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Anti-myeloma activity of endogenous and adoptively transferred activated natural killer cells in experimental multiple myeloma model

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Objective. **Despite advances in autologous stem cell transplantation and chemotherapy, multiple myeloma (MM) remains an incurable disease. Due to the role of natural killer (NK) cells in host resistance against several tumors, it is of interest to explore the anti-MM activity of NK cells. For this reason, we aimed to determine if NK cells provide anti-MM activity following interleukin-2 (IL-2) administration, and if *ex vivo* activated and intravenously administered NK cells prolong survival in MM-bearing C57BL/KaLwRij mice.**

Methods. **The anti-MM effect of IL-2 was tested by intraperitoneal injection into the 5T33MM-inoculated mice. Subsequently, *in vivo* effector cell depletions were performed by administration of anti-NK1.1 or anti-CD8 monoclonal antibodies. Finally, magnetically separated and activated NK cells from splenocytes of C57BL/KaLwRij mice were adoptively transferred to tumor-bearing mice in conjunction with IL-2 treatment.**

Results. **IL-2 administration into MM-bearing mice significantly prolonged their survival. This effect was diminished by *in vivo* depletion of NK cells. Adoptive transfer of activated NK cells showed a significant *in vivo* anti-MM effect that was dependent on cell dose. Biodistribution of the marked adoptively transferred NK cells correlated with MM cells' homing sites.**

Conclusion. **These data suggest that activated NK cells have a promising potential in adoptive immunotherapy for MM.**

Multiple myeloma (MM) is a plasma cell neoplasm characterized by clonal proliferation of plasma cells in the bone marrow (BM). It accounts for 20% of all deaths caused by hematological malignancies [1]. Although allogeneic stem cell transplantation [2–5] and novel drugs like thalidomide, lenalidomide, and bortezomib are tested [6], autologous stem cell transplantation with high-dose melphalan is currently the standard therapy for MM [7–9], but it still does not offer the prospect of a cure. Tumor-specific immunotherapy offers considerable potential in the management of patients with malignant disease. However, so far, efforts to target MM by immunotherapy such as immunoglobulin idiotype vaccination and dendritic cell therapy have had limited or no clinical impact [10–14]. Therefore, novel modalities to enhance the success rate of currently used treatments are needed.

One of the emerging possibilities is use of natural killer (NK) cells as cellular immunotherapy for MM. NK cells are cytotoxic lymphocytes of the innate immune system, clearly distinguishable from T and B lymphocytes [15], and initially discovered because of their ability to spontaneously kill certain tumor cells [16,17]. They have shown capacity to control growth and metastatic spread of certain tumors *in vivo* [18]. However, the role of NK cells in MM remains unclear.

In order to study the *in vivo* effects and the role of autologous-activated NK cells in MM, their effects on the disease and the interaction between them have to be studied in experimental models. Several models for MM have been developed to date, yet many of them are not optimal for evaluating immunotherapeutic approaches. A good model for such studies, however, is a model that comprises

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syngeneic tumor cells and a host with a functional immune system. The C57BL/KaLwRij murine myeloma model fulfills these criteria. About 1% of aging C57BL/KaLwRij spontaneously develop MM [19–21], with characteristics very similar to the human disease [22], and transfer of BM cells derived from MM-bearing mice into syngeneic recipients is being used to trigger MM [23]. MM cell lines, such as 5T33MM, derived from C57BL/KaLwRij mice can likewise be used to induce MM.

In this study, we specifically aimed to characterize possible NK-cell cytotoxic response against syngeneic MM cells following interleukin (IL)-2 treatment in the C57BL/KaLwRij model. Furthermore, we investigated the potential of adoptive immunotherapy using *ex vivo* cultured and activated NK cells in this experimental MM model.

Materials and methods

Cell lines

The 5T33 murine myeloma cell line, derived from C57BL/KaLwRij mice, was kindly provided by Dr. J. Radl (TNO Institute, Rijswijk, The Netherlands). eGFP-5T33 cells that express enhanced green fluorescent protein (eGFP) were prepared as described previously [24]. Both cell lines were maintained at 0.5×10^6 cells/mL concentrations and cultured in minimal essential medium containing 2 mM L-glutamine, 100 μM nonessential amino acids, 1 mM sodium pyruvate, 0.5 μM 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum (FCS). The YAC-1 cell line (mouse lymphoma, susceptible to NK cell lysis *in vitro*) was grown in RPMI-1640 medium with 10% FCS (all components were purchased from GIBCO-Invitrogen, Invitrogen AB, Stockholm). The Phoenix GP retrovirus packaging cell line (used with permission from Dr. G. P. Nolan, Department of Molecular Pharmacology, Stanford University School of Medicine, Stanford, CA, USA) was cultured in Dulbecco's modified Eagle medium with 4.5 mg/mL glucose, pyridoxine, sodium pyruvate, and Glutamax-II with 10% heat-inactivated FCS. Cells were incubated at 37°C, 5% CO_2, and 95% humidity and medium was changed every 2 to 4 days. Aliquots of early passaged cells were frozen in 10% dimethyl sulphoxide (Sigma-Aldrich, St. Louis, MO, USA), 90% FCS and stored at −150°C for later reconstitution. All cell populations were observed at regular intervals using an inverted microscope (Olympus CK40) equipped with a UV module and were monitored for viability with trypan blue dye exclusion and for mycoplasma contamination.

Mice

Female and male C57BL/KaLwRij mice were purchased from Harlan CPB (Horst, The Netherlands). C57BL/6 mice were from the Department of Microbiology, Tumor and Cell Biology breeding unit at the Karolinska Institutet. Both strains were housed in our animal facilities at the Clinical Research Centre at Karolinska University Hospital Huddinge, under conventional conditions, including access to tap water and standard chow *ad libitum.* All mice were 8 to 10 weeks old at the beginning of each experiment. The study was approved by the local animal ethics committee in South Stockholm, Sweden.

Tumor-cell injection and induction of MM

C57BL/KaLwRij mice were injected intravenously (i.v.) with 10^5 5T33MM cells (for survival studies) or 10^5 eGFP-5T33MM cells (for biodistribution studies) suspended in a total volume of 100 μL sterile phosphate-buffered saline (PBS) per mouse. Control mice were injected with an equal volume of PBS i.v. Animals were examined twice daily for development of paraplegia. For biodistribution studies, at weekly intervals and at the time of disease development, mice were sacrificed by CO_2 inhalation followed by cervical dislocation. Spleens, livers, thymi, and lymph nodes were then excised and kept in PBS until processing for preparation of single-cell suspensions. BM from femora and tibiae were obtained by flushing PBS into the cavities of bones.

IL-2 administration into tumor-bearing mice

rIL-2 (Proleukin, Chiron, Emeryville, CA, USA) was injected intraperitoneally (i.p.) at a concentration of 600 μg/kg ($\sim 9.83 \times 10^6$ IU/kg) and administered twice daily for a total of 14 days starting 1 day after MM inoculation to mimic minimal residual disease. Mice were then monitored for disease development.

In vivo depletion of mouse NK1.1$^+$ and CD8$^+$ cells

To deplete NK1.1$^+$ cells *in vivo*, mice were injected starting 2 days before MM challenge with 200 μg anti-NK1.1 monoclonal antibodies i.p., and every 5 days thereafter, until termination of the experiment. Control mice were injected with a similar volume (0.2 mL) and dose of mouse IgG isotype control antibody (Sigma). To deplete CD8$^+$ cells *in vivo*, mice were injected starting 2 days before MM challenge with 500 μg anti-CD8 monoclonal antibody (clone 2.43), and every 5 days thereafter, until termination of the experiment. The efficacy of depletion of NK and CD8$^+$ T cells was monitored by flow cytometric analysis of spleen cells at the endpoint of the experiment. Also, the NK-T cell counts were analyzed at the same time points to be able to observe if NK1.1. depletion had an effect on NK-T cells. Animals exhibiting $>1.0\%$ NK1.1$^+$ or CD8$^+$ cells in the spleen were excluded from the study ($n = 1$ for NK1.1, $n = 3$ for CD8 depletion).

Preparation and separation of NK cells

Single-cell suspensions of spleen cells from mice were pooled in serum-free RPMI-1640 medium by filtering the suspension through mesh with the aid of a homogenizer to exert gentle pressure on the spleen fragments. Erythrocytes were lysed in ammonium chloride solution (0.15 mol/L NH_4Cl; 10 mmol/L $KHCO_3$; 0.1 mmol/L ethylenediamine tetraacetic acid, pH 7.2) or were separated using Lympholyte M (Cedarlane Labs, Ontario, Canada). After single-cell suspension, mouse NK cells were separated using a CD49b (DX5) Mouse Microbeads Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer's instructions. Briefly, single-cell suspensions from different organs were prepared as described above, and the cell number was determined. After cell centrifugation at 300*g* for 10 minutes, the supernatant was removed completely. The cell pellet was resuspended in 90 μL rinsing buffer (PBS, 0.5% bovine serum albumin, 2 mM ethylenediamine tetraacetic acid). Cells were mixed with 10 μL CD49b (DX5) [25] microbeads per 10^7 total cells and incubated for 15 minutes at 4°C. Prior to magnetic separation, cells were washed with 2 mL rinsing buffer per 10^7 cells, centrifuged at 300*g* for 10 minutes to remove supernatant and resuspended in 500 μL rinsing buffer. Meanwhile, magnetic columns were placed in the

magnetic field of a suitable magnetic-activated cell sorting separator and prepared for sorting by rinsing with 3 mL rinsing buffer three times. Finally, the cell suspension was placed into the column. All the unlabeled (negative) cells were rinsed out and collected into a 15-mL Falcon tube. To collect the labeled NK effector cells ($DX5^+$ cells), the column was removed from the magnetic field; 5 mL rinsing buffer was applied to the column and the plunger was used to flush out the positive cell fraction into a clean 15-mL Falcon tube. Obtained cells were washed twice with PBS and resuspended in the complete RPMI-1640 medium (RPMI-1640 medium supplemented with 10% inactivated FCS, 2 mmol/L L-glutamine, 25 mmol/L $NaHCO_3$, 1 mmol/L sodium pyruvate, 25 mmol/L HEPES) with Trace Elements A (Mediatech Inc., Herndon, VA, USA). Proleukin (rIL-2; 500 U/mL) in fresh complete RPMI medium was added on day 0 and every other day afterward. For expansion, the effector cells were cultured for 5 days in a concentration of 10^6 cells/mL and cell density was determined daily. The $DX5^+$ separated NK cells were used for cytotoxicity assay, retroviral transduction, and adoptive transfer, as we will describe here.

Cytotoxicity assay

Cytotoxic functions of NK cells were measured in a ^{51}Cr-release assay in triplicate. Briefly, 1×10^6 target cells were labeled with 100 μL ^{51}Cr (specific activity of 1 mCi/mL) and were incubated for 1 hour at 37°C. 5T33, YAC-1, and fresh autologous cells were used as targets. Nonactivated or IL-2–activated NK cells were mixed with target cells to obtain effector-to-target ratios of 10:1, 3:1, 1:1, and 0.3:1. RPMI medium alone was used as negative control, and for a positive control, cells were incubated with 1% of Triton X. Target cells with RPMI medium only were used for spontaneous release. After incubation in a V-bottom 96-well plate for 4 hours at 37°C, 70 μL supernatants were aspirated from each well and counted using a Packard Cobra Auto-Gamma 5000 Series Counting System (Meriden, CT, USA). The percentage of specific release was calculated from the following formula: % specific ^{51}Cr release = (sample release-spontaneous release) / (maximum release – spontaneous release) × 100.

Retroviral vector production

The retroviral vector plasmid pSF91-DsRed2-N1-g was created by cloning the DsRed2 gene from pDsRed2-N1 (BD Clontech, Mountain View, CA, USA) as an EcoRI-MfeI fragment into the EcoRI site of pSF91-MCSg [24]. Phoenix GP cells were transiently transfected with 3 μg vector plasmid and 1 μg pMD-G (encoding vesicular stomatitis virus envelope glycoprotein, kindly provided by Dr. D. Trono, Department of Genetics and Microbiology, University of Geneva, Geneva, Switzerland) per 35-mm cell culture well. For transfections, Fugene 6 reagent (Roche Boehringer Mannheim, Germany) was used according to manufacturer's instructions. Briefly, pSF91-DsRed2-N1-g and the Fugene reagent were mixed at 1:2 mass-to-volume ratio in 100 μL volume of cell culture medium and added to cells 15 minutes later. When cells were subconfluent, new fresh medium was added and incubated for 24 hours, after which the supernatant was collected, filtered through a 0.45-μm filter (Millipore, Billerica, MA, USA), and frozen at −70°C. The resulting supernatant had a mean titer of around 0.5×10^6 virus particles/mL titrated on HeLa cells.

Retroviral transduction of NK cells

Previously separated $DX5^+$ NK cells from C57BL/KaLwRij mice were cultured at a concentration of 10^6 cells/mL and transduced with the DsRed2 retroviral vector mentioned previously on day 2 of *ex vivo* culture. All transductions were carried out by replacing the media with the retrovirus-containing supernatant at a multiplicity of infection (MOI) of 3, in the presence of 8 μg/mL polybrene (Sigma) and 500 IU/mL IL-2 by centrifugation at 1000*g*, at room temperature for 2 hours. The following day, cells expressing DsRed2 were sorted by FACS Vantage sorter (BD). After sorting, an aliquot of the sorted cells was stained with fluorescein isothiocyanate-conjugated NK1.1 antibody (BD Pharmingen, San Jose, CA, USA) and run on a FACSCalibur to analyze their purity. Transduced cells were cultured until day 5. Both negative and positive populations were included in the adoptive transfer study, and biodistribution of transduced cell injection was analyzed.

Adoptive transfer of NK cells

After transduction, $DsRed^+$ (transduced) and $DsRed^-$ (untransduced) NK cells were sorted by a FACS sorter (BD DIVA; BD Biosciences). These two effector populations, as well as NK cells that were cultured without transduction only, were separately injected i.v. at doses of 2×10^5 (n = 25) or 20×10^6 cells/mouse (n = 28), into C57BL/KaLwRij mice that had been inoculated with 10^5 5T33MM or eGFP-5T33MM cells on the previous day. Mice were then monitored for survival and flow cytometric analysis was performed.

Flow cytometry

Along with cultured unmodified NK cells, 2×10^7 transduced and sorted NK cells expressing the DsRed marker gene, as well as the negative fraction of sorted cells, were injected into tumor-bearing mice, as described above. Cells from the spleen, bone marrow, and liver were isolated for flow cytometry at time points indicated.

For the flow cytometric analysis of 5T33MM cells, 0.5×10^6 cells were washed once with PBS and incubated at 4°C for 30 minutes with appropriate amounts of following monoclonal antibodies: anti-$H\text{-}2D^b$ (KH95; BD Pharmingen), anti-$H\text{-}2K^b$ (AF6-88.5; BD Pharmingen), anti-MULT1 (237104; R&D Systems). Labeled cells were then washed with PBS and fixed in 4% paraformaldehyde prior to data acquisition.

Flow cytometric analysis was performed using a FACSCalibur along with CellQuest 3.3 analysis software (Becton-Dickinson, Palo Alto, CA, USA). In each sample, at least 10,000 cells were acquired in the analysis region of viable cells, defined by side and forward scatter.

Statistics

The nonparametrical Kruskal-Wallis test was used to compare absolute cell counts, NK cell percentages, and cytotoxicity in all cultures. Log-rank test was performed using Graph Pad Prism version 4.03 for Windows (Graph Pad Software, San Diego, CA, USA) to analyze statistical significance ($p < 0.05$) of the Kaplan-Meier survival curves.

Results

Induction of MM

C57BL/KaLwRij mice were injected i.v. with 10^5 5T33MM cells and monitored on a daily basis for the onset of

paraplegia due to tumor spread. 5T33 cells as well as eGFP-transduced 5T33 cells were highly tumorigenic and induced MM-associated symptoms, including primarily paralysis in the hind paws in C57BL/KaLwRij mice, within 22 to 43 days after inoculation (Fig. 1). No difference in terms of tumorigenicity or survival was observed between the groups of mice injected with wild-type or eGFP-transduced 5T33 cells.

IL-2 administration prolongs survival of 5T33MM-bearing mice in an NK-cell–dependent manner

To test if IL-2 prolongs survival of 5T33MM-bearing mice, they were inoculated with IL-2 twice daily, starting 1 day after tumor challenge. IL-2 administration into 5T33MM-bearing C57BL/KaLwRij mice significantly prolonged their survival ($p < 0.05$) (Fig. 1). This observation led us to test, by *in vivo* depletion, if prolongation of survival observed after IL-2 treatment was dependent on NK cells, $CD8^+$ T cells, or both. Interestingly, depletion of NK cells ($p < 0.05$), but not $CD8^+$ T cells ($p > 0.1$), abolished the IL-2–dependent anti-tumor effects in 5T33-inoculated mice, suggesting a role of NK cells in the anti-MM response. Although depletion of both populations did not give a statistically different curve ($p > 0.1$) when compared to depletion of NK cells, we did observe a slight decrease in survival, suggesting that $CD8^+$ T cells might have a supportive effect to NK cells in the anti-MM response. Overall, these results indicate that NK cells are the major and necessary effectors for 5T33MM cell killing following *in vivo* IL-2 administration to C57BL/KaLwRij mice (Fig. 2).

IL-2 activated NK cells are cytotoxic against 5T33 myeloma cells

In order to confirm the killing of syngeneic 5T33MM cells by IL-2–activated NK cells, as suggested by the *in vivo* depletion experiments, the *in vitro* cytotoxicity of NK cells isolated and cultured with IL-2 against 5T33MM cells was examined

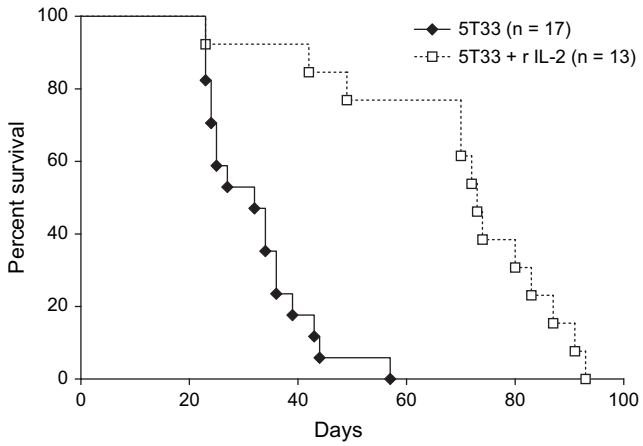

Figure 1. Survival kinetics of 5T33-inoculated C57BL/KaLwRij mice receiving interleukin (IL)-2 therapy ($p < 0.05$).

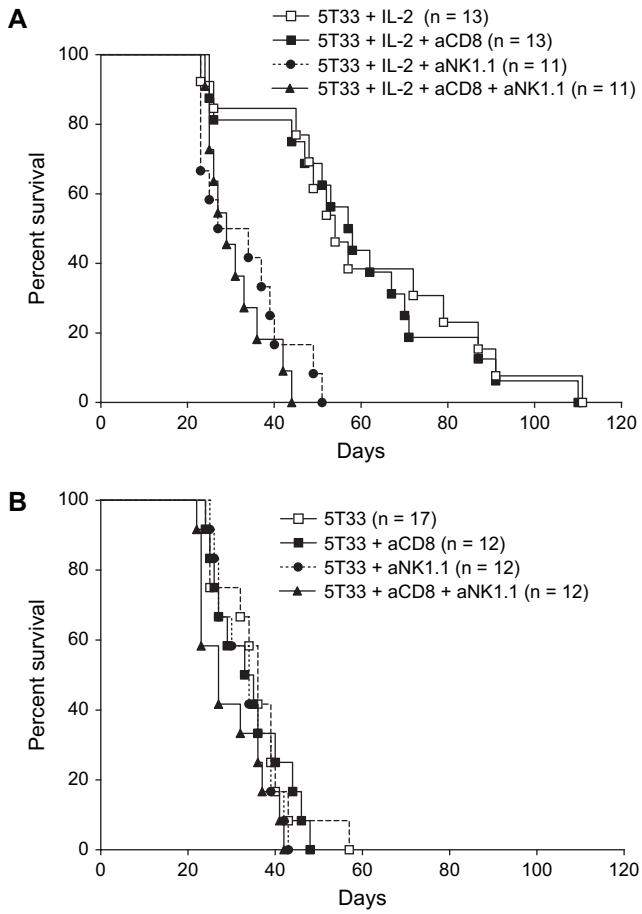

Figure 2. *In vivo* depletion of effector cells in 5T33-inoculated and interleukin (IL)-2–treated C57BL/KaLwRij mice. (**A**) IL-2–treated mice ($p < 0.05$ for natural killer cell [NK] depletion compared to no depletion and $p > 0.1$ for CD8 depletion compared to no depletion). (**B**) Untreated control mice. Injection of isotype controls showed no effect on depletion (not shown).

by a ^{51}Cr release assay. IL-2–activated NK cells from non-challenged C57BL/KaLwRij mice showed marked cytotoxicity against syngeneic 5T33MM cells (Fig. 3). In contrast, no significant cytotoxicity was detected against syngeneic lipopolysaccharide blasts, splenocytes, liver, and normal BM cells (Fig. 3A; data not shown). Moreover, IL-2–activated NK cells from the tumor-bearing C57BL/KaLwRij also displayed significant antitumor activity *in vitro* against 5T33MM cells (Fig. 3B). These results clearly show that IL-2–activated NK cells have capacity to kill syngeneic MM cells. In an attempt to clarify the mechanisms of NK-cell–mediated killing, we have also checked the expression of major histocompatibility complex class I molecules and the NKG2D ligand MULT1 (Fig. 4). We have observed that 5T33MM cells heterogeneously expressed these molecules.

Adoptive transfer of NK cells to tumor-bearing mice prolongs their survival

Given the ability of NK cells to kill 5T33MM cells *in vitro* and the role of NK cells in prolongation of survival following

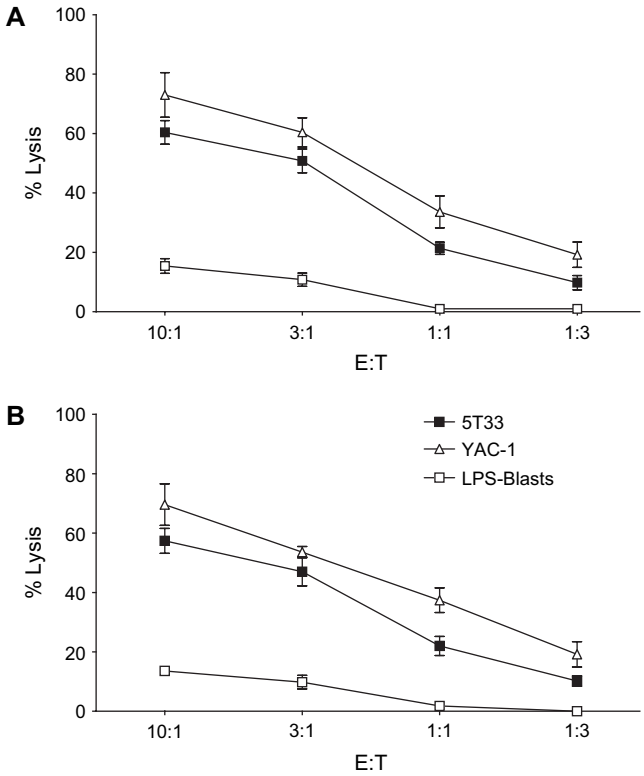

Figure 3. Cytotoxicity of natural killer (NK) cells assessed by ^{51}Cr-release against 5T33 and YAC-1 control cell line as well as lipopolysaccharide blasts. (**A**) NK cells derived from unchallenged mice. (**B**) NK cells derived from 5T33MM-injected mice 5 days after inoculation. Results are presented as mean ± SD.

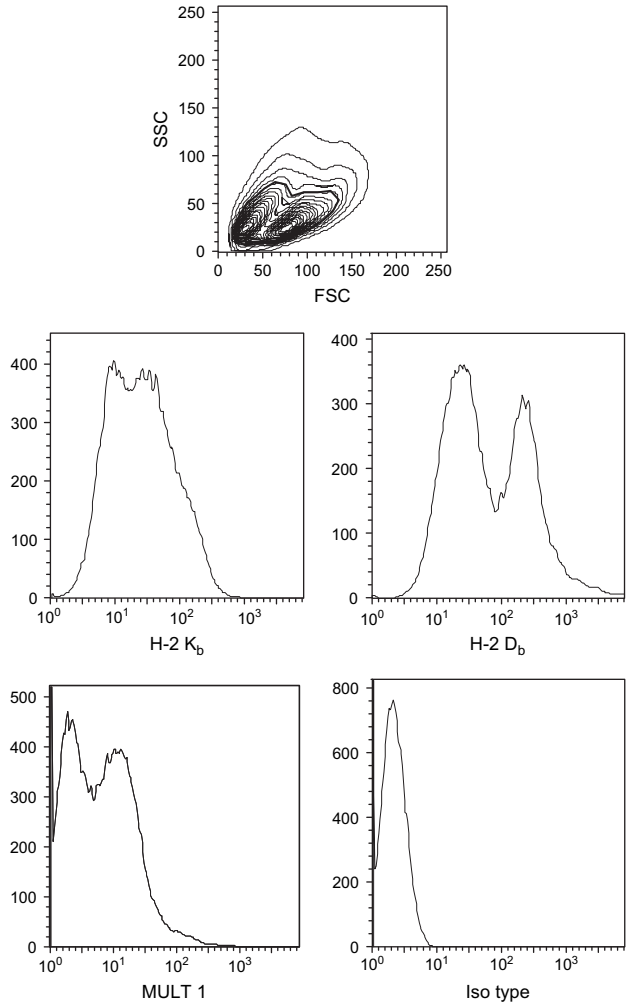

Figure 4. Analysis of the surface expression of major histocompatibility complex class I molecules and the NKG2D ligand MULT1 on 5T33 cells. We have observed a heterogeneous expression of all three molecules.

IL-2 treatment of tumor-bearing mice, we investigated if adoptively transferred IL-2–activated NK cells could prolong survival of 5T33MM-bearing C57BL/KaLwRij mice. Tumor-bearing mice were given two different doses (2×10^5 or 2×10^7) of IL-2–activated NK cells from syngeneic mice. Adoptive transfer of IL-2–activated NK cells without i.p. IL-2 administration prolonged survival of MM-bearing mice only to an insignificant extent ($p = 0.07$). However, when IL-2–activated NK cells were administered in conjunction with IL-2 treatment of mice, a significant prolongation of survival, compared to administration of only IL-2–activated NK cells or only IL-2, was observed ($p < 0.05$ for 2×10^5 NK cells, and $p < 0.01$ for 2×10^7 NK cells) (Fig. 5). Notably, the increase in survival was dependent on the number of cells adoptively transferred. Administration of 2×10^7 cells resulted in a relative increase in survival over that observed upon administration of 2×10^5 cells. These results show that IL-2–activated NK cells exert an anti-MM response *in vivo*, and that this effect is dependent on the presence of IL-2 administration as well as cell dose.

NK cells colocalize to sites of myeloma growth in vivo

To simultaneously analyze the biodistribution of adoptively transferred NK cells and MM cells *in vivo*, magnetically separated NK cells were retrovirally marked with a vector coding DsRed2. The median retroviral transduction rate of primary NK cells was 15.3%. A purity of 92.4% transduced cells was achieved after FACS sorting. The positive and negative fractions (2×10^7 cells/mouse) were injected into eGFP-5T33MM–bearing mice. Flow cytometry analysis of cells derived from animals inoculated with DsRed$^+$ NK cells showed significant numbers of NK cells in the organs where MM cells resided. That is, colocalization of marked NK and MM cells in the liver, spleen, and BM suggested that adoptively transferred cells were homing to the tumor cell–rich organs. In an effort to observe if this homing pattern is due to MM development, we have injected NK cells into non–tumor-bearing mice and observed that they were located mainly in the lungs and liver for the first week and were subsequently undetectable. By taking into account the total number of cells in these organs, and the corresponding percentage of the NK-cell population, we deduced that the latter were proliferating at the tumor site (Fig. 6).

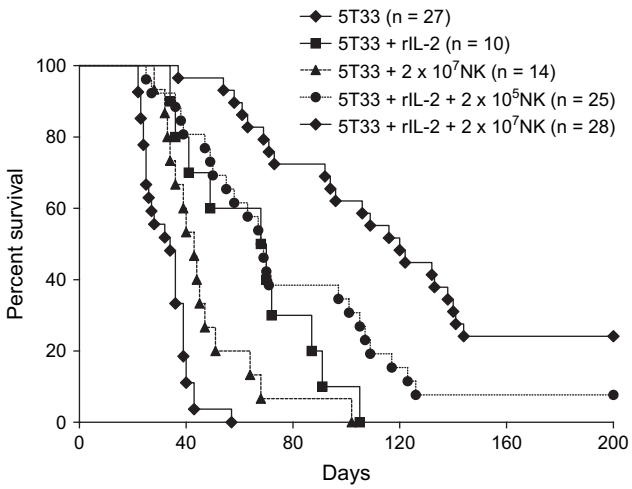

Figure 5. *In vivo* therapeutic effects of adoptively transferred interleukin (IL)-2–activated natural killer (NK) cells in IL-2–treated or control 5T33-inoculated C57BL/KaLwRij mice ($p = 0.07$ for NK transfer without IL-2 compared to 5T33MM alone, $p < 0.05$ for low-dose NK cell transfer and $p < 0.01$ for high-dose NK cell transfer compared to 5T33MM administration alone).

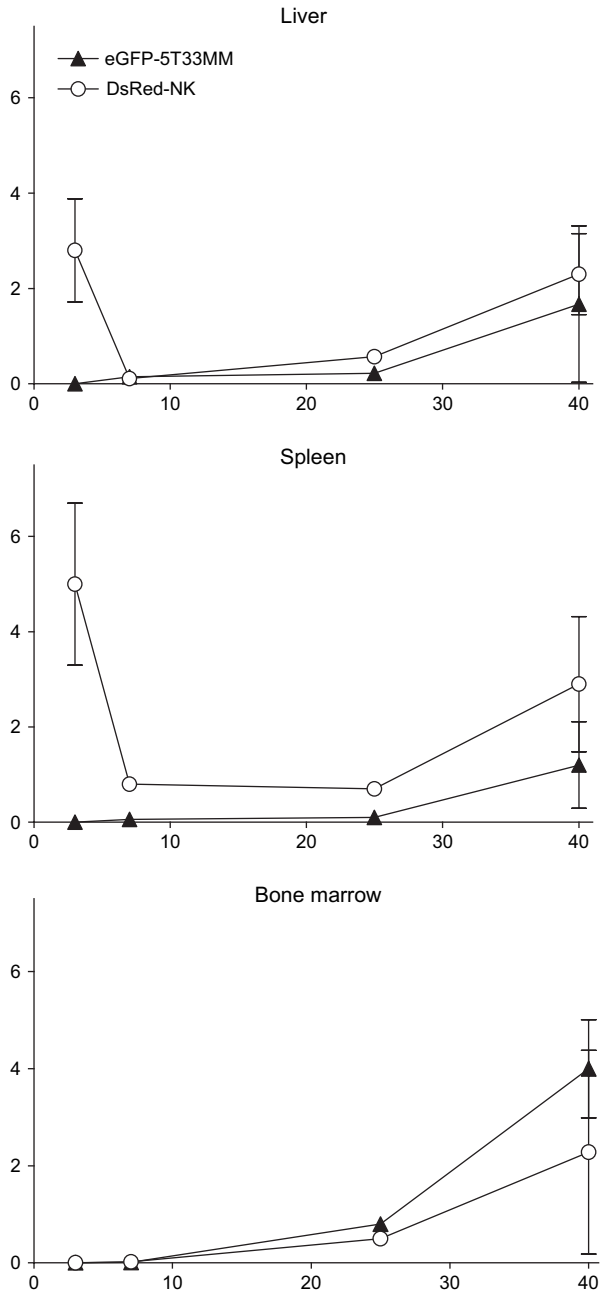

Figure 6. Biodistribution and quantification of the adoptively transferred interleukin-2 (IL-2)–activated DsRed transduced natural killer (NK) cells in the organs of eGFP-5T33MM–bearing C57BL/KaLwRij mice assessed by flow cytometry.

Discussion

In the present study, we have evaluated the *in vitro* and *in vivo* anti-MM activity of activated NK cells from C57BL/KaLwRij mice. Furthermore, we have demonstrated that adoptive transfer of activated NK cells together with IL-2 administration prolongs survival of MM-bearing mice. These findings document, for the first time, that adoptive transfer of activated NK cells may have autologous anti-myeloma effect.

A few studies have explored the role of different effector cells on anti-MM activity. In a previous study assessing anti-MM activity of cytokines, cytotoxic drugs, and effector-cell populations in C57BL/KaLwRij mice, it was reported that although NK cells from the mice were cytotoxic against the YAC-1 cell line, there was no cytotoxicity observed against the 5T33MM cells [26]. The authors concluded that $CD8^+$ cells in lymphokine-activated killer cell cultures [27], but not NK cells, exerted the myeloma killing. Because the literature suggests that MM-cell recognition by lymphocytes is mainly major histocompatibility complex class 1– and, to a much lesser extent, class 2–restricted, and is dependent on perforin-mediated pathway [28], we focused our efforts on $CD8^+$ T cells and NK cells. Our results clearly suggest that IL-2–activated NK cells are efficient effectors for syngeneic MM-cell killing in C57BL/KaLwRij and this effect is dependent on the presence of IL-2. Depletion of NK cells had the most serious negative effect on survival rates of mice with MM, while IL-2 administration did not significantly improve the killing mediated by $CD8^+$ lymphocytes in NK-depleted mice. However, $CD8^+$ T cells might show a supportive effect to NK cells in the anti-MM response, because we have observed a slight decrease in survival ($p > 0.1$) when both populations were depleted at the same time. With respect to the NK-cell depletion performed, we chose to use the NK1.1 monoclonal antibody because previous reports have shown that treatment with anti-NK1.1 using the abovementioned doses yields only partial removal of $NK1.1^+$ T cells, but virtually complete elimination of classical NK cells [29,30].

In an attempt to make use of the observed anti-MM response of IL-2–activated NK cells and their immunotherapeutic potential, we adoptively transferred these cells to MM-bearing mice, along with IL-2 administration, in a minimal residual disease setting and subsequently observed an improved survival. Most importantly, the increase in survival was much better when a higher dose of NK cells was administered, which suggests that the anti-MM response of adoptively transferred NK cells is dose-dependent. Therefore, the feasibility of using autologous *ex vivo* expanded NK cells for management of MM is worthy of being explored further. A minor point is that, although IL-2 may have an indirect role in dampening immune responses through stimulation of regulatory T cells [31,32], we have not observed an apparent inhibitory effect on NK cells in this model.

Another critical dispute for application of this approach is the *in vivo* survival and biodistribution of adoptively transferred NK cells. It could be claimed that adoptively transferred cells arrest, in lungs with subsequent degradation, might take place immediately after administration, thus preventing any therapeutic effect outside of the lungs. If not retained in the lungs, the transfused cells may migrate to preferred organs. Migration of adoptively transferred NK cells to tumor sites is considered a prerequisite for therapeutic efficacy. There have been various studies using dyes (i.e., carboxyfluorescein succinimidyl ester) or radioactive labeling for biodistribution studies. However, these methods can be used for short-term analysis only, because cells proliferate and labels become difficult to detect. Therefore, we chose to use gene-marked NK and MM cells. Using the eGFP-5T33MM system [24] together with $DsRed^{+}$ NK cells, we verified that they reached tissues infiltrated by MM cells, resulting in significant MM-cell clearance and reduced mortality.

In conclusion, we have seen anti-MM activity of activated NK cells from C57BL/KaLwRij mice both *in vitro* and *in vivo*. Furthermore, we have demonstrated a significant delay in MM development with adoptively transferred activated NK cells.

Therefore, we suggest that the present results may be important for implementation of immunotherapeutic treatment strategies complementing autologous stem cell transplantation, based on infusions of autologous NK cells for control or eradication of minimal residual disease. Thus, we believe that further investigations on the effect of adoptively transferred autologous NK cells in patients with MM are warranted.

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