Focal adhesion kinase and paxillin : mediators of breast cancer cell migration

Verkoeijen, S.

CHAPTER 2

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

Maroesja J. van Nimwegen[1], Saertje Verkoeijen[1], Peter J.K. Kuppen[2], Jurjen H.L. Velthuis[1] and Bob van de Water[1].

[1]Division of Toxicology, Leiden/Amsterdam Center for Drug Research (LACDR), Leiden University, Leiden, the Netherlands
[2]Department of Surgery, Leiden University Medical Center (LUMC), Leiden, the Netherlands

Running title: Improved syngeneic model for experimental metastasis

ABSTRACT

To study the tumor cell autonomous processes of metastasis formation, 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.

INTRODUCTION

In the ongoing search for an anticancer 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. The complex environment including the 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 types of animal model: 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 *vs.* 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 knockout of the Rag-gene and the common gamma chain ($Rag^{-/-}$ $Yc^{-/-}$), 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 into 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. T cells belong to the adaptive immune system and respond to foreign antigens presented in self MHC, while 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 secretion 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 breast-to-lung metastases was named MTLn3. This cell line turned out to be highly metastatic: when MTLn3 cells were injected into the fat pad of female Fischer 344 rats, the lungs of the rats were burdened with metastases within 3 weeks. 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, suggesting that the immune system plays an important role in the declining metastasis formation, rather than a MTLn3 cell-intrinsic phenomenon. Using nude mice, no macro-metastases and only few micrometastases in the lungs could be found five weeks after injection of the MTLn3 cells into the fat pad (5), probably due to 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 (6,7,8) dating back to 1985 (9).

To study the cellular biological processes involved in metastasis formation, 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 (FAK) in processes involved in tumorigenesis and metastasis formation 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 efficiently 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 taking place in tumor cells that are essential in the formation of distant metastases.

MATERIALS AND METHODS

Animals

Female Fischer 344 rats weighing 70-80 grams were purchased from Charles River. They were fed *ad libitum* and kept at a 12 hour light-dark cycle and housed 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 Roche (Base, Switzerland). Annexin-V was labeled 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; it then expresses FAK-related non-kinase (FRNK) (13). During the experiments described in this paper, no doxycycline was 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 IL-2 per ml and cultured in R73-coated 175 cm^2 culture flasks (6 μg/ml in PBS, 6 ml per TC175, o/n, 4°C). Two days later, non-adherent cells were removed and complete medium containing 100 Cetus units 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 labeled 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-wells U-bottom plate at a density of $1x10^5$ 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-wells plate was centrifuged for 3 minutes at 1500 rpm (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 minutes, 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 shortly before measurement 10 μl propidium iodide (PI, 100 μM) was added. Samples were analyzed on the FACS-Calibur (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 goat-anti mouse-Alexa 488 in binding buffer. Finally, the cells were washed, suspended in 0.2 ml binding buffer and shortly before measurement 10 μl PI (100 μM) was added. MHC I expression was analyzed on the FACS-Calibur.

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. $1x10^5$ 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 fixed 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 fixed 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, protein 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 minutes, 2000 rpm, room temperature, no brake). The interphase 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-labeled mAb3.2.3 in binding buffer. Cells were washed twice with binding buffer and incubated with PI prior to flow cytometric analysis. PI-negative (= non-necrotic) cells were gated and using forward scatter-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 formation, we used a syngeneic model consisting of the rat mammary adenocarcinoma cell line MTLn3 and Fischer 344 rats. Initially, $1x10^5$ MTLn3 cells were injected into the lateral tail vein of female Fischer 344 rats. However, after 3 weeks, no lung surface tumors were detectable (data not shown). Increasing the number of MTLn3 cells to $5x10^5$ and $1x10^6$ and prolonging the period to form metastases to 5 weeks did not result in the formation of lung tumors. Only when $1x10^6$ cells were injected, a few large lung tumors could be observed 6 weeks post-injection. Importantly, H&E-stained lung sections did not harbour any dormant tumor cells or micrometastases; the metastases that were present on the surface of the lungs were large. This implicated that even when $1x10^6$ 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 into the mammary fat pad, tumors were palpable after two weeks and they were able to grow out into large tumors in four weeks time (13). Thus, the absence of experimental metastases results from the lack of survival of MTLn3 cells in the circulation.

Syngeneic NK cells but not T cells kill MTLn3 cells.

A possible explanation for 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. The tumor cells are apparently very rapidly killed in the circulation, making an antigen-specific response by T cells within that time-frame unlikely. 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). Next, an *in vitro* cytotoxicity assay was performed. For this 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 labeled with a fluorescent dye, calcein-AM, prior to the co-incubation, allowing discrimination by FACS analysis (Fig. 1A). After co-incubation of the target and effector cells, cells were stained with PI, which can only enter necrotic cells, and annexin-633, which recognizes phosphatidyl serine, an early apoptotic marker. PI-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 PI-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 I, the observations could be explained by a lack of MHC I expression on the outer membrane of target cells. However, the MTLn3 cells did express MHC 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 MTLn3 cells.

NK cell-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 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 to 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 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 MTLn3 cells
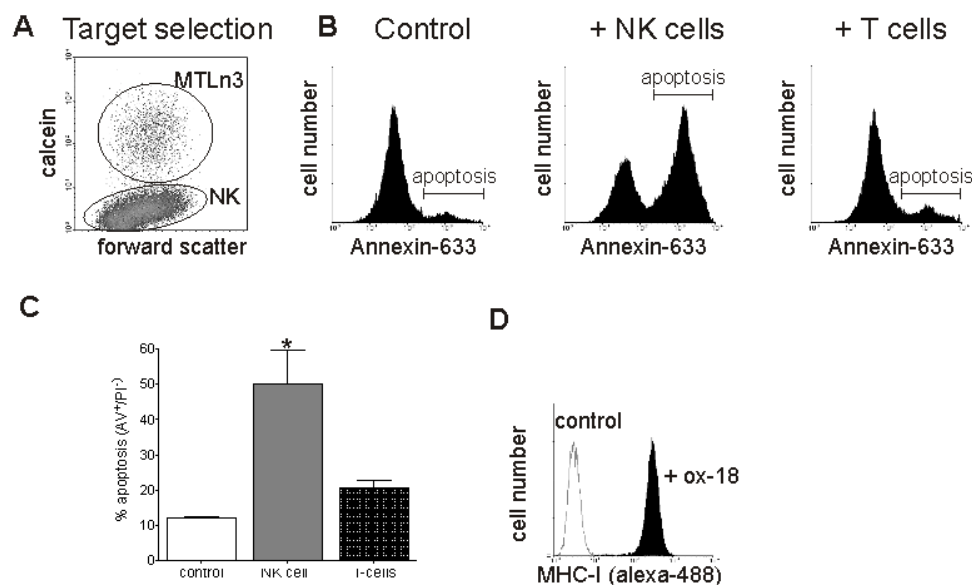
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Figure 1. NK cells, but not T cells, induce apoptosis in MHC I-expressing MTLn3 cells. MTLn3 cells were labeled with calcein-AM and co-incubated with NK or T cells (E/T 10) for 6 hours. (A) By flow cytometric analysis, calcein-AM positive MTLn3 cells were gated. 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). (D) MTLn3 cells were incubated with OX-18 and the presence of MHC I molecules was determined by flow cytometric analysis.

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 inhibitors reduced apoptosis almost completely although some residual cell death was present. Again, no differences between experimental and control incubations in PI-positive cells was found. Thus, Fischer 344 rat NK cells kill MTLn3 cells by induction of apoptosis, predominantly mediated via the granzyme B pathway.
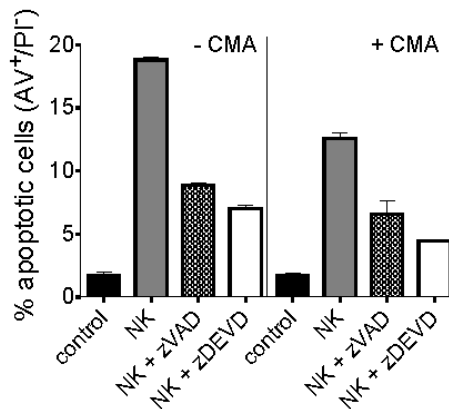
Figure 2. NK cells induce apoptosis of MTLn3 cells via the granzyme-B/perforin pathway. MTLn3 and NK cells were co-incubated as described in figure 1, after 3 hours apoptosis was determined. Where indicated, NK cells were pre-incubated with CMA to prevent granzyme-B/perforin-mediated apoptosis and MTLn3 cells were pre-incubated with 100 μM zVAD-fmk (pan-caspase inhibitor) or 100 μM zDEVD-fmk (caspase-3 inhibitor).

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

The data shown above indicate that MTLn3 cells are efficiently killed by NK cells isolated from 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 formation *in vivo*, NK cells were depleted from Fischer 344 rats. For this purpose, rats were injected intraperitoneally on three consecutive days with 150 μg of the NK-depleting antibody, mAb3.2.3 (17). To determine NK cell depletion, blood was isolated from the rats via a heart puncture one day after the third injection. Mononuclear 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 (Figure 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 less than 0.5%, indicating an efficient depletion of circulating NK cells.
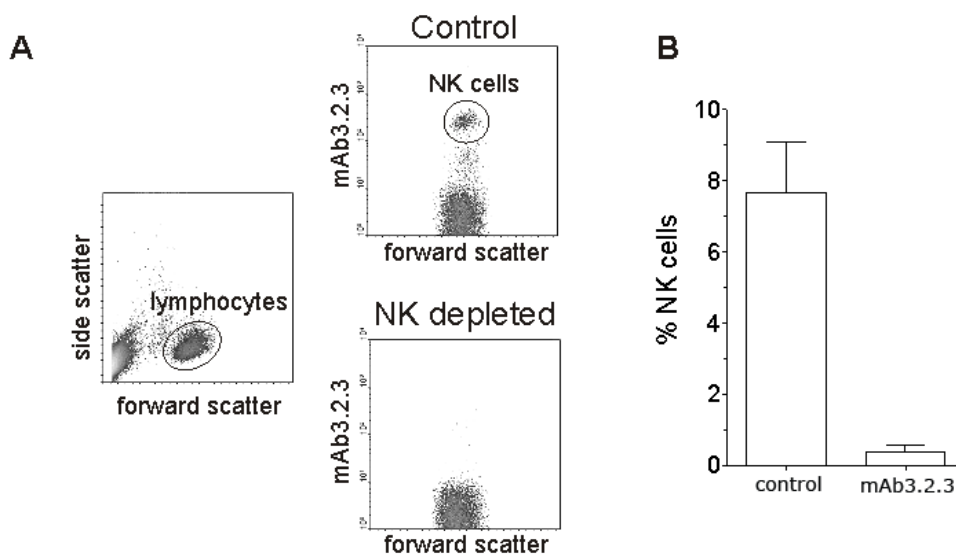

Figure 3. Pre-treating rats with mAb3.2.3 depletes NK cells from the circulation. Fischer 344 rats (90-110 gram) were intraperitoneally injected with 150 μg mAb3.2.3 on three consecutive days. One day after the last injection, mononuclear cells were isolated from the blood and stained for the NK1.1 specific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

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 $1x10^5$ 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

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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 to efficiently colonize the lungs.
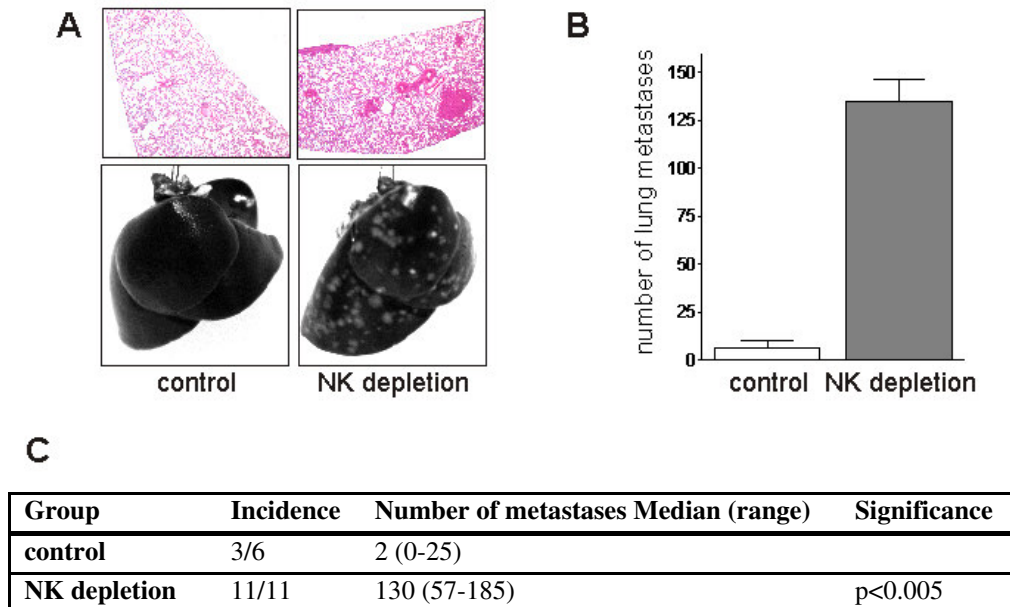

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Group	Incidence	Number of metastases Median (range)	Significance
control	3/6	2 (0-25)	
NK depletion	11/11	130 (57-185)	p<0.005

Figure 4. Depleting NK cells from the circulation enables MTLn3 cells to form experimental metastases. Rats were pre-treated with mAb3.2.3 to deplete NK cells before 1×10^5 MTLn3 cells were injected into the tail vein. After 4 weeks, lungs were isolated and (A) intact lungs were injected with ink or lung sections were stained with H&E. (B) The number of surface metastases were counted. (C) The incidence (the number of animals with visible lung surface metastases divided by the total number of animals in the group), median and significance were determined. Statistical analysis was performed using the Mann-Whitney Rank Sum test.

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 $1x10^5$ cells, providing an important tool to study the different steps involved in experimental metastasis formation.

Circulating NK cells were depleted by repetitive treatment of the rats with the NK cell- depleting antibody mAb3.2.3. Unfortunately, no other highly specific NK cell-depleting antibody was available and therefore the presence of NK cells was determined by studying the presence of fluorochrome-conjugated mAb3.2.3-positive cells in the peripheral blood. This approach can not 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 $1x10^5$ 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 $1x10^6$ MTLn3 cells in nude mice resulted in 26 lung metastases (5). Also, intravenous injection of $2x10^6$ 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 $1x10^5$ 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 $1x10^6$ cells.

To exclude the possibility that the observed lack of metastases after inoculation of $1x10^6$ 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 into the mammary fat pad, the cells were able to grow out into large tumors within 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 formation in a syngeneic model, no experiments in immunodeficient mice were performed. However, injection of MTLn3 cells into 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 NK cells in the prevention of tumor progression was extensively shown by Ben-Eliyahu and co-workers (6,7,8). Using the MADB106 tumor model in Fischer 344 rats they provide evidence for the crucial role of 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 either NK or T cells, our data clearly point towards the NK cells. Firstly, our *in vitro* killing assays show that NK cells, but not polyclonally-activated T cells effectively kill MTLn3 cells. Secondly, the mammary fat pad experiment (13) shows that intra-organ MTLn3 cells are no longer a target for the immune system. Thirdly, depletion of NK cells by equivalent injection of mAb3.2.3 by Van den Brink *et al.* (17) shows re-appearance of NK and 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. No antigen-specific response of T cells towards the MTLn3 cells can be elicited within this limited time-frame. As T cells could potentially 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

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MTLn3 cells from the circulation of Fischer 344 rats, our data strongly point towards NK 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 cell-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 a 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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