Original Paper

High Frequencies of Anti-Host Reactive CD8⁺ T Cells Ignore Non-Hematopoietic Antigen after Bone Marrow Transplantation in a Murine Model

Asmae Gassa^{a,b} Halime Kalkavan^{a,c} Fu Jian^d Vikas Duhan^a Vishal Khairnar^a
Namir Shaabani^a Nadine Honke^a Alexander Carpinteiro^e Lacramioara Botezatu^e
Pietro Crivello^f Sebastian Dolff^{d,h} Stanislav Ferencik^a Dieter Häussinger^g
Cyrus Khandanpour^e Katharina Fleischhauer^f Oliver Witzke^{d,h} Thorsten Wahlers^b
Cornelia Hardt^a Philipp A. Lang^{g,i} Karl S. Lang^{a,g}

^aInstitute of Immunology, University Hospital Essen, University of Duisburg-Essen, Essen, ^bDepartment of Cardiothoracic Surgery, Cologne University Heart Center, University of Cologne, Cologne,

^cDepartment of Medical Oncology, West German Cancer Centre, University of Duisburg-Essen, Essen,

^dDepartment of Nephrology, University Hospital Essen, University of Duisburg-Essen, Essen,

^eDepartment of Haematology, West German Cancer Centre, University of Duisburg-Essen, Essen,

^fInstitute for Experimental Cellular Therapy, University Hospital Essen, University of Duisburg-Essen, Essen,

^gDepartment of Gastroenterology, Hepatology and Infectious Diseases, Heinrich-Heine-University of Düsseldorf, Düsseldorf, ^hDepartment of Infectious Diseases, University Hospital Essen,

University of Duisburg-Essen, Essen, ⁱDepartment of Molecular Medicine II, Medical Faculty,

Heinrich-Heine-University of Düsseldorf, Düsseldorf, Germany

Key Words

Bone Marrow Transplantation (BMT) • Minor Antigen Mismatch • Tolerance • Ignorance • LCMV • Mouse model

Abstract

Background: Graft versus host disease (GvHD) occurs in 20% of cases with patients having an MHC I matched bone marrow transplantation (BMT). Mechanisms causing this disease remain to be studied. **Methods:** Here we used a CD8⁺ T cell transgenic mouse line (P14/CD45.1⁺) and transgenic *DEE* mice bearing ubiquitously the glycoprotein 33-41 (GP33) antigen derived from the major lymphocytic choriomeningitis virus (LCMV) epitope to study mechanisms of tolerance in anti-host reactive CD8⁺ T cells after BMT. **Results:** We found that anti-host reactive CD8⁺ T cells (P14 T cells) were not negatively selected in the thymus and that they were present in wild type (WT) recipient mice as well as in *DEE* recipient mice. Anti-host reactive CD8⁺ T cells ignored the GP33 antigen expressed ubiquitously by host cells but they could be activated *ex vivo* via LCMV-infection. Lipopolysaccharides (LPS) induced transient cell damage in *DEE* mice bearing anti-host reactive CD8⁺ T cells after BMT, suggesting that induction of

A. Gassa, H. Kalkavan, P.A. Lang and K.S. Lang share authorship.

Karl S. Lang

Institute of Immunology, University of Duisburg-Essen
Hufelandstr. 55, 45147 Essen, (Germany)

Tel. +49-201 723 4273, Fax +49-201 723 5410, E-Mail karlsebastian.lang@uk-essen.de

host inflammatory response could break antigen ignorance. Introducing the GP33 antigen into BM cells led to deletion of anti-host reactive CD8⁺ T cells. **Conclusion:** We found that after BMT anti-host reactive CD8⁺ T cells ignored host antigen in recipients and that they were only deleted when host antigen was present in hematopoietic cells. Moreover, LPS-induced immune activation contributed to induction of alloreactivity of anti-host reactive CD8⁺ T cells after BMT.

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Introduction

Hematopoietic stem cell transplantation (HST) is an important therapy for patients with acute myeloid leukemia (AML), myelodysplastic syndrome, refractory relapsed lymphoma and very severe aplastic anemia. Preparative regimens are critical as treatment of the indicated disease and as prevention against graft rejection. Common conditioning regimens are either myeloablative or non-myeloablative defined by the pancytopenia caused. During the last 20 years, an alternative preparative regimen has been developed. This therapy is called reduced intensity conditioning (RIC) and is associated with less GvHD [1]. RIC is an intermediate category of regimens and is commonly used for elderly patients to improve the non-relapse mortality by reducing treatment toxicity of either whole body irradiation or chemotherapy [2-4]. Still limitation to successful BMT comes from severe GvHD, which occurs in up to 40% of cases [5]. GvHD is clinically grouped in an acute and chronic GvHD defined by the time of disease onset after HST with a cutoff of 100 days. Mechanistically chronic GvHD (cGvHD) usually involves several factors including B cell activation, production of autoantibodies and absence of T-regulatory cells (Treg) [6].

Acute GvHD (aGvHD) is characterized by the reaction of CD8⁺ T cells which are activated by antigens presented on host MHC class I [7, 8]. Therefore, T cell depletion of the transplant before BM transfer is performed in cases of HLA mismatch or unrelated donors and significantly reduces aGvHD [9-11]. Due to the high number of prospective bone marrow donors most of BMT are done in complete MHC class I match (HLA-A, -B, -C, DRB1, DQB1). Nevertheless, around 20% of patients develop aGvHD despite complete MHC class I matching [12] and in cases of T cell depletion [13] suggesting that anti-host reactive T cells develop in the host and are then activated under certain circumstances. The high incidence of lethal aGvHD is thought to be due to minor antigenic differences between host and donor [14]. However, around 80% of patients having a minor antigen mismatched BMT do not develop aGvHD suggesting that still insights concerning the conditions leading to tolerance by anti-host reactive CD8⁺ T cells are pending. To mimic the clinical situation of minor antigen mismatch, we focused on the LCMV model to investigate the tolerance in a minor antigen mismatched mouse model. We found that BM transfer of P14 cells led to engraftment of anti-host (GP33) reactive CD8⁺ T cells (P14 T cells) in GP33 expressing mice (*DEE* mice). Lack of negative selection was mainly due to absence of host antigen in the hematopoietic system. Naïve P14 BM cells transferred in irradiated mice were not activated by GP33 expressing host cells, and no GvHD developed despite high frequencies of anti-host reactive CD8⁺ T cells.

Materials and Methods

Mice

All mice used in this study were maintained on the C57BL/6 genetic background. P14 mice express an LCMV-GP33-41 specific T-cell receptor (TCR) on CD45.1 positive CD8⁺ T cells and were used for adoptive transfer experiments and as bone marrow donors [15]. *DEE* mice are transgenic mice that express ubiquitously *LCMV-GP* under the H-2k promotor [16]. P14 mice were crossed with *DEE* mice to create an F1-generation of P14 x *DEE*. Six- to eight-weeks-old, age- and sex-matched mice were used for all studies. All animals were housed in single ventilated cages. During survival experiments, the health status of mice

was checked twice daily. Animal experiments were authorized by the Nordrhein Westfalen Landesamt für Natur, Umwelt und Verbraucherschutz (Recklinghausen, Germany), and in accordance with the German and Canadian law for animal protection.

Bone marrow chimera

For the generation of bone marrow chimera mice, C57BL/6 (WT) mice and *DEE* mice were lethally irradiated with 9.5Gy on day -1. 24 hours later 1×10^7 bone marrow cells were injected intravenously for BMT. In case of mixed BMT, bone marrow cells from different donor mice were transplanted in a ratio of 1:1 to recipient mice. The body weight was determined weekly.

Virus

LCMV strain WE was originally obtained from F. Lehmann-Grube (Heinrich Pette Institut, Hamburg, Germany) and was propagated in L929 cells. C57BL/6 mice were infected by intravenous injection of 200 plaque forming units (PFU) of LCMV-WE.

FACS analysis

Tetramers were provided by the National Institute of Health (NIH) Tetramer Facility. In short, 20μl blood was stained with allophycocyanin (APC)-labeled GP33 MHC class I tetramers (GP33/H-2Db) for 15 minutes at 37°C. After incubation, the samples were stained with anti-CD8 peridinin-chlorophyll-protein-complex (PerCP; BD Biosciences, Franklin Lakes, NJ) for 30 minutes at 4°C. Erythrocytes were then lysed using 1ml BD lysing solution (BD Biosciences); cells were washed once and analyzed by flow cytometry (LSR-Fortessa). Absolute numbers of GP33-specific CD8⁺ T cells per/μl blood were calculated from FACS analysis using fluorescent beads (BD Biosciences). For intracellular interferon (IFN)-γ staining, splenocytes and peripheral blood lymphocytes were stimulated with GP33 in the presence of Brefeldin A. After 6 hours, cells were stained for CD8 for 30 minutes at 4°C, fixed with 2% Formaldehyde for 10 minutes and permeabilized with 1% Triton-X solution, and stained for IFN-γ with anti-mouse IFN-γ antibody (eBioscience) and analysed by flow cytometry.

In vivo proliferation

Splenocytes from P14 mice and chimera mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) and injected intravenously in 4 groups of mice. On day 3, day 6 and at indicated time points, proliferation of CFSE labeled P14 splenocytes was measured by flow cytometry and presented as histograms.

Depletion of CD8⁺ T cells

For CD8⁺ T cell depletion, 500μg/100μl of anti-CD8 antibody clone YTS 169.4 (Bioxcell) was injected intraperitoneally on day -3 and -1 in bone marrow donor mice.

Quantitative LCMV RT-PCR

Total RNA was purified with the TRIzol isolation method. The RNA was reverse-transcribed into cDNA with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The total number of copies of LCMV RNA was calculated as previously described [17].

ALT and LDH

Biochemical analysis of alanine transferase (ALT) and lactate dehydrogenase (LDH) in sera (1:10 dilution in PBS) were done by the central laboratory, University Hospital Essen, University of Duisburg-Essen, Germany.

Statistical analysis

If not stated differently data are expressed as means and S.E.M. Student's t-test was used to detect statistically significant differences between groups. Significant differences between several groups were detected by two-way analysis of variance (ANOVA). The level of statistical significance was set at * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

Results

Anti-host reactive CD8⁺ T cells are not negatively selected after BMT

To get insight into the fate of anti-host reactive CD8⁺ T cells after BMT, we established a single minor-histocompatibility antigen (mHag) mismatched but MHC-matched mouse model. Therefore we used a CD8⁺ T cell transgenic mouse line (P14/CD45.1⁺), which is specific for LCMV-GP 33-41 (GP33) and served as bone marrow (BM) donor. As recipient we used transgenic mice (*DEE* mice), which express ubiquitously *LCMV-GP* under the H2k-promoter [18, 19]. First, we analyzed whether P14 T cells recognized the LCMV specific epitope expressed in the *DEE* mice when P14 mice were crossed to *DEE* mice. Indeed, all P14 T cells were centrally deleted in the thymus by negative selection (Fig. 1A). Additionally, we confirmed GP33-expression in thymus by quantitative RT-PCR (Fig. 1B). When we transferred CFSE labeled naïve P14 splenocytes intravenously into naïve *DEE* mice and WT mice, P14 T cells proliferated significantly in *DEE* mice whereas in WT mice no proliferation could be detected (Fig. 1C). Therefore we concluded that the P14-*DEE* system is useful to study GvHD. To investigate whether anti-host reactive CD8⁺ T cells were detectable after BMT, we transferred 1 × 10⁷ P14 bone marrow cells intravenously into lethally irradiated (9.5Gy) *DEE* and WT mice. P14 T cells were present in blood of *DEE* recipient mice as well as in WT recipient mice during an observation period of 60 days *post-transplant* (Fig. 1D). Even 100 days *post-transplant* we detected statistically significant ($p < 0.01$) differences in P14 T cell numbers at a late time point (Fig. 1E), but without any symptoms of illness even after 6-7 months (data not shown). To rule out that mature anti-host reactive CD8⁺ T cells were present in recipients due to transfusion of donor BM cells contaminated with donor peripheral blood cells, we treated donor P14 mice with anti-CD8⁺ mAbs 3 days and 1 day before harvesting the bone marrow. Again P14 T cells developed from transferred P14 bone marrow cells in irradiated *DEE* and WT recipient mice (Fig. 1E). Together, these data suggest that P14 T cells, despite their potential alloreactivity, were not negatively selected in the thymus after BMT.

Anti-host reactive CD8⁺ T cells are naïve and show normal functionality

Next, we investigated the activation status of P14 T cells, which were tolerant in *DEE* mice after BMT. Expression analysis of activation markers like CD44, CD62L, CD25 and CD122 revealed that circulating anti-host reactive CD8⁺ T cells were mainly naïve in transplanted *DEE* mice (Fig. 2A). To further test the functionality of P14 T cells which engrafted in *DEE* mice, we analyzed the proliferation of transplanted P14 T cells *ex vivo*. Therefore, we labeled splenocytes obtained from P14 BM→*DEE* and P14 BM→WT chimera mice with CFSE and transferred them intravenously either into naïve WT mice, *DEE* mice or LCMV infected mice. Splenocytes from either P14 BM→*DEE* mice or P14 BM→WT mice proliferated comparably in LCMV infected WT mice. Furthermore, both splenocyte groups expanded in *DEE* host mice significantly less compared to LCMV infected WT mice (Fig. 2B). Next, we determined the capacity of engrafted CD8⁺ T cells to produce IFN- γ after *ex vivo* re-stimulation. We observed similar extent of IFN- γ production in P14 T cells independent of their engraftment in WT mice or *DEE* mice (Fig. 2C). We concluded that anti-host reactive CD8⁺ T cells are potentially alloreactive and can be activated *ex vivo*.

Introduction of host antigen on hematopoietic derived cells breaks immunological ignorance and deletes anti-host reactive CD8⁺ T cells

In our model we found that after minor antigen mismatched BMT, anti-host reactive CD8⁺ T cells were present but ignorant despite of minor antigen expression in the host. Therefore we hypothesized that only antigen expressed in the hematopoietic derived cells migrated in the thymus is recognized by CD8⁺ T cells in this setting. To test this hypothesis, we mixed P14 BM cells with *DEE* BM cells in a 1:1 ratio and transferred them into lethally irradiated *DEE* and WT recipient mice. In both groups anti-host reactive CD8⁺ T cells were extremely reduced and deleted (Fig. 3A). FACS analysis of thymus single cell suspension

Fig. 1. Anti-host reactive CD8⁺ T cells are not negatively selected after BMT. (A) Naïve P14 mice (P14), *DEE* mice and P14 crossed with *DEE* mice (P14x*DEE*) were analyzed for MHC class I TET-GP33 positive CD8⁺ T cells. The total number of GP33 specific CD8⁺ T cells per µl blood was determined by tetramer staining using flow cytometry. On the left Y-axis, the absolute number of CD8⁺ T cells and on the right Y-axis, the percentage of tetramer positive CD8⁺ T cells are shown (n=4). (B) RT-PCR analysis of LCMV-GP mRNA harvested from thymus of WT (n=2), *DEE* (n=3) mice and *Tcrb* deficient mice which were infected with 2×10^6 PFU of LCMV-WE representing positive control of LCMV-GP expression due to chronic infection at day 10 *post-infection* (LCMV; n=3). Values show fold change to expression in WT mice. (C) CFSE labeled splenocytes from a naïve P14/CD45.1⁺ mouse injected intravenously into GP33 expressing mice (*DEE*) and WT mice. The histogram represents the count of proliferated CFSE labeled P14 cells gated on total CD45.1⁺ CD8⁺ T cells from blood at day 3, 15 and 29. One histogram is shown as a representative of three individual experiments. (D) WT and *DEE* mice were lethally irradiated at day -1, after 24h 1×10^7 P14 bone marrow (BM) cells were injected intravenously. P14 BM cells transferred to WT mice (P14 BM→WT; black square) and *DEE* mice (P14 BM→*DEE*; white square) are shown in the left panels of Fig. C. Development of peripheral GP33 specific CD8⁺ T cells in blood was assessed by tetramer staining using flow cytometry at different time points *post-transplant*. As control, we transferred 1×10^7 WT bone marrow cells to WT and *DEE* mice (WT BM→WT; black circle and WT BM→*DEE*; white circle), shown in the right panels of Fig. C. Blood was analyzed and absolute numbers of CD8⁺ T cells and percentage of tetramer positive CD8⁺ T cells are shown (n=4). (E) The donor P14 mice were treated with 100µg of anti-CD8 depleting mAbs by intraperitoneal injection at days -3 and -1 before bone marrow transfer. 1×10^7 bone marrow cells of these anti-CD8 mAbs treated P14 mice were used for bone marrow transfer into lethally irradiated mice and analyzed for the development of TET-GP33 positive CD8⁺ T cells in peripheral blood in WT mice (P14 BM→WT) and *DEE* mice (P14 BM→*DEE*) at day 100 *post-transplant*. Same groups of mice are shown without anti-CD8 mAb treatment as control (n=5).

Fig. 2. Anti-host reactive CD8⁺ T cells are naïve and show normal functionality. (A) After 100 days of BMT, P14 T cells in peripheral blood, which developed in WT and *DEE* recipient mice, were analyzed for different activation markers (CD44, CD62L, CD25 and CD122). Histogram blot showing the peripheral blood cells gated on total GP33⁺CD8⁺ T cells from the group of WT mice receiving P14 BM (upper panel) and *DEE* mice receiving P14 BM (lower panel) are shown in red lines. For a positive control, peripheral blood T cells from WT mice infected with LCMV-WE (200 PFU) were measured after day 20 and shown in dotted black lines. Isotype control is shown in grey filled area. One representative histogram for each group is shown (n=4). (B) 1×10^7 splenocytes of 40-days-old P14 BM→WT mice and P14 BM→*DEE* mice were labeled with CFSE and transferred into naïve WT mice, *DEE* mice and WT mice infected intravenously with 2×10^4 PFU of LCMV-WE 1 day after splenocyte transfer (n=2-4). Proliferation of CD45.1⁺ CD8⁺ T cells (P14 T cells) was assessed by CFSE dilution in spleen 6 days after transfer. Histograms show cells gated on CD45.1⁺ CD8⁺ T cells in correlation to CFSE. One representative set of data is shown. (C) Next, these splenocytes were analyzed for IFN- γ production after re-stimulation with the LCMV peptide GP33-41 *in vitro*. An intracellular cytokine staining was set and CD45.1⁺ CD8⁺ specific T cells (P14) of either P14 BM→WT or P14 BM→*DEE* mice were characterized and counted in percentage of lymphocytes (n=2-4).

revealed that GP33-specific CD8⁺ T cell deletion occurred in the thymus as well as in spleen and blood (Fig. 3B) which correlated with higher expression of GP33 in thymus of mixed bone marrow chimera mice (Fig. 3C). Next, we examined whether reduced number of P14 T cells in mixed chimera mice were also functionally deleted. Therefore we re-stimulated splenocytes from P14+*DEE* BM→*DEE* mice and P14 BM→*DEE* mice with GP33 and measured intracellular cytokines (ICS). P14 BM→*DEE* splenocytes but not mixed P14+*DEE* BM→*DEE* splenocytes showed IFN- γ production (Fig. 3D). In conclusion, we found that introducing LCMV-GP antigen into the hematopoietic system resulted in anergy and partial deletion of anti-host reactive GP33-specific CD8⁺ T cells.

Fig. 3. Introduction of host antigen on hematopoietic derived cells breaks immunological ignorance and deletes anti-host reactive CD8⁺ T cells. (A) Mixed bone marrow cells were transferred in 1:1 ratio (P14 BM + DEE BM) into WT and DEE mice 24 hours after irradiation. Development of peripheral LCMV GP33 specific CD8⁺ T cells in blood was assessed by tetramer staining using flow cytometry at different time points (n=5). (B) At day 100, development of P14 T cells was analyzed in thymus, spleen and blood. As control P14 bone marrow chimera mice are shown. Thymocytes, splenocytes and blood cells were characterized by flow cytometry after tetramer staining (n=5). (C) RT-PCR analysis of LCMV-GP mRNA harvested from thymus of P14 BM→WT and P14 BM→DEE mice (n=5), and of mixed chimera mice, P14+DEE BM→WT and P14+DEE BM→DEE mice (n=5) at day 35 after BMT. Values show fold change to expression in WT mice. (D) Anti-host reactive CD8⁺ T cells in spleen were analyzed for IFN- γ production after re-stimulation with the anti-host reactive peptide (GP33). Splenocytes have been examined in P14 bone marrow chimera mice (P14 BM) and mixed chimera mice (P14+DEE BM) at day 100 after BMT. Absolute number of IFN- γ positive CD8⁺ T cells are shown in WT and DEE mice recipients (n=4-6).

High frequencies of anti-host reactive CD8⁺ T cells induce limited GvHD after stimulation with LPS

We found that absence of antigen presentation on bone marrow derived immune cells led to development of anti-host reactive CD8⁺ T cells after BMT. Most of anti-host reactive CD8⁺ T cells ignored peripheral antigen and stayed in a naïve status. Previous studies showed that despite lack of negative selection due to low avidity to host antigen in the thymus, peripheral tolerance can be maintained by T regulatory cells, tolerogenic dendritic cells, or costimulatory factors such as CTLA-4 and PD-1 [20]. To analyze organ damage after BMT we analyzed serum for ALT and LDH *post-transplant*. We did not detect any difference between P14 BM→DEE mice and P14 BM→WT mice (Fig. 4A). Moreover, we analyzed the body weight to look for signs of immunopathology. Also there was no obvious sign of weight loss between P14 BM→DEE mice and P14 BM→WT mice considering limited GvHD (Fig. 4B), and neither P14 BM→DEE nor P14 BM→WT died in an observation period of 60 days (Fig. 4C). Next we hypothesized, that an exogenous trigger might break the immune tolerance. Innate immune activation via Toll-like Receptor (TLR) can impact on adaptive auto-reactive immune response [21, 22]. It is described that LPS antagonism reduces GvHD [23]. Therefore we tested whether an innate immune trigger could influence tissue damage in P14 BM→DEE

Fig. 4. High frequencies of anti-host reactive CD8⁺ T cells induce limited GvHD after stimulation with LPS. (A) ALT and LDH were measured in the sera of bone marrow chimera mice (P14 BM→WT and P14 BM→DEE mice) at several time points (n=4). (B) Weight of bone marrow chimera mice are shown measured once a week (n=4). (C) Survival of mice after P14 bone marrow cell transfer is shown as percentage (n=5). (D) To trigger the innate immune activation via TLR4 signaling the chimera mice were treated with 50μg lipopolysaccharide (LPS) intraperitoneally at day 100 after BMT. The liver enzymes ALT and LDH were measured in the sera of mice at days 1 and 2 (n=4-5).

chimera mice. We injected LPS intraperitoneally in P14 BM→WT mice and P14 BM→DEE mice 200 days after BMT. Serologically, we tested LDH as general marker for cell damage and transaminase as specific marker for liver disease. We found that LDH was enhanced when LPS was administered in P14 BM→DEE mice but not in P14 BM→WT mice, indicating cell damage due to activation of P14 T cells (Fig. 4D). The liver specific enzyme was in normal range. This suggests that LPS impacts on anti-host reactive CD8⁺ T cells. Together these data show that even high precursor frequencies of CD8⁺ T cells are not able to induce GvHD in this model of BMT but show some disease after innate immune activation with LPS.

Discussion

In this murine minor antigen mismatched BMT model we transplanted P14 BM cells into DEE mice to analyze the activation of these anti-host reactive CD8⁺ T cells. The recipients developed high frequencies of GP33-specific CD8⁺ T cells despite antigen expression in peripheral organs. Nevertheless, they did not develop lethal GvHD. To confirm that central tolerance is induced by recognition of GP33 minor antigen we crossed P14 mice with DEE mice. Almost no GP33-specific CD8⁺ T cells were detected. DEE mice express ubiquitously GP33 and transferring CFSE labeled P14 T cells into DEE mice led to significant proliferation, suggesting that the antigen is detected by the transgenic TCR of P14 cells. As described in

previous publications proliferation of anti-host reactive CD8⁺ T cells could be induced by minor antigen presentation in the host [24].

Our results confirm earlier studies showing that microchimerism can induce tolerance in mice [25-27]. It remains unclear, whether presentation of minor antigens in the host can lead to ignorance and finally to reduced immunopathology in patients after BMT. We demonstrate that triggering the innate immune system with LPS could lead to significant cell damage in *DEE* recipients whereas WT recipients remained unaffected by GP33-specific CD8⁺ T cells. This is in line with the clinical situation where bacterial or viral infection (e.g. CMV) often induces GvHD [28]. Especially for the human cytomegalovirus (HCMV), preexisting data show an association of viral infection with increased risk of GvHD [29-31]. Interestingly, in a clinical trial it was shown that early replicative HCMV infection reduces the risk of leukemic relapse in patients with AML [32]. Though the underlying mechanism is not completely understood yet; a virus-induced abrogation of immune ignorance comparable to our murine model and even induction of graft versus leukemia (GvL) is a possible explanation for reduced risk of leukemic relapse after early replicative HCMV infection. Thus, beyond genetic haplotype matches the immune status on bacterial and viral infections of bone marrow donors might be helpful for GvHD risk evaluation, which is already performed for herpes-viruses, especially HCMV [33].

Recent studies showed that reduced intensity conditioning (RIC) before allogeneic BMT is a potential alternative to conventional myeloablative conditioning [2-4, 34]. Patients develop macrochimerism after BMT and show a similar survival curve and less non-relapse mortality. In correlation to our data and to previous clinical trials, macrochimerism after BMT assumes tolerance of potential anti-host reactive T-lymphocytes [35, 36]. Further analysis of anti-host reactive T cells from bone marrow of RIC treated patients would give a better understanding of tolerance development.

Our data are in contrast to current models, suggesting that low frequencies of anti-host reactive CD8⁺ T cells result in development of GvHD [6, 37]. We found that in the absence of secondary stimulation even high numbers of host-reactive T cells are hardly activated. GvHD is often associated with GvL. GvL can result in complete control of malignant cells after minor HLA mismatch. Interestingly efficient GvL can occur even in the presence of mild GvHD [38]. The reasons for that are not well explained. From our data we would suggest that the presentation of leukemic cells in primary lymphoid organs is crucial for the recognition by anti-host reactive CD8⁺ T cells and for early elimination. Therefore, a promising approach to treat GvHD would be the inhibition of T cell egress to peripheral organs. This would potentially limit GvHD, but still allow GvL. Such a preparation is Fingolimod (FTY720) which is licensed for treatment of relapsing remitting multiple sclerosis. Fingolimod prevents the egress of lymphocytes from secondary lymphoid organs thereby preventing migration of immune cells to the central nervous system [39]. Fingolimod is not clinically tested in BMT. Even though its application seems attractive, Fingolimod has first to be evaluated in animal models since it reduces the peripheral lymphocyte count and acts as an immunosuppressant. In conclusion we demonstrated that high frequencies of anti-host reactive CD8⁺ T cells can remain ignorant and tolerant after engraftment in recipient and do not induce GvHD.

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Disclosure Statement

The authors of this manuscript have no conflicts of interest to disclose as described by the journal of Cellular Physiology and Biochemistry.

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