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ORIGINAL RESEARCH

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CD4+ T cell-derived IL-22 enhances liver metastasis by promoting angiogenesis

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ABSTRACT

Metastasis is a cancer-related systemic disease and is responsible for the greatest mortality rate among cancer patients. Interestingly, the interaction between the immune system and cancer cells seems to play a key role in metastasis formation in the target organ. However, this complex network is only partially understood. We previously found that IL-22 produced by tissue resident iNKT17 cells promotes cancer cell extravasation, the early step of metastasis. Based on these data, we aimed here to decipher the role of IL-22 in the last step of metastasis formation. We found that IL-22 levels were increased in established metastatic sites in both human and mouse. We also found that Th22 cells were the key source of IL-22 in established metastasis sites, and that deletion of IL-22 in CD4+ T cells was protective in liver metastasis formation. Accordingly, the administration of a murine IL-22 neutralizing antibody in the establishment of metastasis formation significantly reduced the metastatic burden in a mouse model. Mechanistically, IL-22-producing Th22 cells promoted angiogenesis in established metastasis sites. In conclusion, our findings highlight that IL-22 is equally as important in contributing to metastasis formation at late metastatic stages, and thus, identify it as a novel therapeutic target in established metastasis.

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Introduction

Metastasis is a leading cause of mortality in cancer patients, accountable for approximately 90% of cancer-related death.[1,2] More than half of the patients suffering from colorectal cancer (CRC) develop metastasis during the disease course, with the liver or the lungs being most frequently affected.[3] Unlike the primary tumor, metastasis is a systemic disease that cannot be locally removed through surgery or radiation.[4] The whole metastatic cascade process starts from the invasive migration of the primary tumor, during which cancer cells disseminate into the circulation, followed by extravasation into the

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metastatic niche in distant organs, and subsequently, establishment of metastasis.[5,6] Thus, therapeutic approaches targeting the whole metastatic cascade, from primary tumor invasion, to micro-metastatic growth in the distant organ, are of high scientific interest.[4,7] Therefore, identifying and targeting the contributing factors of metastasis is crucial for designing effective treatment strategies, and improving disease outcome and prognosis for patients suffering from cancer.

Several studies have highlighted the essential role of the immune system in metastasis formation.[8,9] Tumor-infiltrating immune cells were shown to actively contribute to cancer progression.[8] Importantly, evidence indicates that the immune system plays a dual role in carcinogenesis. Depending on the immune response pattern mounted every time, it could facilitate or restrain tumor development.[9–11] Therefore, identifying the role of the implicated immune components, including both immune cells and secreted cytokines, is crucial for developing novel and efficient therapeutic approaches. As a major cellular component of the immune system, T cells have been shown to play a central role in metastasis formation.[12,13] For example, infiltrating cytotoxic CD8+ T cells were found to mediate antitumor immunity within the metastatic site, which could be enhanced by immune checkpoint blockade (ICB) via impediment of exhaustion and promotion of their renewal.[14,15] Regulatory T cells (Treg) are characterized as an immunosuppressive population in this setting, that are known to inhibit cancer cell elimination and induce tolerance.[12] Taken together, this evidence suggests that T cells present in metastatic organs play an important role in tumor progression and suppression.

IL-22 is a cytokine produced by immune cells, e.g. IL-22 +IL17- T cells (Th22), IL22+ IL-17+ T cells (Th17), innate lymphoid cells type 3 (ILC3), and natural killer T cells (NKT).[16–19] IL-22 acts on nonimmune cells, such as mucosal and skin epithelial cells, hepatocytes, endothelial cells, etc.[17,20] Together with its receptor IL22RA1, IL-22 plays a dual role in primary cancer development.[21] On the one hand, IL-22 is involved in tissue integrity maintenance via promoting epithelial proliferation and tissue regeneration.[22] On the other hand, uncontrolled IL-22 functions as a tumor-promoting cytokine in various cancers.[23] Recently, the role of IL-22 in metastasis has been studied in great detail. We have previously reported that tissue resident iNKT17-cell-derived IL-22 promoted cancer cell extravasation at early metastatic stages in colon cancer-derived liver metastasis.[19] On the other hand, γδ-T cells were the key source of IL-22 during cancer cell extravasation in the lung.[19] Furthermore, Kobold and colleagues found that constitutional and T cell-specific deletion of *Il22* reduced metastases in murine models of lung and breast cancer. Furthermore, they described that Th22 cells promote lung metastasis by hindering the anti-tumor immune response in lung metastasis.[24] However, whether IL-22 contributes to metastasis formation in colon cancer-derived liver metastasis, specifically during the establishment of metastasis has not yet been revealed. Also, the source and potential mechanisms of IL-22 during late metastatic stages in the liver remain unclear. Of note, such a time- and organ-dependent distinction of IL-22 function is essential to allow an accurate treatment choice based on the disease stage during metastasis formation.[4,25]

Here, we showed that IL-22 was upregulated in established human and mouse colon cancer-derived liver metastasis. Of note, IL-22 in established metastatic sites was prominently produced by Th22 cells. Furthermore, *Il22* deletion in T cells, but not in IL-17A positive cells, led to decreased liver and lung metastasis in mouse models. Importantly, neutralizing IL-22 in the establishment of metastasis formation resulted in a decreased metastatic burden. Mechanistically, we found that IL-22 promotes angiogenesis. Thus, IL-22 might serve as an immune therapy in cancer patients, to not only prevent but also to treat liver metastasis formation.

Materials and methods

Cancer cells

Colon adenocarcinoma cells (MC38) were cultured in DMEM containing 10% FBS, 100 mg/ml streptomycin, and 100 U/ml penicillin. Cells were maintained in the incubator at 37°C in 5% CO_2-95% air.

Animals

C57BL/6J, IL-22^{sgBFP} x IL-17A^{Katushka} x Foxp3^{mRFP}, Il22$^{flox/flox}$; CD4^{Cre+},Il22$^{flox/flox}$;Il17a^{Cre+} and *Il22ra1$^{flox/flox}$;Cdh5^{Cre+}* mice were kept at the University Hamburg Medical Center Eppendorf.[26–28] *IL-22^{sgBFP} x IL-17A^{Katushka} x Foxp3^{mRFP}* mice were kindly provided by Dr. Richard Flavell. *Il22$^{flox/flox}$* mice were kindly provided by Genentech and Lexicon. Mice were bred and housed in a 12-h light-dark cycle in the animal facility of the University Medical Center Hamburg Eppendorf under specific pathogen-free conditions. Age- and sex-matched 8–12-week-old littermates were used for forced metastasis formation as described below.

Isolation of immune cells from human liver and murine liver and lung

Fresh human liver samples, including peri-metastatic liver and liver metastasis, were obtained from the Surgical Department of the University Hamburg Medical Center Eppendorf, after surgeries of patients diagnosed with CRC and resectable liver metastasis. Murine liver and lung tissues were harvested 21 days after liver or lung metastasis induction. All samples were processed immediately after collection for immune cell isolation. To isolate the immune cells, samples were cut into small pieces after scaling. Subsequently, tissues were incubated at 37°C on a shaker in RPMI 1640 medium containing Collagenase (1 mg/ml), DNase I (10 U/ml), and HBSS (with Ca^{2+} and Mg^{2+}) for 25 min. Then, tissues were smashed using a metal strainer and immune cells were subsequently enriched by Percoll gradient centrifugation at a concentration of 67% and 40%. White cells in the interphase were collected and washed in 1% FBS-PBS, ready for antibody staining as mentioned below.

Isolation of liver sinusoidal endothelial cells (LSECs)

The *C57BL/6J* mice were euthanized and 5 mL RPMI 1640 medium containing 0.05% collagenase was injected into the

portal vein and vena cava before dissection. The liver was then digested using Collagenase and DNase I as mentioned above. Then, the liver was smashed into singe cell solution through a 200-µm cell strainer and centrifuged at 40 × g for 4 min to get rid of hepatocytes. The supernatant was then collected. After repeating 2–3 times, the supernatant was centrifuged at 400 × g for 20 mins and the pellet was processed using an Optiprep (Sigma, Kawasaki, Japan) gradient to enrich LSECs. At the end, MACS sorting using anti-CD146 was used to isolate LSECs. Isolated LSECs were subsequently cultured on collagen-coated plates using IMDM medium (10% FBS) for 5 days before IL-22 (PeproTech) stimulation (50 ng/ml). 2 days after IL-22 stimulation, LSECs were harvested for RNA isolation and qPCR.

Flow cytometry and cell sorting

Immune cells from human and mouse tissues were collected as described above. Fc-block was used to block Fc-γ receptors, followed by extracellular staining for 15 min at 4°C. Flow cytometry data were acquired with the BD LSRFortessa and analysis was performed using the FlowJo software (FlowJo, Ashland, OR). Cell sorting was carried out using FACSAria (BD Biosciences, San Jose, CA).

Forced liver metastasis induction

To induce liver metastasis in mice, MC38 cells were harvested and counted. Subsequently, 3×10^5 MC38 cells diluted in 250 ul PBS per mouse were injected intrasplenically (i.s.). To perform the intrasplenic injection, a small incision in the left upper abdomen of the mice was made under anesthesia to expose the spleen. Then, the spleen was divided into two parts via ligation, MC38 cells were injected into one half of the spleen, and a splenectomy was performed 3 min later. The mice were closely monitored and sacrificed after 3 weeks. Livers were harvested for metastatic burden assessment, including liver weight and number of macroscopic metastases.

For IL-22 neutralization, each mouse received 50 ug of an IL-22 blocking antibody (Genentech) intraperitoneally twice from day 14–21 post liver metastasis induction. Metastatic burden was monitored and mice condition was scored accordingly every day. Mice were sacrificed on day 21 after metastasis induction.

Forced lung metastasis induction

To induce lung metastasis in mice, MC38 cells were harvested as mentioned above. Each mouse received 2.5×10^5 MC38 cells in 100 ul PBS intravenously (i.v.). All mice were sacrificed after 3 weeks and the lungs were harvested for metastatic burden assessment.

RNA extraction and qPCR

Total RNA from lung and liver tissues, as well as from sorted cells, was extracted using Trizol (Invitrogen) according to the manufacturer's instructions. The high-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) was used for cDNA synthesis after RNA concentration measurement. qPCR was then performed on the StepOne Plus system (Applied Biosystems) using primer sets acquired from Taqman. For result analysis, both human and mouse readouts (ΔCt) were normalized to house-keeping gene.

Immunofluorescence

Sections (5 µm) were washed with PBS and incubated in PBS-Triton 0.3% for 5 min. After washing, sections or cells were incubated for 60 min in blocking buffer. Samples were stained overnight with specific antibodies at 4°C. After washing, secondary antibody staining was performed (1 h, RT) followed by 5 min staining with Hoechst 33,258 (1:5000). As control, the primary Ab was omitted. Bright-field and fluorescent microscopy was carried out using either an Axio Vert.A1 (Zeiss, Jena, Germany) inverted microscope or an SP5 (Leica, Heidelberg, Germany) confocal microscope.

TUNEL staining

Assessment of apoptosis was performed with *in situ* Cell death Detection Kit (Merck, Germany) according to the manufacturer' s instruction.

Statistics

All statistical analysis was performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). All data obtained from each experiment are presented as the mean ± SEM of more than three independent experiments. The differences between two groups were assessed using Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Study approval

All animal experiments were performed in accordance with the Institutional Review Board "Behörde für Soziales, Familie, Gesundheit und Verbraucherschutz" (Hamburg, Germany). Human samples were obtained under the approval codes PV-3578 and PV-3548, which were approved by the local ethical committee "Ethik-Kommission der Ärztekammer Hamburg". Written informed consent was received from all participants prior to inclusion in this study.

Results

IL-22 levels are increased in human liver metastasis

To explore the role of IL-22 in human liver metastasis, we analyzed the mRNA expression of *IL22* and *IL22RA1*, the specific IL-22 receptor, in healthy liver tissue controls (obtained from patients with suspicion of nonalcoholic fatty liver disease, who showed normal histological findings) and liver metastasis from colorectal cancer (CRC) patients.[21,29] We found increased levels of *IL22* in established liver metastasis compared to healthy tissue controls (Figure 1a,b, and Figure S1A, B), while the levels of *IL22RA1* were similar between the groups (Figure 1b). We confirmed that IL-22 is increased at protein level using flow cytometry. Specifically, samples of

human peri-metastatic liver, liver metastasis from patients with primary CRC, and newly diagnosed liver metastasis (M1, TNM staging) were analyzed (Figure 1c,d). To characterize the liver metastatic inflammatory environment, we went on to phenotypically analyze the IL-22-producing cells. We showed that CD45+CD3+CD4+IL-17A-IL-22+ T cells, from now on referred to as 'Th22' cells, are the major source of IL-22 in established liver metastasis in humans (Figure 1e,f).

Taken together, IL-22 is increased in human liver metastatic sites and is mainly produced by Th22 cells.

Th22 cells are the main source of IL-22 in established liver metastasis in mice

We next aimed to assess the cellular source of IL-22 in established metastasis using a forced liver metastasis induction mouse model. Additionally, we also analyzed lung metastasis. In line with our observations in patients (Figure 1), *Il22* mRNA levels were significantly higher in the mouse metastatic liver and lung, compared to the respective normal tissue (Figures 2a, b,3a,b, and Figure S2). We measured *Il22* expression in different cell populations isolated from the liver and found that CD4 T cells express high *Il22* levels (Figure 2c). To identify the cellular source of IL-22, we used an IL-22 reporter mouse model (*IL-22*^{sgBFP} *x IL-17A*^{Katushka} *x Foxp3*^{mRFP}). Using this IL-22 reporter mouse, we demonstrated that CD3+CD4+ T cells were a main source of IL-22 in already established liver and lung macro-metastasis (Figures 2d,e and 3c,d, S2, and S3). We confirmed this finding using qPCR of sorted CD4+ and CD8+ T cells (Figure 2f). Further flow cytometric analysis of liver and lung metastatic sites indicated that CD4 +IL-17A-IL-22+, Th22 cells, were the main source of IL-22 (Figures 2g,h and 3e,f).

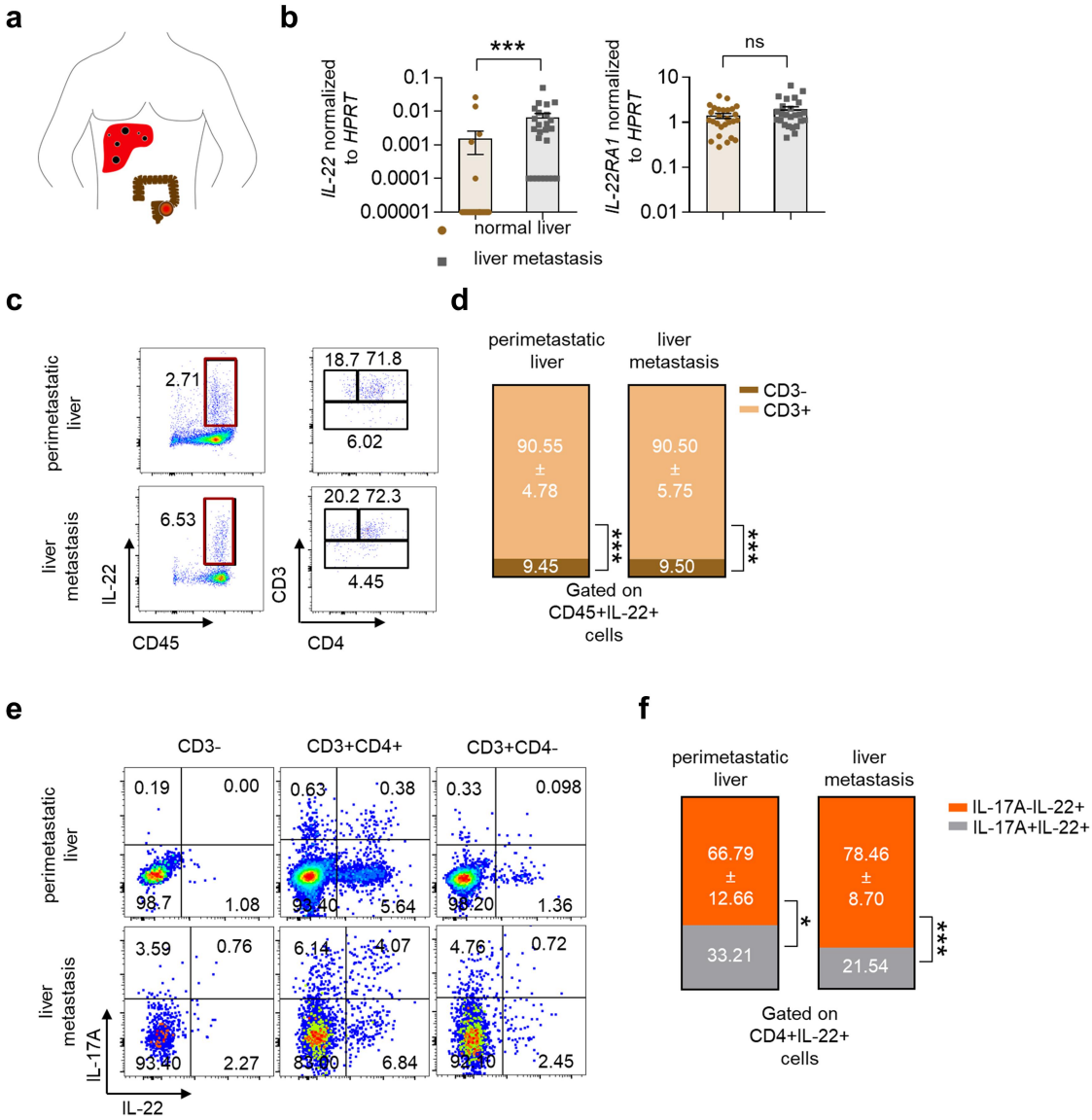

Figure 1. Increased IL-22 levels in human liver metastasis. (a) Schematic overview of human liver metastasis. (b) *IL22* and *IL22RA1* mRNA levels in total liver were measured by qPCR. $n \geq 25$ patients per group. (c) Flow cytometry of peri-metastatic and metastatic human liver. (d) Diagram showing the proportion of CD45+IL-22+ cells in peri-metastatic and metastatic human liver. (e) Cells were isolated from fresh peri-metastatic and metastatic liver tissue and analyzed by flow cytometry. (f) Diagram showing the proportion of CD4+IL-22+ cells in peri-metastatic and metastatic human liver. $n = 7$-10 patients per group. See also Figure S1. Data presented as mean ± SEM. ns > 0.05; ***:$p \leq 0.001$ as assessed by the Mann-Whitney U test.

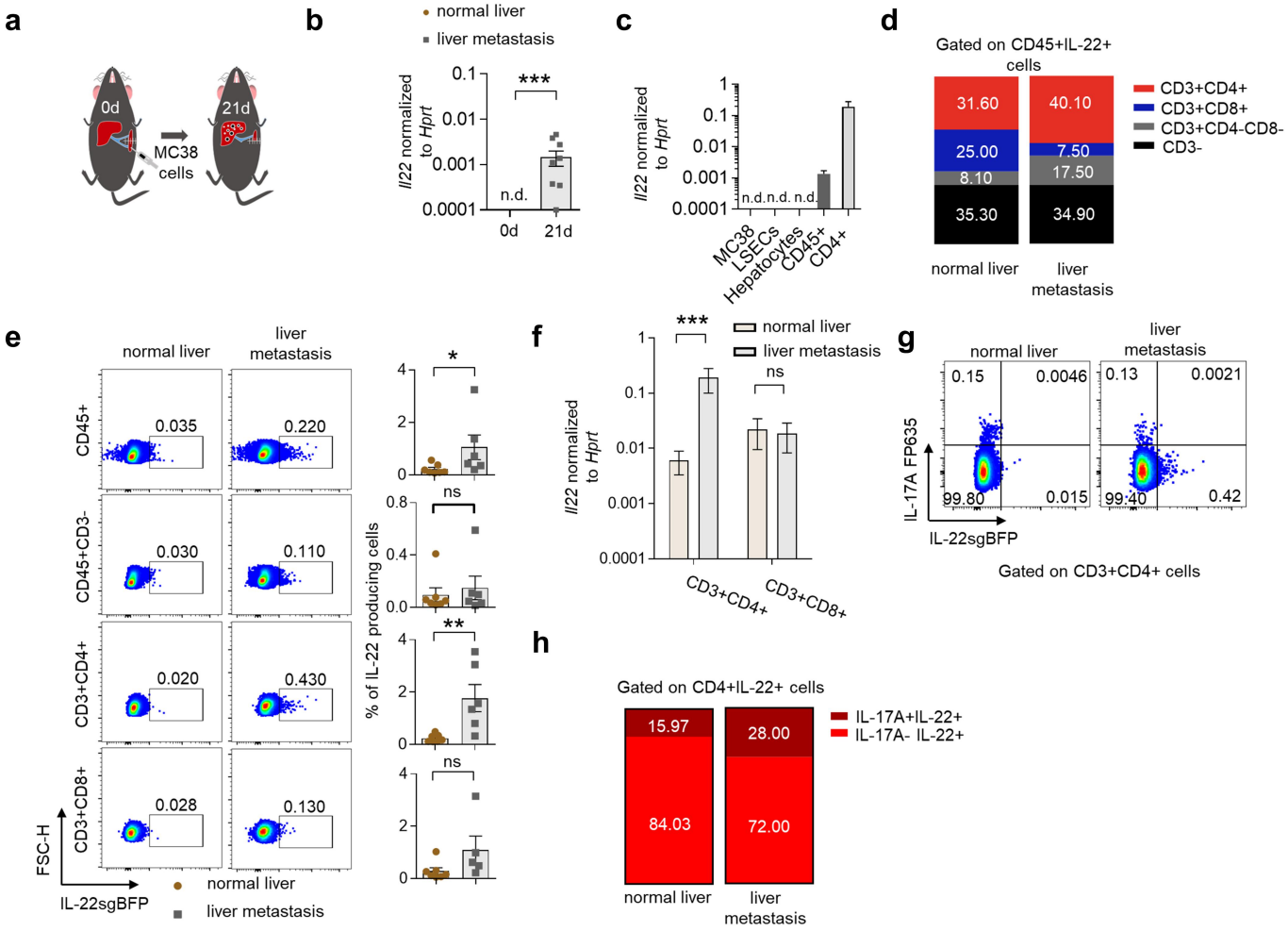

Figure 2. Th22 cells are the main source of IL-22 in murine-established liver metastasis. (a) Schematic overview of intrasplenic injection of MC38 cells for forced liver metastasis induction. (b) *Il22* mRNA levels in total liver with or without metastasis were measured by qPCR. $n \geq 8$ mice per group. n.d, not detected. (c) *Il22* mRNA levels in different cell populations. $n \geq 4$ mice per group. n.d, not detectable. (d) Diagram showing the proportion of CD45+IL-22+ cells in healthy and metastatic murine liver. (e) Flow cytometry of isolated lymphocytes of healthy and metastatic murine liver 21 days post intrasplenic injection of MC38 cancer cells. (f) *Il22* RNA levels of sorted CD3+CD4+ and CD3+CD8+ cells of normal and metastatic murine liver 21 days post-intrasplenic injection of MC38 cancer cells. (g) Cells were isolated from fresh healthy and metastatic liver tissue and analyzed by flow cytometry. (H) Diagram showing the proportion of CD4+IL-22+ cells in healthy and metastatic murine liver 21 days post-intrasplenic injection of MC38 cancer cells. See also Figure S2. Data presented as mean ± SEM. ns > 0.05; *:$p < 0.05$; **:$p \leq 0.01$ as assessed by one-way ANOVA with Bonferroni post hoc tests or Mann-Whitney U test.

Innate lymphoid cells (ILCs) are another potential source of IL-22. Therefore, we also analyzed ILCs in the non-metastatic liver and liver metastasis by flow cytometry (Figure 4a). The majority of IL-22-producing cells in liver metastasis are not ILCs (Figure 4b). We further analyzed whether ILCs-derived IL-22 increased in liver metastasis compared to the non-metastatic liver (Figure 4c). No significant IL-22 upregulation in CD45+Lin- (ILCs) was detectable in the metastatic liver (Figure 4d). However, more IL-22-producing CD45+Lin+ cells were observed in liver metastasis (Figure 4d). Similarly, we examined IL-22 in ILCs in murine lung metastasis (Figure 5a). ILCs were still not the major IL-22-producing immune cell population in this setting (Figure 5b). In the metastatic lung, IL-22 was increased in both ILCs and non-ILCs compared to the non-metastatic lung (Figure 5c,d).

Taken together, Th22 is a predominant source of IL-22 in established liver metastasis in mice.

T-cell-specific deletion of IL-22 protects against liver metastasis

It was confirmed by us that in a forced liver metastasis model, IL-22-deficient innate cells did not alter hepatic metastatic burden without the presence of T cell and B cells compared to controls[19]. To further investigate the functional role of T cell-derived IL-22, we used mice carrying the conditional *Il22flox/flox* allele combined with specific Cre recombinase drivers for T cells (*CD4Cre*), and as control, IL-17A-producing cells (*Il17aCre*). Upon intrasplenic injection of MC38 cancer cells, the $Il22^{flox/flox}$;$CD4^{Cre+}$ mice lacking IL-22 production in CD4+ and CD8+ T lymphocytes, showed reduced liver and lung metastatic sites (Figures 6a,b & 6d,e) compared to littermate controls. Based on our results indicating that IL-17A- CD4+ T cells are the main source of IL-22 in established metastasis, $Il22^{flox/flox}$;$Il17a^{Cre+}$ mice showed comparable liver and lung metastasis burden to littermate controls (Figure 6c,f).

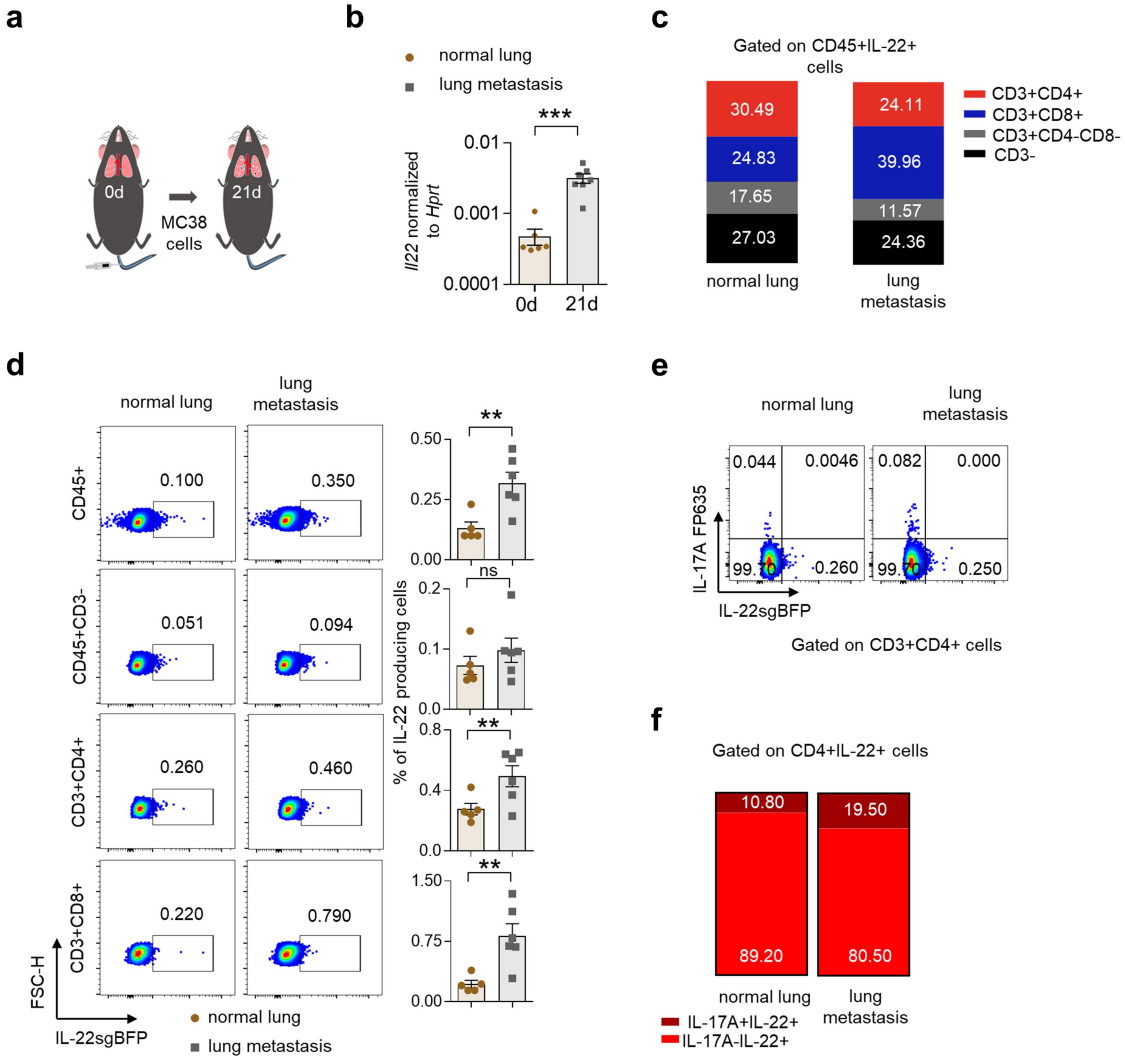

Figure 3. Th22 cells are the main source of IL-22 in established murine lung metastasis. (a) Schematic overview of MC38 cell intravenous injection for forced lung metastasis induction. (b) *Il22* mRNA levels in total lung with or without metastasis were measured by qPCR. $n \geq 6$ mice per group. (c) Diagram showing the proportion of CD45+IL-22+ cells in healthy and metastatic mouse lungs. (d) Flow cytometry of isolated lymphocytes of healthy and metastatic mouse lung 21 days post-intravenous injection of MC38 cancer cells. (e) Cells were isolated from fresh healthy and metastatic lung tissue and analyzed by flow cytometry. See also Figure S2. (f) Diagram showing the proportion of CD4+IL-22+ cells in healthy and metastatic murine lung 21 days post MC38 cancer cell intravenous injection. Data presented as mean ± SEM. ns > 0.05; *:$p < 0.05$; **:$p \leq 0.01$; ***:$p \leq 0.001$ as assessed by Mann-Whitney U test.

Taken together, Th22 cells are the main source of IL-22 in established liver and lung metastasis. Specifically, deletion of T-cell-derived IL-22, but not Th17 cell-specific deletion of IL-22, protects against metastasis formation.

IL-22 promotes angiogenesis in established liver metastasis

We previously found that IL-22 promotes cancer cell extravasation, the early step of metastasis. To test if IL-22 contributes to liver metastasis formation after extravasation, we used an IL-22 neutralizing antibody. Indeed, blocking IL-22 at the establishment of metastasis, e.g. from day 14 post-injection of cancer cells, significantly decreased liver metastasis burden (Figure 7a,b). To further decipher the mechanisms underlying the role of IL-22 in established metastasis, we performed immunofluorescence of $Il22^{wt/wt}$;$CD4^{Cre+}$ and $Il22^{flox/flox}$;$CD4^{Cre+}$ mice. We had shown before that the endothelial cells can react to IL-22 stimulation via STAT3 activation.[19] More importantly, ablation of IL-22Ra1 in endothelial cells ($Il22ra1^{flox/flox}$;$Cdh5^{Cre+}$) significantly reduced liver metastasis.[19] We thus focused our analysis on endothelial cells. Indeed, we found that mice lacking IL-22 production in T cells had less angiogenesis (Figure 7c,d). Furthermore, and in line with a previous publication,[30] IL-22 upregulated the expression of angiogenesis-related genes in liver sinus endothelial cells (LSECs) *in vitro* (Figure 7e), namely *Cxcl16*, *Vegfa*, and *Nrp1*. To understand how IL-22 affects angiogenesis specifically during the establishment of metastasis, we examined these genes in established liver metastasis from $Il22ra1^{wt/wt}$;$Cdh5^{Cre+}$ and $Il22ra1^{flox/flox}$;$Cdh5^{Cre+}$ mice. In established liver metastasis, *Cxcl16* and *Vegfa* were downregulated in $Il22ra1^{flox/flox}$;$Cdh5^{Cre+}$ mice compared to littermate controls, while *Nrp1* was comparable between the two groups (Figure 7f). Indeed, $Il22ra1^{flox/flox}$;$Cdh5^{Cre+}$ mice exhibited less angiogenesis, less proliferation but comparable apoptosis in established liver metastasis compared to littermate controls (Figure 7g, Figure S4A, S4B). We next aimed to assess the

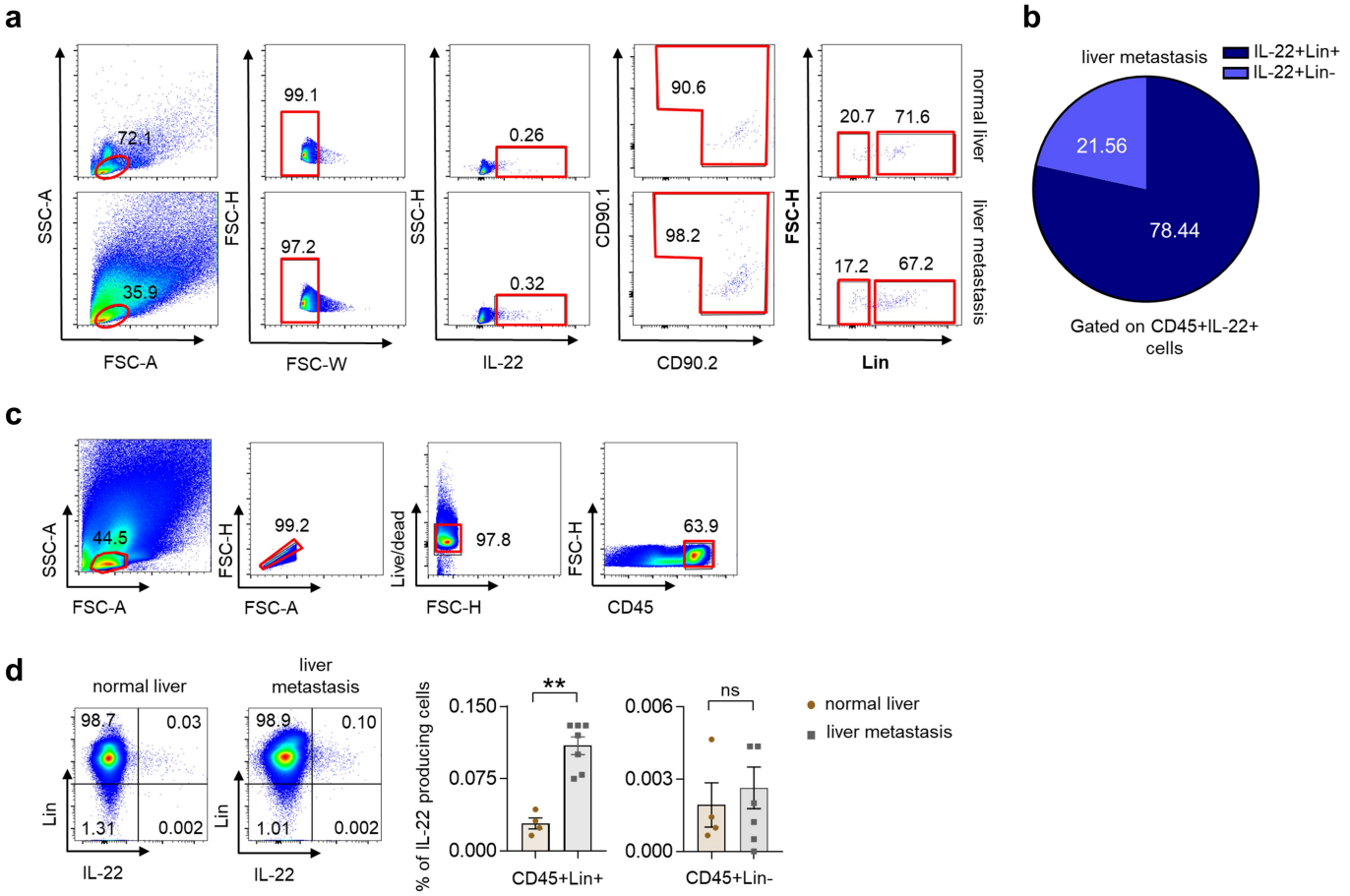

Figure 4. Lin- cells are not the main source of IL-22 in established murine liver metastasis. (a) Gating strategy used to assess the source of CD45+IL-22+ cells isolated from mouse healthy and metastatic liver upon i.s. injection of MC38 cells. (b) Diagram showing the proportion of CD45+IL-22+ cells in healthy and metastatic murine liver. (c) Gating strategy used to identify lymphocytes isolated from mouse healthy and metastatic liver upon i.s. injection of MC38 cells. (d) Flow cytometry of isolated Lin+ and Lin- lymphocytes of healthy and metastatic mouse liver upon i.s. injection of MC38 cells and respective quantification. $n \geq 4$ mice per group. Data presented as mean ± SEM. ns > 0.05; **:$p \leq 0.01$ as assessed by Mann-Whitney U test.

involved signaling pathways downstream of IL-22, namely STAT3 and Erk1/2. We have previously shown that IL-22 can activate STAT3 in murine endothelial cells *in vitro*.[19] Based on this, we assessed the activation of STAT3 and Erk1/2 *in vivo*. We found both to be decreased in *Il22ra1*$^{flox/flox}$;*Cdh5*$^{Cre+}$ mice in liver sinusoid endothelial cells in the context of liver metastasis compared to control (Figure 7h,i). Of note, these data are in line with a previous report indicating that IL-22 promotes angiogenesis via activation of STAT3 and Erk1/2 *in vitro*.[30]

Taken together, our findings indicate that IL-22 promotes angiogenesis in established liver metastasis.

Discussion

Metastasis emerges as the end-product of a multi-step cascade of events, including cancer cell invasion, intravasation, circulation, extravasation, and colonization into the target organs[25]. It is responsible for the majority of cancer-related deaths worldwide. Recent studies indicate that communication among the immune system, the tissue, and cancer cells is key in tumor and metastasis development.[8,31] However, this communication and the mediators affecting the various metastatic steps remain unknown to a large extent. Here, we studied the role of the cytokine IL-22 in the establishment of liver metastasis formation. Overall, we found that IL-22 at this stage is mainly produced by Th22 cells. Moreover, T cell-derived, but not Th17 cell-derived, IL-22 plays a crucial role in metastasis formation by promoting angiogenesis. Finally, blocking IL-22 in mice with established metastasis reduced metastasis burden.

It was shown before that IL-22 is upregulated in the primary tumor, e.g., in colon cancer.[32,33] Within these reports, IL-22 was shown to promote carcinogenesis by acting on the cancer cells themselves. Specifically, IL-22 was reported to promote cancer cell stemness[32] and facilitate tumor growth.[33] Likewise, a recent publication showed an upregulation of IL-22 in established lung metastasis.[24] On the basis of these findings, we aimed to study IL-22 in established liver metastasis in mouse models and human tissue. Indeed, we found that IL-22 is upregulated in liver metastasis compared to healthy liver tissue, both in mouse models and human samples.

To identify the cellular source of IL-22 in the pro-metastatic tissue environment, we performed flow cytometry. Notably, several immune cells can produce IL-22, for example, Th22, Th17, ILC3, and NKT cells.[19,34,35] We previously showed that NKT cells and γδ T cells are the key sources during the early

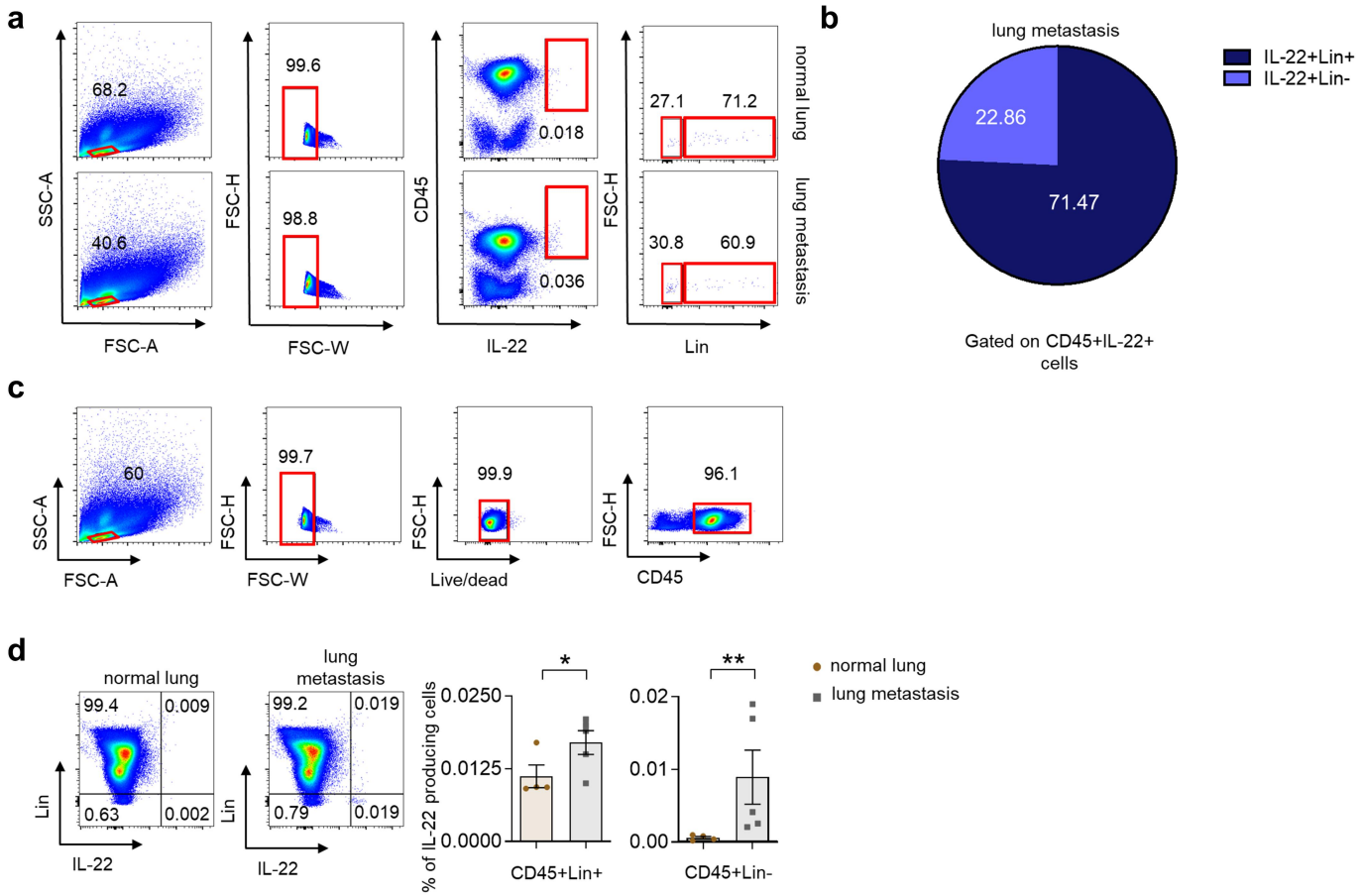

Figure 5. Lin- cells are not the main source of IL-22 in established murine lung metastasis. (a) Gating strategy used to assess the source of CD45+IL-22+ cells isolated from mouse healthy and metastatic lungs upon i.v. injection of MC38 cells. (b) Diagram showing the proportion of CD45+IL-22+ cells in healthy and metastatic murine lung. (c) Gating strategy used to identify lymphocytes isolated from mouse healthy and metastatic lung upon i.v. injection of MC38 cells. (d) Flow cytometry of isolated Lin+ and Lin- lymphocytes of healthy and metastatic mouse lung upon i.v. injection of MC38 cells and respective quantification. $n \geq 4$ mice per group. Data presented as mean ± SEM. ns > 0.05; **:$p \leq 0.01$ as assessed by Mann-Whitney U test.

step of metastasis, namely extravasation, in liver and lung metastasis, respectively.[19] Interestingly, in established lung metastasis, Th22 cells were identified as the key source of IL-22 in the lung.[24] However, the source of IL-22 in established liver metastasis was unknown. We identified CD4+ T cells to be the major source of IL-22 in human and murine-established liver metastases. We focused here on liver metastasis. However, it is worth mentioning that the source of IL-22 seems to be different in liver and lung metastasis, e.g. CD8+ T cell-derived IL-22 was elevated significantly in lung but not liver metastasis. To clarify whether CD4+ T cell-derived IL-22 is sufficient for promoting lung metastasis, we engrafted *Rag-/-;Il-22-/-* mice with IL-22+/+ and IL-22-/-CD4+ T cells, followed by lung metastasis induction.[24] We found that the engrafted *Rag-/-;Il-22-/-* mice with IL-22+/+CD4+ T cells showed increased lung metastases compared to mice engrafted with IL-22-/-CD4+ T cells[24]. Thus, our findings demonstrate that CD4+ T cell-derived IL-22 can promote liver and lung metastasis formation. However, a potential role of other cellular sources, e.g innate cells and CD8+ T cells, in lung metastasis formation can not be excluded and will be studied in future experiments. Within CD4+ T cells, Th22, but not Th17 cells, were the main source of IL-22. Moreover, the frequency of IL-22+ ILCs, another potential major IL-22-producing cell subtype, was not altered in the liver metastatic sites. In spite of an increase of IL-22+ ILCs in lung metastasis, ILCs were not the major source of IL-22 on site. This finding indicates that the stage of the metastatic cascade, as well as the organ, may impact the source of IL-22. Throughout the study, we used flow cytometry on dissociated cells to quantify IL-22 producing cells. This is appropriate to quantify expression, but does not allow us to assess the location of IL-22-expressing cells. We therefore aim in future studies to address this important point.

Next, we aimed to study the function of T cell-derived IL-22 in metastasis formation. To this end, we used mouse models with cell-specific IL-22 deletion. We found that *Il22* deficiency in T cells, but not in Th17 cells, resulted in reduced metastatic burden in the liver. Of note, metastasis is a process involving multiple steps. As we have previously demonstrated, NKT cell-derived IL-22 can promote cancer cell extravasation via ANPEP upregulation within endothelial cells in the liver.[19] Together with the presented data here, it seems that the source of IL-22 varies during the different steps of metastasis formation. This might be attributed to the fast immune response mounted by liver NKT cells during cancer cell extravasation, and a relatively late infiltration of Th22 cells in the metastatic niche. However, IL-22 maintains its pro-metastatic function during all these steps. Thus, to better decipher the role of IL-22

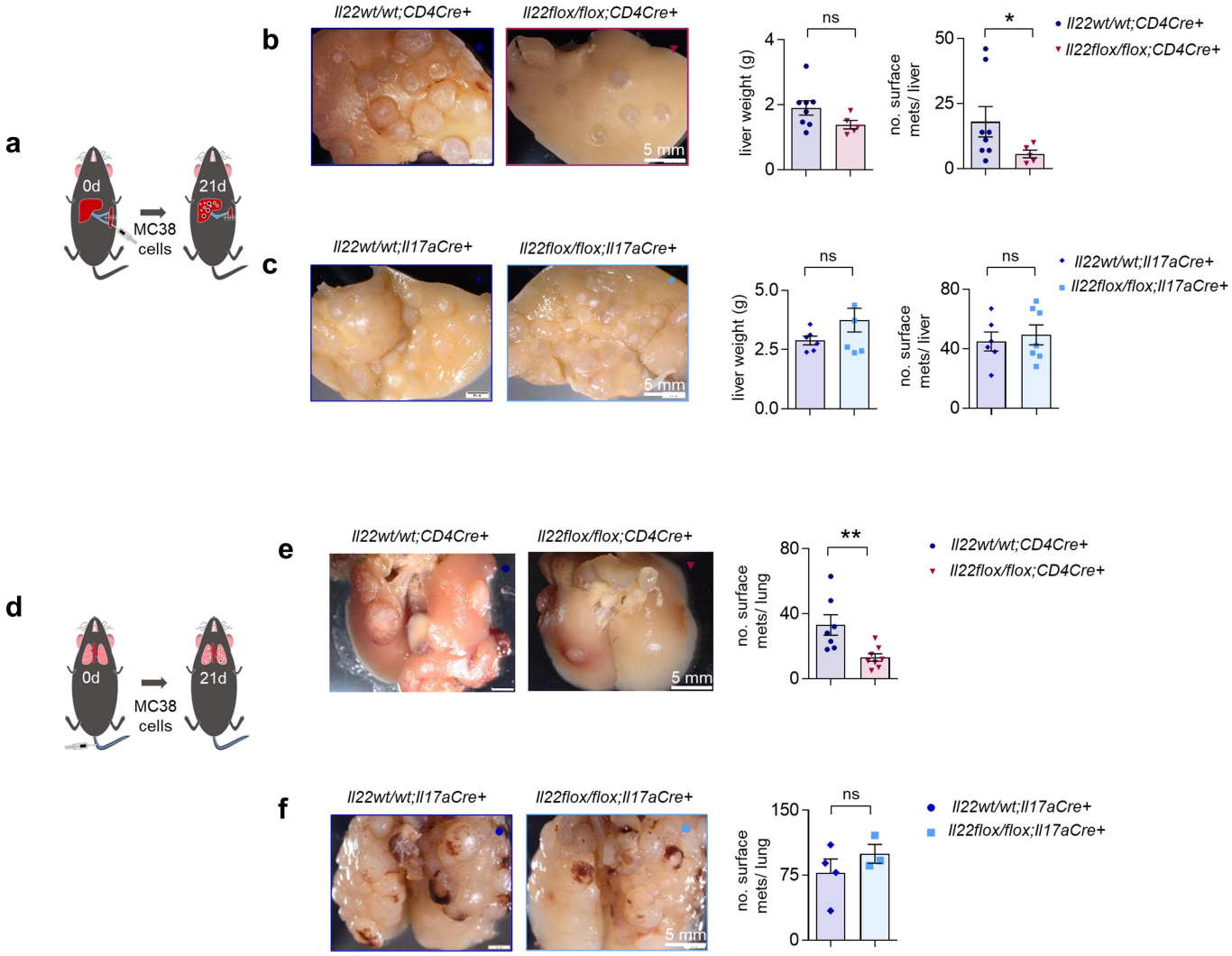

Figure 6. T cell- but not Th17-derived IL-22 plays a key role in liver and lung metastasis formation. (a) Schematic overview of i.s. injection of MC38 cells. (b) Liver weight and number of macroscopic liver metastases post intrasplenic injection of MC38 cells in $Il22^{wt/wt}$;$CD4^{Cre+}$ and $Il22^{flox/flox}$;$CD4^{Cre+}$ mice. $n \geq 9$ mice per group. (c) Liver weight and number of macroscopic liver metastases post intrasplenic injection of MC38 cells in $Il22^{wt/wt}$;$Il17a^{Cre+}$ and $Il22^{flox/flox}$;$I17a^{Cre+}$ mice. $n \geq 9$ mice per group. (d) Schematic overview of i.v. injection of MC38 cells. (e) Number of macroscopic lung metastases post intravenous injection of MC38 cells in $Il22^{wt/wt}$;$CD4^{Cre+}$ and $Il22^{flox/flox}$;$CD4^{Cre+}$ mice. $n \geq 8$ mice per group. (f) Number of macroscopic lung metastases post intravenous injection of MC38 cells in $Il22^{wt/wt}$;$Il17a^{Cre+}$ and $Il22^{flox/flox}$;$I17a^{Cre+}$ mice. $n \geq 3$ mice per group. Data presented as mean ± SEM. ns > 0.05; **:$p \leq 0.01$ as assessed by Mann-Whitney U test.

selectively in the establishment of metastasis, we used an IL-22-neutralizing antibody. Specifically, blocking IL-22 in the establishment of metastasis significantly reduced the liver metastatic burden. Collectively, the major cellular source of IL-22 during metastasis formation in the liver appears to shift from tissue-resident NKT cells to Th22 cells. However, in both phases, IL-22 had pro-metastatic functions.

The target cells of IL-22 consist of mostly nonimmune cells, such as hepatocytes, endothelial cells and cancer cells[18]. Of note, we have demonstrated that impaired IL-22 signaling in cancer cells did not alter liver metastatic burden in Wt mice. Furthermore, deletion of *Il-22ra1* resulted in less liver metastasis. Thus, IL-22 can in principle act on tumor cells. However, in our model system the effect of IL-22 appears to be mediated via non-tumor cells.[19] As metastasis formation is a complex cascade from primary tumor cell invasion to distant organ metastatic colonization, defining the exact cells and events that were affected by IL-22 during all steps of the metastatic cascade is essential.[36,37] We previously, reported that IL-22 at early stages promotes cancer cell extravasation in the liver and lung by acting on endothelial cells.[19] Furthermore, it was shown in murine models of lung and breast cancer, that T cell-derived IL-22 drives the expression of CD155 by cancer cells to suppress NK cell function and promote metastasis.[24] However, it was unknown how IL-22 may impact established liver metastasis. IL-22 was also reported to promote angiogenesis in murine primary tumors, induced by subcutaneous injection of EL4 (lymphoblast) and GL261 (glioma) cell lines[30]. Additionally, we found that the deletion of IL-22Ra1 on endothelial cells led to reduced liver metastasis[19]. We next aimed to examine whether IL-22 signaling in endothelial cells not only affected extravasation, but also the establishment of metastasis. Therefore, we measured angiogenesis-related genes, namely *Cxcl16*, *Vegfa* and *Nrp1*, in LSECs after IL-22 stimulation, and tested the role of IL-22 in angiogenesis. We found that *Cxcl16*, *Vegfa* and *Nrp1* were all increased in LESCs upon IL-22 stimulation. In line with this, we found that angiogenesis was decreased in mice with *Il22*-deficiency in T cells in the context

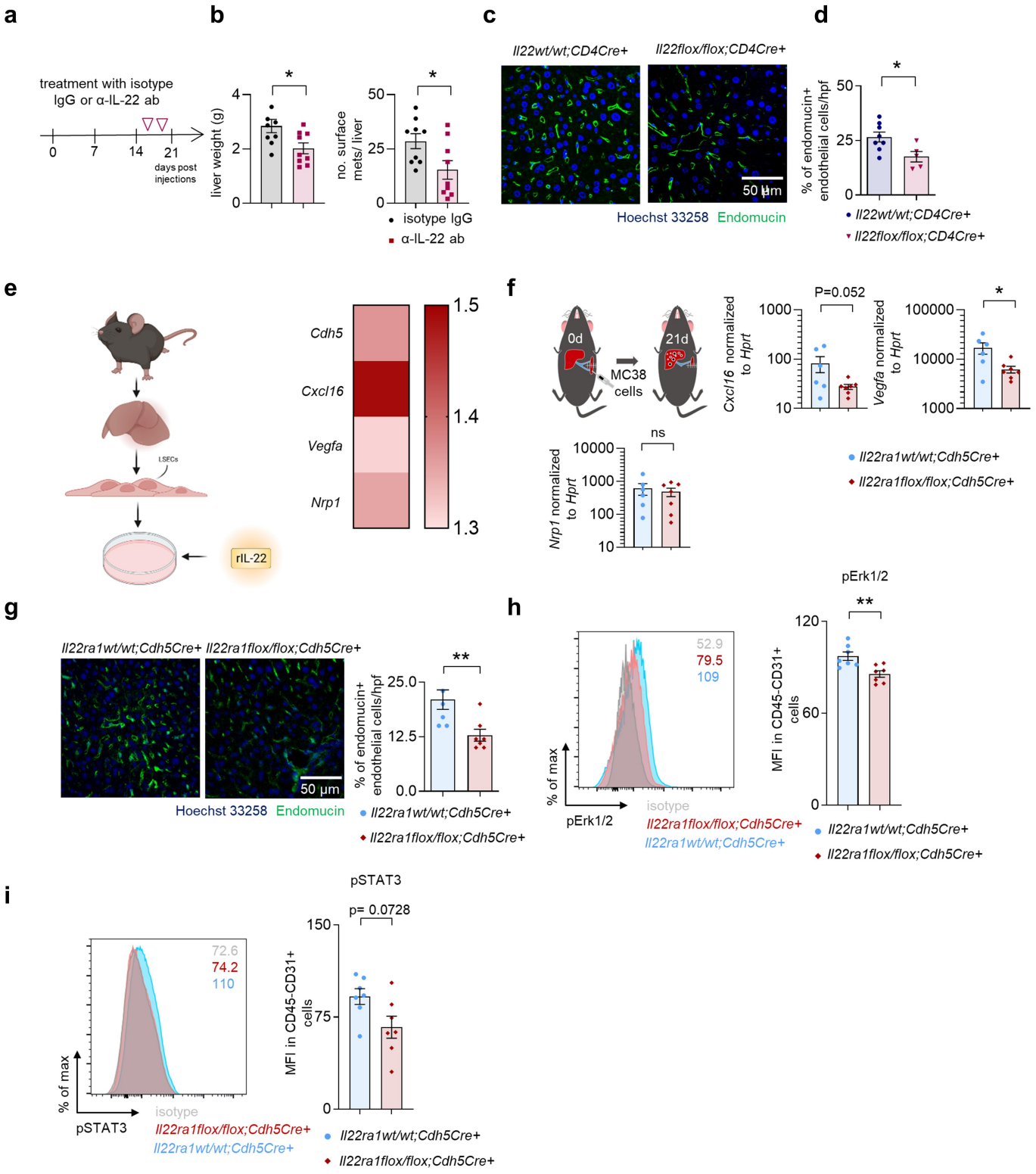

Figure 7. IL-22 promotes angiogenesis in established liver metastasis. (a) Schematic overview of i.s. injection of MC38 cells for forced liver metastasis induction in mice receiving an α-IL-22 or IgG control antibody. (b) Liver weight and number of macroscopic liver metastases post intrasplenic injection of MC38 cells in wildtype mice receiving IL-22 blocking antibody. One group was administrated IL-22 blocking antibody and the other IgG antibody from day 14 post injection. $n \geq 9$ mice per group (c) Immunofluorescence of endomucin+ endothelial cells from established liver metastasis in $Il22^{wt/wt};CD4^{Cre+}$ and $Il22^{flox/flox};CD4^{Cre+}$ mice. (d) Percentage of vessels 21 days post i.s. injection in $Il22^{wt/wt};CD4^{Cre+}$ and $Il22^{flox/flox};CD4^{Cre+}$. $n \geq 6$ mice per group. (e) Schematic overview of LSECs isolation and culture with rIL-22 (50 ng/ml). Angiogenesis-related gene expression was analyzed. (f) Schematic overview of i.s. injection of MC38 cells for forced liver metastasis induction in mice. mRNA levels of angiogenesis-related genes in total liver were analyzed between $Il22ra1^{wt/wt};Cdh5^{Cre+}$ and $Il22ra1^{flox/flox};Cdh5^{Cre+}$ mice. $n \geq 6$ mice per group. (g) Immunofluorescence of endomucin+ endothelial cells from established liver metastasis in $Il22ra1^{wt/wt};Cdh5^{Cre+}$ and $Il22ra1^{flox/flox};Cdh5^{Cre+}$ mice. $n \geq 5$ mice per group. (H, I) Flow cytometry of p-STAT3 and p-Erk1/2 from liver endothelial cells (CD45-CD31+) in liver metastasis of $Il22ra1^{wt/wt};Cdh5^{Cre+}$ and $Il22ra1^{flox/flox};Cdh5^{Cre+}$ mice. $n \geq 7$ mice per group. Data presented as mean ± SEM. ns > 0.05; **:$p \leq 0.01$ as assessed by Mann-Whitney U test.

of liver metastasis. We previously reported that the number of extravasated cancer cells is reduced in mice with *Il22*-deficiency in tissue-resident NKT cells[19]. However, T-cell-specific deletion of IL-22 did not impact cancer cell extravasation, which is in line with the observation that T cells are not the major source of IL-22 at this stage of metastasis. Further strengthening these data, we observed less angiogenesis in murine metastatic sites with impaired IL-22 signaling in endothelial cells. Interestingly, angiogenesis-related genes were regulated by IL-22 in a time-dependent manner during metastasis formation. Specifically, IL-22 induced the upregulation of *Nrp1* during early metastatic stages,[19] while *Cxcl16* and *Vegfa* were not affected. In contrast, *Cxcl16* and *Vegfa* were increased in established liver metastasis, rather than *Nrp1*. Previous studies reported that IL-22 promotes angiogenesis via the activation of Erk1/2 and STAT3 *in vitro*.[30] We found reduced phosphorylation of Erk1/2 and STAT3 in endothelial cells with IL-22RA1 deficiency from established liver metastasis. *In vitro*, we have previously demonstrated the phosphorylation of STAT3 in LSECs.[19] This shows that endothelial cells are not only the target cells during cancer cell extravasation, but also play a critical role in angiogenesis during established metastatic sites.

In conclusion, we identified IL-22 as a critical cytokine mediating the pathogenesis of metastasis formation and identified Th22 cells as its major cellular source in established metastatic sites in the liver. We would like to point out that the IL-22 pro-metastatic effect can in fact be targeted therapeutically via a neutralizing antibody in the establishment of metastasis formation *in vivo*. Antibody-mediated IL-22 neutralization could indeed restrict metastasis formation in the target organ. Based on these data, we suggest IL-22 as a potential candidate for future immuno-therapeutic interventions for cancer patients with established distant metastasis.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

T.Z., R.W. conceived, designed and carried out most experiments, analyzed data, and wrote the paper; D.E.Z., JÖ.L., A.M.S., J.K., L.Z., T.A., A. P.M., I.B., R.J., J.T., P.S., SW.Z. carried out *in vivo* experiments, immunofluorescence and flow cytometry assays; O.G. carried out statistical analysis and provided critical intellectual input and edited the paper H.S., J.K. G., B.M., L.S., M.F.A., P.S., J.W., M.K., B.B., A.D., K.B., P.B., R.G., T.G., M. T., N.M:, E.G.A., F.N, T.H, O.M., J.R.I., J.L. provided the human liver metastasis specimens, provided critical intellectual input and edited the paper; P.M.L., A.P., E.G., P.S., N.L., M.V.G, G.E.G, I.C.M, P.C.A, R.N., V. G.P and A.W.T. provided critical intellectual input and edited the paper; N.G. designed experiments, provided critical intellectual input and edited the paper; S.H. and A.D.G conceived the idea and supervised the study, designed and carried out experiments, analyzed the data, and wrote the paper. All authors reviewed and concur with the submitted manuscript.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article and its supplementary materials.

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