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RESEARCH PAPER

An improved method to study NK-independent mechanisms of MTLn3 breast cancer lung metastasis

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Abstract To study the tumor cell autonomous processes of metastasis, an in vivo tumor metastasis model is required that excludes the involvement of the innate immune system. For this purpose we used the established syngeneic MTLn3 cell – Fischer 344 tumor model. MTLn3 cells are efficiently eradicated by NK cells in vivo. Using isolated cell systems, we provide evidence for apoptosis-induction by IL-2 activated NK cells, but not T-cells, despite the expression of MHC class I. This is largely mediated by the perforin/granzyme B pathway in MTLn3 cells in a caspase-dependent manner. Temporal in vivo depletion of NK cells by an antibody-based method, dramatically improved colonization of the lungs by MTLn3 cells, from 5 metastases in the untreated animals to 130 metastases in the NK-depleted animals. Thus, we improved the syngeneic MTLn3-Fischer 344 tumor model by temporal depletion of NK cells of which the advantages over the use of immunodeficient animals are evident.

Keywords MTLn3 cells · Fischer 344 rats · NK cells · Metastasis

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Abbreviations

ADCC	antibody dependent cellular cytotoxicity
CMA	concanamycin A
CTL	cytotoxic T-lymphocyte
E/T	effector target cell ratio
FACS	fluorescence activated cell sorter
FAK	focal adhesion kinase
FRNK	FAK-related non-kinase
IL-2	interleukin 2
MHC	Major histocompatibility complex
NK cell	natural killer cell
PI	propidium iodide

Introduction

In the ongoing search for an anti-cancer drug, numerous in vitro and in vivo models have been generated to study tumor cell biology. Although in in vitro models many aspects of tumor cell biology can be studied, relevant in vivo models are essential to define the ultimate biological relevance of in vitro findings for the intact organism. Thus, the complex environment including local presence of growth factors and chemokines, host immune system and angiogenesis are essential determinants of tumor development and progression including the biological processes essential in metastasis formation [1].

In vivo tumor growth is studied in two different animal models: syngeneic models and immunodeficient models. As in syngeneic models the transplanted cells, the tumor microenvironment, and the host are not only from the same species, but from the same strain of inbred animals, no alloreactivity should occur. Given the importance of tumor

versus immune cell interactions as principal requirements for tumor progression and metastasis formation, syngeneic models are preferred. However, prolonged culturing of syngeneic tumor cells in vitro may affect the expression of adhesion molecules or antigen expression molecules such as the Major Histocompatibility Complex (MHC) families, rendering these syngeneic cells a target for the immune system. Therefore, other in vivo models, such as the use of immunodeficient mice are being employed. The most used immunodeficient mice (SCID, $Rag^{-/-}$) lack the adaptive immune response, but still harbour large numbers of cells of the innate immune system. Additionally, mice are being used that, due to combined knock out of the Rag-gene and the common gamma chain ($Rag^{-/-}$ $\gamma c^{-/-}$), lack both the adaptive and innate immune response.

Two important disadvantages of the use of these immunodeficient animals are the lack of tumor-immune cell interactions and the use of cancer cells originating from other species, i.e. injection of rat cancer cells in mice. In immunocompetent animals, the cytotoxic response of the immune system is mediated by two distinct subsets of lymphocytes: the T-lymphocytes and the Natural Killer (NK) cells. The T-cells belong to the adaptive immune system and respond to foreign antigens presented in self MHC. The NK-cells are an important component of the innate immune system and function in an antigen-non-specific manner [2]. NK cell-mediated killing of target cells is controlled by a balance between activating and inhibitory signals, of which the latter are mediated by expression of the appropriate MHC I molecules [2]. Both types of lymphocytes kill their targets by excretion of perforin and granzyme B on one hand and by activation of death-receptors on the other hand. All signals finally converge in activation of the executioner caspase-3, leading to induction of apoptosis in the target cells.

The rat mammary adenocarcinoma cell line MTLn3 is an excellent model to mimic breast cancer and its treatment. In the early eighties, Neri and Welch isolated a founder cell line out of the Fischer 344 rat, which was named 13762NF [3]. Injection of this cell line into the fat pad of the Fischer 344 rat resulted in the formation of lung metastases. Several lung metastases were isolated and one of the cultured breasts to lung metastases was named MTLn3. This cell line turned out to be highly metastatic: when injected into the fat pad of female Fischer 344 rats, within 3 weeks the lungs of the rats were burdened with metastases. However, after several passages of in vitro culturing, this cell line started to lose its metastatic potential [4]. To circumvent this problem, immunodeficient mice can be used to study these cells under in vivo conditions, indicating an important role for the immune system in declining metastasis rather than a cell intrinsic phenomenon. Using nude mice, no macro-metastases and only few micrometastases in the lung could be found five weeks after injection of the MTLn3 cells in the fat pad [5], probably due to the NK-mediated killing of metastasizing cells. The importance of NK-cell mediated killing of tumor cells is well established in the MADB106 syngeneic tumor model [6–8] dating back to 1985 [9].

To study the cellular biological processes involved in metastasis, it is important to develop an improved model that excludes interference by the immune system. We have previously used MTLn3 cells to study the mechanisms of cytostatic-induced apoptosis [10, 11]. In addition, we studied the role of the non-tyrosine kinase focal adhesion kinase in the processes involved in tumorigenesis and metastasis under in vitro circumstances [12]. The goal of the current study was to improve the MTLn3 cell – Fischer 344 rat syngeneic breast tumor metastasis model, to allow the unbiased analysis of tumor cell dependent metastasis programs, independent of the innate immune system surveillance.

A model was set up in which killing of the circulating MTLn3 cells by the innate immune system of Fischer 344 rats was prevented during the metastasis process. In vitro MTLn3 cells are subject to IL-2 activated NK cell-, but not T-cell-mediated killing. Killing depended on granzyme-B/perforin mediated caspase activation and in vivo depletion of NK cells enabled tail vein injected MTLn3 cells to highly efficient colonize the lungs. In conclusion, by depleting NK cells in Fischer 344 rats, we have created an improved syngeneic model that can be used to study the biological steps taken place in tumor cells that are essential in the formation of distant metastasis.

Materials And Methods

Animals

Female Fischer 344 rats weighing 70–80 g were purchased from Charles River. They were fed ad libitum and kept at a 12 hour light-dark cycle and kept for at least 2 weeks at the local animal facility before use.

Reagents

Human recombinant interleukin-2 was obtained from Chiron (Amsterdam, The Netherlands), concanamycin A from Fluka Chemistry (St. Louis, MO, USA) and annexin-V from Roch (Base, Switzerland). Annexin-V was labelled with allophycocyanin (a 652 nm fluorescent probe) (Prozyme, San Leandro, CA, USA). Molecular Probes (Leiden, The Netherlands) supplied the calcein-AM and the propidium-iodide was purchased from Sigma (St. Louis, MO, USA). Alpha modified minimal essential medium (α-

MEM), Fetal Bovine Serum (FBS), phosphate buffered saline (PBS) and trypsin were from Life Technologies (Rockville, MD, USA). All other chemicals were of analytical grade.

Cell lines

MTLn3-tetFRNK cells were used. This cell line is derived from the MTLn3 cell line and differs only from the founder cell line when doxycycline is present; in then expresses a FAK-related non kinase (FRNK) protein [13]. During the experiments described in this paper, no doxycycline is present and therefore, we will refer to this cell line as MTLn3. MTLn3 rat mammary adenocarcinoma cells [3] were cultured as previously described [10].

NK and T-cells

NK cells were isolated as described previously [14]. Briefly, spleens were aseptically removed from rats and placed in 10 ml culture medium, consisting of RPMI 1640 (L-glutamine, 25 mM HEPES) supplemented with 10% heat-inactivated FBS, 100 μg/ml streptomycin, 100 IU/ml penicillin, 5 μl lipopolysaccharoids 100 μg/ml, 6.66 ml 7.5% (w/v) $NAHCO_3$, 0.1 ml 100 mM β-mercapto-ethanol (complete medium). A single cell suspension was prepared by pressing the spleen through a sterile open filter chamber (Embrarad, Sao Paulo, Brasil). The splenocytes were depleted of B-cells, macrophages and monocytes by allowing these cells to adhere to nylon-wool for 2 hours (Fenwal laboratories, Morton Grove, IL, USA) at 37°C in complete medium. The non-adherent cells were collected and cultured in 175 cm^2 culture flasks (Greiner, Langentha, Switzerland) in 35 ml culture medium containing 1000 Cetus units human recombinant IL-2 per ml. After 24 hours, the culture medium was transferred to a centrifuge tube and the non-adherent cells were washed three times with 10 ml culture medium without IL-2. The conditioned medium, obtained after centrifugation, was re-added and the cells were cultured in this medium for 6 days. To obtain T-cells, the remaining pellet was resuspended in complete medium containing 100 Cetus units recombinant IL-2 per ml and cultured in R73 coated 175 cm^2 culture flasks (6 μg/ml PBS, 6 ml per TC175, o/n, 4°C). Two days later, non-adherent cells were removed and complete medium containing 100 Cetus units human recombinant Il-2 per ml was added. Cells were cultured in this medium for 4 days.

Cytotoxicity assays

MTLn3 cells were harvested and washed twice. They were labelled by incubation with 1 μM calcein-AM for 15 minutes in culture medium. For some cytotoxicity assays, MTLn3 cells were pre-incubated with zVAD-fmk (100 μM) or DEVD-fmk (100 μM) and these inhibitors were also added during the co-incubation period. Subsequently, these cells were washed and seeded in a 96-well-U-bottom plate at a density of 100,000 cells in 0.1 ml/well. The effector cells (NK or T-cells) were added in a volume of 0.05 ml. For some experiments, to block induction of apoptosis by the granzyme B/perforin pathway, NK cells were pre-incubated for 2 hours with 20 nM concanamycin A (CMA) which was previously proven to be the optimal concentration to inactivate perforin [14]. After three or six hours, the 96-well plate was centrifuged for 3 minutes at 1500 rpm at 4°C. The supernatant was removed, the cells were washed with 0.1 ml PBS and the detached cells were transferred to a new plate. Next, 0.1 ml of trypsin was added to the original plate and this plate was incubated for 10 minutes at 37°C. After trypsinization, the detached cells and the cells in the PBS were pooled and the plate was centrifuged (3 min., 1500 rpm 4°C). Next, the cells were suspended in 200 μL binding buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2.6H_2O$, 1.8 mM $CaCl_2.2H_2O$ in Milli Q Water, pH 7,4) containing 0.5 μl annexin-V (0.5 μg/μl). The cells were incubated (20 minutes, 4°C) and subsequently washed twice with binding buffer. Finally, the cells were suspended in 0.2 ml binding buffer and 10 μl propidium-iodide (PI, 100 μM) was added shortly before measurement. Samples were analyzed on the FACScalibur (Becton Dickinson, San Jose, CA). Target cells were selected by gating the calcein-AM positive cells. Viable cells are annexin-V-APC and PI-negative, apoptotic cells are annexin-V-APC positive but PI-negative and necrotic cells are annexin-V-APC- and PI-positive.

MHC-I staining

MTLn3 cells were harvested and incubated for 30 minutes with 25 μl culture supernatant of mAb OX18 IgG (binding to rat MHC class I [15]). The cells were washed with PBS and incubated for 1 hour with the secondary antibody Gt-anti mouse-alexa 488 in binding buffer. Finally, the cells were washed and suspended in 0.2 ml binding buffer and 10 μl propidium-iodide (PI, 100 μM) was added shortly before measurement. MHC-I expression was analyzed on the FACScalibur (Becton Dickinson).

In vivo metastasis formation

Cells were injected into the lateral tail vein of Fischer 344 rats as described previously [13]. Briefly, MTLn3 cells were trypsinized, counted, washed twice and resuspended in PBS. 1×10^5 Viable cells in 0.2 ml PBS were injected into the lateral tail vein. After 4 weeks, the animals were

anesthetized with pentobarbital and the lungs were excised and rinsed in ice-cold PBS. The right lung was cut into three pieces and fixated in isopentane (used to prepare tissue homogenates for immunoblot analysis), 4% paraformaldehyde or Carnoy's (10% (v/v) acetic acid, 30% (v/v) chloroform in 60% ethanol). The left lung was injected with ink solution and thereafter destained in water and fixated in Fekete's (4.3% (v/v) acetic acid, 0.35% (v/v) formaldehyde in 70% ethanol). Lung tumor burden was determined by counting the number of surface metastases.

Purification of mAb3.2.3 antibody

Culture supernatant was obtained using centrifugation of the hybridoma culture, followed by filtering through a 0.2 μM pore size filter. Supernatant was passed through a protein G column (Pharmacia, Uppsala, Sweden). Bound immunoglobulin was eluted using a pH gradient from pH 7.4 (PBS) to pH 2.7 (0.1 M Glycin-HCl). Each of the peak fractions obtained was pooled and then dialyzed against PBS. Finally the protein concentration was diluted to 2 mg/ml in PBS (protein concentration measured against IgG standard).

Depletion of NK cells

To deplete NK cells, rats were treated with the monoclonal anti-CD161A antibody, called mAb3.2.3, on 3 consecutive days (150 μg in 0.5 ml PBS, i.p.). The next day, rats were anesthetized with pentobarbital and at least 1 ml blood was isolated by a heart puncture. After addition of heparin, 2 ml RPMI was added to the blood and this mixture was carefully pipetted on top of 3 ml Ficoll Hypaque and centrifuged (20 min. 2000 rpm, room temperature, no brake). The inter phase that contained the white blood cells was isolated and washed 2 times with RPMI. White blood cells were transferred to a V-bottom 96 wells plate, blocked with 5% BSA in PBS for 30 minutes and incubated with alexa-633 labelled mAb3.2.3 in binding buffer. Cells were washed twice with binding buffer and prior to flowcytometric analysis incubated with propidium iodide. Propidium iodide negative (= non-necrotic) cells were gated and using forward-side scatter plots the lymphocytes were gated. By gating the alexa-633 positive cells, the percentage of NK cells was determined in the lymphocyte groups.

Statistical analysis

Student's t test was used to determine significant differences between two means ($p < 0.05$). For the in vivo lung metastasis experiments, statistical significance was determined by the Mann-Whitney Rank Sum test.

Results

Intravenous injection of MTLn3 cells does not result in experimental metastasis formation. To study the process of metastasis we used a syngeneic model consisting of the rat mammary adenocarcinoma cell line MTLn3 and the Fischer 344 rats. Initially 1×10^5 MTLn3 cells were injected into the lateral tail vein of the female Fischer 344 rats. However, after 3 weeks, no lung surface tumors were detectable (data not shown). Increasing the number of MTLn3 cells to 5×10^5 and 1×10^6, and prolonging the period to form metastases to 5 weeks did not result in the formation of lung tumors. Only when 1×10^6 cells were injected, a few large lung tumors could be observed 6 weeks post-injection. Importantly, HE stained lung sections did not harbour any dormant tumor cells or micrometastases and those metastases that were present at the surface of the lungs were large. This implicated that even when 1×10^6 tumor cells were injected, few cells survived in the circulation allowing outgrowth into lung metastases. Thus, in our hands, the MTLn3 cell line was not able to colonize the lungs after tail vein injection. Importantly, when these cells were injected subcutaneously in the mammary fat pad, after two weeks, the tumors were palpable and were able to grow out into large tumors in four weeks [13]. Thus, the absence of experimental metastases results from the lack of survival of MTLn3 cells in the circulation.

Syngeneic NK- but not T-cells kill MTLn3 cells

A possible explanation of the lack of lung metastases could be that, despite the use of a syngeneic model, circulating MTLn3 cells are efficiently eradicated by cells of the immune system shortly after injection. As the cytotoxic component of the immune response, responsible for eradication of cellular threats comprises the cytotoxic T-cells and NK-cells, we tested both cell types for their potency to kill MTLn3 cells. Since the tumor cells are very rapidly killed in the circulation, no antigen-specific response could be elicited by the T-cells within that time-frame and therefore, only the possibility of cross-reactivity was tested by examining the cytotoxic potential of polyclonally activated T-cells. To do so, NK cells and T-cells were isolated from the spleen of Fischer 344 rats, activated by culturing in IL-2 enriched medium (and anti-CD3 for T-cells), and followed by an in vitro cytotoxicity assay. For this cytotoxicity assay, the cells were co-incubated for 6 hours at an effector (= NK cells or T-cells)/target (= MTLn3 cells) ratio (E/T) of 10. The target cells were labelled with a fluorescent dye, calcein-AM, and prior to the co-incubation, allowing discrimination using FACS analysis (Fig. 1A). After the co-incubation of the target and effector

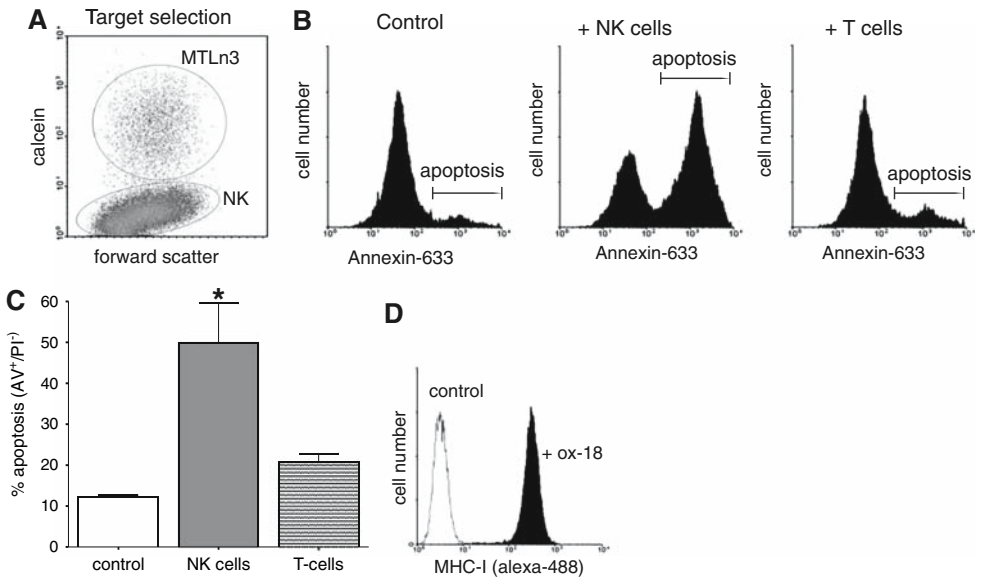


Fig. 1 NK cells, but not T-cells, induce apoptosis in MHC-I expressing MTLn3 cells. MTLn3 cells were labeled with calcein-AM and co-incubated with NK or T-cells (E/T 10) for 6 hours, by using flowcytometric analysis, calcein-AM positive MTLn3 cells were gated (**A**). The flow cytometer was programmed to detect only annexin-633 and PI-staining of calcein-positive MTLn3 cells and the percentage apoptosis (annexin-633$^+$/PI$^-$) was determined in the absence (**B**, left panel and **C**) and in the presence of NK or T-cells (**B**, right panels and **C**). MTLn3 cells were incubated with ox-18 and the presence of MHC-I molecules was determined by flow-cytometric analysis (**D**)

cells, cells were stained with propidium iodide, which can only enter necrotic cells, and annexin-633, which recognizes phosphatidyl serine: an early apoptotic marker. Propidium iodide negative target cells were gated and the percentage annexin-633 positive (= apoptotic) cells was determined (Fig. 1B). Exposure of the MTLn3 cells to IL-2 activated NK cells resulted in 50% apoptosis (Fig. 1C), whereas T-cells did not induce apoptosis of MTLn3 cells. Importantly, no difference in propidium iodide positive cells (i.e. necrotic cells) was found between the experimental and control incubations. As NK-cell mediated killing is controlled by a balance between activating and inhibitory signals, of which the latter is mediated by expression of MHC class I, the cytotoxic observations could be explained by a lack of MHC-class I expression on the outer membrane of target cells. However, the MTLn3 cells did express MHC class I, as shown by staining with OX-18 (Fig. 1D). Thus, under in vitro conditions, regardless of the presence of MHC-I, syngeneic NK cells are capable of killing the MTLn3 cells.

NK cells-induced apoptosis of MTLn3 cells is perforin- and caspase-3 dependent

Since MTLn3 cells do express MHC-I, we wondered whether the cells were indeed killed by the NK cells via the classic perforin/granzyme B mediated caspase activation. To study this, the perforin/granzyme B route was disabled by pre-treatment with CMA, which irreversibly binds perforin. Since co-incubation of the target and effector cells for 6 hours resulted in massive cell death, the co-incubation period for this set of experiments was reduced to three hours. Background apoptosis did not exceed 2% and NK cell induced apoptosis was approximately 19% (Fig. 2); inhibition of the perforin/granzyme B pathway caused a significant reduction of NK cell-mediated cytotoxicity. To verify that the killing of MTLn3 cells by the

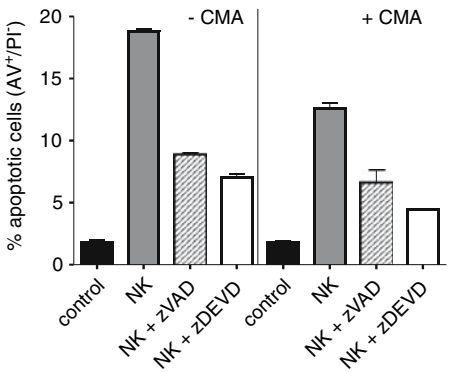


Fig. 2 NK cells induce apoptosis of MTLn3 cells via the granzyme-B/perforin pathway. MTLn3 and NK cells were co-incubated as described in figure 1, after 3 hours apoptosis was determined. Where indicated, NK cells were pre-incubated with CMA to prevent granzyme-B/perforin mediated apoptosis and MTLn3 cells were pre-incubated with 100 μM zVAD-fmk (pan-caspase inhibitor) or 100 μM zDEVD-fmk (caspase-3 inhibitor)

NK cells involved the induction of apoptosis, the apoptotic machinery of the MTLn3 cells was abrogated by using a pan-caspase inhibitor (zVAD-fmk) or an inhibitor of the effector caspase-3 (zDEVD-fmk). Previously, we have shown the apoptosis-inhibiting capacity of these caspase inhibitors in cytostatic-induced apoptosis of MTLn3 cells [16]. Pre-treatment of the MTLn3 cells with the caspase inhibitors zDEVD-fmk and zVAD-fmk significantly reduced the percentage of NK cell induced apoptosis. Finally, the combined addition of CMA and caspase inhibition reduced apoptosis almost completely albeit that some residual cell death was present. Again, no differences between experimental and control incubations in propidium iodide positive cells was found. Thus, Fischer 344 NK cells kill MTLn3 cells by induction of apoptosis, predominantly mediated via the granzyme B mediated pathway.

Depletion of NK cells in the Fischer 344 rat enables MTLn3 cells to form metastases

The above data indicate that MTLn3 cells are efficiently killed by NK cells of the Fischer 344 rats by means of apoptosis. Therefore, the lack of lung metastases may well be related to efficient NK cell mediated killing of circulating MTLn3 cells. To confirm the potency of NK cells to prevent metastasis in vivo, NK cells were depleted from the Fischer 344 rats. For this purpose, rats were injected intraperitoneally for three consecutive days with 150 μg of the NK depleting antibody, mAb3.2.3 [17]. To determine NK cell depletion, one day after the third intraperitoneally injection blood was isolated from the rats via a heart puncture. Mono-nuclear cells were isolated and stained for the NK-cell specific marker NK1.1, followed by flow cytometric analysis to determine the amount of NK cells. First, using the forward scatter-side scatter plot, the lymphocytes were gated (Fig. 3A, left panel) and subsequently, by gating the NK1.1 positive population, the percentage of NK cells could be determined (Fig. 3A, right panels). The lymphocytes isolated from control animals consisted for approximately 8% of NK cells (Fig. 3B). Treatment of the rats with the NK depleting antibody reduced the percentage of NK cells to <0.5%, indicating an efficient depletion of circulating NK cells.

Next, this method was used to determine the role of NK cells in MTLn3 cell killing in vivo, and, consequently, the lack of lung metastasis formation. Therefore, after NK-cell depletion, Fischer 344 rats were injected with only 100,000 MTLn3 cells. All animals were sacrificed after four weeks and the lungs were isolated and injected with ink, as described previously [13]. In control animals, neither lung surface inspection nor microscopical analysis of H&E stained lung sections showed lung tumors and micrometastases, respectively. Yet, in the lungs of the NK cell depleted animals large numbers of tumors were found (Fig. 4A). In the control group hardly any surface lung metastases were present. However, in the rats that were treated with the NK depleting antibody on average more than 130 lung metastases were present (Fig. 4B and C). In conclusion, temporal depletion of NK-cells from the Fischer 344 rats causes MTLn3 cells to escape immunosurveillance and enables them efficiently to colonize the lungs.

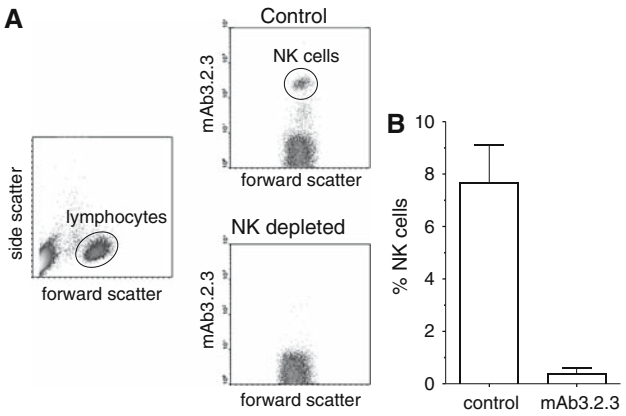
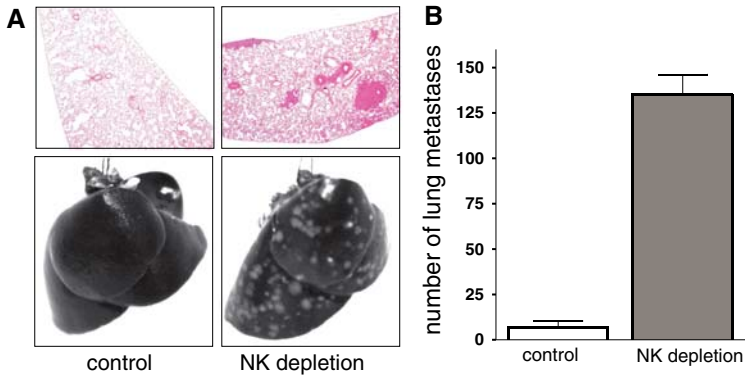


Fig. 3 Pre-treating rats with mAb3.2.3 depletes NK cells from the circulation. Fischer 344 rats (90–110 g) were intraperitoneally injected with 150 mg mAb3.2.3 on three consecutive days. One day after the last injection, mononuclear cells were isolated from the blood and stained for the NK1.1 specific marker. Using FACS analysis, lymphocytes were gated in a forward scatter-side scatter plot (**A**, left panel) and the percentage NK cells was determined (**A**, right panels and **B**)

Discussion

The goal of our study was to improve the MTLn3 cell – Fischer 344 rat breast tumor metastasis model, to allow the unbiased analysis of tumor cell dependent metastasis programs in a syngeneic environment. In our hands, MTLn3 cells were not able to form experimental metastases after tail vein injection into syngeneic Fischer 344 rats. We show that MTLn3 cells are efficiently killed by NK cells in a granzyme B and caspase-3 dependent manner. Eradication of NK cells by using the NK cell depleting antibody mAb3.2.3 enables MTLn3 cells to circumvent NK cell killing in the circulation and allows efficient homing of the tumor cells to the lungs followed by outgrowth into metastases. Using this approach, the number of experimental lung surface metastases increased from 5 to 130 upon injection of only 1×10^5 cells, providing an important tool to study the different steps involved in experimental metastasis formation.



C

Group	Incidence[1]	Number of metastases Median (range)	Significance[2]
Control	3/6	2 (0-25)	
NK depletion	11/11	130 (57-185)	P < 0.005

Fig. 4 Depleting NK cell from the circulation enables MTLn3 cells to form experimental metastases. Rats were pre-treated with mAb3.2.3 to deplete NK cells before 100,000 MTLn3 cells were injected into the tail vein. After 4 weeks, lungs were isolated, injected with ink or lung sections were stained with H&E (**A**) and the number of surface metastases were counted (**B**). The incidence (number of animals with visible lung surface metastsases/total number of animals in the group), median and significance were determined (**C**). Statistical analysis was performed by using the Mann-Whitney Rank Sum test

Circulating NK cells were depleted by repetitive treatment of the rats with the NK-cell depleting 3.2.3 antibody. Unfortunately, no other highly NK-cell specific antibody was available and therefore the presence of NK-cells was determined by studying the presence of fluorochrome-conjugated 3.2.3-positive cells in the peripheral blood. Thus this approach cannot formerly exclude the possibility that the NK-cells were coated with the 3.2.3 antibody in vivo. Importantly, validation of this NK-depletion method by van den Brink et al. [17] not only showed absence of NK-cells by phenotyping, but also a complete lack of NK-cell mediated cytotoxicity as well as antibody dependent cellular cytotoxicity (ADCC), functionally proving the absence of NK-cells. Therefore, we are confident that in our model NK-cells are also depleted.

Only 130 experimental lung surface metastases were formed after inoculation of 1×10^5 cells in NK cell-depleted animals. This indicates that the formation of experimental metastasis upon injection of MTLn3 cells is a very inefficient process. In comparison, implantation of 1×10^6 MTLn3 cells in nude mice resulted in 26 lung metastases [5]. Also, intravenous injection of 2×10^6 selected, highly metastatic CC531-m2 tumor cells resulted only in several hundred experimental metastases in fully immunocompetent animals [18]. Thus 130 lung metastases after inoculation of no more than 1×10^5 MTLn3 cells in NK-cell depleted animals can be regarded as efficient and fully dependent on the lack of NK-cells as in non-treated animals almost no metastases were seen, even after injection of 1×10^6 cells.

To exclude that the observed lack of metastases after inoculation of 1×10^6 cells was a supplier-specific phenomenon, the experimental metastasis experiments were performed in animals ordered at two different suppliers, Harlan and Charles River. Both experiments did not result in experimental metastases (data not shown). However, when the cells were injected subcutaneously in the mammary fat pad, the cells were able to grow out into large tumors in four weeks [13]. Thus, the lack of experimental metastases could neither be attributed to the used Fischer 344 rats nor to the preparation of the cells. Moreover, the mammary fat pad experiment indicates that the lack of experimental metastases is due to killing of tumor cells in the circulation and not after extravasation into the target organ. Since we think it is very important to study the process of metastasis in a syngeneic model, no experiments in immunodeficient mice were performed. However, injection of MTLn3 cells in the fat pad of nude mice lacking T- but not NK-cells resulted in only low numbers of lung metastases [5]. Thus also in these nude mice, NK-cell mediated killing seems to be responsible for the lack of metastases. The importance of Natural Killer cells in the prevention of tumor progression was extensively shown by Ben-Eliyahu and co-workers [6–8]. Using the MADB106 tumor model in Fischer 344 rats they provide evidence for the crucial role for NK-cells in resistance to metastasis and clearly show that external factors influencing susceptibility to malignancies, such as alcohol intoxication, diverse pharmacological agents and stress cause a marked decrease in NK-cell activity [7,8]. Concordant with their findings, in

our experiments, NK-cell mediated killing of circulating tumor cells is responsible for the lack of experimental metastases. Although the eradication of circulating tumor cells could be mediated by NK-or T-cells, our data clearly point toward the NK-cells: First, our in vitro killing assays show that NK-cells, but not polyclonally activated T-cells effectively kill MTLn3 cells. Second, the mammary fat pad experiment [13] shows that intra-organ MTLn3 cells are no longer a target for the immune system. Third, depletion of NK-cells by equivalent injection of 3.2.3 mAb by Van den Brink et al. [17] shows re-appearance of NK- and antibody dependent cellular cytotoxicity (ADCC) activity after 7 days. As the experiments last 28 days, NK cell activity is fully restored one week after tumor cell inoculation. Taken together, these observations show that the MTLn3 cells are a target for the cellular immune system directly upon injection into the circulation, probably lasting no more than 24 hours. Thus, no antigen-specific response of the T-cells towards the MTLn3 cells can be elicited within this limited time-frame. As the T-cells could respond to the MTLn3 cells by cross-reactivity of a memory population, the cytoxocity of polyclonally activated T-cells was tested and disproven. Although these experiments do not formally exclude involvement of T-cell mediated processes in the eradication of MTLn3 cell from the circulation of Fischer 344 rats, our data strongly point towards the Natural Killer cells as the metastases-preventing factor.

NK cell action is controlled by a balance between activating and inhibitory signals, the latter mediated by expression of the appropriate MHC I molecules on the target cells [2]. Flow cytometric analysis showed that MTLn3 cells do express MHC-I molecules. Thus either MTLn3 cells express ligands for the NK cell-activating receptors tipping the balance towards NK reactivity, or the MHC molecules are altered and therefore no longer able to trigger the inhibitory receptors. DNA micro-array data show that MTLn3 cells express RAE-1 (unpublished observations), which is a ligand for the activating receptor NKG2D [19]. The rat homolog of NKG2D, NKR-P2 was shown to bind a RAE-1 like molecule [20]. In conjunction, recently it was shown that NKG2D expression protects the host from tumor initiation [21].

Additionally, a good interaction between target and effector cell is a prerequisite for efficient NK-mediated killing, since blocking antibodies against LFA-1 or ICAM-1 strongly inhibited the cytotoxicity of human NK cell clones [22]. Prolonged culturing of the MTLn3 cells may have resulted in the expression of alternative adhesion molecules. Since these surface molecules are important for the target-effector interactions, their changed expression could be responsible for the observed NK cell mediated killing.

Prevention of the granzyme B/perforin mediated apoptotic pathway partly reduced the NK cell-induced cytotoxicity. The remaining apoptosis is probably not mediated via TRAIL or FAS-ligand pathways, since exposure of MTLn3 cells to effector cells expressing either functional TRAIL or FAS-ligand did not result in apoptosis (data not shown); in our hands these effector cells efficiently kill colon tumor cells [23]. The caspase dependency of NK cell-induced cell death in MTLn3-cells was confirmed by addition of zVAD-fmk and DEVD-fmk. Interestingly, prevention of granzyme B-mediated apoptosis in combination with the pan-caspase inhibitor zVAD-fmk, did not completely prevent apoptosis. This might indicate that other proteases, such as cathepsins, may be involved in apoptosis induction by rat NK cells.

Eradication of the NK cells by pre-treating the rats with an NK cell depleting antibody resulted in the formation of more than 130 lung metastases within four weeks after injection of the cells; these results are comparable to in -vivo lung metastasis results obtained with earlier passages of the MTLn3 cells [24]. Therefore, the susceptibility of the MTLn3 cells to NK cell cytotoxicity can be regarded as the cause of the lack of lung metastases after tail vein injection of the MTLn3 cells. This model is now used to study cell biological pathways of metastasis. Inducible expression of FRNK, an inhibitor of Focal adhesion kinase (FAK), prevented metastasis formation, providing evidence that this method can indeed be used to study the role of signaling molecules on the process of metastasis [13]. By using inducible expression of the proteins of interest, the importance of proteins in the distinct steps of metastasis can be determined.

In conclusion, we show that NK cells can prevent metastasis in a syngeneic Fischer 344 model, which could be overcome by temporal in vivo depletion of the NK cells, resulting in an improved tumor formation model. The advantages of a syngeneic model over immunodeficient models, such as being more representative for the human situation, are evident.

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