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Authors

Wolf, Dietlinde
Barreras, Henry
Copsel, Sabrina N
et al.

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Improved NK cell recovery following the use of PTCy or Treg expanded donors in experimental MHC-matched allogeneic BMT

Dietlinde Wolf, PhD[1,3,*], **Henry Barreras, MPH**[2], **Sabrina N. Copsel, PhD**[2], **Krishna V. Komanduri, MD**[1,2,3], **Robert B. Levy, PhD**[1,2,3]

[1]Sylvester Comprehensive Cancer Center; University of Miami, Miller School of Medicine, Miami, FL

[2]Department of Microbiology & Immunology; University of Miami, Miller School of Medicine, Miami, FL

[3]Division of Transplantation and Cellular Therapy, Department of Medicine; University of Miami, Miller School of Medicine, Miami, FL

Abstract

Allogeneic hematopoietic stem cell transplantation (aHSCT) is complicated by Graft Versus Host Disease (GVHD) which causes immune dysfunction and further delays immune reconstitution via effects on primary and secondary lymphoid organs. Treatments to prevent GVHD and improve immune recovery following aHSCT are needed. Post-transplant cyclophosphamide (PTCy) is a well-established and clinically widely used method for GVHD prophylaxis after HLA-matched as well as haploidentical aHSCT, and also a promising strategy in the setting of mismatched unrelated donor aHSCT. Recently, regulatory T cells (Tregs), a critical subset for immune homeostasis and tolerance induction, are being evaluated for use as GVHD prophylaxis in experimental models and clinical trials. Natural killer (NK) cells are one of the first lymphoid populations to reconstitute following aHSCT and are important mediators of protective immunity against pathogens and also critical for limiting post-transplant relapse of hematologic cancers. Several reports have noted that a delay in NK cell recovery may occur following allogeneic experimental mouse HSCT as well as after clinical aHSCT. Here we examined how two treatment strategies, PTCy and donor expanded Tregs (TrED), in an experimental MHC-matched

[*]Corresponding author: Dietlinde Wolf, Ph.D., Division of Transplantation and Cellular Therapy, Department of Medicine, University of Miami, Miller School of Medicine, 1501 NW 10th Ave, Suite 926 B, Miami, FL 33136, Phone: 305-243-6060, 305-243-6118, FAX: 305-243-5522, dwolf@med.miami.edu.

Author contributions. DW designed and performed experiments, analyzed and interpreted data sets and wrote the manuscript. HB performed experiments. SC edited the manuscript. KVK provided research support, assisted with data interpretation, and edited the manuscript. RBL designed experiments, wrote the manuscript, interpreted data sets, and provided research support. All authors contributed to the article and approved the submitted version.

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allogeneic HSCT affected NK recovery. Experiments showed that both strategies improved NK cell numbers, with PTCy slightly better vs TrED, early after aHSCT (1 month) compared to untreated aHSCT recipients. Importantly, NK cell interferon gamma (IFNγ) production and cytotoxic function, reflected by CD107 expression as well as *in vivo* killing of NK-sensitive tumor cells, were improved using either PTCy or TrED vs. control aHSCT recipients. In conclusion, both prophylactic treatments were found to be beneficial for NK recovery and NK cell function following MHC-matched minor antigen mismatched experimental HSCT. Improved NK recovery could help provide early immunity towards tumor and pathogens in these transplant recipients.

Keywords

aHSCT; GVHD; PTCy; Treg; TL1A; IL-2; NK cells

Introduction

In addition to providing immunity against infections and anti-tumor activity dependent on the specific cancer, NK cells eliminate various populations of host cells which diminish HVG, promote engraftment as well as reduce GVHD[1–6]. Allogeneic HSCT (aHSCT) requires GVHD prophylaxis. Following aHSCT, NK cells are one of the earliest lymphoid populations to recover quantitatively, which make them critical early mediators of post-aHSCT outcomes, including opportunistic infection[7–9]. Within the first month following aHSCT, NK cells reach levels approximating normal in the blood of aHSCT patients, but it takes several months to acquire phenotypic and functional characteristics found in healthy donors[10, 11]. Moreover, the incidence of acute GVHD was reported to be associated with a delayed expansion of the NK compartment, therefore, treatments which promote NK cell recovery could be useful to improve these transplants[8].

The use of post-transplant cyclophosphamide (PTCy) was initially reported to diminish HVG responses and promote engraftment in mouse studies[12]. Subsequent work by Luznik and colleagues demonstrated that administration of cyclophosphamide on post-aHSCT days 3 and 4 was an effective GVHD prophylactic treatment for both MHC-matched and MHC-mismatched aHSCT; this approach is now widely utilized in many centers for transplants involving a variety of donor and recipient genetic disparities[13–17]. Moreover, PTCy is becoming a viable option in patients as a sole GVHD prophylactic treatment after ablative conditioning[18–23].

$CD4^+Foxp3^+$ Treg cells are a non-redundant regulatory population critical for immunologic homeostasis and self-tolerance[24]. The suppressive capacity of these cells has made them an attractive option for regulation of allogeneic transplants including HSCT where they are highly effective at ameliorating GVHD[25–28]. Our laboratory has reported *in vivo* manipulation of Tregs through stimulation of the TNFRSF25 receptor alone and together with low dose IL-2[29]. Recently we analyzed the use of both PTCy and Tregs in murine aHSCT models and reported both diminished GVHD and prolonged survival[30]. Interestingly, although both treatments ameliorated GVHD, we found that the use of expanded donor Tregs facilitated earlier thymopoetic recovery compared to PTCy therapy[30].

Russo et al., evaluated NK cell reconstitution following PTCy administration in patients receiving unmanipulated haploidentical grafts (containing high numbers of mature NK cells)[31]. They observed a robust proliferation of donor derived NK cells immediately after aHSCT. Infusion of PTCy led to a marked reduction in NK cells with subsequent recovery to approximately pre-HSCT numbers by day 30. Recently, Rambaldi et al., showed significant delays in NK cell recovery in the first 3 months after haplo-HCT with PTCy compared with matched related/unrelated HCT with conventional GVHD prophylaxis[32]. Importantly, McCurdy et al. reported that early NK cell recovery is the main determinant for overall survival after haploidentical and HLA-matched BMT using PTCy as GVHD prophylaxis[33].

Based on the above studies and our own findings involving thymus-derived immune reconstitution, we were interested in assessing NK recovery in murine aHSCT to evaluate how these two GVHD prophylaxis strategies may affect reconstitution within the NK compartment. Notably, both strategies were found to improve reconstitution of NK cell numbers, with PTCy tending to facilitate greater recovery early post-transplant (within 4 weeks), when compared to untreated recipients; these augmented numbers of NK cells were functional, reflected by *in vivo* killing of NK-sensitive tumors.

Materials and Methods

Mice.

C3H.SW (Stock: 000438), C57BL/6J (B6, Stock: 000664), BALB/c (Stock: 000651) mice and B6-CD45.1 breeders (Stock: 002014) were purchased from The Jackson Laboratory. All mice were maintained in specific pathogen-free housing at the University of Miami and given autoclaved food and water *ad libitum*. Mice were used at 12–16 weeks of age. All animal use procedures were approved by the University of Miami institutional animal care and use committee.

Cell lines.

RMA and RMA-S cell lines were kindly provided by Dr. Eli Gilboa (University of Miami, Miami FL)[34,35]. Tumor cells were maintained in IMDM media supplemented with 10% FCS plus antibiotics (PS) and used for *in vivo* cytotoxicity assays.

Bone marrow transplantation.

For MHC-matched (C3H.SW to B6) and syngeneic (B6 to B6) transplants, B6-WT mice received 10.5 Gy total body irradiation (TBI) (Cs^{137} source) on day 0. Three hours later, irradiated mice were injected iv with 7×10^6 TCD-BM and pooled splenocytes plus lymph node cells containing 2×10^6 $CD8^+$ T cells from C3H.SW mice (MHC-matched) or 10×10^6 non-TCD BM plus 5×10^6 splenocytes from B6 mice (syngeneic). T cell depletion was performed using HO134 hybridoma supernatant (αThy1.2) and rabbit complement (Cedarlane Labs, Burlington, NC). Mice were monitored 3x per week for weight loss and clinical score as previously described[29].

Cyclophosphamide treatment.

Cyclophosphamide (50 mg/kg) was administered ip on days +3 and +4 following aHSCT[14, 15].

Treg expansion protocol.

TL1A-Ig was generated as described previously[36]. TL1A-Ig (50 μg) was administered ip on post-aHSCT days 1–4 and free human IL-2 (10,000 U) was given ip on days 4–6[29]. Mice were sacrificed on day 7 and Treg expansion confirmed by Foxp3 staining (flow cytometry). Spleen and LN cells were collected from donors and used in HSCTs.

Flow cytometry.,

Peripheral blood, lymphoid tissue and BM were collected from transplant recipients at indicated time points post-HSCT. Lymphoid organs and BM were prepared into single-cell suspensions. Peripheral blood was collected in heparinized tubes. Peripheral blood mononuclear cells (PBMC) were isolated by standard Ficoll density gradient centrifugation. Next, 10^6 cells were pre-blocked with anti-mouse CD16/CD32 and stained with different antibody combinations. The following commercial mAbs from BD Biosciences, Biolegend or eBioscience/ThermoFisher to the indicated molecules were used for flow cytometry: CD4, CD8, CD3, CD19, NK1.1 (B6), Nkp46, CD122, CD27, CD11b, CD107, Ki67, Foxp3, IFNγ. For intracellular cytokine staining, splenic single cell suspensions were stimulated in complete media containing 10% FBS, CD107, PMA (50 ng/mL), ionomycin (500 ng/mL) and brefeldin A for 5h. After surface staining, cells were fixed using Cytofix/Cytoperm and Perm/Wash Buffers (BD Biosciences) according to the manufacturer's instructions. Permeabilized samples were then stained for 1h for cytokine detection. Intracellular staining for Foxp3 and Ki67 was performed using the Foxp3 Transcription Factor Staining Set from eBioscience/ThermoFisher Scientific according to the manufacturer's instructions. Flow cytometry analyses was performed on a BD LSRII Flow Cytometer. The data was analyzed using FlowJo version 10 software (TreeStar).

In vivo cytotoxicity assay for NK cells.

One-month post-aHSCT mice were inoculated iv with 10×10^6 RMA plus 10×10^6 RMA-S (ratio 1:1) cells labeled with low (0.4 μM) and high (4 μM) CellTrace Violet (CTV) (Invitrogen/Thermo Fisher Scientific), respectively. 5h later the mice were sacrificed, and cytotoxicity was assessed in spleen and blood by gating on the CTV positive cells. Cytotoxicity (% specific killing) was calculated using the following formula: $[1-(\text{RMA-S}^{\text{sample}}/\text{RMA}^{\text{control}})/(\text{RMA-S}^{\text{control}}/\text{RMA}^{\text{sample}})]\times100$[37]. As controls, CTV labeled cells cultured *in vitro* for the same amount of time were used.

Statistical analysis.

Numbers of animals per group and statistical tests utilized are described in the figure legends. All figure panels include data sets obtained from individual animals. Graphing and statistical analysis were done using GraphPad Prism 9 (La Jolla, CA). Statistical differences between two experimental groups were determined using two-tailed unpaired *t* tests. For experiments comparing more than two groups, data were analyzed using a one- (bar graphs)

or two-way (kinetic graphs) ANOVA with Bonferroni correction for multiple comparisons. For survival analyses, a Log-rank (Mantel-Cox) test was performed. Significance indicated by * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns=non-significant. Data shown are means ± SD.

Results and Discussion

To begin to assess the NK compartment in recipients following aHSCT, we utilized an MHC-matched ($H2k^b$), non-MHC-mismatched donor – recipient strain combination (C3H.SW→B6) that results in mild GVHD and no lethality (Fig S1A, B). Nonetheless, we observed a clear deficit in the NK compartment early post-aHSCT as defined by both the percentage and numbers of NK cells (defined by an $NKp46^+CD122^+CD3^-CD19^-$ phenotype) in the spleens of B6 recipients compared to syngeneic (B6→B6) transplant recipients (Fig 1A, B). This defect was observed 2–4 weeks post-aHSCT (Fig 1A, B).

Next, to examine the effect of GVHD prophylaxis on the NK compartment, groups of animals were transplanted with either donor cells from Treg expanded mice (TrED) or cells from unmanipulated mice that received cyclophosphamide on days 3 and 4 post-transplant (PTCy) at 50mg/kg/d (Fig 1). Notably, both frequency and numbers of total NK cells were elevated in recipients administered either PTCy or receiving TrED cells (Fig 1A,B).

Although both treatment strategies augmented the NK compartment, PTCy treatment yielded improved NK recovery as reflected by significantly increased frequencies and total numbers (Fig 1A, B). The percentages and numbers of NK cells also increased over time post-aHSCT in the PTCy and TrED transplanted recipients compared to untreated aHSCT recipients or syngeneic HSCT recipients (Fig 1A, B – right panels). Since one potential explanation for why PTCy-treated recipients had higher NK cell levels would be higher proliferation rates, we assessed NK proliferation by examining Ki67 staining at both time-points. First, Ki67 expression in NK cells was higher in both PTCy and TrED mice at Day 14 compared with their levels at Day 28, consistent with the increased numbers of NK cells in both groups of recipients. Second, the higher levels of Ki67 expression were present after two weeks in NK cells of PTCy treated vs TrED recipients (Fig 1C–left panels). This latter observation is consistent with the findings of greater percentage and numbers of NK cells in the former groups of recipients. Investigation of the marrow compartment revealed a similar pattern of percentage and numbers of donor NK cells two weeks post-aHSCT in the groups examined (Fig S1C).

One prior clinical study noted that PTCy treatment of recipients of haploidentical and HLA-matched transplants caused an early (~1 month) delay in NK recovery in the former group[32]. In the experimental studies here using MHC-matched recipients, PTCy treatment (and as noted above, use of TrED donors) increased recovery of the NK compartment compared to untreated aHSCT recipients. Interestingly, the findings here using the MHC-matched experimental aHSCT regarding NK cell proliferation post-transplant identified elevated proliferation within several weeks following PTCy treatment as reported following haplo-matched clinical transplants[31].

To more granularly assess the NK compartment, established subsets of NK cells were evaluated as defined by phenotypic markers identifying Immature ($CD27^+CD11b^-$), M1 ($CD27^+CD11b^+$) and M2 ($CD27^-CD11b^-$) populations[38] (Fig 2). Two weeks post-HSCT, only syngeneic transplant recipients had greater levels and numbers of M2 versus immature NK cells in contrast to all aHSCT recipients whose compartments consisted of primarily immature NK cells (Fig 2A, B). Analysis of NK subsets indicated that untreated aHSCT recipients had lower numbers of all NK subsets compared to syngeneic recipients as well as PTCy and TrED treated groups (Fig 2B). M2 NK cells, the most mature and cytolytic subset, are important in reducing GVHD[39]. Notably, the M2 NK frequency and numbers increased between 2 and 4 weeks post-HSCT in PTCy and TrED treated allogeneic recipients as well as syngeneic transplanted animals in contrast to untreated aHSCT recipients. (Fig 2C). Examination of the immature and M1 subset numbers indicated a trend toward higher levels in the treated groups compared to untreated aHSCT recipients although differences did not reach statistical significance (Fig S1D). One implication of these observations was that the functional capabilities of the total NK compartment in these groups likely differ due to the overall numbers and perhaps function of individual NK cells.

To address this hypothesis, we assessed the function of NK cells present in untreated and treated groups of recipient mice post-HSCT by examining interferon gamma (IFNγ) production and expression of the degranulation marker CD107[40]. Following *in vitro* stimulation with PMA + ionomycin, the frequency of splenic IFNγ-producing NK cells was significantly greater in allogeneic vs syngeneic HSCT recipients two and four weeks post-HSCT (Fig 3A). Since immature NK cells dominated the early reconstituting compartment (Fig 2B), their IFNγ production was assessed, and this same pattern in the allogeneic and syngeneic groups was detected (data not shown). The frequency of $IFN\gamma^+$ NK cells increased over the first month post-transplant; therefore, NK cells from all groups of transplanted mice were capable of producing this effector cytokine (Fig 3A). However, at day 30 we did not detect functional cytokine (IFNγ) or cytotoxic impairment (see below) in PTCy or TrED treated MHC-matched recipients following HSCT compared to untreated HSCT recipients. Within this context, Rambaldi et. al. reported immature NK cells from PTCy-treated MHC-mismatched and MHC-matched recipients were functionally impaired (based on IFNγ, TNFα production) two months following clinical aHSCT, although these responses were compared to healthy donor NK cells[32]. It should be noted that another study reported that reconstituting NK cells - after *in vitro* stimulation - were capable of cytokine production similar to healthy controls beginning at one-month post-HSCT (HLA-matched reduced intensity conditioning)[41].

Next, CD107 expression was examined in unstimulated and PMA + ionomycin stimulated NK cells. While unstimulated cells expressed low levels which decreased further between 2 weeks and one-month post-HSCT, the CD107 levels in stimulated cells significantly increased in NK cells from all groups of transplanted mice (Fig 3B). To evaluate CD107 expression after *in vivo* stimulation, we used NK-sensitive RMA-S cells. We posit the upregulation of CD107 on NK cells examined resulted from RMA-S cell presence in all transplanted groups (Fig 3C). These observations support the notion there is no endogenous defect in cytotoxic function in NK cells from all groups of transplanted mice one-month post-HSCT.

To directly assess overall NK function in each group of transplanted mice, *in vivo* cytotoxicity was examined (Fig 3D). Therefore, CellTrace Violet labeled NK-sensitive (RMA-S; MHC I deficient) and NK-resistant (RMA) cells were adoptively transferred at a ratio 1:1 into recipients and after 5h the relative levels of RMA-S/RMA were evaluated to determine the level of killing. These studies demonstrated that the cytotoxic capacity of the NK compartments (in spleen and peripheral blood) in both PTCy and TrED treated recipients were greater versus that in untreated transplant recipients (Fig 3D). These findings correlated with the numbers of NK cells detected in the three groups of recipient animals, i.e. untreated animals had the lowest numbers of total NK cells and both PTCy and TrED treated mice contained significantly higher numbers of all splenic NK cell subset compartments (Fig 1B, 2B). The reconstitution of the peripheral NK compartment in an independent experimental MHC-matched aHSCT was previously reported to be delayed during the first month post-transplant in untreated animals as observed in the present studies[42]. Regarding NK cytotoxic function in those untreated recipients, cytotoxicity was examined 2 weeks post-HSCT. Results in the MHC-matched model examined here clearly indicated that PTCy and TrED treatment improved cytotoxic NK function by one-month post-aHSCT.

In addition to GVHD prophylaxis treatment, the presence of virus and tumor post-HSCT has been reported to impact the NK compartment. For example, studies have been reported examining the effect of CMV reactivation which accelerated the emergence of mature NK cells following HLA-haploidentical TCD HSCT[43]. Tumor presence in un-transplanted patients has also been reported to affect NK cells. A relationship of NK cells to myeloid malignancies (CML, AML, MDS) was detected, observing an inverse correlation of NK levels and tumor burden and some studies reported a selective loss of immature NK cells[(reviewed in 44)]. Additionally, transcriptomic analysis recently indicated NK function was affected by AML tumor presence[45]. While the studies here did not examine NK reconstitution in the presence of tumor, further investigation will be required to begin dissecting the individual contributions of tumor presence plus treatment for GVHD prophylaxis.

In summary, the NK cell compartment in the experimental model studied here, as in the clinical setting, recovers robustly following PTCy treatment[31, 32]. Notably, our studies suggest that the total numbers and functional capability of NK cells post-aHSCT were improved compared with untreated HSCT recipients using either PTCy or TrED as GVHD prophylaxis, thereby strengthening anti-pathogen and tumor immune function. These observations further suggest that since mouse NK and Treg cells can promote donor hematopoietic engraftment, these treatments likely facilitate beneficial transplant outcomes mediated by these critical cellular compartments[3, 25, 46, 47].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability:

Data reported in the manuscript will be shared under the terms of a Data Use Agreement and may only be used for approved proposals. Requests may be made to: dwolf@med.miami.edu.

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Highlights

- PTCy and Treg treatment for GVHD prophylaxis improves NK reconstitution post-MHC matched aHSCT
- Effector function of the NK compartment is augmented after PTCy or Treg treatment

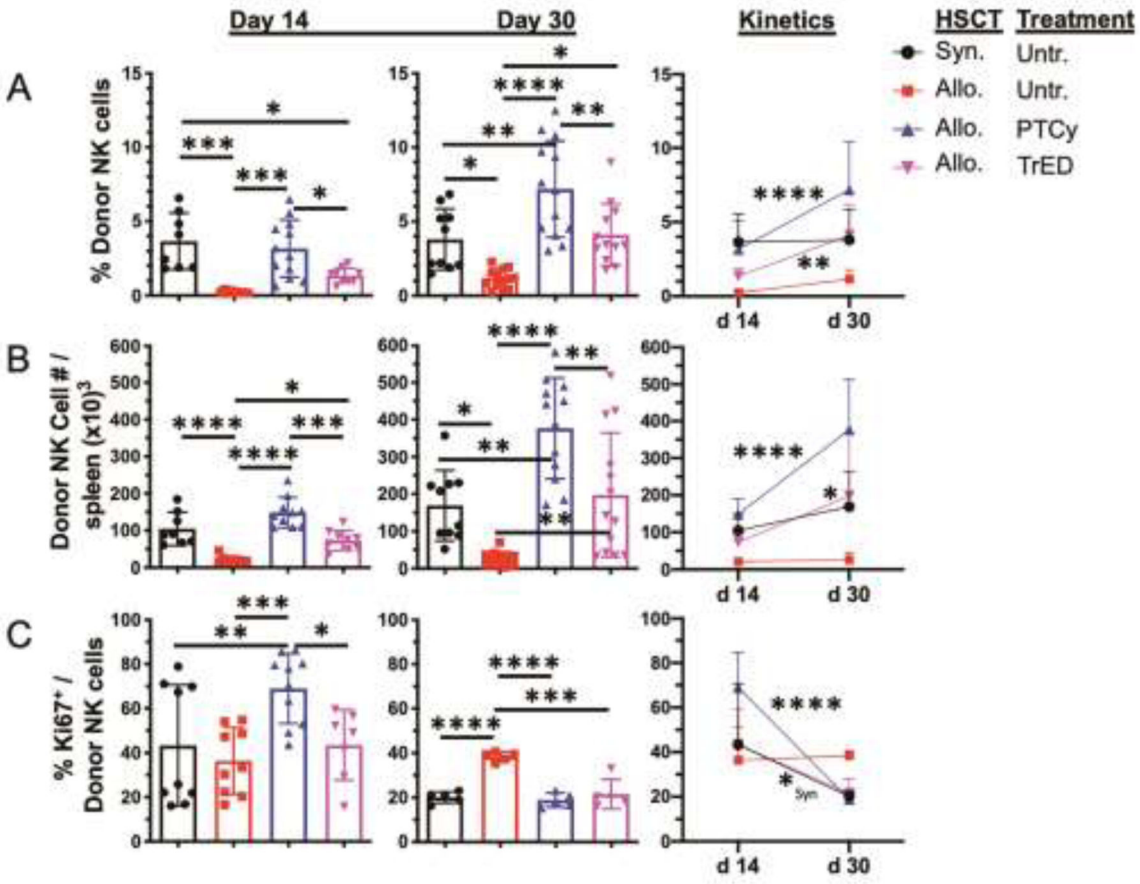

Fig. 1.: NK cell recovery in recipients treated with expanded donor Tregs or PTCy is improved following aHSCT.

A HSCT utilizing a C3H.SW → B6 donor/recipient mouse model involving a minor MHC mismatch was performed on day 0. Lethally irradiated (10.5 Gy on day 0) B6 mice received 7×10^6 TCD C3H.SW BM cells and spleen+LN cells from expanded (TL1A-Ig/IL-2; TrED group) or untreated C3H.SW (GVHD and PTCy group) donor mice adjusted to contain 2×10^6 CD8 T cells. Cyclophosphamide was given on day 3 and 4 post-HSCT at 50 mg/kg ip. For the syngeneic HSCT 10×10^6 non-TCD BM cells plus 5×10^6 spleen cells from B6 mice were transplanted. **(A)** Percent (%) donor derived NK cells ($CD3^-CD19^-NKp46^+CD122^+$) on day 14 and 30 post HSCT in spleens are depicted. **(B)** Donor derived NK cell number (#) per spleen is shown on day 14 and 30 post HSCT. **(C)** Proliferation of donor NK cells (% Ki67) in spleen on day 14 and 30. n = 5–12 per group per time point. Data in column graphs are expressed as means ± SD and were analyzed by one-way ANOVA with Bonferroni correction for multiple comparisons. Data in kinetics graphs were analyzed by two-way ANOVA with Bonferroni correction for multiple comparisons. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. Data are pooled from 3 independent experiments, respectively.

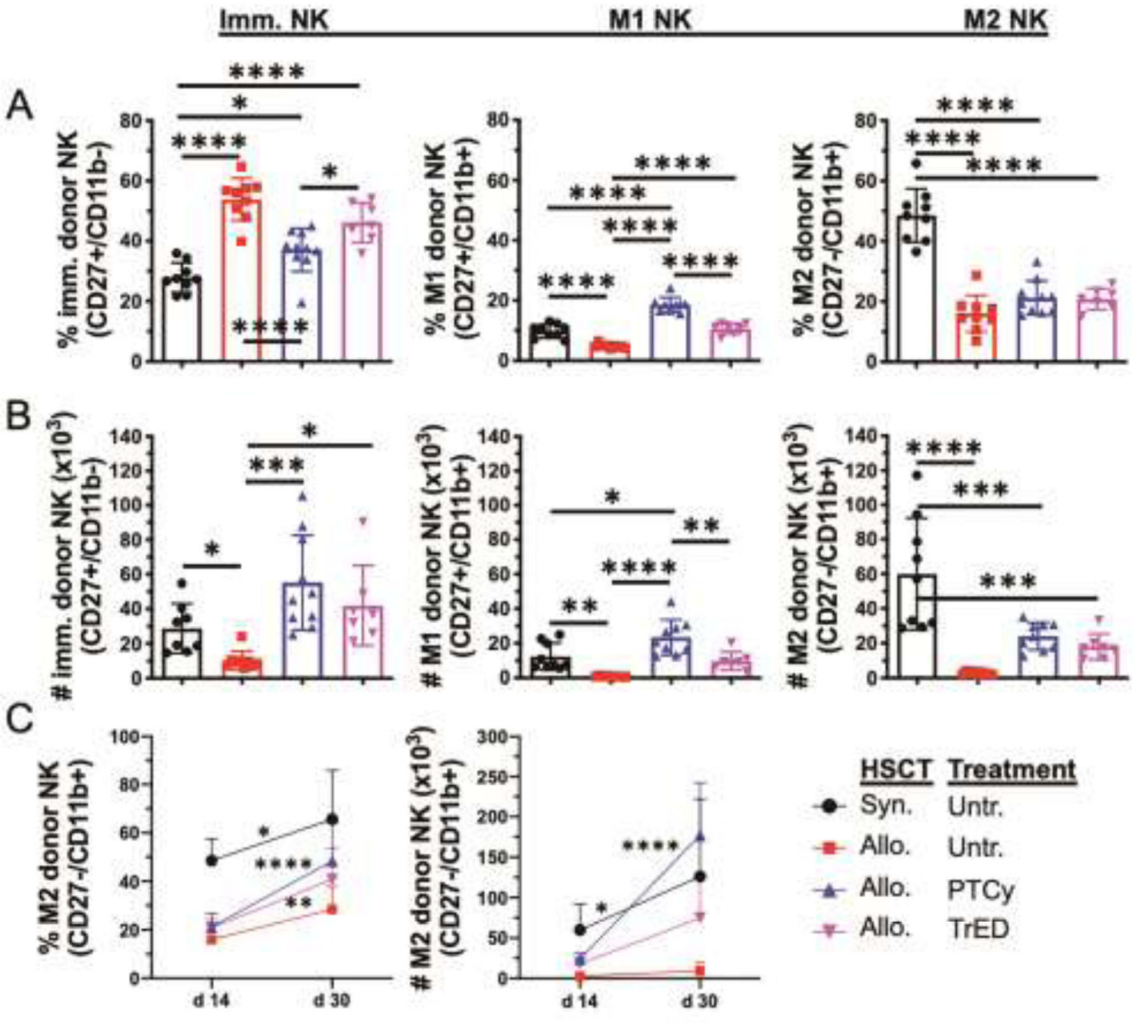

Fig. 2.: Donor NK cell subset recovery.

A HSCT was performed as in Fig 1. Donor NK cell subsets (immature [imm.] NK cells = $CD27^+/CD11b^-$; M1 NK cells = $CD27^+/CD11b^+$; M2 NK cells = $CD27^-/CD11b^+$) were examined in recipient spleens 14 days post HSCT (C3H.SW → B6). **(A)** Percent (%) and **(B)** Numbers (#) per spleen are shown **(C)** Kinetics of the M2 subset over time (% and #). Data are pooled from 3 independent experiments with n = 7–13 per group per time point. Data in column graphs are expressed as means ± SD and were analyzed by one-way ANOVA with Bonferroni correction for multiple comparisons. Data in kinetics graphs were analyzed by two-way ANOVA with Bonferroni correction for multiple comparisons. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

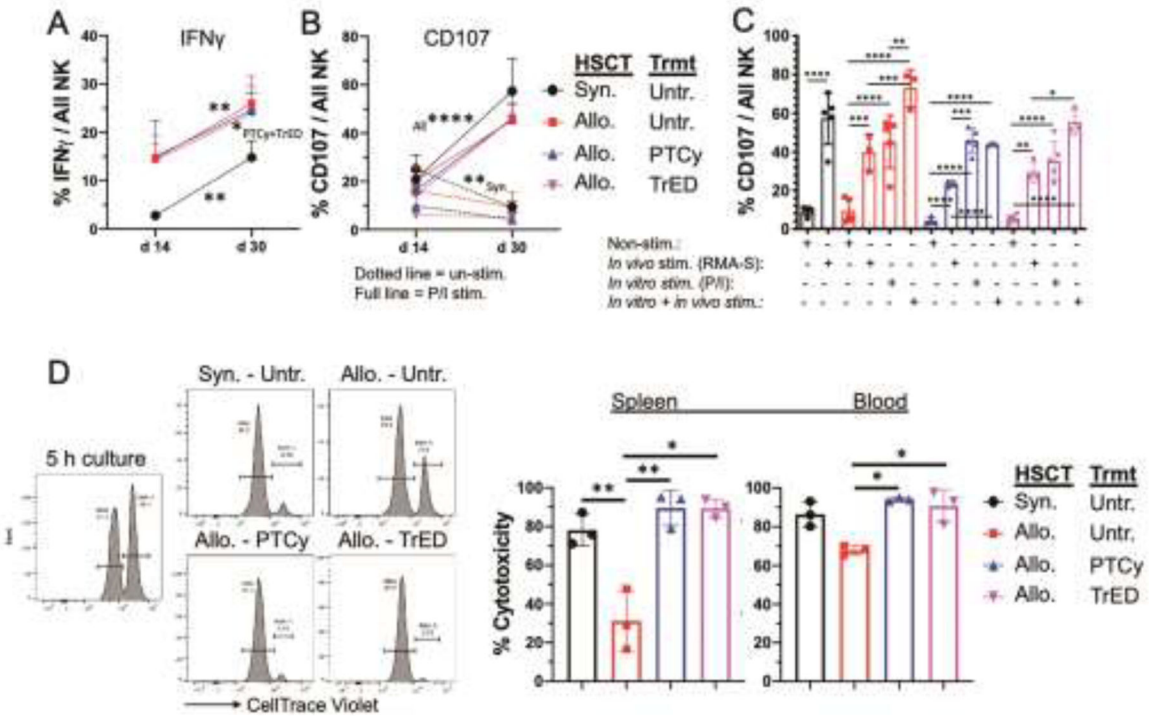

Fig. 3.: Functional assessment of NK cells *in vitro* and *in vivo* following minor mismatch aHSCT. The (C3H.SW → B6 experimental transplant model was performed as in Fig 1. **(A) Kinetics of IFNγ – producing** NK cells on day 14 and day 30 is shown after 5 h of *in vitro* stimulation with PMA (50 ng/ml) and Ionomycin (500 ng/ml) in the presence of 1 μl/ml BD GolgiStop. n = 5 per group. **(B,C) Expression of the degranulation marker CD107 after *in vitro* and *in vivo* stimulation. (B)** CD107 expression on unstimulated (dotted line) and *in vitro* stimulated NK cells (full line; 5 h, PMA+Ionomycin stimulation as in A) on day 14 and 30. n = 4–5 per group. **(C)** CD107 expression on NK cells on day 30 after *in vivo* (1×10^6 iv injected RMA-S cell for 18 h), *in vitro* (5 h PMA + Ionomycin as in A) or double stimulation (*in vivo + in vitro*). n = 3–5 per group. Data are pooled from 2 independent experiments (A-C). **(D) *In vivo* cytotoxicity assay**. On day 30 post HSCT, 10×10^6 NK resistant RMA and 10×10^6 NK sensitive RMA-S cells, respectively, were labeled with low (0.4 uM) and high (4 uM) amounts of CellTrace Violet (CTV) and injected iv into recipients. 5 h later, the killing of the NK sensitive RMA-S cells was quantified in blood and spleen. Representative histograms and graphs are shown from one of 2 experiments. n = 3 per group. Data in kinetics graphs are expressed as means ± SD and were analyzed by two-way ANOVA with Bonferroni correction for multiple comparisons. Data in column graphs are expressed as means ± SD and were analyzed by one-way ANOVA with Bonferroni correction for multiple comparisons. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.