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Scientific report: Identifying the impact of the T cell cytokine micromilieu in different organs on cell adhesion molecules in metastasis formation

The central hypothesis of our submitted proposal was that the interaction of T cell-derived cytokines with endothelial cell adhesion molecules is tissue-specific thereby driving cancer cells and metastasis formation in distinct organs. To test this hypothesis, it is necessary to specifically identify the expression pattern of T cell-derived cytokines as well as cell adhesion molecules in endothelial cells in the main metastatic target organs of colon cancer. **The objectives of our submitted proposal were the following:**

1. **To characterize the functional impact of the T cell-derived cytokines on cancer cell adhesion and therefore metastasis formation.**
2. **To identify the expression pattern of endothelial adhesion molecules in lung and liver, the two most common organ targets of colon cancer metastasis.**

According to our objectives we had the following results until now:

a) IL-22 promotes extravasation by inducing endothelial ANPEP upregulation

Since cancer cell adhesion and extravasation are crucial steps during metastasis formation, we hypothesized that IL-22 contributes to the metastatic process by interacting with the endothelial barrier, thereby facilitating cancer cell transmigration into the organ parenchyma and thus establishment of the metastatic niche. In a first step, we aimed to investigate whether IL-22 signaling in LSECs promotes cancer cell endothelial adhesion. We used an *in vitro* adhesion assay to test the retention of the MC38 colorectal cancer cells onto a monolayer of LSECs in the presence or absence of IL-22. As a positive control for the laminar flow assay, we used IL-1alpha, a cytokine that is known to increase the adhesion of cancer cells to endothelial layers. Contrary to the effect of IL-1alpha, the IL-22 treatment did not increase MC38 cell absorption to the LSEC monolayer. Thus, IL-22 does not seem to promote cancer cell adhesion in LSECs. Second, we tested whether IL-22 would be able to promote cancer cell transmigration into the liver parenchyma. To this end, *Il22*$^{-/-}$ or *Il22ra1*$^{-/-}$ mice or Wt mice were injected with GFP expressing MC38 cells i.s. and 24h later the number of extravasated GFP+ cancer cells in the liver were assessed by flow cytometry. Interestingly, significantly reduced GFP+ cells were found in the livers of *Il22*$^{-/-}$ or *Il22ra1*$^{-/-}$ mice, suggesting that IL-22 promotes cancer cell transmi-

gration from the vasculature into the liver. Likewise, the administration of a neutralizing IL-22 antibody into Wt mice prior to injection of GFP+ cancer cells blocked the transmigration of cancer cells into the liver parenchyma *in vivo*. In line with these data, *Il22ra1*$^{flox/flox}$*;Cdh5*$^{Cre+}$ mice lacking IL-22 signaling on the endothelium showed reduced extravasation. On the basis of these data, we next wanted to identify the underlying molecular mechanism. We exposed LSECs to recombinant (rm) IL-22 and determined the expression of an endothelial-specific gene array, via qPCR. LSEC exposure to rmIL-22 specifically induced the expression of three transcripts, namely *Anpep*, *Epas1* and Fgfr3. However, we could only verify the upregulation of *Anpep* in LSECs by both qPCR and immunoblotting upon IL-22 exposure. Indeed, ANPEP was reported before to be expressed by pericytes (Kloc et al., 2015). For this reason, we first analyzed whether LSEC can in principle, also express ANPEP *in vivo*. Accordingly, immunostaining of freshly isolated LSEC from wild type mice revealed that CD31+ endothelial cells express ANPEP. Finally, we tested the functional relevance of this finding *in vitro*. Indeed, the exposure of human endothelial HUVEC cells to rmIL-22, promoted the permeability in a vascular permeability assay and the transmigration of human colorectal cancer cells (HT-29) in a cancer cell transmigration assay. Silencing of ANPEP expression in HUVEC cells resulted in reduced cancer cell transmigration compared to respective controls. Finally, treating C57BL/6 mice with ubenimex, a general antagonist of aminopeptidase activity before intra-splenically administering MC38 cells, resulted in reduced metastatic sites *in vivo*.

Taken together, these findings indicate that IL-22 directly acts on LSECs. It induces ANPEP aminopeptidase, thereby increasing endothelial permeability and promoting cancer cell transmigration and thus extravasation.

***Il22*-deficient mice are protected against liver and lung metastasis**

We next examined the functional role of IL-22 in metastasis. To this end, we employed forced and spontaneous metastatic mouse models (Giannou et al., 2017; Soares et al., 2014). Firstly, C57BL/6 syngeneic colorectal cancer cells (MC38 cell line) were intra-splenically (i.s.) injected into *Il22*$^{-/-}$ mice and littermate controls. To avoid the formation of splenic tumors, the injected splenic part was resected 3 min after injection (hemi-splenectomy) and the mice were left for liver metastasis to occur. After 21 days the mice were sacrificed and their livers were examined for macroscopic metastatic burden and microscopic metastatic foci. Interestingly, *Il22*$^{-/-}$ mice were largely protected from metastasis formation compared to littermate controls. Next, we employed a spontaneous metastasis model by implanting C57BL/6 Lewis lung adenocarcinoma syngeneic cancer cells (LLC cell line) into the caecum of *Il22*$^{-/-}$ mice and littermate controls. Indeed, we could confirm that *Il22*$^{-/-}$ mice were largely protected from liver metastasis. We furthermore confirmed the pro-metastatic function of IL-22 in spontaneous and forced models of lung metastasis using different tumor cells. In order to further strengthen these data, we used a gain of function approach. To this end, we used *Il22tg8* transgenic mice, which overexpress IL-22 under the albumin promoter. These mice overexpress IL-22 specifically in the liver and have also increased serum IL-22 levels (≈ 6000pg/ml). In line with our data obtained using *Il22*$^{-/-}$ mice, we found increased number of liver metastases in *Il22tg8* transgenic mice compared to littermate controls upon induction of forced liver metastasis formation via intra-splenic (i.s.) injection of MC38 cells. These data were confirmed in a model of forced lung metastasis. Strikingly, 60% of these mice also developed liver metastasis in this model of forced lung metastasis, while this was not the case in any of their littermate controls. We further confirmed the pro-metastatic function of IL-22 using hydrodynamic injection of Balb/c mice with an *Il22* expression plasmid. On the basis of these data, we next used a genetic model which spontaneously develops CRC and liver metastasis. In *Apc*^{15lox}*;Kras*^{G12D} mice (Hung et al., 2010; Robanus-Maandag et al., 2010; Sakai et al., 2018) a specific mutation

was induced using adenovirus, Ad-Cre injection. Indeed, 60% of these mice developed liver metastases upon hydrodynamic overexpression of IL-22, compared to 0% of the mice with control (*Egfp*) gene transfer. Finally, we aimed to test the therapeutic potential of IL-22. To that end, we used a neutralizing IL-22 antibody and the model of forced liver metastasis, which mimics a possible application in patients, e.g. to prevent liver metastasis upon resection of the primary tumor. Strikingly, IL-22 blockade protected mice from developing liver metastasis.

Taken together, IL-22 plays a crucial role in metastasis formation. Accordingly*, Il22*-deficieny and IL-22 blockade using a neutralizing antibody is protective, while overexpression of IL-22 promotes metastasis formation.

b) *Using* Tie2;BacTRAP mice to elucidate the regulation of endothelial genes during metastasis formation

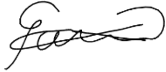
Based on our preliminary findings supporting that colorectal cancer spontaneously metastasizes into the liver, we aimed to elucidate the underlying mechanism that drives liver metastasis formation likely by affecting cancer cell adhesion on liver endothelium. We used an unbiased approach to identify the molecular mechanism mediating the adhesion procedure of cancer cells on the endothelium and finally the extravasation process. To this end, we introduced in our lab a methodology that allows us to profile the entire translated mRNA complement of any genetically defined cell population. This methodology, known as translating ribosome affinity purification or TRAP, combines cell-type-specific transgene expression with affinity purification of translating ribosomes. TRAP can be used to study the cell type-specific mRNA profiles of any genetically defined cell type. Unlike other methodologies that rely upon micro-dissection, cell panning, or cell sorting, the TRAP methodology bypasses the need for tissue fixation or single-cell suspensions (and potential artifacts these treatments introduce), and reports on mRNAs in the entire cell body. This method was combined with bulk RNA sequencing of the whole tissue, thereby allowing us to correlate the expression pattern of the cell adhesion molecules with the associated cytokine gene expression pattern during the cancer cell adhesion step of the metastatic cascade. Firstly, we injected Tie2;BacTRAP (Tie2; endothelial specific promoter) mice with cancer cells by having 2 groups. In the first group, the mice received PBS and in the second, cancer cells. The experiment performed and we analyzed the data after the sequencing. After the analysis we identified upregulated genes such as *klrd1, Olfr2, Olfr1261*. We currently create knockout mice for these genes or knocking down these genes by using targeting of adeno-associated viral vectors in collaboration with Dr. Körbelin in UKE to perform functional experiments of metastasis. We are going to use these data as preliminary data for an ERC Starting grant application in October 2024.

Finally, we have also recently published 3 protocols in ISTAR Protocols (Cell Press) describing models of forced and spontaneous liver and lung metastasis and a detailed protocol focusing on orthotopic lung transplantation in mice. All protocols are currently in press (attached).

We recently published that Interleukin-22 (IL-22) is produced by immune cells and promotes tissue repair and regeneration; however, in malignancy, IL-22 can promote tumor growth. Giannou et al. found that tissue resident iNKT17 cells produce IL-22 and promote cancer cell extravasation through regulation of aminopeptidase N. Neutralization of IL-22 inhibits metastasis formation, suggesting therapeutic avenues for cancer treatment. These finding were published in Immunity (publications attached) and a part of them is under revision in Oncoimmunology (manuscript and email from the journal attached). Additionally, we investigated the role of IL-10 in liver metastasis formation and decipher its therapeutic potential in affecting immunotherapy effectiveness. Our data identified IL-10 as a pro-metastatic factor in liver metastasis formation and characterize this cytokine as a regulator of PD-L1. This provides an

affirmation for future monitoring and targeting of IL-10 in CRC-derived liver metastasis. Our findings was reviewed in Journal of Hepatology (manuscript and email from the journal attached) and we are currently working on the revision. The role of other cytokines such as IL-17A and IL-17F is the aim of other ongoing projects.

Sincerely Yours,



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