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Alloreactive Natural Killer cells have anti-tumor capacity against disseminated human multiple myeloma in Rag2-/-γC-/-mice when combined with low dose cyclophosphamide and total body irradiation

Subhashis Sarkar[1], Willy Noort[2,3], Catharina HMJ Van Elssen[1], Richard Groen[2,3], Louis van Bloois[4], Michel van Gelder[1], Harry C Schouten[1], Marcel GJ Tilanus[5], Wilfred TV Germeraad[1], Lotte Wieten[5], Anton CM Martens[2,3,6] and Gerard MJ Bos[1]*

[1]Department of Internal Medicine, Division of Hematology, Maastricht University Medical Center, Maastricht, The Netherlands

[2]Department of Cell Biology, University Medical Center Utrecht, Utrecht, The Netherlands

[3]Department of Hematology, VU University Medical Center, Amsterdam, The Netherlands

[4]Department of Pharmaceutical Sciences, Utrecht University, The Netherlands

[5]Department of Transplantation Immunology, Maastricht University Medical Center, Maastricht, The Netherlands

[6]Department of Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

Abstract

Immunotherapy with alloreactive Natural Killer (NK) cells could be a new treatment option for the incurable plasma cell malignancy Multiple Myeloma (MM). In the present study, we explored the anti-MM potential of alloreactive NK cells using the RAG2-/-γc-/-immunodeficient disseminated mouse model. In this model, we studied NK cell-mediated Graft versus MM (GvM) responses while concurrently examining the risk of developing Graft versus Host Disease (GvHD). First, luciferase expressing human U266 MM cells were intravenously (i.v.) injected in RAG2-/-γc-/- mice, and human KIR-ligand alloreactive NK cells or PBMC were injected (i.v.) after Bioluminescent Imaging (BLI)-confirmed tumor formation. BLI of tumor growth kinetics in a preliminary experiment revealed that infusion of resting NK cells decreased MM burden in one out of four treated mice receiving 3 injections of $8x10^6$ NK cells, with no signs of GvHD. Although infused PBMCs efficiently eradicated established MM in all treated mice, this concurrently induced lethal GvHD. rhIL-2 activated NK cells, injected in subcutaneously (s.c.) growing MM tumors, proved to be superior to resting NK cells and anti-MM responses occurred NK cell dose dependently. Since MM is a dispersed malignancy spread throughout the body, we investigated the therapeutic potential of rhIL-2 activated NK cells, from either cord blood or peripheral blood origin, in the disseminated MM mouse model. Whereas activated NK cells were unable to trigger efficient GvM responses when given alone, the infusion of activated NK cells following a non-curative conditioning treatment with cyclophosphamide and total body irradiation, did prolong progression free survival (p=0.003) without any signs of GvHD. This study illustrates the therapeutic potential and safety of NK cell therapy in MM and is an important step towards cell-based immunotherapy for MM patients. It also establishes the RAG2-/-γc-/- MM model as a clinically relevant pre-clinical model to further develop NK cell immunotherapy.

Keywords: Cancer; Immunotherapy; NK cell; Multiple Myeloma

Introduction

Multiple Myeloma (MM) is a hematological malignancy characterized by clonal expansion of plasma cells in the bone marrow [1]. Despite new options, state-of-the-art treatment results only in a median survival of 5-7 years [2]. New treatment strategies with curative potential with reduced toxicity are therefore urgently needed. Autologous-Stem Cell Transplantation (auto-SCT) combined with high dose chemotherapy has improved overall survival when compared to treatment with conventional chemotherapy alone; still the great majority of patients eventually relapse [3]. Allogeneic-SCT (allo-SCT) has the potential to cure MM by the so-called graft versus tumor response of donor immune cells. However, an important drawback of allo-SCT is the high risk of life-threatening Graft-versus-Host Disease (GvHD) mediated by donor T cells responding to mismatched histocompatibility antigens [4,5]. Moreover, treatment related mortality after allo-SCT is high, and patients surviving the procedure still show progression of disease in the majority of cases.

When allo-SCT is performed over HLA barriers, both Natural Killer (NK) cells and T cells can mediate GvM responses, but an important advantage of NK cells is that they do not seem to mediate GvHD [6]. NK cells are capable of targeting virally infected or malignantly transformed cells and become activated by tilting the delicate balance of inhibitory and activating receptor signals [7]. An important class of inhibitory receptors is the Killer Immunoglobulin-like Receptor (KIR) family interacting with the highly polymorphic HLA class I molecules expressed by virtually every healthy cell. The most important inhibitory KIRs are KIR3DL1

*Corresponding author: Gerard MJ Bos, Department of Internal Medicine, Division of Hematology, Maastricht University Medical Center (MUMC), PO Box 5800, 6202 AZ Maastricht, The Netherlands, Tel: +31 433877026; Fax: +31 433875006; E-mail: Gerard.Bos@mumc.nl

that binds HLA-Bw4 epitopes, KIR2DL2 and KIR2DL3 that interact with HLA-group C1 alleles and KIR2DL1 that binds HLA-group C2 alleles [8-10]. Upon engagement of inhibitory KIRs with the HLA class I molecules of a target cell, suppressive signals are transmitted, prohibiting NK cells to kill the target cell. This mechanism of self-recognition can be used in anti-tumor therapy, by transplantation of NK cells with a licensed KIR repertoire that mismatches for the donor HLA according to the "missing self" principle [11,12]. Ruggeri et al., showed that the anti-tumor effect KIR-mismatched haplo-identical SCT setting in Acute Myeloid Leukemia (AML) is attributed to the presence of "alloreactive NK cells" [6]. AML patients treated with a KIR-ligand mismatched allogeneic bone marrow transplant show increased survival over patients transplanted without such a mismatch [13].

There is accumulating evidence to support a role for NK cells in controlling the progression of MM [14-20]. Therapeutic interventions like lenalidomide and thalidomide increase NK cell reactivity and thereby improve survival. Additionally, there is evidence that alloreactive NK cells may be relevant in MM. In a group of 10 patients with MM that were treated with alloreactive NK cells, fifty percent demonstrated a response, with some patients having a complete remission [18]. Recently, these authors expanded their observations in more patients, showing that freshly prepared NK cells could continue to expand *in vivo* after infusion, but only in two of eight patients a clinical response could be attributed to the infused NK cells [19]. Kroger et al., analyzed patients that were transplanted with an unrelated donor, which included donors with a mismatch for HLA-C groups, thereby allowing NK cell alloreactivity. The group of patients that received a transplant with the alloreactive NK cells indeed showed a far lower incidence of disease recurrence [21].

Unfortunately, since MM is a disease of the elderly, the majority of MM patients is not eligible for KIR mismatched haplo-identical SCT due to the severe conditioning regime and transplantation related complications and thus indicating that other modalities have to be developed. Our previous research lead us to the idea that infusion of only KIR-ligand mismatched NK cells is an interesting treatment option to provide curative effects that bypasses the risks that are associated with a full HLA mismatched allo-SCT. *In vitro*, NK cells have been shown to efficiently kill MM cells [16,17]. Recently, we showed that KIR-ligand mismatched NK cells are better in doing this than matched cells [22]. We could demonstrate that it is also possible to kill MM cells under hypoxia, which is an immunosuppressive condition relevant in this disease [23]. Before performing clinical trials, immunodeficient mouse models provide an excellent opportunity to test novel treatment strategies. The RAG2$^{-/-}$γc$^{-/-}$MM mouse model was originally developed by some of us to study GvHD development after injection of PBMCs or purified T cells [24]. In this model, we previously showed that human PBMC xenografts did not only induce GvHD but also GvM [25]. In the present study, we used this RAG2$^{-/-}$γc$^{-/-}$MM mouse model to obtain the proof of concept for the effect of alloreactive KIR-ligand mismatched NK cells on GvM and GvHD in MM bearing mice.

Materials and Methods

Cell lines and cell cultures

The human MM cell lines studied were U266, RPMI-8226/S, UM-9, LME-1, XG-1, OPM-1, and L-363, all retrovirally transduced with the luciferase gene as described previously [20]. U226 had been

transfected with GFP previously. All MM cell lines were cultured in RPMI-1640 (Gibco, Breda, The Netherlands) except for XG-1 and LME-1, which were cultured in IMDM medium (Gibco), supplemented with 10% fetal calf serum (Integro, Zaandam, The Netherlands), 100 U/ml penicillin (Gibco), 100 μg/mL streptomycin (Gibco). The XG-1 cell line was additionally cultured in the presence of 20 μg/ml Transferin (Sigma) and 500 pg/ml IL-6 (Biosource). The cultures were maintained at 37°C with 5% CO_2 in humidified atmosphere. Presence and absence of HLA epitopes (HLA-C1, -C2 and -Bw4) and of HLA-A in cell lines (Table 1) and healthy control donors, were routinely determined by Sequence-Specific Oligonucleotides (SSO) analysis and Luminex® according to manufacturer's guidelines (One Lambda, Montpelier, France).

Cell line	HLA genotype
U266	C1+ C2- Bw4-
L363	C1+ C2- Bw4-
LME-1	C1+ C2- Bw4-
UM-9	C1+ C2- Bw4-
RPMI-8226/s	C1+ C2+ Bw4-
OPM-1	C1+ C2+ Bw4-
XG-1	C1+ C2+ Bw4+

Table 1: Matched and mismatched KIRs based on genotypic expression of HLA epitopes. Genotypic expression of HLA-class I epitopes as determined by luminex-SSO.

Mice

The RAG2$^{-/-}$γc$^{-/-}$ mice were originally obtained from the Amsterdam Medical Center (Amsterdam, The Netherlands). They were bred and maintained in filter top cages under specified pathogen-free conditions at the Central Laboratory Animal Institute (Utrecht University, Utrecht, The Netherlands) and received sterile water and irradiation sterilized food pellets ad libitum. All animal experiments were approved by the Ethical Committee for Animal Experimentation of Utrecht University and in accordance with current Dutch Law on Animal Experiments.

Generation of Multiple Myeloma (MM) RAG2$^{-/-}$γc$^{-/-}$mice

Generation of MM in RAG2$^{-/-}$γc$^{-/-}$mice was performed as described in more detail in [25]. In brief, twenty-four hours before the injection of freshly cultured MM cells, mice received total body irradiation conditioning treatment (TBI; 3.0 Gy, 200kV X-rays). $5×10^6$ U266 cells were injected intravenously (i.v.) via the lateral tail vein. For the experiments with subcutaneous (s.c.) MM tumors, $0.3×10^6$, $1×10^6$, $3×10^6$ or $9×10^6$ U266 cells in 100 μl phosphate-buffered saline were injected s.c. in the flank of the mice. In all experiments, tumor load was determined by Bioluminescence Imaging (BLI) measurements (Biospace Φ-Imager and Acquisition software) on the ventral and dorsal sides of the mice. The BLI images were analyzed with M3Vision software (Biospace, Paris, France). In case of paralysis of the hind limbs or when the mice became moribund, they were sacrificed by cervical dislocation. To determine progression free survival mean BLI signal (ventral BLI signal + dorsal BLI signal)/2 for each time point was calculated. Progression was defined as a mean BLI signal that was greater than 2x the mean BLI signal at start of treatment on day 34 when applicable.
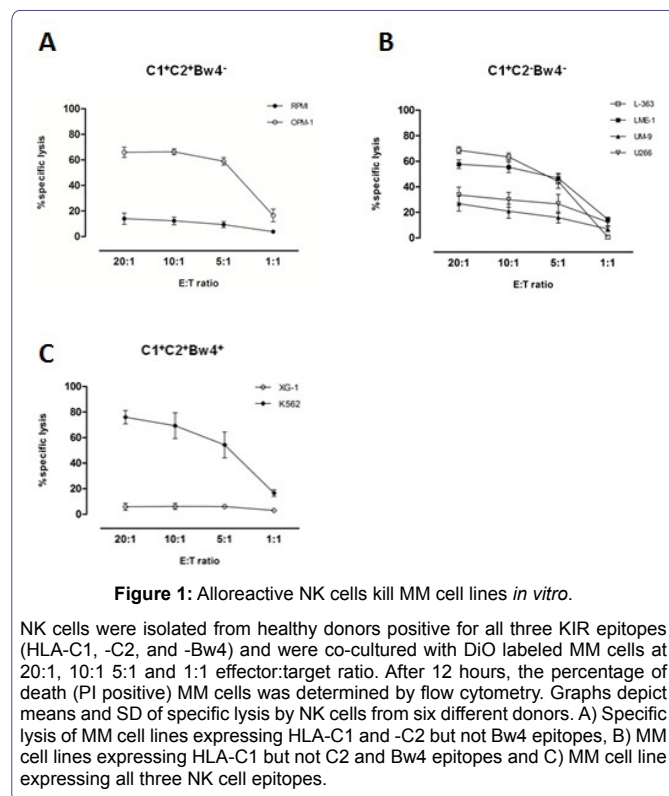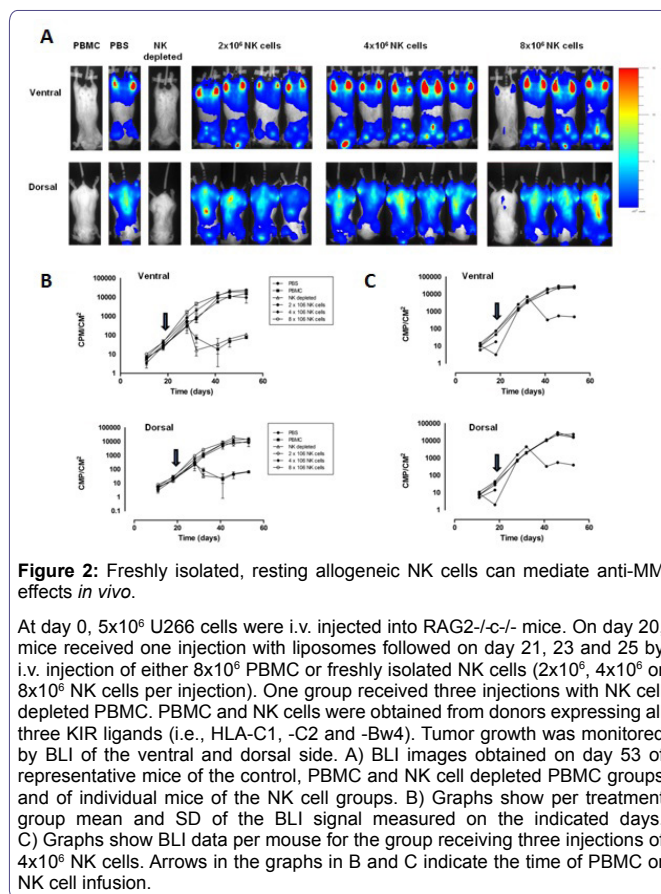
Figure 1: Alloreactive NK cells kill MM cell lines *in vitro*.

NK cells were isolated from healthy donors positive for all three KIR epitopes (HLA-C1, -C2, and -Bw4) and were co-cultured with DiO labeled MM cells at 20:1, 10:1 5:1 and 1:1 effector:target ratio. After 12 hours, the percentage of death (PI positive) MM cells was determined by flow cytometry. Graphs depict means and SD of specific lysis by NK cells from six different donors. A) Specific lysis of MM cell lines expressing HLA-C1 and -C2 but not Bw4 epitopes, B) MM cell lines expressing HLA-C1 but not C2 and Bw4 epitopes and C) MM cell line expressing all three NK cell epitopes.

Preparation and transplantation of human PBMCs and peripheral blood derived NK cells

Buffy coats were obtained from healthy human blood donors that were HLA-genotyped (all donors were HLA-C1, C2 and Bw4 positive) and KIR genotyped (all donors expressed KIR2DL1, KIRDL2/3 and KIR3DL1) by Sequence-Specific Oligonucleotides (SSO) analysis and Luminex® according to manufacturer's guidelines (One Lambda). Mononuclear cells from peripheral blood of these healthy donors were isolated by density gradient separation. NK cells were either negatively (for NK cell groups) or positively (for NK-depleted group) selected by immunomagnetic cell separation according to manufacturer's protocol (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purity of isolated populations typically exceeded 95% as determined by flow cytometry. Unactivated NK cells were used directly upon isolation. For the experiments with IL-2 activated NK cells, the isolated NK cells were cultured overnight with 1000U/ml of IL-2 (Proleukin). Cord blood derived NK cells were expanded and differentiated into NK cells from umbilical cord blood CD34+ cells (HLA-C1, C2 and Bw4 positive) as described before [26,27] and were kindly provided by Dr. Spanholz and Dr. Groenewegen (Glycostem, Nijmegen, The Netherlands). Before injection, cells were counted and resuspended at the concentration indicated in the results section and legends. Suspensions of PBMC or NK cells were injected in a volume of 0.2 ml either intravenously into the mice via the tail vein or intratumorally. Mice received 0.2 ml of the liposomal suspension intravenously one day before injection of the cells. Control mice received liposomes as well but no PBMC or NK cell infusion. Liposomes were prepared as described previously [25]. GvHD was scored (4-point scale) on the parameters poor physical condition, ruffled fur, arched back, and a body weight loss greater than 10 percent.

Figure 2: Freshly isolated, resting allogeneic NK cells can mediate anti-MM effects *in vivo*.

At day 0, 5x10⁶ U266 cells were i.v. injected into RAG2-/-c-/- mice. On day 20, mice received one injection with liposomes followed on day 21, 23 and 25 by i.v. injection of either 8x10⁶ PBMC or freshly isolated NK cells (2x10⁶, 4x10⁶ or 8x10⁶ NK cells per injection). One group received three injections with NK cell depleted PBMC. PBMC and NK cells were obtained from donors expressing all three KIR ligands (i.e., HLA-C1, -C2 and -Bw4). Tumor growth was monitored by BLI of the ventral and dorsal side. A) BLI images obtained on day 53 of representative mice of the control, PBMC and NK cell depleted PBMC groups and of individual mice of the NK cell groups. B) Graphs show per treatment group mean and SD of the BLI signal measured on the indicated days. C) Graphs show BLI data per mouse for the group receiving three injections of 4x10⁶ NK cells. Arrows in the graphs in B and C indicate the time of PBMC or NK cell infusion.

Flow cytometry

Cell isolates from hind limbs were analyzed using flow cytometry for the presence of human cells in the total nucleated cell fraction. Bone marrow was obtained by flushing the bones with PBS. Single cell suspensions from the soft tissues were obtained by passaging through 70 μm cell strainers (Becton Dickinson Biosciences, Erembodegem, Belgium). Fluorochrome conjugated monoclonal antibodies specific for surface molecules were used: human-CD3, human-CD45, mouse-CD45, human-CD56 all obtained from BD Biosciences. Cells were incubated with antibodies at proper dilutions for 30 minutes on ice. Samples were analyzed on a FACS Canto II (BD) with FACS Diva (BD) software.

NK cell Cytotoxicity assay

Enriched NK cells, isolated as described in the preparation and transplantation of huPBMCs section, were used in cytotoxicity assays. Target cells (MM cells) were labeled with 3'-Dioctadecyloxacarbocyanine (DiO) according to the manufacturer's instructions (Sigma) and 2×10^4 target cells were incubated with NK cells at various Effector:Target (E:T) ratios for 12 hours, each ratio in triplicate. Percentage of killed target cells (%PI positive and DiO positive cells) was determined by flow cytometry. Percentages specific lysis was calculated as follows: ((% PI positive target cells - % spontaneous PI positive cells)/ % of viable cells) × 100.
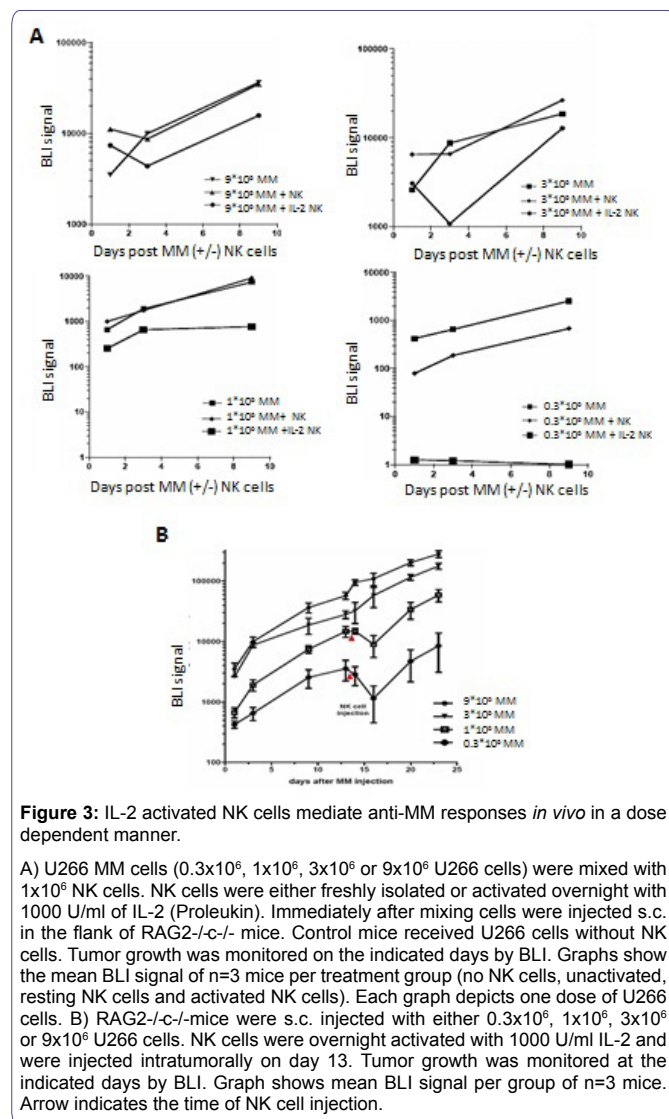
Figure 3: IL-2 activated NK cells mediate anti-MM responses *in vivo* in a dose dependent manner.

A) U266 MM cells (0.3×10^6, 1×10^6, 3×10^6 or 9×10^6 U266 cells) were mixed with 1×10^6 NK cells. NK cells were either freshly isolated or activated overnight with 1000 U/ml of IL-2 (Proleukin). Immediately after mixing cells were injected s.c. in the flank of RAG2-/-c-/- mice. Control mice received U266 cells without NK cells. Tumor growth was monitored on the indicated days by BLI. Graphs show the mean BLI signal of n=3 mice per treatment group (no NK cells, unactivated, resting NK cells and activated NK cells). Each graph depicts one dose of U266 cells. B) RAG2-/-c-/-mice were s.c. injected with either 0.3×10^6, 1×10^6, 3×10^6 or 9×10^6 U266 cells. NK cells were overnight activated with 1000 U/ml IL-2 and were injected intratumorally on day 13. Tumor growth was monitored at the indicated days by BLI. Graph shows mean BLI signal per group of n=3 mice. Arrow indicates the time of NK cell injection.

Statistics

Differences in progression free survival (Figure 1 and 2) were calculated with Graphpad Prism software version 5.01 using log rank test (Mantel Cox) test.

Results

Alloreactive NK cells kill MM cell lines *in vitro*

To test whether freshly isolated alloreactive KIR-ligand mismatched NK cells could eliminate MM cells *in vitro*, NK cell alloreactivity against a panel of eight MM cell lines was determined. These cell lines were first genotyped for HLA-A,-B and-C to determine which KIR ligands were present and absent. Two of the cell lines, i.e., RPMI-8226/s and OPM-1, expressed HLA-C1 and -C2 but not the Bw4 epitope (Table 1). U266, LME-1, L-363 and UM-9 expressed only the HLA-C1 and not the C2 and Bw4 epitopes. XG-1 was the only cell line expressing all three epitopes. Then, cell lines were co-cultured with freshly isolated NK cells derived from healthy donors. To avoid hyporesponsiveness of NK cells due to lack of licensing, donors expressing all three epitopes (i.e., positive for HLA-C1, -C2 and -Bw4) were used. After 12 hours of co-culture, the
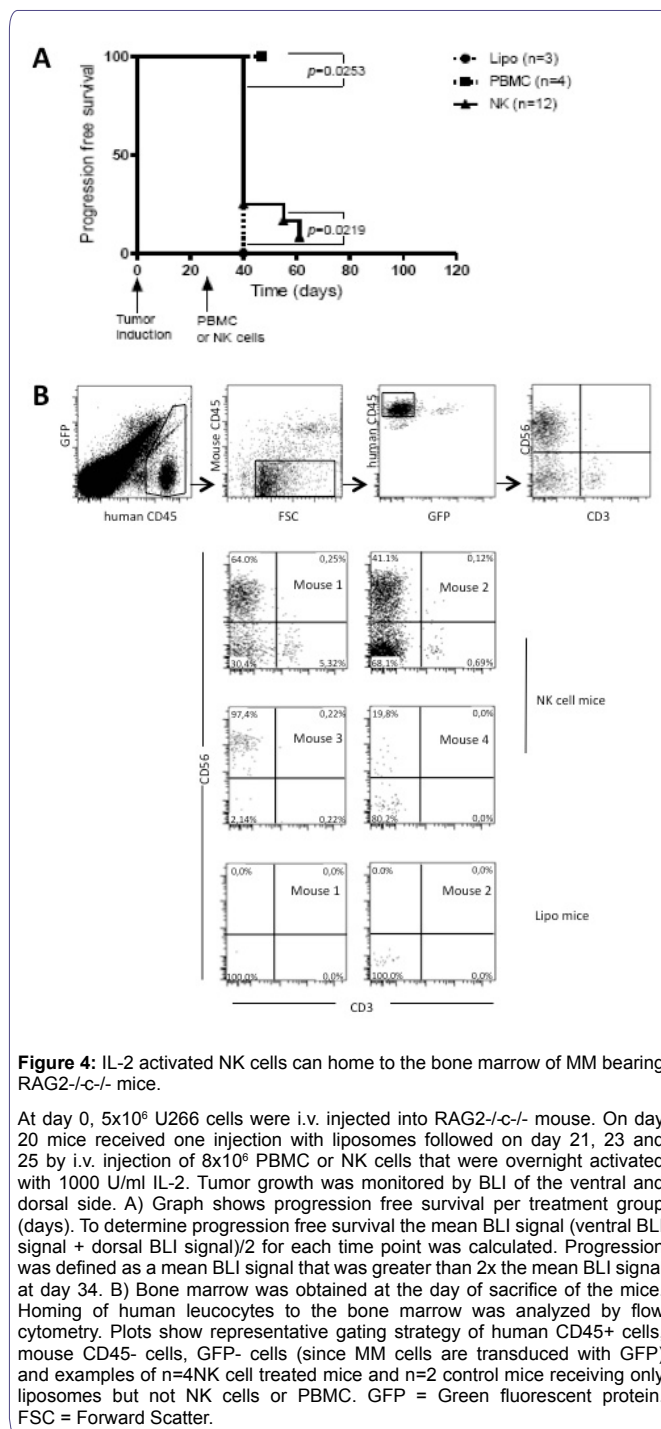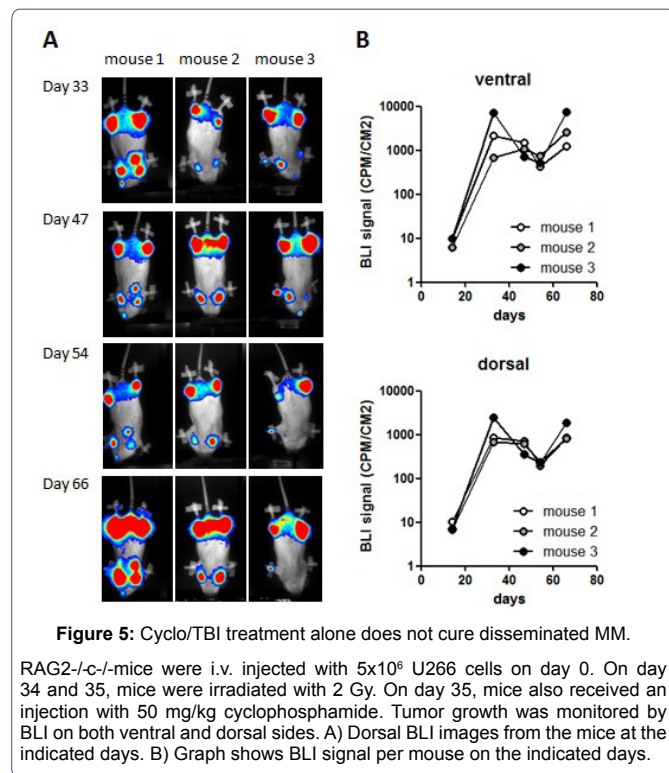

Figure 4: IL-2 activated NK cells can home to the bone marrow of MM bearing RAG2-/-c-/- mice.

At day 0, 5×10^6 U266 cells were i.v. injected into RAG2-/-c-/- mouse. On day 20 mice received one injection with liposomes followed on day 21, 23 and 25 by i.v. injection of 8×10^6 PBMC or NK cells that were overnight activated with 1000 U/ml IL-2. Tumor growth was monitored by BLI of the ventral and dorsal side. A) Graph shows progression free survival per treatment group (days). To determine progression free survival the mean BLI signal (ventral BLI signal + dorsal BLI signal)/2 for each time point was calculated. Progression was defined as a mean BLI signal that was greater than 2x the mean BLI signal at day 34. B) Bone marrow was obtained at the day of sacrifice of the mice. Homing of human leucocytes to the bone marrow was analyzed by flow cytometry. Plots show representative gating strategy of human CD45+ cells, mouse CD45- cells, GFP- cells (since MM cells are transduced with GFP) and examples of n=4NK cell treated mice and n=2 control mice receiving only liposomes but not NK cells or PBMC. GFP = Green fluorescent protein. FSC = Forward Scatter.

percentage of death MM cells was determined by flow cytometric analysis of the percentage PI positive MM cells. This revealed that killing of all MM lines and of K562, a leukemia cell line devoid of HLA class I expression and used as positive control cell line for NK mediated lysis, occurred in a NK cell concentration dependent manner (Figure 1 A-C). Killing efficiency differed between the lines; LME-1, L363, OPM-1 were almost as sensitive to NK cell killing as K562 (Figure 1 A-B) whereas the C1+C2+Bw4+ cell line XG-1 was quite resistant to killing by alloreactive NK cells (Figure 1C). Together this shows that MM cell lines, though at different efficiencies, can by killed by alloreactive NK cells *in vitro*.

Figure 5: Cyclo/TBI treatment alone does not cure disseminated MM.

RAG2-/-c-/-mice were i.v. injected with 5x10⁶ U266 cells on day 0. On day 34 and 35, mice were irradiated with 2 Gy. On day 35, mice also received an injection with 50 mg/kg cyclophosphamide. Tumor growth was monitored by BLI on both ventral and dorsal sides. A) Dorsal BLI images from the mice at the indicated days. B) Graph shows BLI signal per mouse on the indicated days.

Freshly isolated, resting alloreactive NK cells can mediate anti-MM effects *in vivo*

To investigate whether NK cells mediate anti-MM responses *in vivo*, the $RAG2^{-/-}\gamma c^{-/-}$ mouse disseminated MM model was used with the U266 cell line. This cell line was killed by NK cells *in vitro* (Figure 1), and in a previous study we showed that infusion of PBMC could cure mice from established MM induced by injection of U266 cells [25]. In the current study, 5x10⁶ U266 cells were i.v. injected on day 0 followed by i.v. injection of either PBMC or freshly isolated, resting NK cells at different doses on day 21, 23 and 25. To test whether NK cells were required for the anti-tumor response after PBMC injection, one group of mice received PBMC depleted from NK cells. Both PBMC and NK cells were obtained from peripheral blood of donors expressing all three KIR ligands (i.e., HLA-C1, -C2 and -Bw4). Monitoring of tumor growth by BLI revealed that all treatment groups had developed MM at the day of PBMC or NK cell injection (Figure 2 A-B). As expected from our previous studies, injection of PBMC subsequently completely cured the mice from MM. NK cells were not required for this curative effect as injection of NK cell depleted PBMC was as effective as injection of the complete PBMC fraction. NK cell treatment was performed by three times injection (days 21, 23 and 25) with either 2x10⁶, 4x10⁶ or 8x10⁶ NK cells per injection. In none of the two groups receiving the lower NK cell doses this resulted in a robust anti-MM response and tumor load of the NK cell groups was comparable to the tumor load in the control group (i.e., group w.o. PBMC or NK cells; Figure 2 A-B). However, in the group receiving the highest dose of NK cells one mouse apparently showed a reduction in tumor burden upon infusion of NK cells hinting that purified NK cells can mediate anti-MM responses in this model (Figure 2C). In the groups receiving PBMC or NK cell depleted PBMC all mice developed signs of GvHD as observed by visual inspection of the mice and by weight loss. Importantly, none of the NK cell treated mice showed signs of GvHD demonstrating the safety of NK cell infusion.

IL-2 activated NK cells mediate anti-MM responses in a dose dependent manner *in vivo*

NK cells can eliminate MM cells *in vitro*, however *in vivo* no anti-myeloma effect is seen. Therefore, we hypothesized that the bone marrow micro-environment could suppress NK cell function. To test if NK cells were actually able to function in the $RAG2^{-/-}\gamma c^{-/-}$, we performed an *in vivo* kill assay. We have previously shown that IL-2 activation can potentiate the anti-MM response of NK cells *in vitro*. As in this case the number of NK cells needed for the *in vivo* kill is expected to be lower, U266 MM cells (0.3x10⁶, 1x10⁶, 3x10⁶ or 9x10⁶ U266 cells) were mixed with 1x10⁶ IL-2 activated NK cells followed by immediate s.c. injection in the flank of $RAG2^{-/-}\gamma c^{-/-}$ mice. Monitoring of tumor growth showed that the BLI signal increased between day 0 and day 9 in the groups of mice receiving any of the four doses of U266 cells but not NK cells (Figure 3A). This increase in BLI signal was also observed for the groups receiving U266 cells and non-activated, resting NK cells. For all groups injected with U266 cells and IL-2 activated NK cells the BLI signal was lower than that of the corresponding control groups and in the group receiving the lowest number of U266 cells the effect was already detectable by *in vivo* imaging on day 1. Co-injection of activated NK cells reduced outgrowth of the tumor showing NK cells are active in this model (Figure 3A).

The above-mentioned data demonstrated that the effect of IL-2 activated NK cells occurred in a dose dependent manner. To investigate whether IL-2 activated NK cells could eliminate established MM *in vivo* as well, mice were s.c. injected with either 0.3x10⁶, 1x10⁶, 3x10⁶ or 9x10⁶ U266 cells. Upon development of a s.c. tumor on day 13, IL-2 activated NK cells were injected intratumorally. In the two groups receiving the highest number of U266 cells (3x10⁶ or 9x10⁶ cells), NK cell injection did not reduce the tumor load (Figure 3B). However, in the two groups receiving the lowest number of U266 cells (0.3x10⁶ or 1x10⁶ cells) a reduction in the BLI signal could be observed after NK cell injection. Together these data demonstrate that IL-2 activated NK cells can mediate anti-MM responses *in vivo* but that the effector to target ratio is very important for *in vivo* responses.

IL-2 activated human NK cells can home to the bone marrow of MM bearing $RAG2^{-/-}\gamma c^{-/-}$ mice

As we established that IL-2 activated NK cells are indeed effective in $RAG2^{-/-}\gamma c^{-/-}$mice after subcutaneous injection, we next investigated the effect of IL-2 activated KIR-ligand mismatched NK cells in the disseminated MM model. To this end, 5x10⁶ U266 cells were i.v. injected, followed by i.v. injection of PBMC on day 21, 23 and 25, (n=4 mice) or 8x10⁶ NK cells that were overnight activated with IL-2 (on day 21, 23 and 25, n=6 mice). As an alternative source of activated NK cells, we used NK cells expanded from cord blood and six mice received on day 21 one injection with 24x10⁶ cord blood derived NK cells. Since it has previously been shown that IL-15 administration alone promotes *in vivo* survival and expansion of NK cells while it does not have an anti-tumor effect [28], IL-15 was injected i.p. three times a week for two weeks after NK cell infusion in this experiment. Around day 40, all control mice (mice receiving no NK cells or PBMC) were sacrificed due to high tumor burden. For the NK cell treated mice progression free survival was longer than for control mice (p=0.02; Figure 4A). Nevertheless, also the NK cell treated mice died due to high tumor load. In line with our experiment in Figure 2, injection of PBMC reduced tumor burden but these mice had to be sacrificed

due to severe GvHD around day 50. Symptoms of GvHD were not observed in NK cell treated mice. Importantly, flow cytometric analysis of the bone marrow harvested at the day of sacrifice of the mice revealed that cells expressing the human leukocyte marker CD45 and CD56 but not CD3 (hCD45+CD56+CD3- is indicative of NK cells) were present in the bone marrow (Figure 4B). These hCD45+CD56+CD3- NK cells were only present the IL-2 activated NK cell treated groups indicating that NK cells could actually home to the bone marrow. In the mice receiving NK cells obtained from peripheral blood hCD45+CD56-CD3+ T cells (range 0 -5.32%) and cells expressing hCD45+ but not CD56 or CD3 were also present (Figure 4B) in the bone marrow.

Cyclophosphamide (cyclo)/Total Body Irradiation (TBI) treatment alone does not cure disseminated MM

In parallel studies in a mouse model for breast cancer, we obtained evidence that cyclo/TBI conditioning is important for *in vivo* anti-tumor responses of alloreactive NK cells against 4T1 breast cancer tumor cells [29]. In the current study, we therefore aimed to investigate whether addition of cyclo/TBI to the treatment regime resulted in prolonged progression free survival in the RAG2$^{-/-}$γc$^{-/-}$ MM model. To confirm that cyclo/TBI treatment was performed at a dosage that by itself was not sufficient to eliminate the tumor, we

Figure 6: Combination treatment with cyclo/TBI and IL-2 activated NK cells provides anti-MM responses in the disseminated model.

RAG2-/-c-/-mice were i.v. injected with 5x10⁶ U266 cells on day 0. On day 34 and 35, mice were irradiated with 2 Gy. On day 35, mice also received an injection with 50 mg/kg cyclophosphamide. NK cell treated mice also received activated 8x10⁶ NK cells (blood or cord blood derived) on days 36, 38 and 42 by i.v. injection. Control mice received neither cyclo/TBI nor NK cells. Tumor growth was monitored by BLI on both ventral and dorsal sides. A) Dorsal BLI images from the mice at the indicated days. B) Graph shows progression free survival per treatment group. To determine progression free survival means BLI signal (ventral BLI signal + dorsal BLI signal)/2 for each time point was calculated. Progression was defined as a mean BLI signal that was greater than 2x the mean BLI signal at day 34.

performed a pilot experiment in which RAG2$^{-/-}$γc$^{-/-}$mice were i.v. injected with 5*10⁶ U266 cells on day 0. After development of MM, mice were irradiated with 2 Gy on day 34 and day 35. On day 35 mice also received 50 mg/kg cyclophospamide. Monitoring of tumor growth revealed that although cyclo/TBI treatment initially reduced the tumor burden, it was not sufficient to cure the mice as tumors started growing again in these mice (Figure 5).

Combination treatment with cyclo/TBI and IL-2 activated NK cells can provide anti-MM responses in the disseminated MM model

To investigate the effect of treatment with IL-2 activated NK cells in combination with cyclo/TBI, MM was induced in RAG2$^{-/-}$γc$^{-/-}$ mice by i.v. injection of 5x10⁶ U266 cells on day 0. In line with our pilot, cyclo/TBI treatment was done at days 34 and 35 and tumor growth was monitored by BLI. This confirmed that cyclo/TBI treatment only slightly, and not significantly, increased progression free survival as compared to control mice (p=0.06; Figure 6). However, chemo/radio therapy in combination with injection of 8x10⁶ IL-2 activated blood derived NK cells (on day 36, 38 and 42) or 24x10⁶ of cord blood-derived NK cells on day 36 prolonged progression free survival as compared to control mice (p=0.003; Figure 6). No difference was noted between the two sources of NK cells. Two mice in the NK cell treated group died for unknown reason while having a very low tumor load and without having signs of GvHD. In the other NK cell treated mice we also did not observe signs of GvHD by visual inspection. Although MM eventually progressed, this shows that combination treatment with cyclo/TBI and IL-2 activated alloreactive NK cells are able to provide anti-MM effects against established disseminated MM.

Discussion

Despite novel treatment options in the last decade, MM remains largely incurable illustrating the need to develop novel therapeutic treatment options. In the present proof-of-concept study, we investigated the effect of alloreactive KIR-ligand mismatched NK cells on GvM and GvHD in the RAG2$^{-/-}$γc$^{-/-}$ mouse MM model. This revealed that IL-2 activated NK cells in combination with low dose conditioning with cyclo/TBI can mediate anti-MM responses. Importantly, we did not observe a single sign of GvHD in any of the experimental treatment protocols upon NK cell injection. In line with these data, a first clinical study in MM showed that KIR-ligand mismatched NK cells could be infused into MM patients without causing toxicity and GvHD [18] and comparable observations have been made in AML. Altogether, this illustrates the feasibility and clinical potential of the approach.

Thus far, the clinical efficacy of NK cell infusion in cancer patients has been somewhat inconsistent emphasizing that optimization of therapeutic protocols is required [30]. NK cells can kill only a limited number of target cells. In the current study, we observed that NK cell responses against s.c. MM occurred in a NK cell dose dependent manner (Figure 3) and with unactivated, resting NK cells, we possibly observed a biological effect of NK cells in one of the four mice (25%) with disseminated MM in the group receiving the highest NK cell dose (Figure 2). Dose dependency has also been shown in the 5T33M MM model were the anti-MM response of transplanted mouse NK cells could be enhanced by infusion of a higher number
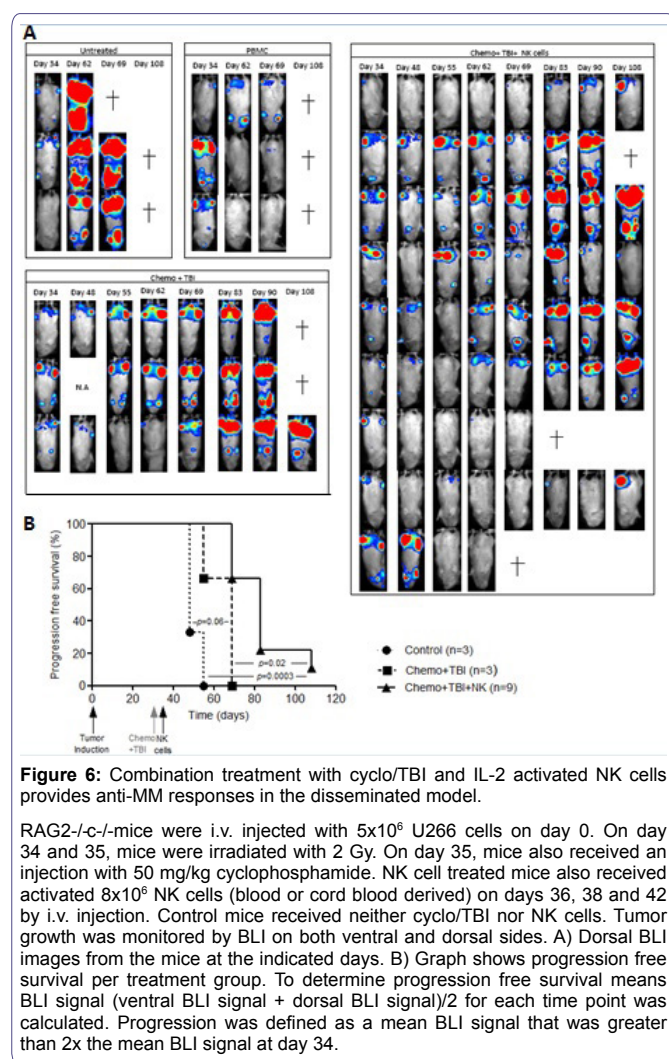
of NK cells [31]. In another study, with human NK cells expanded on K562-mb15-41BBL feeder cells, adoptive transfer of 160x10⁶ NK cells inhibited growth of OPM2 while transfer of 40x10⁶ NK cells was not sufficient [32]. These data suggest that infusion of really high numbers of NK cells are needed to improve clinical efficacy of NK cell therapy. Numbers that can be obtained using a bioreactor (our unpublished data).

In our model, mice having established MM were treated with NK cells. In that way, our model resembles the clinical situation were plasma cells are dispersed over multiple sites encompassing the entire skeletal system. Because of this high tumor load, a major caveat is the number of NK cells required for clinically effective therapy that can be obtained by isolation from peripheral blood. *Ex vivo* expanded NK cells could provide a solution and are an interesting alternative for NK cells derived from apheresis products. An additional advantage is that the *ex vivo* culturing period would allow for extra NK cell activation. In the current study, we used two sources of NK cells; IL-2 activated NK cells isolated from blood of healthy individuals and NK cells that were generated and expanded from cord blood [26,27]. With both sources we observed reactivity against established, disseminated MM in combination with chemo/TBI (Figure 6). This is in line with two previous studies showing that NK cells expanded on K562-mb 15-41BBL feeder cells [32] and the NK cell lines NK-92 or KHYG-1 [33] reduced disease burden *in vivo* (in mouse models for MM), together demonstrating that different sources of NK cells could be further tested for clinical application.

In the current study, NK cell donors were selected based on the genotypic presence of a mismatch between inhibitory KIRs on the NK cells and the HLA epitopes on the U266 cell line. Phenotypically, however, the infused NK cell population was heterogeneous because NK cells express one or a combination of inhibitory receptors on their cell surface hence leading to different NK cells subsets; i.e., subsets exclusively expressing KIRs mismatched with the HLA ligands present on U266 target cells, subsets exclusively expressing KIRs matched with the ligands and subsets expressing both types of KIRs. Therefore, only a small percentage of the infused NK cells exclusively expressed KIRs mismatched with the U266 HLA ligand (HLA-C1) and the majority of NK cells (co-)expressed NKG2A. We recently showed, that *in vitro* KIR-ligand mismatched NK cells are more effective than matched NK cells in mediating myeloma cell killing [22]. In addition, we demonstrated that U266 cells in this disseminated MM model expressed HLA-E at a level that is sufficient to inhibit NKG2A expressing NK cells [22]. Based on these data, NK cells expressing mismatched KIRs and lacking expression of matched KIRs or NKG2A could be expected to be the most potent effector cells against MM *in vivo* as well. Future experiments with HLA-E gene knock-down in U266-luc cells in the RAG2-/-γc-/-mice might enable us to determine the efficacy-blocking effect of HLA-E expression in NK cell therapy. Ruggeri et al., have demonstrated that, in an AML mouse model, indeed the mismatched NK cells mediated *in vivo* anti-tumor effects while matched NK cells did not [6]. Therefore, it is highly relevant to study whether KIR-ligand mismatched NK cells are indeed more potent against MM *in vivo* because acquiring high enough numbers of these subsets for clinical application will be challenging and it is probably worthwhile to develop strategies to promote expansion or development of NK cell subsets with the highest anti-MM reactivity.

Our data show that U266 cells are sensitive for NK cell killing *in vitro* (Figure 1) while *in vivo* these cells seemed to be more difficult to eliminate (Figure 2). An explanatory factor could be that only a fraction of the NK cells actually homes to the bone marrow resulting in a less favorable E:T ratio as compared to *in vitro*. Phenotypic analysis of bone marrow biopsies at the time of sacrifice of the mice revealed that activated NK cells derived from peripheral blood and cord blood indeed can home to the site of tumor location in our model (Figure 4). This is in line with a previous study showing that cord blood derived NK cells migrate to the bone marrow of NSG mice and functionally express CXCR4 [28], a chemokine receptor described to be important for homing to the bone marrow [34], and with the observation that primary blood derived NK cells express CXCR4 [35]. In addition to NK cells, we observed some T cells (huCD45+CD3+CD56-) and human leukocytes that were neither NK cells nor T cells (huCD45+CD3-CD56-) in mice receiving NK cells from peripheral blood. Presumably this was the result of the minor impurity of the peripheral blood derived NK cell product. Although, in principle contaminating T cells in the peripheral blood product can mediate an anti-MM activity as well, the actual numbers of T cells contaminating our cell preparations were not sufficient to explain the anti-MM response. To induce a xenogeneic GvHD response with infused PBMC cells at least 6x10⁶ T cells are required in pre-conditioned mice (TBI + toxic liposomes) [25]. In the experiment where we combined cyclo/TBI and NK cell treatment, we did not observe any human lymphocytes in the bone marrow at the time of sacrifice, which presumably was due to the longer follow-up period 108 days vs 60 days in the experiment with activated NK cells without cyclo/TBI.

In the RAG2-/-γc-/-model, MM cells grow in their natural environment, i.e., the bone marrow, making it an excellent model for preclinical testing of immunotherapy reflecting MM pathophysiology. This is important because it is exactly this environment that, through the presence of factors like hypoxia [23] and stromal cells [36], or soluble factors like PGE2 [37] can suppress development of effective anti-tumor immunity making it extremely important to test the anti-MM capacity of NK cells in such relevant models. An interesting other pr clinical model recently developed by some members of our group is a scaffold-based model where patient derived MM cells grow on s.c. implanted scaffolds [38]. In such a model, it would be highly i teresting to address the synergistic potential of a combination of a proaches targeting immunosuppressive bone marrow associated factors with alloreactive NK cell therapy to achieve maximal efficiency of the response. One such approach can be the combination of chemo/radiotherapy and activated NK cells that, in our study, resulted in a better anti-MM response as compared to treatment with activated NK cells alone. Here, we did not study the mechanism by which the conditioning regime enhanced NK cell functioning but, several previous studies demonstrated that either chemo or radiotherapy could render tumor cells more susceptible for NK cells by up regulation of activating ligands [39-41]. It can be hypothesized that donor dendritic cells are killed by the host NK cells, thus a T cell mediated GvHD cannot be induced [42]. On the other hand, the conditioning treatment could trigger the release of NK cell promoting cytokines such as IL-2, IL-15 and IL-21 [43] or promote recruitment or survival of the NK cells at the tumor site [44,45]. Also, it would be interesting to combine the immunomodulatory drug Pomalidomide or ADCC triggering agents such as Elotuzumab to boost the NK cell activity, in this model.

In the current proof-of-concept study, we showed the biological potential of alloreactive NK cells against disseminated MM. In addition, we demonstrated that IL-2 activated NK cells can home to

the bone marrow of MM bearing mice and that anti-MM responses occurred in a dose dependent manner. The latter suggests that infusion of a higher number of alloreactive NK cells can help to improve clinical efficacy and those patients should, preferably, be treated in remission or at least with low residual disease. Our data also demonstrate that combination therapy of alloreactive NK cells and chemo/radiotherapy could help to improve the anti-MM effect. The RAG2$^{-/-}$γc$^{-/-}$ mouse MM is a pathophysiologically relevant model to further develop and optimize NK cell treatment protocols and to test *ex vivo* expanded/activated NK cells ultimately leading to enhanced clinical efficacy.

Acknowledgements

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