Characterization of Human Natural Killer Cell Activity in Solid Tumors

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Abstract—Natural Killer (NK) cells are major contributors of the human body’s protection against cancer development [1]. Targeting these cells for immunotherapy has shown promising results in the treatment of hematologic cancers but NK cell associated therapy against solid tumors have given little response [2], [3]. In order to develop means of targeting these cells in the fight against all cancer types, the mechanisms of NK cell-tumor cell interactions need to be understood. In vitro models, mimicking the in vivo tumor environment can be used for these kinds of interaction studies [4]. Three dimensional (3D) in vitro cultivation models generating micro size tumors provide a good setting when trying to resemble in vivo tumor environments [5]. One recently developed 3D cultivation model utilizes the forces generated by ultrasound to aggregate cells into micro tumors [6], [7]. This project aimed to increase the understanding of how NK cells are affected when co-cultured with cells of a solid tumor cancer type deploying this 3D cultivation model. Appropriate cultivation protocols using renal carcinoma cell line A498 and primary NK cells were developed, including control generation. Effects on cell surface receptor expression was characterized using flow cytometric analysis. A subpopulation of NK cells showed an increased expression of inhibitory Killer Cell Immunoglobulin-like Receptors (KIRs) following 3D co-culture. A KIR negative subpopulation of NK cells expressing inhibitory receptor TIGIT was also enlarged. NK cells expressed activation marker CD69 to a higher extent following 3D co-culture compared to 2D co-culture. Induced expression of NK- and T-cell associated receptor NK2D on tumor cells was indicated following 3D co-culture. The upregulation of inhibitory NK cell receptors could serve as a mean of tumor cell escape from NK cell mediated killing. Using this cultivation and analysis model, further explorations of NK cell responses to solid micro tumor environments can be performed.

I. INTRODUCTION

Kidney and renal pelvis cancer represents the twelfth most common cancer type in the world and over 330,000 people were diagnosed with the disease in 2012 [8]. The majority of these kidney cancers are of the Renal Cell Carcinoma type, where the Clear Cell Renal Carcinoma (ccRCC) histology subtype is most prevalent [9]. ccRCC is highly associated with mutations of a tumor suppressor gene, the von Hippel-Lindau gene (VHL), coding for a protein that is central for the cell response to hypoxia and inactivation of this protein leads to enhanced angiogenesis [10]. Anti-ccRCC therapies used today targets proteins that are expressed as a result of this mutation, although patient response rate is still limited [11], [12]. Due to the high number of tumor infiltrating immune cells associated with the cancer type, the spontaneous regression rate of the disease as well as occasional benefits from immunoreactive agents, ccRCC is considered an immunogenic cancer [13], [14]. Several attempts to treat the disease with immune check point inhibitors have been performed, however the success rate remains low, and new therapies are desperately needed for the management of the disease [15].

Natural killer cells are innate cytotoxic lymphocytes constituting 5-20% of peripheral blood lymphocytes [1]. These cells are divided into two major subgroups based on their surface expression level of CD56 and they have the ability to kill target cells without prior priming, a function mainly performed by CD56<sup>Dim</sup> NK cells [16]. CD56<sup>Dim</sup> NK cells are recognized by a low CD56 expression and a high CD16 and inhibitory killer cell immunoglobulin-like receptors (KIRs) expression. Apart from the cytotoxic effector function, NK cells, especially CD56<sup>Bright</sup> cells, are also important cytokine producers and act as regulators of other immune cells [17]. These cells are characterized by a high expression of receptors such as CD56, CD62L and inhibitory receptor CD94/NKG2A [18]. NK cells have been shown to play a key role in the defense against tumor development and viral infections acting in a complementary fashion to T-cells and NK cell deficiency has been shown to result in severe infections [17], [19], [20], [21]. Their function is tightly regulated by both activating, for example Natural Cytotoxicity Receptors (NCRs) (NKp30, NKp44 and NKp46), NK2D and DNAM1, and inhibitory receptors, such as KIRs, TIGIT and NKG2A/CD94 [22], [23]. In order to avoid NK cell mediated cytolysis, cancer cells have developed several mechanism of escaping recognition, for example by upregulation of non-classical MCH class I molecules resulting in NK cell inhibition by interactions with inhibitory NK cell receptor NKG2A/CD94, induction of NK cell downregulation of activating receptors such as NCRs, DNAM1 and NK2D and release of soluble NK2D ligands acting in a NK cell inhibitory fashion compared to their membrane bound counterparts [24], [25], [26], [27]. Activation of NK cells results in expression of several markers, such as CD69, CD25 and NCRs NKP44 and NKP30, expression that can be utilized when investigating cancer immune response [28], [29], [30], [31].

In vitro models are an important tool when investigating immune cell interactions with tumor cells [4]. These models are used in order to gain knowledge about the in vivo cancer progression, and models should, although of course simplified from the in vivo condition, resemble their natural counterparts.
to the highest degree possible [32]. Although considered an immunogenic cancer, renal carcinoma often resist NK cell mediated killing in vivo while cancer cells are killed during in vitro studies [3], [33], [34], [35]. Most of the in vitro cell cultivation models used does however implement a two dimensional (2D) cancer cell growth. The limitations of such 2D models in mimicking the cell to cell interaction seen in in vivo tissues have recently been appreciated [5]. It is for example challenging to orchestrate the Therefor more complex models, utilizing cell growth in three dimensions (3D), are developed [36]. One such 3D cultivation technique deploys acoustic forces to waft cells together, creating cell clusters leading to micro tumor formation [6]. This method has previously been used in studies of tumor and NK cells interactions and the model gives a mean of gently creating cell to cell interactions for time spans up to a week [6], [7].

In this project, I aimed to investigate interactions between peripheral NK cells and primary ccRCC cells A498, co-cultured in this 3D model. Creation of 100 micro tumors, one single tumor per micro well in a phosphorylcholine and organosilane copolymer surface coated microchip using ultrasound was performed. Tumors were examined using confocal and light sheet microscopy and effects on surface protein expression in tumor cells and NK cells following co-culture was analyzed using flow cytometry.

II. Results
A. Tumor cell susceptibility to NK cell mediated killing and tumor expression of NK cell ligands

Although most often avoiding NK cell recognition, renal carcinoma cells have occasionally shown to be susceptible to NK cell mediated killing in vivo [3]. Previous research in this group has also shown that primary renal carcinoma cell line A498 is killed in vitro by NK cells (Fig.1). In order to understand with what mechanisms this killing is exerted, NK cell activating and inhibitory ligands expressed by A498 cells were examined after 2D and 3D cultivation of A498 cells alone. Ligand expression was quantified using flow cytometry. Tumor cells expressed three out of five tested ligands, PVR, ICAM1 and HLA-1, and expression seemed to be altered in the 3D setting compared to traditional 2D cultivation (Fig.2). PVR was downregulated in 3D, both after 48 and 96 hours, compared to 2D. This downregulation was in accordance with previous experiments performed by the group (Fig.3).

B. Renal cell carcinoma cell line A498 expression of NK cell receptors

Before proceeding with development of an A498 and NK cell co-culture model appropriate for flow cytometry analysis, NK cell receptor expression by A498 cells was investigated. This was done in order to identify NK cell receptors that were not expressed by A498 cells and thereby could be used for NK cell identification during flow cytometry analysis of co-cultures. Cells were analyzed both after 2D and 3D cultivation, 48 hours. A498 cells were positive for CD56 both when cultivated in 2D and 3D (Fig.4: only results from 2D cultivation). A498 cells appeared to express NKG2D following 3D culture, but not 2D culture (Fig.5). The fraction of cells positively stained for NKG2D was however larger when including cells dimly positive for live/dead AF700 stain, possibly representing apoptotic cells (not shown). As no fluorescence minus one controls but only single fluorescence controls were used in this experiment, it could not be excluded that NKG2D positive cells were only an artefact caused by bad fluorescence compensation. Therefore, the experiment was repeated using fluorescence minus one controls. The panel size was also reduced in order to minimize spillover from other fluorophores. A control using stressed cells (starved over night in PBS supplemented with 2% FBS) was also included in order to elucidate weather NKG2D expression was a result of 3D culture or cell stress. This experiment showed no expression of NKG2D on A498 cells neither in 3D nor 2D culture, (Fig.6).
Fig. 3: NK cell receptor ligand expressed by A498 cells following cultivation in 2D, 3D 48 hours and 3D 96 hours. Experiment has been performed by group prior to start of this project.

Fig. 4: Expression of CD56 in 2D cultivated A498 cells in blue. Grey demonstrates fluorescence minus one control.

Fig. 5: Expression of NKG2D in A498 cells following 2D culture (left) and 3D culture (right) for 48 hours. Fraction of NKG2D positive cells is enhanced from 28% (2D) to 58% (3D). Single stained controls were used as negative controls.

Fig. 6: Expression of NKG2D in A498 cells following 2D culture (left), starved cell in 2D culture (middle) and 3D culture (right) for 48 hours. Fluorescence minus one controls used as negative controls.

with the tumor cells during the ultrasound cultivation, light sheet microscopy was performed as it reduces issues with loss of signal. Experiment was performed by coworkers. Some NK cells appeared to be trapped in the tumor, (Fig.6).

An additional experiment performing live cell time lapse imaging of co-cultures following a 24 hours cultivation using confocal microscopy were conducted. Both NK cells and A498 cells appeared to be proliferating (not shown).

2) Flow cytometry panel 1: For information about flow cytometry antibody fluorophore complexes used, see experimental procedure. An example of gating strategy for tumor cells and CD56+ NK cells can be seen in supplementary material, (Fig.21). After establishing a system where both NK and A498 cells survived during 3D co-culture, analysis of NK response to 3D co-culture was examined using flow cytometry. Cells were co-cultured at a ratio of 1:5 NK cells per A498 cells. Controls of 2D co-cultures using uncoated microchips resulting in A498 cells adhering to chip surface, and 2D co-cultures using polymer coated microchips, resulting in formation of several small irregular micro tumors per well, were used. Single cell type controls were also used. Five experiments using NK donor 2 were conducted examining the response of CD56Bright and CD56Dim NK cells following 3D co-culture with A498. For the first experiment, NK cell differentiation marker CD57 was also added to the panel. No increase in CD57 expression after 48 hours of 3D co-culture could be seen (not shown). The percentage of CD56 positive NK cells

C. Co-culture of A498 and peripheral NK cells

1) Imaging: The first NK cell and A498 cell co-culture experiment to be performed was analyzed with confocal microscopy after 48 hours of cultivation. A NK cell to tumor cell ratio of 1:6 was used. NK cells from NK donor 1 were stained with CSFE prior to co-culture and all cells were stained with NucBlue right before imaging. NK cells appeared to be located mainly at bottom of well whereas micro tumors were floating above (Supplementary materials 1, Fig.20). It was not possible to resolve the top or the core of the tumors due to loss of signal. This made it difficult to elucidate whether some NK cells were located inside, or in direct connection to the tumor. In order to determine if NK cells were in fact trapped
was reduced in both 3D and 2D co-cultures as well as in 2D microchips with NK cells grown in absence of tumor cells, (not shown). The fraction of NK CD56Bright cells were reduced in all five experiments, also in controls (Fig.8). No differences in Nkp46 or CD16 expression between 3D and 2D cultures could be observed (not shown). Expression levels of CD56 on A498 cells fluctuated between experiments both in co-cultures and in controls (not shown). No conclusions could be drawn regarding this fluctuation.

Three experiments were conducted adding CD62L and KIRs (KIR2DL1 and KIR2DL2) to the panel. This was done in order to clarify the differentiation behavior of NK cells in 3D and to confirm CD56Bright loss during microchip cultivation. Of these experiments, one (24 hours) used NK donor 4 and two (24 and 48 hours) used NK donor 3. CD62L expression on NK cells was completely lost in both co-cultures and NK cell controls (not shown). The fraction of CD56Bright cells was reduced but not entirely lost as in the case when using NK donor 2. The percentage of KIR positive NK cells was constant between co-cultures and controls (Fig.9). The expression level of KIRs within the KIR positive population was increased in NK cells grown in 3D after 24 hours and stable after 48 hours. KIR expression was reduced in NK cells grown alone and reduced in 2D co-cultures after 24 hours and stable after 48 hours (Fig.10).

An additional experiment using NK donor 4 co-cultured with A498 for 48 hours analyzed with a separate antibody panel but including KIRs was performed. The expression level of KIRs within the KIR positive population was increased after 3D co-culture, slightly increased in NK cells co-cultured in 2D and reduced in NK cells grown alone (Fig.11).
Fig. 11: Expression levels of KIR in KIR+ NK cells following. Cells are gated on CD45+, live/dead-, KIR+. Cells are derived from donor 4.

Fig. 12: Expression of NK cell receptors after 48 hours of activation using IL15 (upper figure) or after 48 hours of 3D co-culture (lower figure). Grey histograms represents fluorescence minus one controls.

3) Flow cytometry panel 2: Two experiments, 24 and 48 hours, were performed using NK donor 5. Cells were stained for inhibitory and activating NK cell receptors. Fig. 12 shows the flow cytometry results after 48 hours of cultivation for IL15 activated NK cells and 3D co-cultured NK cells. The percentage of KIR positive NK cells was decreased in all samples, however to a larger extent in NK cells grown alone (Fig.13). The expression level of KIRs within the KIR positive population was increased in NK cells grown in 3D after 24 hours and stable after 48 hours. KIR expression was increased in NK cells co-cultured in 2D and decreased in NK cells cultured alone in both experiments (Fig.14). The percentage of NK cells expressing inhibitory receptor TIGIT but not KIRs was increased in NK cells cultured in 3D co-culture, especially after 48 hours (Fig.15). Major differences of expression of DNAM1, NKG2D and NKp30 could not be observed (not shown). Panel 2 included an anti-NKG2D antibody. A498 cells stained positively for NKG2D after 3D co-culture and after culture alone in coated microchip (Fig.16). It should be noted that A498 forms tumors in coated microchips also without any applied ultrasound, although smaller, more irregular and most often they form many tumors per well instead of a single aggregate as in the case when acoustic forces are utilized.
Fig. 17: Expression of NK cell receptors after 48 hours of activation in IL15 (upper figure) or after 48 hours of 3D co-culture (lower figure). Grey histograms represents fluorescence minus one controls.

4) Flow cytometry Panel 3: NK cells from donor 3 were co-cultured with A498 in 3D for 48 hours and analyzed by flow cytometry staining for receptors expressed in response to NK cell activation. Fig. 17 shows the flow cytometry results for IL15 activated NK cells and NK cells co-cultured with A498 in 3D. KIR2DL1 was not used for this experiment but only KIR2DL2 potentially explaining the different appearance of KIR expression compared to panel 1 where the same NK cell donor was used. The percentage of CD69 positive NK cells was increased in both co-culture samples but to a higher extent in the 3D co-cultured sample (Fig. 18). The percentage of CD69 positive cells was reduced in both samples with NK cells cultured alone. The expression level of CD69 in living CD45+ NK cells was largely increased in cells co-cultured in 3D (Fig. 19). The expression level was also increased in NK cells co-cultured in 2D, but decreased in NK cells cultivated alone. No major differences in expression of NKp30, NKp44, NKp46 and KIR2DL2 could be observed.

III. CONCLUSION

NK cells play a crucial part in protecting the body against cancer development but occasionally fail their role as defenders and cancer progression occurs[20]. Immunotherapy using NK cells have shown efficacy as a treatment of hematological cancers but the success for treatment of solid tumors has been limited [3]. In order to understand how NK cells could be utilized in the management of solid tumors, in vitro models closely mimicking in vivo systems are needed [5]. As of today, most in vitro studies of NK cell interaction with tumor cells are carried out in a two dimensional setting. Solid tumors developing in vivo however, propagate in a three dimensional fashion. This way of growing largely affects cell to cell interactions and cell contact susceptibility, two facts that are not reproduced when analyzing cell interactions in two dimensions.

This project sought to enhance the understanding of how NK cells interact with tumors grown in a more in vivo-like in vitro model. The model used, gentle micro tumor formation using acoustic forces, has previously been developed and utilized in dynamic studies of NK cell tumor immune surveillance. The focus of this project was to develop an assay allowing for analysis of NK cell responses to co-culture with three dimensional solid tumors using flow cytometry. A suitable protocol including i.a cell ratios, cell seeding procedures...
and generation of appropriate controls was developed. Three panels containing a maximum of eight different antibody fluorophore complexes were used for investigation of NK cell responses. The first panel included CD56, a marker which expression level characterizes the two main subsets of NK cells, CD56\text{Bright} and CD56\text{Dim} cells. The proportion of CD56\text{Bright} NK cells was largely reduced during cultivation in all experiments, including 3D and 2D co-cultures and single NK cell controls using donor 2. This cell population was also reduced in single NK cell controls but not co-cultures for donor 3 and 4. It has previously been shown that CD56\text{Bright} NK cells can differentiate to become CD56\text{Dim} cells. Whether this is what occurred in these experiments or if CD56\text{bright} NK cells died to a larger extent is not determinable. In order to investigate such a question, one would need to perform cell sorting prior to experiment setup and co-culture only one subclass of NK cells with tumor cells.

KIR receptors KIR2DL1 and KIR2DL2 monitored in these experiments are inhibitory NK cell receptors. They inhibit NK cell-mediated killing by binding to HLA-C on the target cell. Because of their function as inhibitory receptors these molecules could represent a suitable target for blockade in immunotherapy. In fact, anti-KIR antibodies have been tested in phase I and phase II studies of Multiple Myeloma and Acute Myeloid Leukemia (ALM). KIR appeared to be upregulated in all experiments performed during this project. No statistical significance between 3D culture and 2D culture compared to NK cells newly derived from PBMC could be established, however it should be noted that the number of replicate experiments per donor was only at maximum two. All donors did show similar expression patterns during all experiments. The effect appeared to be larger after 24 hours of cultivation compared to 48 hours and longer experiments are needed in order to reveal weather this change of KIR expression is long lasting, potentially representing a tumor escape mechanism of NK cells. Experiments using panel 3 and 2 were only performed one and two times respectively. Results from these experiments indicated an upregulation of both TIGIT and CD69 in NK cells after 3D co-culture with A498. Although no conclusions can be drawn from these single experiments, they reveal the utilization of the 3D in vitro cell model used in this project.

These results display a good example of how NK cell responses to solid tumors can be investigated, as they reflect the differences in expression patterns between a common 2D culture model and a more in vivo-like 3D model. Moreover, not only can this model be used for investigation of simple co-cultures containing two cell types as the ones presented in this project, but it also has potential for more multifaceted scenarios. One could for example create more complex tumor environments using i.a fibroblasts, monocytes, NK cells etc. Eventually, this model could even be used for co-culture of patient-derived tumor and NK cells for determination of patient-specific NK cell responses, information that could be utilized in determination of treatment type. A major difficulty during the project has been the creation of appropriate controls for all experiments. Cell number and concentration is a crucial factor for experiments of this type, and comparing cell numbers for adherent cells growing in a monolayer with cells expanding in a 3D fashion as in the case with micro tumors is difficult. The usage of low NK cell numbers, a necessary measure in order to mimic a more in vivo like situation, also creates problems when cultivating the NK cells in microchips with no tumors as the total cell concentration per well becomes very low. Further thought needs to be put into the development of appropriate controls for future experiments.

Due to the limited time span of this project, most co-cultures were short, 24 or 48 hours. It is likely that effects on NK cells function will be acquired over time. One future perspective is to analyze cell populations over multiple time points spanning the culture time. If responses like up-regulation of inhibitory receptors are long lasting, one could investigate these effects on NK cell-mediated tumor killing by blocking the receptors or their corresponding ligands.

Conclusively, the project has revealed exciting insights into changes of NK cells receptor expression as a result of co-culture with micro tumors in a three-dimensional system.

IV. EXPERIMENTAL PROCEDURE

A. Cell lines and cell culture

Adherent human primary renal carcinoma cells A498 was grown in cell culture medium RPMI-1640 supplemented with 10 % fetal bovine serum (FBS) and 1 % non-essential amino acids in 75 ml culture flasks. Cultures were split using a Trypsin/EDTA solution every 48 hours and cultivation times were kept at a maximum of two weeks.

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood derived from healthy donors using density gradient separation (Ficoll-Hypaque, Ge Healthcare) and frozen at a number of 100 million cells per freezing vial (-80°C) in 90 % FBS and 10% DMSO. NK cells were isolated from thawed PBMC by negative selection using magnetic bead separation (NK cell isolation kit, Miltenyi) at the day of experiment setup. NK cell purity was controlled by flow cytometry analysis of CD3, CD56 and NKP46 expression.

Co-cultures of A498 cells and NK cells as well as controls were established in 100 well microchips, see [6], [7] for detailed description of microchips. All microchips used for tumor generation by ultrasound were coated with a mix of phosphorylcholine and organosilane copolymers in order to reduce cell attachment to surface of chip [37]. Coated microchips were used approximately four times following coating and were thereafter re-coated again. Polymer coated microchips as well as non-identical but similar to shape uncoated microchips were used as controls for the different experiments.
Controls included: Uncoated and coated microchip co-culture of A498 and NK cells, uncoated and coated microchip 2D A498 cells alone, uncoated and coated microchip 2D NK cells alone. All microchip cultures were grown in RPMI-1640 supplemented with 10% FBS and 1% non-essential amino acids and media exchanged were performed for cultures cultivated for more than 48 hours. A T25 flask containing 0.6 million A498 cells at a concentration of 0.1 million cells per ml was prepared as a 2D control when investigating NK cell ligand and receptor expression on A498 cells. NK cells used for fluorescence controls in flow cytometry experiments were grown in 24 well plates (one million cells per ml) in RPMI-1640 supplemented with 10% FBS and 1% non-essential amino acids. IL15 activated NK cells were used as additional controls when investigating panel 2 and 3. These cells were cultivated in 96 well plates (1.25 million cells per ml) in RPMI-1640 supplemented with 10% FBS, 1% non-essential amino acids and 10 ng/ml IL15 for 48 hours. All cell cultures were incubated at 37°C and CO₂ 5%.

Cell concentrations used for different experiments: A498 expression of NK cells ligands: 0.3 million A498 cells per ml. Confocal and light sheet imaging of co-cultures: 0.3 million A498 cells per ml, 0.05 million NK cells per ml. A498 expression of NK cell receptors: 0.3 million A498 cell per ml. Co-culture models for flow cytometry analysis: 0.5 million A498 cells per ml, 0.1 million NK cells per ml.

B. Microchip preparation, cell seeding and ultrasound cultivation

Microchips (stored in Milli-Q water) were prepared by an initial cleaning with distilled water and 70% ethanol and left in ethanol for approximately 15 minutes. Microchips were then transferred to Petri dishes and 100 ul cell culture medium was added and removed from the chip five times in order to remove all excess ethanol. Cell suspensions of either single cell types or a mix of A498 cells and NK cells of appropriate cell concentrations were prepared in falcon tubes and cells were seeded to microchips by adding 100 ul of cell suspension to microchip. Microchips were stored in the incubator for approximately 15 minutes in order for cells to sediment to the bottom of the microchip. Cells remaining in suspension were removed by gently adding and removing 50 ul of cell culture medium to the upper part of the supernatant five times. A pre-washed circular 10 mm in diameter glass cover slip was placed on top of the microchip and microchips were incubated in regular incubator or placed in ultrasound for tumor formation for 24 hours. Microchip was removed from ultrasound after 24 hours and placed in a regular incubator for the remaining incubation period.

C. Flow cytometry and antibody panels used

Antibodies specific for the following proteins were used during this project: Panel 1: CD56, CD45, CD16, NKp46, CD62L, KIR2DL1, KIR2DL2, CD57. Panel 2: CD45, NKp30, NKG2D, DNAM1, TIGIT, KIR2DL1, KIR2DL2. Panel 3: CD45, NKp30, NKp44, NKp46, KIR2DL1, CD25, CD69. NK ligand panel: PVR, MIC/A/B, NECTIN2, ICAM1, HLA-1. NK cell receptors on A498: NKp46, NKG2D, CD56, CD16, LFA-1. NK cell purity after isolation: CD56, CD3, NKp46. Amine binding live/dead fixable aqua dead cell stain kit AlexaFlour700 (Invitrogen) was used in order to exclude dead cells. Appropriate concentrations of antibody fluorophore complexes were decided through titration on PBMC and/or cytokine activated NK cells. Expression levels in cells prior to experimental setup (day zero) was examined for all panels tested during the project. In order to reduce occurrence of false positives, fluorescence minus one (FMO) controls were used for all co-culture experiments as well as for the last experiment of A498 expression of NK cell receptors. Fluorescence compensation for signal acquired from spillover signals using compensation beads (BD Biosciences) was generated before each experiment. A498 cells used for FMOs were harvested from T75 culture flask using Trypsin/EDTA solution. 100 ul cell suspension containing 50 000 NK cells and 50 000 A498 cells was prepared for FMO controls. Cells were collected from microchips using 500 ul of Accumax cell dissociation solution (Accumax Sigma-Aldrich). All antibody staining was performed in 100 ul PBS supplemented with 2% bovine serum albumin (BSA).

D. Confocal and light sheet microscopy

NK cells were stained with celltrace cell proliferation kit, CSFE (ThermoFisher) prior to co-culture and all cells were stained with NucBlue Live ReadyProbes Reagent (ThermoFisher) right before imaging with confocal microscopy. For light sheet microscopy, NK cells were stained with CSFE prior to co-culture setup and all cells were stained with CellTrace Far Red Cell Proliferation Kit (ThermoFisher) and NucBlue right before imaging. Cell preparation and staining for light sheet microscopy was performed by a coworker.

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V. Supplementary Material

Fig. 20: Confocal image of a co-culture model with NK cells in green, A498 cells in dark grey (brightfield out of focus). NK cells are in focus on the bottom of the well whereas micro tumors are floating above (out of focus).

Fig. 21: Example of flow cytometry gating strategies for A498 (upper row of figures) and for CD56+ NK cells (two lower row of figures) applied for co-culture experiments.