Combination of Antibody-Targeted Amanitin Conjugates (ATAC) with Immune checkpoint inhibitors shows a synergistic therapeutic effect *in vivo*

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Introduction

While cancer therapy has relied mainly on the use of systemic cytotoxic substances such as chemotherapeutic agents for the last decades, the focus of cancer therapy has shifted recently towards target-specific drugs as well as the manipulation of patients´ immune system[1]. Among the latter, immune checkpoint inhibitors (ICI) have proven promising as in many cancer entities, tumor cells can suppress T-cell immune responses by upregulating immune checkpoint molecules, such as PD-L1 and CTLA4. ICIs bind to these molecules and thereby suppress T-cell inhibition[2].

Among the class of target-specific therapeutics, antibody-drug conjugates (ADCs) show promise as they combine the target specificity of antibodies with the potent anti-tumor activity of the toxin. While most ADCs are based on a few cytotoxic compounds with mainly microtubule- or DNA-targeting toxins as payload, Heidelberg Pharma (HDP) focuses on amanitin based ADCs, so called **ATAC**s (Antibody Targeted Amanitin Conjugates), comprising a new class of ADCs with amanitin as payload[3]. Amanitin is the well-known toxin of the amatoxin family and binds specifically to the eukaryotic RNA polymerase II thereby inhibiting the cellular transcription process[4]. We have previously demonstrated the potent anti-tumor activity of ATACs in various tumor types *in vivo*[5].

In the present study we show that treatment with ATACs induces immunogenic cell death (ICD) *in vitro* and immunity towards tumor re-challenge *in vivo,* suggesting an involvement of the immune system. Furthermore, NK cells play an important role in the anti-tumor effect of ATACs. The activation of the immune system by ATAC treatment translates into a synergistic anti-tumor effect of ATACs and ICIs *in vivo.* Hence, combination of ATACs with ICIs provides a promising approach for potential further cancer therapy.

Methods

Cell lines: JIMT-1, BT474, and Raji cells were obtained from DSMZ, NCI-N87 from ATCC, MM1.S-GFP(luc) from the Max Delbrück Zentrum in Berlin and human PBMCs from DRK Blutspendedienst Mannheim.

ATACs: Anti-HER2 (variable domains of trastuzumab; cysteine engineered monoclonal antibody, HDP), chimeric anti-CD19 (DKFZ Heidelberg, Germany; cysteine engineered monoclonal antibody, HDP) and anti-CD79 were conjugated site-specifically to cysteine reactive linker-amanitin constructs synthesized at HDP.

Flow cytometry: FACS staining was performed on spleen cell suspensions of NMRI nude mice using anti-mCD45-APC/Cy7, anti-mNKp46-FITC, anti-mCD3-APC antibodies and 7-AAD and was analysed on a BD FACSLyric.

Immunohistochemistry: Tumors were fixed with 10% formalin, embedded in paraffine and stained with the anti-HER2 antibody Trastuzumab (Proteinase K antigen retrieval) and an anti-human IgG- HRP antibody as secondary antibody.

Immunofluorescence: Tumors were snap frozen, embedded in OCT. 5 µm sections were fixed with 10% formalin and stained with an anti-mNKp46-FITC antibody.

Animal models: 5x10^6 JIMT-1 or NCI-N87 cells were injected s.c. into 6-8 weeks-old female NMRI Nude mice. When tumor volumes reached ca. 150mm^3, mice were treated with a single i.v. dose of an anti-HER2 ATAC. 2-4 weeks after complete tumor remission (CR), mice were re-inoculated with 5x10^6 tumor cells. Anti-Asialo GM1 treatment (Walko; 50 µl i.p. q5d) was started one day before.

HER2low TNBC PDX model studies were performed at XenTech (Evry, France). Mice were treated with a single dose of anti-HER2 ATAC i.v. at a mean tumor volume of 170 mm^3.

2.5x10^6 Raji cells alone or in a mix with 1x10^7 human PBMCs were injected s.c. into 6-8 weeks old NOD Scid mice. On the same day, treatment was initiated with either a single i.v. dose of anti-CD19 ATAC, Avelumab given i.v. q3dx6 or a combination of both.

Results

1. Anti-HER2 ATAC treatment *in vivo* results in complete tumor remission in heterogeneous TNBC PDX models

A HER2 low expressing (1+), heterogeneous patient derived xenograft (PDX) model of TNBC growing subcutaneously in athymic nude mice was treated with a single i.v. dose of an anti-HER2 ATAC. ATAC treatment resulted in a significant tumor growth delay as compared to control tumors and complete tumor remission (CR) in 10/10 mice (Figure 1).

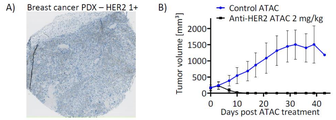


Figure 1: Anti-HER2 ATAC treatment results in CR in a HER2 1+ breast cancer PDX model.
A) Immunohistochemistry straining for HER2 protein expression in a s.c. TNBC PDX model.
B) Anti-tumor efficacy of an anti-HER2 ATAC in the same breast cancer PDX. At a mean tumor volume of ~170 mm^3, the mice were treated with either a non-targeting control ATAC or an anti-HER2 ATAC. (n= 10 mice; Mean +SD)

2. ATAC treatment *in vivo* leads to immunity towards tumor re-challenge

Mice that were re-challenged with the same tumor cell line after CR achieved by ATAC treatment developed tumors at a significant lower rate than wildtype mice upon first inoculation (Figure 2).

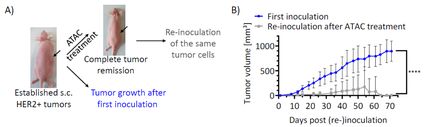


Figure 2: Anti-HER2 ATAC treatment results in significantly reduced tumor growth of HER2+ NCI-N87 cancer cells after re-challenge
A) Scheme of the experimental set-up of the re-challenge experiment
B) The tumor growth of NCI-N87 cells in untreated mice was compared the to tumor growth in mice that were re-challenged with NCI-N87 cells after CR by ATAC treatment. (n=15; Mean + SD; ****: p-value < 0.00001 (unpaired Welch t-test, Holm-Sidak correction))

3. ATAC treatment induces immunogenic cell death

Intrigued by the finding that ATAC treatment reduces the take rate of tumor cells upon re-challenge, it was investigated if ATAC treatment can activate the immune system. Therefore, the secretion of the immunogenic cell death (ICD) markers Calreticulin, HMGB1 and ATP was measured in HER2+ BT474 breast cancer cells *in vitro*. Treatment with an anti-HER2 ATAC resulted in an increase in all three markers in a target dependent manner while α-Amanitin and an anti-CD79b ATAC did not have any effect as CD79b is not expressed on BT474 cells (Figure 3).

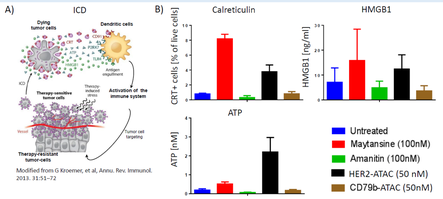


Figure 3: ATACs induce secretion of ICD markers in a target dependent manner
A) Scheme of the molecular consequences of ICD in tumors.
B) HER2+ BT474 breast cancer cells were either left untreated or treated with Maytansine, α-Amanitin, an anti-HER2 ATAC or an anti-CD79b ATAC. The number of tumor cells expressing Calreticulin (CRT) on their cell membrane (upper left) was analysed by flow cytometry. The HMGB1 and ATP concentration in the supernatant was measured by ELISA (upper right and lower left). (Mean + SD; n = 3)

4. NK cells are involved in the anti-tumor effect mediated by ATACs

Immune-deficient mice were used in all *in vivo* experiments, eliminating T- and B-cells as effector cells of the observed effects, while all mouse strains retained NK-cells (Figure 4 A).

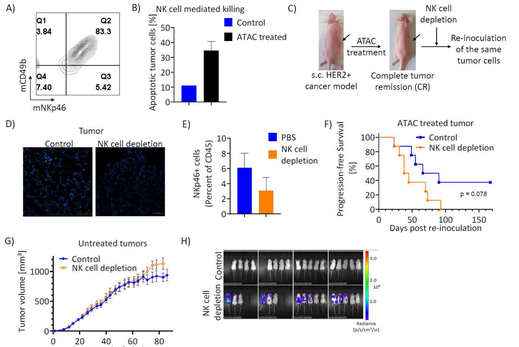


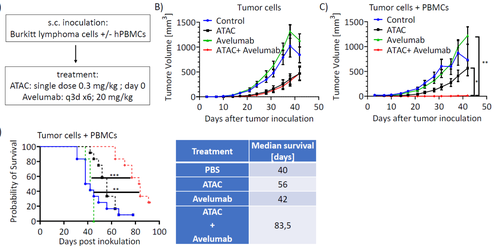
Figure 4: NK cells are involved in the anti-tumor effect of ATAC treatment
A) Flow cytometry of spleens from NMRI Nude mice using anti-mCD49b and anti-mNKp46 antibodies.
B) MM1.S tumor bearing mice were either treated with PBS or with an ATAC. NK cells isolated from spleens of these mice were co-cultured with MM1.S cells for 4h. Cell death of cancer cells was measured by flow cytometry.
C) Scheme of the re-challenge experiment with NK cell depletion in a s.c. HER2+ cancer model.
D) Expression of the murine NK cell marker mNKp46 (green) in PBS or anti-Asialo treated JIMT-1 tumors.
E) Percentage of mNKp46 positive cells in the mCD45+ cell population in spleens of control and anti-Asialo treated NMRI Nude mice was quantified by flow cytometry. (Mean +SD; n =3)
F) Progression free survival of control and NK cell depleted mice that were re-inoculated with JIMT-1 cells after CR by ATAC treatment. (n=8; Log-rank test)
G) s.c. tumor growth of HER2+ cancer cells in control or anti-Asialo treated mice. (Mean +SEM; n=15)
H) Bioluminescence imaging of mice that were re-inoculated with 1x10^7 MM1.S-luc cells i.v. after CR by ATAC treatment and either treated with control serum or with anti-Asialo.

First we tested *in vitro* if ATAC treatment can potentiate NK cell mediated tumor cell killing. NK cells from MM1.S tumor bearing mice, that were treated with PBS or ATAC, were isolated and co-cultured with MM1.S cells for 4h. Apoptosis of cancer cells was measured by flow cytometry. NK cells isolated from mice that were treated with an ATAC induced higher cell death rates in tumor cells than NK cells isolated from control mice (Figure 4 B), confirming the activation of NK cells by ATAC treatment.

To investigate a possible role of NK cells in the anti-tumor effect of ATACs *in vivo,* the re-challenge experiment with HER2+ JIMT-1 tumors was combined with NK cell depletion by anti-Asialo treatment. One day before tumor re-inoculation, NK cell depletion was initiated (anti-Asialo q5d; Figure 4 C). 50% NK cell depletion was confirmed in JIMT-1 tumors by IF staining (Figure 4 D) and in the spleens of treated mice by flow cytometry (Figure 4 E). NK cell depletion resulted in reduced progression free survival (Figure 4 F) and better tumor take as compared to control mice. Interestingly, NK cell depletion did not affect the growth of HER2+ cancer cells in mice that were not previously treated with an anti-HER2 ATAC (Figure 4 G). Similar results were obtained in a disseminated multiple myeloma model. NK cell depletion also resulted in an increased take rate of MM1.S-luc cells upon re-inoculation after CR by ATAC treatment (Figure 4 H).

5. Combined treatment of ATACs with an immune checkpoint inhibitor leads to a synergistic anti-tumor effect *in vivo*

The induction of ICD by ATAC treatment and the role of NK cells for in the anti-tumor effect of ATACs suggest activation of the immune system. Consequently, it was investigated whether combination of ATACs and ICIs may have a synergistic effect. Mice were inoculated s.c. with CD19+ Burkitt lymphoma Raji cells either alone or in a 1:4 mix with human PBMCs. On the day of tumor inoculation, the mice were treated with a single dose of an anti-CD19 ATAC, the ICI Avelumab (q3d x6) or the combination of both (Figure 5 A).



Treatment	Median survival [days]
PBS	40
ATAC	56
Avelumab	42
ATAC + Avelumab	83,5

Figure 5: Combined treatment with an ATAC and ICI results in a synergistic anti-tumor effect in a s.c. Burkitt lymphoma model
A) Scheme of the experimental set-up with ATAC and ICI combination treatment.
B) s.c. tumor growth of Raji cells that were treated with PBS, an anti-CD19 ATAC, Avelumab (q3d x6) or a combination of ATAC and Avelumab. (n=12; Mean + SEM)
C) And D) Tumor growth (C) of Raji cells inoculated s.c. in a 1:4 ratio with human PBMCs and survival (D) of the animals. Tumors were treated with PBS, an anti-CD19 ATAC, Avelumab (q3d x6) or a combination of ATAC and Avelumab. (n=12; Mean+SEM) * p<0.05; ** p<0.001; ***p>0.0001 (C) unpaired Welch t-test; Holm-Sidak correction; D) Log-rank test)

Combined treatment of an anti-CD19 ATAC and Avelumab resulted in significantly reduced tumor growth and increased survival as compared to both single treatments (Figure 5 C and D). This effect was caused by activation of human immune cells as combined treatment of ATAC and ICI did not have an synergistic effect in the absence of human PBMCs (Figure 5 B).

Conclusion

ATAC treatment results in a complete and persistent anti-tumor effect *in vivo* in different cancer entities, including heterogeneous PDX models that cannot solely be explained by the toxicity of α-Amanitin. Especially, as a direct bystander effect can be excluded due to the hydrophilic nature of amanitin. Thus, the possibility of immune activation by ATAC treatment was investigated in this study.

ATAC treatment resulted in ICD *in vitro* in multiple tumor models (data shown for breast cancer). Furthermore, if mice that achieved complete and stable tumor remission by ATAC treatment were re-challenged with the same tumor cells, the tumor take rate was significantly reduced compared to the first inoculation. These findings may suggest a possible immune activation by ATAC treatment.

As all mouse strains used in *in vivo* studies were immune deficient but maintained functional NK cells and as it is well known that NK cells can induce tumor cell killing, a possible involvement of NK cells in the anti-tumor effect of ATACs was investigated. NK cell killing assays demonstrated that ATAC treatment can potentiate NK cell activation. 50% depletion of NK cells *in vivo* resulted in increased tumor re-take after ATAC treatment in different cancer models while NK cell depletion did not affect tumor growth in untreated mice. This data suggest that NK cells can potentiate the anti-tumor effect of ATACs.

To investigate if this immune activation can be used for therapeutic purposes, ATAC treatment was combined with immune checkpoint inhibition in a humanized mouse model. Combined treatment with ATACs and ICIs resulted in a synergistic anti-tumor effect in a s.c. Burkitt lymphoma model that was co-injected with human PBMCs. This effect was dependent on the presence of human immune cells as combined treatment did not have a beneficial effect in the absence of human PBMCs.

Taken together, the data presented provide a rationale to the use of ATACs in combination therapy with immune checkpoint inhibitors.

References

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