In comparison to non-depleted F1 hybrids, which did not suffer from GvHD until POD 10.2 (± 1.2), appearance of the first GvHD symptoms was already evident at POD 7.2 (± 1.8), $P < 0.001$. Thus, the mean time period between the first demonstration of clinical GvHD and death comprised 4.3 (± 0.82) days versus 7.3 (± 2.34) days ($P < 0.0001$) in mAb 3.2.3-pretreated and untreated recipients (Table 1). The fact that F1 hosts lacking their initial NK activity are subject to more advanced and extensive GvHD was also mirrored in life expectancy and survival of mAb 3.2.3-depleted animals. Figure 1 shows the survival of mAb 3.2.3-treated and non-treated F1 hybrid rats which received a parental DA small bowel in comparison to syngeneic F1 control animals which were given a F1 small bowel transplant. The mAb 3.2.3-mediated elimination of NKR-P1$^+$ cells led to a significantly shorter survival in comparison to non-depleted animals [Savage (Mantel-Cox) log-rank test, $P < 0.01$]. Whereas non-depleted animals had an average life expectancy of 16.1 (± 0.9) days, mAb 3.2.3-treated hosts surrendered to GvHD after a mean survival time of 11.4 (0.8) days. Table 2 illus-

Table 2 Survival after small bowel transplantation from DA to F^1 hybrid rats

Group	Donor	Recipient	Treatment	*n*	Survival (days)	Mean ± SD (days)
I	DA	F1	None	24	10 × 4; 12 × 2; 13; 14 × 4; 16 × 2; 18 × 2; 19; 20 × 2; 21 × 6	16.1 ± 0.9
II	DA	F1[a]	mAb 3.2.3	10	6; 9; 11 × 3; 12; 13 × 2; 14 × 2	11.4 ± 0.8[b]
III	F1	F1	None	10	> 100 × 10	> 100

[a] F1 recipients were administered 50 µl of anti-NKR-P1 mAb 3.2.3 at days − 2, − 1, and 0 prior to small bowel transplantation

[b] $P > 0.01$ for group I versus group II

Table 3 Flow cytometric analysis of T-lymphocyte subsets in spleen and mesenteric lymph nodes following semiallogeneic parental heterotopic small bowel transplantation. One- and two-color flow cytometric analysis of fresh spleen cells from control, syngeneic, mAb 3.2.3-pretreated, and non-depleted F1 hybrid rats was performed. Splenocytes from six animals in each group were harvested at postoperative day *(POD)* 3 and 10 and submitted to flow cytometric analysis. Results are expressed as mean ± SD of six experiments. Markers were set so that the percentages of PE^+ or $FITC^+$ cells in the isotype controls were < 1 %

Spleen (% positive cells)	DA > F^1 (untreated)		DA > F^1 (mAb 3.2.3 pretreated)		F^1 > F^1 (untreated)		F^1 control (untreated)
	POD 3	POD 10	POD 3	POD 10	POD 3	POD 10	
$CD4^+$	35.0 (± 2.19)	19.0 (± 3.74)	32.0 (± 4.33)	12.8** (± 4.40)	34.8 (± 2.04)	31.5 (± 3.08)	36.3 (± 4.92)
$CD8^+$	19.6 (± 6.53)	22.8 (± 8.95)	17.8 (± 1.94)	7.3*** (± 1.96)	16.1 (± 2.48)	15.1 (± 2.04)	16.0 (± 3.16)
$CD3^+$	49.8 (± 4.62)	37.0 (± 13.17)	46.3 (± 4.84)	24.5 (± 2.07)	49.5 (± 2.58)	45.0 (± 5.17)	51.6 (± 10.21)
NKR-$P1^+$	9.8 (± 1.72)	12.3 (± 9.97)	3.0* (± 0.89)	5.5 (± 2.66)	8.8 (± 2.04)	7.0 (± 1.54)	8.6 (± 2.50)
$OX\text{-}3^+$	40.2 (± 4.53)	18.6 (± 10.09)	42.0 (± 10.11)	33.6*** (± 4.67)	27.5 (± 2.16)	29.8 (± 2.85)	24.3 (± 8.63)
$CD3^+$/ NKR-$P1^+$	4.8 (± 0.98)	1.2 (± 1.5)	1.1* (± 0.75)	1.0 (± 0.89)	4.2 (± 0.75)	2.8 (± 0.75)	4.5 (± 1.05)
$CD4^+$/ $CD8^+$ ratio	2.0 (± 0.7)	0.9 (± 0.3)	1.8 (± 0.2)	1.8*** (± 0.6)	2.2 (± 0.34)	2.1 (± 0.5)	2.3 (± 0.3)

Comparisons between mAb 3.2.3-pretreated and untreated animals were highly significant for:
* the percentage of NKR-$P1^+$ and NKR-$P1^+$/$CD3^+$ double-positive lymphocytes, at POD 3, $P > 0.0001$; ** the percentage of $CD4^+$ lymphocytes at POD 10, $P < 0.05$; and *** the percentage of $CD8^+$, $OX\text{-}3^+$, and $CD4^+$/$CD8^+$ ratios, at POD 10, $P < 0.01$

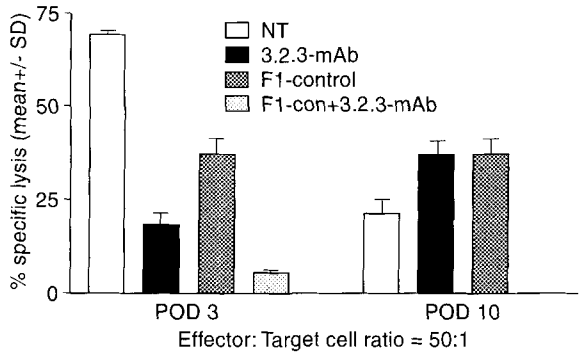

Fig. 2 NK activity of DA small bowel transplanted untreated *(NT)* and mAb 3.2-3-pretreated *(3.2.3-mAB)* F1 hybrid rats and F1 control, and F1 control mAb 3.2.3-pretreated rats was compared and functional assessment was determined as lytic activity of freshly isolated spleenocytes against Yac-1 targets at an effector to target cell ratio of 50:1 at postoperative day (POD) 3 and 10. Each column represents mean NK activity ± SD of four experiments in each group, consisting of two pooled rats per experiment. Assays were performed in three replicates. At POD 3, specific lysis of Yac-1 targets from the untreated F1 host significantly exceeded NK-depleted F1 host activity ($P < 0.001$)

trates the individual survival of each animal in the three different experimental groups. The selective depletion of NK activity in mAb 3.2.3-treated rats strongly affected the cell surface markers associated with NKR-$P1^+$ cells, $CD4^+$, $CD8^+$, $OX\text{-}3^+$, and $CD3^+$/NKR-$P1^+$ (double-positive), after HSBTx. Table 3 summarizes the main significant changes of these T-cell subsets evaluated for recipient spleens. At POD 3, the percentage of NKR-$P1^+$ and NKR-$P1^+$/$CD3^+$ double-positive cells was significantly reduced in mAb 3.2.3-pretreated animals, ($P < 0.0001$). NK cell depletion also affected $CD4^+$ and $CD8^+$ T-cell subsets of mAb 3.2.3-treated animals, which both were significantly diminished compared to non-depleted recipients ($P < 0.05$ and $P < 0.01$, at POD 10, respectively). At this time, the amount of F1-specific $OX\text{-}3^+$ cells was profoundly increased following NK cell depletion, consistent with the findings of others that advanced GvHD mediates the upregulation of MHC class II antigen expression on host target cells [16]. Further evidence for advanced clinical progression of GvHD in these animals was gained by calculating the $CD4^+$/$CD8^+$ ratios within the lymphocyte population of freshly harvested spleen cells from F1 hybrid animals. GvH-mediated immunosuppression is a common sequel in host lymphoid organs and correlates with $CD4^+$/$CD8^+$ ratios which, initially, are decreased during the "proliferation phase", characterized by clonally expanding $CD8^+$ T-lymphocyte subsets [17]. Whereas non-depleted animals still demonstrated inverse $CD4^+$/$CD8^+$ ratios of 0.9 (± 0.3) at POD 10 after HSBTx, mAb 3.2.3-treated recipients revealed a mean ratio of 1.8 (± 0.6) due to substantial depletion of both $CD4^+$ and $CD8^+$ lymphocytes, consistent with severely progressed GvHD.

Beside the finding of significant differences in lymphocyte population characteristics of recipient spleens for mAb 3.2.3-pretreated and untreated F1 hybrid rats,

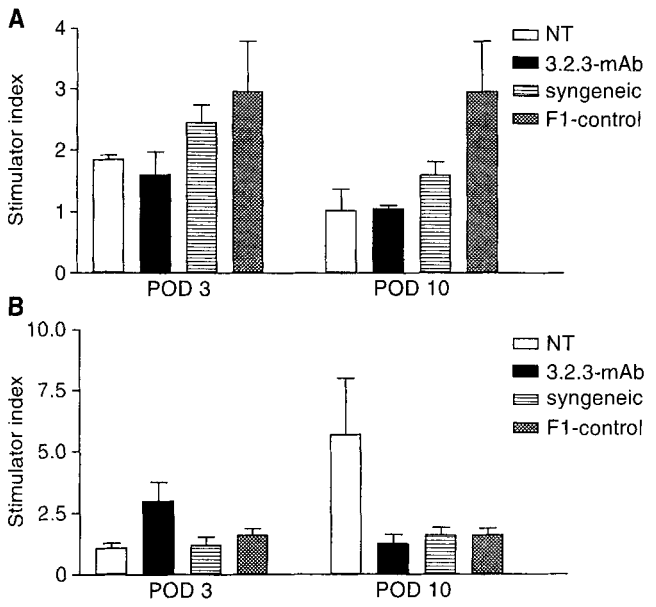

Fig. 3 A, B MLC-Response **A** against DA-stimulator cells and **B** against F1-stimulator cells. One-way MLR was performed with freshly isolated F1 splenocytes used as responder cells and co-cultured with DA or F1 irradiated (200 rad) stimulator cells. Varying numbers of stimulator cells were mixed in triplicate with F1 responder cells. Columns represent the mean of 6 to 10 experiments consisting of two pooled rats per experiment. The stimulator index was calculated as E/C, where E = cpm of [^{3}H]thymidine-treated responder cells co-cultured with irradiated stimulator cells, and C = cpm of [^{3}H]thymidine-treated responder cells not co-cultured with stimulator cells. Notice the increased anti-F1 proliferation response in mAb 3.2.3-pretreated animals at POD 3

the immunofunctional status as determined by Yac-1 target cell lysis and the mixed lymphocyte culture (MLC) response of splenocytes from these animals differed strongly. Figure 2 illustrates the NK activity of both groups ad POD 3 and 10 in comparison to syngeneic and F1 control animals at POD 3 and 10 after HSBTx. Obviously, NK depletion efficiently inhibited the lysis rate of Yac-1 target cells determined in splenocytes harvested at POD 3 ($P < 0.001$) compared to non-depleted animals. Conversely, at POD 10, splenocytes from NK-depleted animals yielded higher NK activity (37.75 % ± 7.5 versus 21.5 % ± 6.7 specific lysis from non-depleted F1 animals, $P < 0.05$). In order to investigate the impact of NK cell depletion on T-cell proliferation, DA and F1 stimulator cells were cultured together with [^{3}H]thymidine-pulsed F1 effector T-cells from mAb 3.2.3-treated and untreated F1 hosts. All experimental groups showed reduced proliferation responses to DA stimulator cells compared to control F1 animals without reaching a level of significance. Interestingly, at POD 3 after HSBTx, splenocytes from NK-depleted recipients responded more strongly to F1 stimulator cells than did undepleted splenic lymphocytes ($P = 0.052$). Besides, lack of host NK activity was correlated with a stronger anti-F1 response, which exceeded the proliferation rate of F1 host cells in response to parental T-cells (Fig. 3). At POD 10, an opposite picture was observed due to the advanced GvHD status of NK-depleted animals, characterized by severe depletion of $CD4^+$ and $CD8^+$ T-cell subsets and concomitant immunosuppression.

Discussion

The major findings of this experimental approach, designed to delineate the role of NK cell-mediated ALC in semiallogeneic solid organ transplantation, give rise to the conclusion that the initial host NK activity plays an important role as a first line defense barrier for allogeneic targets by initiating effective immunoregulatory signals within the afferent sensitization circuit of the host's immune response to the GvH-related immune attack. Animals lacking NK activity after mAb 3.2.3-pretreatment suffered from and succumbed to far more advanced GvHD than did F1 hosts with, initially, activated levels of NK-mediated lysis (Fig. 2). The inherent nonadaptive response and defense mechanisms of NKs appear to be especially important in MHC-disparate models by virtue of their capability to discriminate allogeneic MHC class I specificities [2–5]. In vitro assays for NK alloreactivity by use of an enlarged panel of intra-MHC recombinant rat strains permitted the comparison of different MHC regions and outlined the importance of non-classical MHC class I molecules encoded by the RT1.C region to elicit efficient NK susceptibility [4, 5]. Thus, early events following NK cell activation in the course of ALC can be initiated by non-classical MHC class I cell surface antigens and include: (1) recognition and elimination of allogeneic target cells within hours following graft reperfusion; (2) release of various cytokines, e.g., γ-interferon, tumor necrosis factor-α, and interleukin-2; and (3) selective enhancement of Th1 clones with concomitant downregulation of Th2 clones [18]. Thus, an anti-parental stimulus of the host's immune response is transiently initiated to balance the donor-derived immunogenicity in the course of GvHD. Host NK cell depletion prior to parental HSBTx obviated this important defense mechanism, with a concomitant decrease of the mean survival time. Our results underline the important role of NK-mediated ALC in the framework of solid organ transplantation. The exact role of non-classical MHC class I-encoded cell surface antigens as triggering structures for NK cell engagement and NK target susceptibility are the subject of forthcoming experiments in the experimental setting of solid organ transplantation.

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