EMBO reports

T cell-dependent bispecific antibodies alter organ-specific endothelial cell - T cell interaction

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1st Editorial Decision **18th Jul 2022**

Dear Dr. Ye,

Thank you for the transfer of your research manuscript to EMBO reports. I have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of high interest. However, all referees have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all their points need to be addressed, I will not detail them here.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in a detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

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The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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4) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this in a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

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Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

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13) Please order the manuscript sections like this using these names:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - DAS (data availability section) - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

14) I would suggest uploading the lists/tables in the methods section as 'Reagents and Tools table'. I have attached templates

for that in word or excel format. Please upload the filled in table to the manuscript tracking system as a 'Reagent Table' file. The example below shows how the table will display in the published article and includes examples of the type of information that should be provided for the different categories of reagents and tools. Please list your reagents/tools using the categories provided in the template and do not add additional subheadings to the table. Reagents/tools that do not fit in any of the specific categories can be listed under "Other":
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I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

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Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

This is an elegant study on how endothelial cells in liver versus tumors respond differentially to T cell dependent bispecific antibodies (TDBs) and contribute to differences in T cell trafficking to these tissues. Their findings offer a potential mechanism of toxicity from T cell-engagers. The manuscript will be strengthened by addressing the following comments and concerns:

Specific Comments:

- The tumor implantation procedure is associated with strong local inflammation compared to other methods. This may attract more immunosuppressive cells and prevent antitumor immune cells infiltration. Would injecting cells in the mammary fat pad (MFP) elicit similar response?

- Most studies on differences between liver and tumor are based on a single preclinical model. Would this hold in other solid tumors?

- Large breast tumors tend to have necrotic cores, which would impair the infiltration of T cells. Are similar responses observed in smaller tumors?

- Since the liver is a common metastatic site for breast cancer, would liver metastases elicit more infiltration and respond better to TDB?

- Mobilization of T cells is just part of the immune activation process, and the activation of immune cells triggers further immune recruitment. Is there a difference in T cell activation, effector function, proliferation, etc. between tumors and liver? Or, at least what is the status of engaged T cells in the tumors?

- What was the impact of T-cell engagers beyond tumor size reduction (Fig1A)? Any durable response?

- The connection between cytokines and enrichment of T cells in liver vs. tumor is not quite clear. Were cytokine levels representative of organ damage/systemic inflammation, or strong activation in response to tumors?

- The increase in cytokines (CXCL9/10, IL2/6, TNFa and IFNy) can have good or a deleterious effect. For example, high level of CXCL9 in tumors is desirable as it can attract more TILs, while higher circulating level is linked to worse prognosis in cancer. Moreover, serum levels do not necessarily represent tissue levels. Did the authors measure the same cytokines in the liver versus tumors? Also, the kinetics of immune cell accumulation in the liver versus tumors?

- How are the TDB antibodies eliminated? What is the pharmacokinetics in tumors, livers, and other organs [e.g., kidneys]? If they tend to accumulate in the liver, isn't the local inflammation governing the lymphocyte accumulation?

- Drug-induced hepatotoxicity/DILI is not necessarily linked to cytokine activation. So, it is not possible to rule them out without other specific markers (e.g., histology, liver enzymes). Is it possible to repeat experiment of Fig S1H in non-tumor bearing mice following ip/iv injections?

- Several questions related to Fig. 3 need to be addressed:
o Vessel density alone does not capture the status of tumor vasculature. Is the fraction of mature vessels surrounded by pericytes (e.g., co-expressing aSMA, NG2, desmin etc. along with endothelial cell markers such as CD31) similar in tumors versus liver? Is the amount of ICAM1/VCAM1 expression simply a function of 'functional/mature' vessels? In other words, does the extent of ICAM/VCAM staining correlate with the number of mature vessels?
o Since ANG2 is considered as a marker of endothelial activation, it would be useful to know ANG2 levels in liver versus tumors. Is Ang-2 a biomarker of response to T cell-engagers in tumor or only liver toxicity? What is role of Ang-2 in Kupfer cells?

- Immunofluorescence may not be enough to rule out that endothelium do not express CD9. Is it possible to confirm this by flow cytometry?

- The scRNAseq approach revealed CD9 as a candidate. However, the link remains associative for the following reasons:
o CD9 downregulation nicely confirms that it participates in the adhesion of T cells by ICAM1. What about VCAM1? Is it modulated by other molecule(s)?
o Is there a correlation or enrichment of ICAM1/VCAM1 in endothelial cells expressing CD9 in the scRNAseq data?
o Does CD9 change in response to TNFa, IFNg, CXCL9, etc. in vitro?
o Is there a specificity for T cell subtype?
o Does CD9 account for the differential accumulation in tumors vs liver? If so, inhibition/knocking down CD9 in vivo should blunt tissue differences in infiltration.

Minor
- Please, modify the scale on Figs S1E so it is possible to assess biological variability. Please indicate If any cytokine was not detected.

- The rationale behind the in vitro studies in Fig 2 is not clear. Why were PBMCs treated with anti-CD20/CD3 TDB? Is there any evidence that B cell-mediated release of cytokines triggered accumulation of TDB or immune cells in liver in vivo?

Referee #2:

Immunotherapies with CAR T cells and T cell-engaging bispecific antibodies are very promising therapeutic strategies showing durable treatment responses. Yet, some of their adverse effects results in serious morbidities and even mortalities. Understanding the underlying mechanisms would lead to better management of the toxicities. Using the liver as an example, the study described in the current manuscript showed that the transient acute toxicity resulted from activation of endothelial cells in response to elevated circulating cytokines induced by TDB treatment. The activation in turn led to attachment of T cells to activated endothelial cells and subsequent infiltration in the normal tissues. Furthermore, through single cell transcriptome and subsequent functional analyses, the study identified CD9, a component of the "endothelial adhesive platforms", as a potential target for disrupting T cell - endothelial cell interaction, potentially reducing T cell redistribution selectively in normal tissues.

Major issues:
1. Transient hepatotoxicity has been observed in preclinical studies with TDBs. However, it's not clear whether this toxicity is dose-limiting in humans.
2. It's stated in lines 141-142 that "We confirmed that the effects observed are dependent on the engagement of TDB with T cells and tumor antigen, as non-tumor bearing mice did not show any of these effects (Figure S1E)". Only serum cytokine data are shown in Fig. S1E. Effects on T cell infiltration in the liver of non-tumor bearing mice should be provided.
3. The effect size for T cell adhesion (Fig. 2F) seems to be modest. Would T cell activation prior to the assay induce the expression of the ligands of VCAM-1 and ICAM-1 (e.g. VLA-4), thus making the effect size larger?
4. CD9-specific siRNA efficiently inhibited CD9 expression (Fig. 5E), and yet the effect size for T cell attachment was modest (anti-ICAM-1 had a larger effect size)(Fig. 5F), raising the question whether targeting CD9 would be a robust approach to reduce T cell infiltration in normal tissues.

Minor issues:
1. Statistics should be given in Fig. S1G.
2. In line 479, "44.500" should be "44,500"?

Referee #3:

Dr. Himmels and co-workers the mechanism of toxic off-target effects of bispecific antibodies targeting CD3 and a solid tumor antigen. Their studies show the expected tumor-dependent increase in cytokines after treatment, accompanied by decrease in circulating T-lymphocytes and increase in liver inflammation. However, they find that the increase in liver enzymes is accompanied by liver infiltration by T lymphocytes, exceeding infiltration of the tumor bearing the effector antigen. Through extensive single cell seq studies they demonstrate that a subset of liver endothelial cells upregulate adhesive molecules, ICAM-1 and CD9, in response to the cytokines released. Also, notably, they demonstrate that the expression profile of hepatocytes isn't changed by cytokine exposure. Finally, the authors use a surrogate Lymphocyte-endothelial adhesion assay to demonstrate that cytokine treated endothelial cells use both ICAM1- and CD9 to adhere to T-lymphocytes.

This novel work suggests a largely unexpected mechanism of liver injury, whereby trafficking of T cells to hepatic space along reactive veins leads to injury. It also suggests that toxicity might be attenuated if endothelium-mediated T cell egress was blocked. This may also enhance antitumor efficacy by leaving more effector cells to respond to the tumor.

Overall, I found the work convincing and intriguing. However, I think the impact would be enhanced if the authors demonstrated that blocking, knockdown, cluster prevention, or absence of CD9 actually alters accumulation of T cell in the liver or attenuates liver inflammation. I don't want to minimize the potential complexity in interpreting data from these experiments - but think the demonstration that the absence or attenuation of CD9 activity diminishes hepatic toxicity would substantially enhancethe impact of this work.

The authors appear to assume that hepatic injury is caused by T lymphocytes in response to cytokines and adhesion to endothelium. They appear not to consider the possibility that pre-conditioning by making contact with a tumor cell under the influence of a bispecific antibody may also be required. This could be addressed in the discussion.

A minor issue is that the five symbols in Fig 2 A,B,D are small and distinguished only by color. Those who are partially color-blind (myself included) have difficulty distinguishing the symbols in some places. Might it be possible to use shape and color or make the symbols larger so that they are easier to distinguish.

Point-to-point responses

Referee#1:

This is an elegant study on how endothelial cells in liver versus tumors respond differentially to T cell dependent bispecific antibodies (TDBs) and contribute to differences in T cell trafficking to these tissues. Their findings offer a potential mechanism of toxicity from T cell-engagers. The manuscript will be strengthened by addressing the following comments and concerns:

Specific Comments:

1) The tumor implantation procedure is associated with strong local inflammation compared to other methods. This may attract more immunosuppressive cells and prevent antitumor immune cells infiltration. Would injecting cells in the mammary fat pad (MFP) elicit similar response?

   Answer: In our studies, we have used two different mouse tumor models – a breast (Founder5) and an ovarian (ID8/LYPD1X4) tumor model. The breast cancer model utilized tumor implantation procedure, whereas the ovarian cancer model was through injection of cancer cells (ID8/LYPD1X4) in the mammary fat pad. In both models, we see similar responses upon TDB treatment as shown in Figure 1 and Figure EV1 (Founder5 tumor model) and Figure EV3 (ID8/LYPD1X4 tumor model). We have now added additional clarifying descriptions in the Material and Method section (see lines 599-608).

2) Most studies on differences between liver and tumor are based on a single preclinical model. Would this hold in other solid tumors?

   Answer: We have performed comparable analyses in two different mouse tumor models mentioned in point #1 and reported similar findings in both models regarding circulating cytokines and chemokines (Figure 1C, Figure EV1A (Founder5) and Figure EV3A (ID8/LYPD1X4)), T cell margination (Figure 1D (Founder5) and Figure EV3B (ID8/LYPD1X4)) and T cell accumulation (Figure 1E-H (Founder5) and Figure EV3C,D (ID8/LYPD1X4)). We now also added data reflecting activation of the liver endothelium in the ovarian cancer model (Figure EV3F,G). In addition, we included extensive additional analysis at another timepoint

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(four days post TDB treatment) in both models to address question #8 from this referee (Figure EV1A,C,E-I (Founder5) and Figure EV3A,C-E (ID8/LYPD1X4)).

3) Large breast tumors tend to have necrotic cores, which would impair the infiltration of T cells. Are similar responses observed in smaller tumors?

Answer: This is an important point that we had paid attention to in our studies. Indeed, the breast cancer model (Founder5) shows signs of progressing necrosis correlated with increasing tumor size (see Figure R1). Among the small (250 - 385 mm^3), medium (710 - 1090 mm^3) and large (1300 - 1601 mm^3) tumors, we observed minimal to modest necrosis in the small and medium tumors, which resembles the degree of necrosis seen in most human tumors. Based on this data, we selected the tumor size range of 350 – 500 mm^3 to initiate TDB treatments in all studies reported in this manuscript.

4) Since the liver is a common metastatic site for breast cancer, would liver metastases elicit more infiltration and respond better to TDB?

Answer: This is an interesting idea worthy of further investigation, but it is beyond the scope of our study. We are happy to share an example of our existing data from a different project with the referee, which demonstrates that tumors grown in the liver do not respond well to TDB treatment and do not elicit more tumor-infiltrating T cells. The example shown is a colorectal cancer (CRC) organoid liver metastasis model treated with a TDB targeting a CRC cell surface protein. TDB treatment did not induce a significant increase of infiltrating T cells nor significantly reduced tumor growth (see Figure R2).

5) Mobilization of T cells is just part of the immune activation process, and the activation of immune cells triggers further immune recruitment. Is there a difference in T cell activation, effector function, proliferation, etc. between tumors and liver? Or, at least what is the status of engaged T cells in the tumors?

Answer: This is an excellent question that stimulated us to better differentiate the responses in the tumor versus the liver. As it has been described in the literature, T cell activation depends primarily on two cues: (i) the magnitude of TCR activation and (ii) the pattern (types and concentration) of cytokines available (Barberis *et al*, 2018). When analyzed by flow

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cytometry, we found that T cells ($CD8^+$ and $CD4^+$ T cells) in the tumor showed an activation pattern (upregulation of $CD69^+$ and $GZMB^+$ in T cells) consistent with TCR engagement. However, T cells in the liver did not show this type of activation. In line with this finding, T cells in the tumor shifted towards effector T cells whereas we couldn't observe any difference in the T cell subsets in the liver. Proliferation was only observed in the CD4+ T cell fraction in the tumor. This data indicated that the primary response to TDB treatment occurred in the tumor, while T cell infiltration into the liver is likely a secondary response that correlated with vascular activation in the liver. This data has now been included in the manuscript (see Figure 2 and text on page 8 lines 179-187).

6) What was the impact of T-cell engagers beyond tumor size reduction (Fig1A)? Any durable response?

Answer: This is indeed a very interesting question. Similar to human patients, we observed variable responses in the mice as well – with some mice showing complete tumor regression, some others showing reduction in growth rate and yet others not responding at all (see Figure 1B). The reason for this heterogenous response to treatment, in patients as well as in animal models, is still not very well understood and is an area of active investigation.

7) The connection between cytokines and enrichment of T cells in liver vs. tumor is not quite clear. Were cytokine levels representative of organ damage/systemic inflammation, or strong activation in response to tumors?

Answer: This report is the first step in defining different responses in the tumor versus other organs upon cancer immunotherapies and in attempting to identify molecular players that contribute to the observed differences. By comparing tumor-bearing mice with non-tumor-bearing mice, we have shown that cytokine release requires the presence of tumors (see Figure EV2). In addition, we compared cytokine release between mice harboring different sized tumors, and found that larger tumors generally induced a greater degree of cytokine release into the circulation. This stipulates that cytokine levels are indicative of the degree of T cell activation in tumors because more antigens present in the larger tumors allow more engagement and activation of T cells (see Figure R3).

Furthermore, our tissue cytokine data (now included in the manuscript, see Figure 1I,J and Figure EV1H,I) confirms that the primary action of TDB happens in the tumor as several

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cytokines and chemokines were increased upon TDB treatment. On the contrary, we did not observe cytokine elevation in the liver tissue, supporting the notion that the observed changes in the liver are secondary. Taken together, our data argues that T cell accumulation and endothelial cell activation in the liver is a result of a systemic change induced by the direct action of TDB in the tumor.

8) The increase in cytokines (CXCL9/10, IL2/6, TNFa and IFNy) can have good or a deleterious effect. For example, high level of CXCL9 in tumors is desirable as it can attract more TILs, while higher circulating level is linked to worse prognosis in cancer. Moreover, serum levels do not necessarily represent tissue levels. a) Did the authors measure the same cytokines in the liver versus tumors? b) Also, the kinetics of immune cell accumulation in the liver versus tumors?

Answer: We thank the referee for these excellent questions.

a) We have now included data analyzing tissue cytokine levels in the tumor and liver (see Figure 1I,J and Figure EV1H,I). This comparison provides strong evidence that the response to TDB primarily occurs in the tumors, as the levels of many cytokines and chemokines are increased in the tumor but are largely unchanged in the liver.

b) We have now included a “four-days post TDB treatment” timepoint in Figure EV1A,C,E-I (for the Founder5 breast cancer model) and in Figure EV3A,C-E (for the ID8/LYPD1X4 ovarian cancer model). Similar to other preclinical studies described (lines 57-64), most of the effects observed were transient and acute, as T cell margination and liver enzyme levels were resolved four days post TDB treatment in our studies (Figure EV1C, G, and Figure EV3E). On day four, all circulating cytokines and chemokines returned to baseline except of CXCL9 and CXCL10, which exhibited a slower decrease (Figure EV1A and Figure EV3A). T cell infiltration into the tumor was further increased in the Founder5 tumor model but not yet in the ID8/LYPD1X4 tumor model on day four (Figure EV1E, and Figure EV3C, respectively). Conversely, the T cell accumulation in the liver was resolved in the Founder5 tumor model but remained elevated in the ID8/LYPD1X4 tumor model on day four, albeit to a lesser extent than on day one (Figure EV1F, and Figure EV3D).

Taken together, our data argues that the primary response to TDB occurs in the tumors, which led to a rapid release of cytokines into the circulation that triggered acute T cell accumulation in the liver. The liver reaction gradually declined in the following days. Despite rapid T cell

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activation locally in the tumors, T cell accumulation happened at a slower pace than in the liver.

9) How are the TDB antibodies eliminated? What is the pharmacokinetics in tumors, livers, and other organs [e.g., kidneys]? If they tend to accumulate in the liver, isn't the local inflammation governing the lymphocyte accumulation?

Answer: The primary mechanism of systemic clearance of full length IgGs is widespread fluid-phase pinocytosis into endothelial cells (Rafidi *et al*, 2021; Ryman & Meibohm, 2017; Tabrizi *et al*, 2010). HER2/CD3 TDB biodistribution and catabolism in huCD3ε-TG mice has been previously characterized (Mandikian *et al*, 2018). Data related to this work, but not included in the publication showed that hepatic distribution (Figure R4A) and catabolism (Figure R4B) of TDBs is limited to under 5% ID/g, and not the predominant route of elimination. T cell enrichment in the liver did not drive hepatic distribution because all antibody formats, including non-binding IgG controls, non-targeted CD3 bispecifics and HER2/CD3 TDBs were equally distributed following a single dose of i.v. injection with these antibodies.

It is important to note that the liver does not express the antigen recognized by the TDBs used in this study. Passive distribution of the TDBs in the liver did not lead to inflammation as shown in our non-tumor-bearing mice study (Figure EV2 A-C).

10) Drug-induced hepatotoxicity/DILI is not necessarily linked to cytokine activation. So, it is not possible to rule them out without other specific markers (e.g., histology, liver enzymes). Is it possible to repeat experiment of Fig S1H in non-tumor bearing mice following ip/iv injections?

Answer: Thanks for pointing this out. We have now included analysis of liver enzymes (ALT and AST) in non-tumor-bearing mice following i.v. and i.p. injections of TDB (Figure EV2C). In non-tumor-bearing mice, ALT and AST levels were not increased upon i.v. injection of TDB, and showed an insignificant slight numeric change upon i.p. injection of TDB. Note that all our studies used i.v. injection.

11) Several questions related to Fig. 3 need to be addressed:

a. Vessel density alone does not capture the status of tumor vasculature. Is the fraction of mature vessels surrounded by pericytes (e.g., co-expressing aSMA, NG2, desmin etc. along with endothelial cell markers such as CD31) similar in tumors versus liver?

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Is the amount of ICAM1/VCAM1 expression simply a function of 'functional/mature' vessels? In other words, does the extent of ICAM/VCAM staining correlate with the number of mature vessels?

Answer: We used the combination of NG2, Desmin, and αSma antibodies to quantify pericyte coverage in the tumor and liver, and defined the pericyte-covered vessels as "mature vessels". Indeed, we observed a lower mature vessel density in the tumor than in the liver, but we did not observe a significant change upon TDB treatment (Figure 4E,F, right panel). When directly comparing mature vessel density with ICAM-1 and VCAM-1-positive vessels, we saw a complex pattern: in the liver, vessel maturity correlated with ICAM-1 but not VCAM-1 (Figure 4G), whereas the converse is true in the tumor (Figure 4H). Our data indicated that the regulation of vascular ICAM-1 and VCAM-1 differ between the liver and tumor, therefore, vascular maturation is not the only factor that influences the regulation of vascular activation. This is an intriguing observation that warrants further investigation, but is beyond the scope of this study. We have now added a statement to the manuscript (see lines 230-235).

b. Since ANG2 is considered as a marker of endothelial activation, it would be useful to know ANG2 levels in liver versus tumors. Is Ang-2 a biomarker of response to T cell-engagers in tumor or only liver toxicity? What is role of Ang-2 in Kupfer cells?

Answer: We have now included analysis of ANG2 levels in the liver and tumor tissue (see Figure 4J,K, respectively), and showed that TDB did not cause a change in tissue ANG2 levels. This data indicates that the elevation of serum ANG2 (Figure 4I) is a result of increased release instead of increased synthesis. As discussed above, our data in totality supports the notion that the changes seen in the liver is a secondary response to the circulating cytokines triggered by TDB's direct action within the tumors, and is consistent with the finding that ANG2, stored in Weibel-Palade bodies, is released upon stimulation by inflammatory cytokines.

Regarding the role of Ang-2 in Kupffer cells, we believe this is beyond of the scope of the current study, especially in light of the previous reports that Kupffer cells are a minor contributor of Ang-2 compared to other cell types including endothelial cells (Hu *et al*, 2014; Shimizu *et al*, 2005).

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12) Immunofluorescence may not be enough to rule out that endothelium do not express CD9. Is it possible to confirm this by flow cytometry?

Answer: We have now demonstrated CD9 expression on liver ECs and the lack of signal on tumor ECs by flow cytometry analysis, corroborating our immunofluorescence data (Figure 6E). Note the different scales on the y-axis.

13) The scRNAseq approach revealed CD9 as a candidate. However, the link remains associative for the following reasons:

a. CD9 downregulation nicely confirms that it participates in the adhesion of T cells by ICAM1. What about VCAM1? Is it modulated by other molecule(s)?

Answer: Previous work indicates that ICAM-1 and VCAM-1 leverage different facilitating factors to enhance their function. ICAM-1 preferentially associates with CD9 while VCAM-1 associates with CD151. In addition, the ICAM-1/CD9 pair plays a more important role in mediating EC-lymphocyte interaction under flow conditions (Bailey et al, 2011; Barreiro et al, 2005; Barreiro et al, 2008; Franz et al, 2016; Reyes et al, 2018). Taking these published findings in consideration, in conjunction with the fact that we only saw up-regulation of CD9 but not CD151 in our studies, we only evaluated CD9 in the context of ICAM-1-mediated T cell – endothelial interaction.

b. Is there a correlation or enrichment of ICAM1/VCAM1 in endothelial cells expressing CD9 in the scRNAseq data?

Answer: Unlike CD9, *Vcam-1* and *Icam-1* appeared to be upregulated upon TDB treatment in all EC clusters independent of EC subtype (Figure R5A), consistent with our IHC results. When performing correlation analysis based on our scRNAseq data of *Cd9* and *Icam-1* as well as *Cd9* and *Vcam-1* (Figure R5B,C, respectively), we did not see any striking correlation between mRNA levels of these molecules. Mechanistically, since ICAM-1 and VCAM-1 can support leukocyte adhesion on their own, the facilitating factors may not be needed in all vascular subtypes and/or under any conditions. Therefore, the regulation of the facilitating factors such as CD9 could be different from that for ICAM-1 and VCAM-1.

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c. Does CD9 change in response to TNFa, IFNg, CXCL9, etc. in vitro?

Answer: We have performed extensive additional *in vitro* analysis of primary endothelial cells (HUVEC) stimulated with various cytokines and chemokines as well as with TDB-conditioned PBMC supernatant. We found that the pro-inflammatory cytokines TNFα, IFNγ, and IL-2 as well as the chemokine CXCL9 led to an upregulation of *Cd9* mRNA levels but to a lesser extent compared to TDB-conditioned PBMC supernatant (Figure R6). These results imply that the regulation of *Cd9* expression is multifactorial, involving multiple cytokines/chemokines released from the TDB-mediated T cell killing of target cells. We have included the data for *Cd9* upregulation in HUVEC upon stimulation with TDB-conditioned PBMC supernatant in the manuscript (Figure 6F).

d. Is there a specificity for T cell subtype?

Answer: Please see answers to question #5 above.

14) Does CD9 account for the differential accumulation in tumors vs liver? If so, inhibition/knocking down CD9 in vivo should blunt tissue differences in infiltration.

Answer: Based on the data discussed in multiple points above, we believe that the complex response described in this manuscript involves many factors, therefore, knocking out CD9 alone is very unlikely going to abolish T cell accumulation in the liver. We provide an explanation and rationale for publishing our findings without an *in vivo* knockout study on page one of this letter, and hope that the referee agrees with us that not all questions can be answered in a single manuscript.

Minor

15) Please, modify the scale on Figs S1E so it is possible to assess biological variability. Please indicate If any cytokine was not detected.

Answer: We have adjusted the scale of this Figure now to better assess the biological variability (see Figure EV2A). In non-tumor-bearing mice, IL-2 was not detected as indicated in the figure with the label nd (nd: not detected) (Figure EV2A).

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16) The rationale behind the in vitro studies in Fig 2 is not clear. Why were PBMCs treated with anti-CD20/CD3 TDB? Is there any evidence that B cell-mediated release of cytokines triggered accumulation of TDB or immune cells in liver in vivo?

Answer: Previous studies from our department have shown that when non-tumor-bearing mice were treated with CD20/CD3-TDB, we observed B cell killing by T cells and cytokine release (Sun *et al*, 2015) as well as T cell accumulation around large vessel structures in the liver (Figure R7), similar to what we reported in this study. T-cell mediated killing of B cells can be easily recapitulated *in vitro* by adding the CD20/CD3 TDB to PBMCs, which also triggers cytokine release. We confirmed that the cytokine profile of the *in vitro* system is consistent with the cytokine profile of our *in vivo* studies (Figure 3B). As such, we believe that the *in vitro* model of treating PBMCs with anti-CD20/CD3-TDB is a convenient and excellent surrogate for the *in vivo* models (see lines 203-209).

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Referee #2:

Immunotherapies with CAR T cells and T cell-engaging bispecific antibodies are very promising therapeutic strategies showing durable treatment responses. Yet, some of their adverse effects results in serious morbidities and even mortalities. Understanding the underlying mechanisms would lead to better management of the toxicities. Using the liver as an example, the study described in the current manuscript showed that the transient acute toxicity resulted from activation of endothelial cells in response to elevated circulating cytokines induced by TDB treatment. The activation in turn led to attachment of T cells to activated endothelial cells and subsequent infiltration in the normal tissues. Furthermore, through single cell transcriptome and subsequent functional analyses, the study identified CD9, a component of the "endothelial adhesive platforms", as a potential target for disrupting T cell - endothelial cell interaction, potentially reducing T cell redistribution selectively in normal tissues.

Major issues:

1) Transient hepatotoxicity has been observed in preclinical studies with TDBs. However, it's not clear whether this toxicity is dose-limiting in humans.

   Answer: Liver toxicity alone is not reported to be a dose-limiting toxicity in the clinical trials testing T-cell engaging therapies, however, it has been shown that elevation of liver function test (LFT) is commonly associated with cytokine release syndrome (CRS). As CRS itself is the most significant dose-limiting and occasionally life-threatening toxicity in the context of immunotherapies with CAR T cells and T cell-engaging antibodies (Teachey *et al*, 2016), it is important to manage all contributing symptoms in CRS, including hepatoxicity. Therefore, we believe that understanding and minimizing liver toxicity will contribute to a better patient care. We have included a discussion about this point in the manuscript now (see lines 51-69).

2) It's stated in lines 141-142 that "We confirmed that the effects observed are dependent on the engagement of TDB with T cells and tumor antigen, as non-tumor bearing mice did not show any of these effects (Figure S1E)". Only serum cytokine data are shown in Fig. S1E. Effects on T cell infiltration in the liver of non-tumor bearing mice should be provided.

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Answer: We thank the referee for pointing out this important oversight. We have now included the data demonstrating that TDB did not induce significant T cell infiltration into the liver in non-tumor-bearing mice (Figure EV2B).

3) The effect size for T cell adhesion (Fig. 2F) seems to be modest. Would T cell activation prior to the assay induce the expression of the ligands of VCAM-1 and ICAM-1 (e.g. VLA-4), thus making the effect size larger?

Answer: We thank the referee for the helpful suggestion. In the *in vitro* T cell adhesion assays originally reported in the manuscript, we stimulated the T cells with CD3/CD28 to mimic T cell activation. After evaluating the T cell phenotypes in the tumor and liver based on referee #1's comments, we found that T cells in the liver and tumor differ. In tumors, T cells were activated with evidence of TCR engagement, whereas T cell activation was not seen in the liver, indicating that the liver accumulated non-activated T cells. Therefore, we repeated the *in vitro* T cell adhesion assay previously shown in Figure 2F with unstimulated T cells to recapitulate the *in vivo* finding. Gratifyingly, we saw a more robust response to factors released from the TDB-mediated killing reaction. This data is now included in the new version of the manuscript (see Figure 3F).

4) CD9-specific siRNA efficiently inhibited CD9 expression (Fig. 5E), and yet the effect size for T cell attachment was modest (anti-ICAM-1 had a larger effect size) (Fig. 5F), raising the question whether targeting CD9 would be a robust approach to reduce T cell infiltration in normal tissues.

Answer: Our data in totality indicated that the complex response described in this manuscript involves many factors, and CD9 is only one of the contributing factors. Its modest *in vitro* function confirms it to be a facilitating factor of ICAM-1 as reported in published literature. Therefore, we agree with the referee that targeting CD9 is unlikely going to significantly reduce T cell infiltration in normal tissues.

We used CD9 as an example to illustrate how we characterize hits from our screen. On page one of this letter, we provided an explanation and rationale for publishing our findings without determining which factor(s) can substantially reduce T cell infiltration into normal tissues and hope that the referee agrees with us that a single manuscript cannot address all questions.

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Minor issues:

5) Statistics should be given in Fig. S1G.

Answer: We have added the statistics now (see Figure EV2E).

6) In line 479, "44.500" should be "44,500"?

Answer: We have corrected the number accordingly (now in line 537).

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Referee #3:

Dr. Himmels and co-workers the mechanism of toxic off-target effects of bispecific antibodies targeting CD3 and a solid tumor antigen. Their studies show the expected tumor-dependent increase in cytokines after treatment, accompanied by decrease in circulating T-lymphocytes and increase in liver inflammation. However, they find that the increase in liver enzymes is accompanied by liver infiltration by T lymphocytes, exceeding infiltration of the tumor bearing the effector antigen. Through extensive single cell seq studies they demonstrate that a subset of liver endothelial cells upregulate adhesive molecules, ICAM-1 and CD9, in response to the cytokines released. Also, notably, they demonstrate that the expression profile of hepatocytes isn't changed by cytokine exposure. Finally, the authors use a surrogate Lymphocyte-endothelial adhesion assay to demonstrate that cytokine treated endothelial cells use both ICAM1- and CD9 to adhere to T-lymphocytes.

This novel work suggests a largely unexpected mechanism of liver injury, whereby trafficking of T cells to hepatic space along reactive veins leads to injury. It also suggests that toxicity might be attenuated if endothelium-mediated T cell egress was blocked. This may also enhance antitumor efficacy by leaving more effector cells to respond to the tumor.

Overall, I found the work convincing and intriguing. However, I think the impact would be enhanced if the authors demonstrated that blocking, knockdown, cluster prevention, or absence of CD9 actually alters accumulation of T cell in the liver or attenuates liver inflammation. I don't want to minimize the potential complexity in interpreting data from these experiments - but think the demonstration that the absence or attenuation of CD9 activity diminishes hepatic toxicity would substantially enhance the impact of this work.

Answer: We are grateful for the referee's encouragement.

Our data in totality indicated that the complex response described in this manuscript involves many factors, and CD9 is only one of the contributing factors. Its modest *in vitro* function confirms it to be a facilitating factor of ICAM-1 as reported in published literature. Therefore, we believe that knocking out CD9 is unlikely going to substantially reduce T cell infiltration in normal tissues. We used CD9 as an example to illustrate how we characterize hits from our screen. On page one of this letter, we provided an explanation and rationale for publishing our findings without

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determining which factor(s) can substantially reduce T cell infiltration into normal tissues and hope that the referee agrees with us that a single manuscript cannot address all questions.

The authors appear to assume that hepatic injury is caused by T lymphocytes in response to cytokines and adhesion to endothelium. They appear not to consider the possibility that pre-conditioning by making contact with a tumor cell under the influence of a bispecific antibody may also be required. This could be addressed in the discussion.

Answer: We thank the referee for pointing out this important caveat. We have included a paragraph to discuss this possibility (see lines 440-445).

A minor issue is that the five symbols in Fig 2 A,B,D are small and distinguished only by color. Those who are partially color-blind (myself included) have difficulty distinguishing the symbols in some places. Might it be possible to use shape and color or make the symbols larger so that they are easier to distinguish.

Answer: We apologize for not being very considerate in the making of figures. We have changed the color as well as the symbols and added indicators of treatment conditions on the x-axes to provide a better explanation in the figure legend (see Figure 3B,D,F).

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Figures and Legends for Response Letter - EMBOR-2022-55532-T

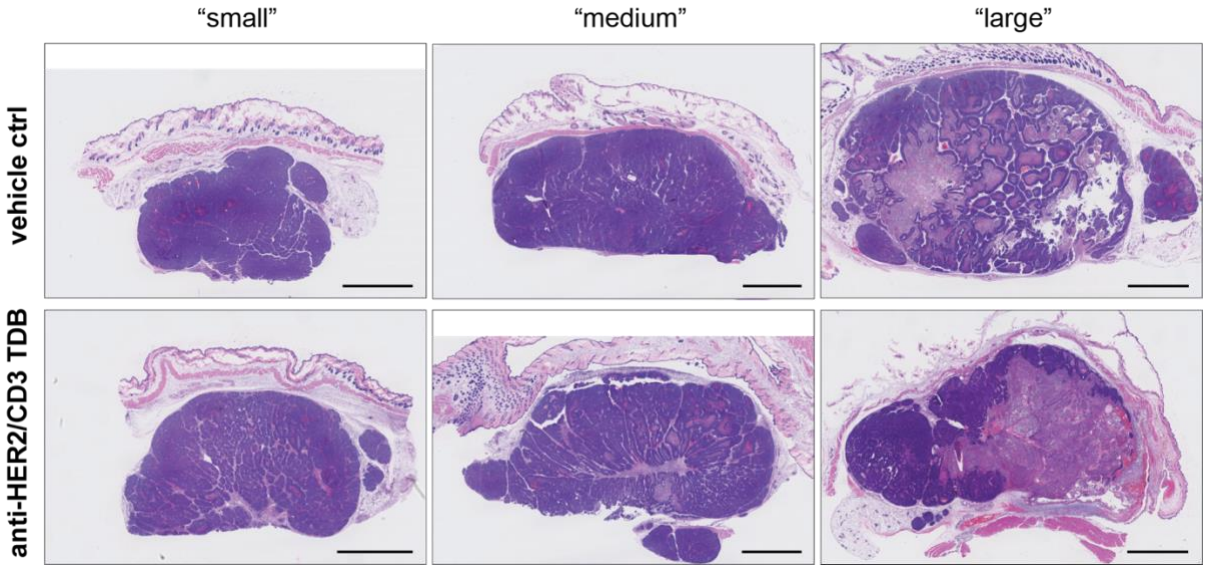

Figure R1. Characterization of necrosis versus tumor sizes
Representative H&E staining of Founder5 tumor sections of small (250 - 385 mm^3), medium (710 - 1090 mm^3) and large tumors (1300 - 1601 mm^3) either treated with vehicle (upper panels) or anti-HER2/CD3 TDB (lower panels). Scale bar = 2000 μm. Dark staining indicates viable tumor cells, light purple/pink staining indicates necrotic area. Tissues on top are skin and muscles from the host animals.

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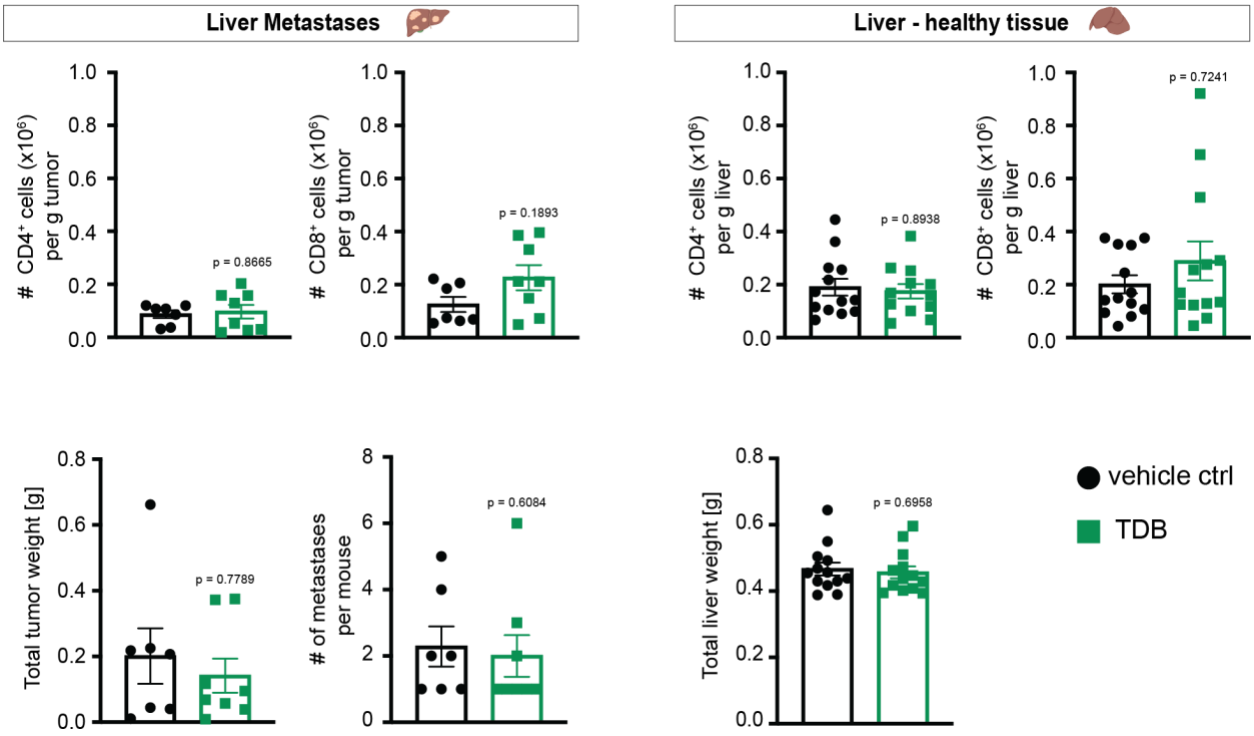

Figure R2. Impact of TDB treatment on the liver lesions of a colorectal cancer (CRC) liver metastasis model

Upper panels: Flow Cytometry analysis of T cells (CD4+ and CD8+) in dissociated liver metastases (left panels, n = 7 mice/treatment) or healthy liver tissue (right panels, n = 13 mice/treatment) normalized to liver weights.

Lower panels: Analysis of tumor burden – total tumor weight (left panel), number of metastases per mouse (middle panel), and total liver weight (right panel).

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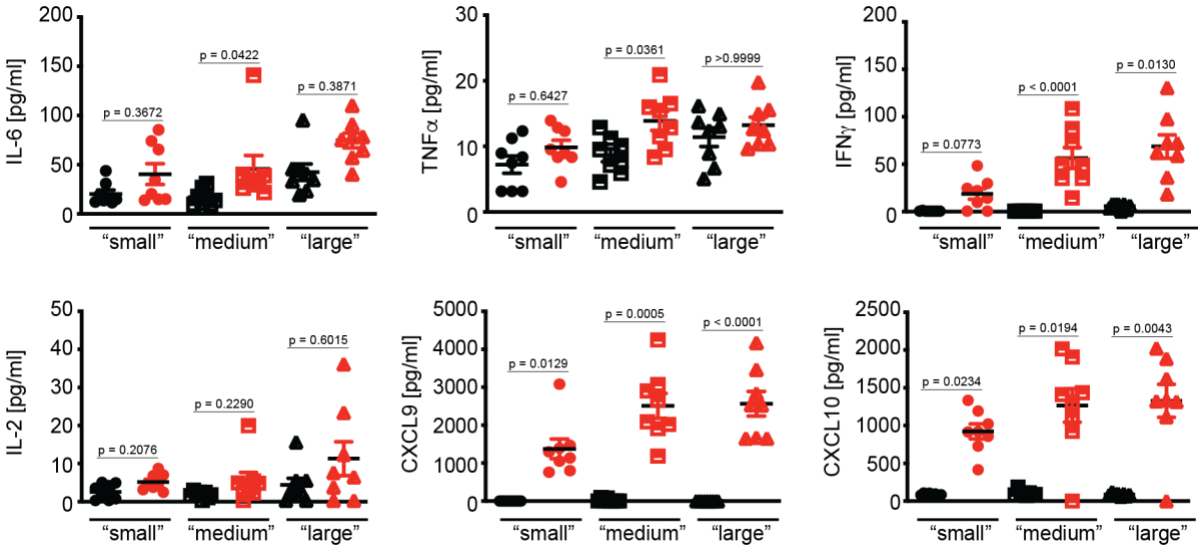

Figure R3. Impact of tumor sizes on cytokine release into the circulation
Sera from mice harboring different size MMTV-HER2 Fo5 mammary tumors and treated with control (black symbols) or anti-HER2/CD3 TDB (red symbols) were collected 24 hours post treatment (n = 7-8 mice/treatment). Cytokine and chemokine levels in the sera were measured by Luminex assay. The tumor size categories indicated on the x-axes are defined in Figure R1.

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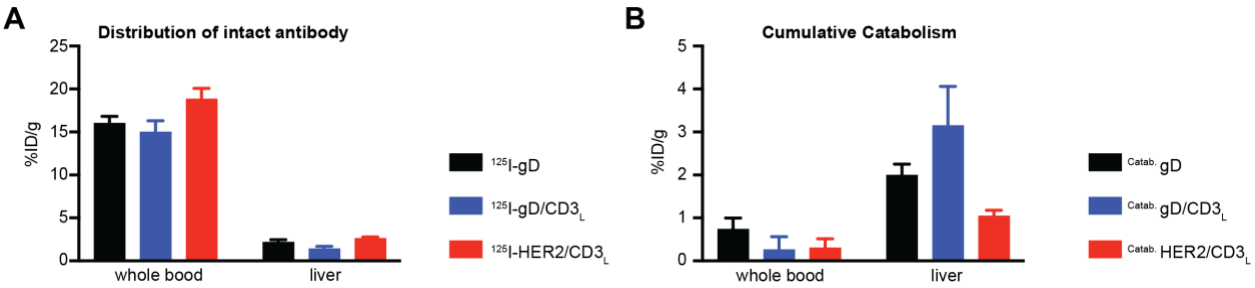

Figure R4. Tissue distribution and catabolism in blood and liver of TDBs in huCD3ε-TG mice

The huCD3ε-TG mice bearing CT26 and huHER2-CT26 tumors on opposite flanks were injected with a single intravenous bolus of ^{125}I- and ^{111}In-labeled anti-gD, anti-gD/CD3 and antiHER2/CD3 TDBs (0.5mg/kg) with low CD3εL affinity to human CD3 *(comparable to the one used in our studies)*. At 72 hours, mice were sacrificed for tissue distribution. Values are reported as a percentage of injected radioactive dose normalized to gram of dry blotted tissue (%ID/g) to convey changes in enrichment. Methods reported in (Mandikian *et al.*, 2018).

A. Tissue distribution of intact antibodies expressed as ^{125}I (ID/g).
B. Catabolized antibody values were determined by subtracting the ^{125}I (%ID/g) from the ^{111}In (%ID/g), and plotted as catabolism (%ID/g).

[gD is a viral antigen that is absent in all mouse tissues. The anti-gD and gD/CD3 bispecific antibodies served as negative controls].

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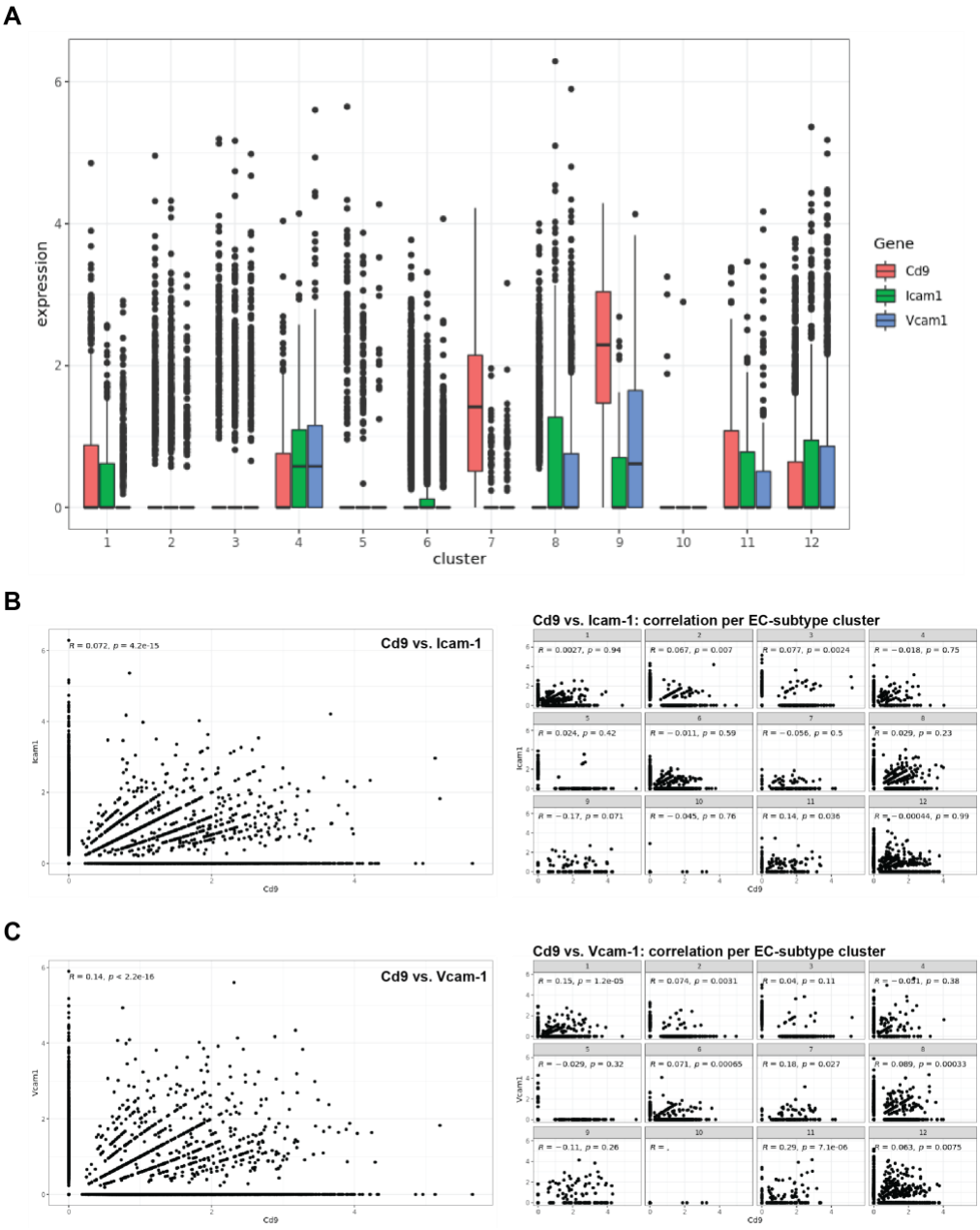

Figure R5. *Cd9, Icam1* and *Vcam1* expression in liver endothelial cells

A. Normalized expression of *Cd9, Icam1,* and *Vcam1* in liver endothelial subtype clusters.
B. Correlation between *Cd9* (x-axis) and *Icam1* (y-axis) normalized expression in all liver endothelial cells (left) and stratified by liver endothelial subtype clusters (right panels)
C. Correlation between *Cd9* (x-axis) and *Vcam1* (y-axis) normalized expression in all liver endothelial cells (left) and stratified by liver endothelial subtype clusters (right panels)
R and p values in B, C were obtained by the Spearman correlation test.

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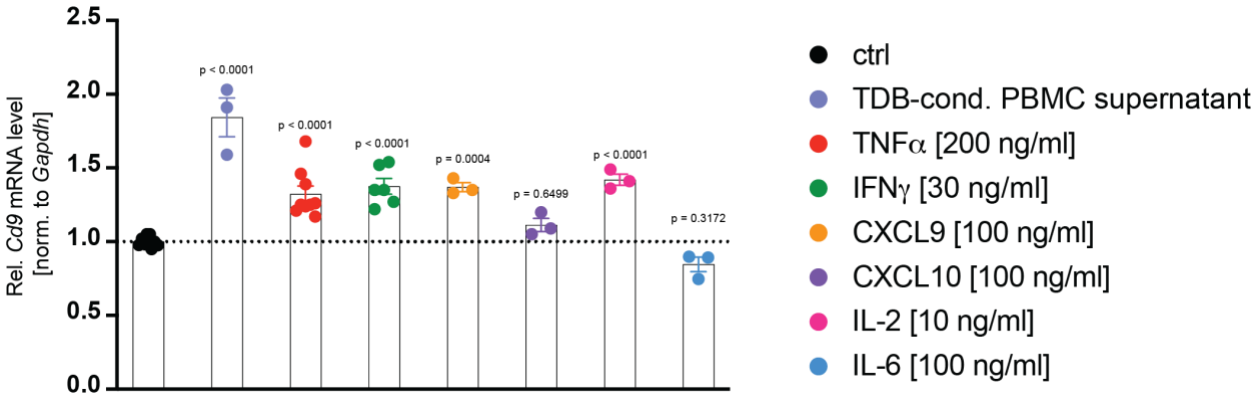

Figure R6. Characterization of *Cd9* expression upon stimulation with various cytokines and chemokines

Quantitative RT-qPCR analysis of relative expression of *Cd9* normalized to *Gapdh* in HUVEC after 24 h stimulation with either control (black, 1st bar) or PBMC TDB-conditioned supernatant (lilac, 2nd bar), 200 ng/ml TNF*a* (red, 3rd bar), 30 ng/ml IFN*γ* (green, 4th bar), 100 ng/ml CXCL9 (yellow, 5th bar), 100 ng/ml CXCL10 (purple, 6th bar), 10 ng/ml IL-2 (pink, 7th bar), or 100 ng/ml IL-6 (blue, 8th bar).

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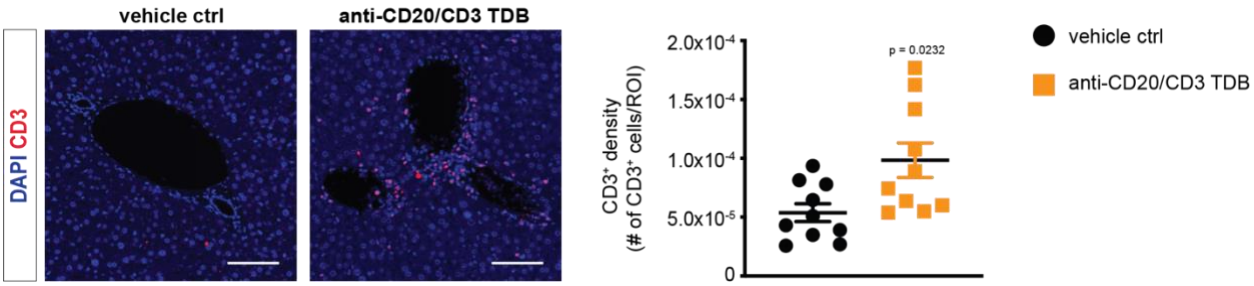

Figure R7. Anti-CD20/CD3 TDB increased leukocyte accumulation in the liver
Representative images of hu.CD3E.tg_hu.CD20.tg.B6N mice liver sections stained with CD3 (red) and DAPI (blue) treated either with vehicle (left) or anti-CD20/CD3 TDB (right). Right panels: quantification of CD3+ cell counts in liver sections normalized to tissue areas. All samples were analyzed 24 h post treatment, n = 10 mice/treatment. Scale bar = 100 μm. ROI: region of interest.

For all Figures: Mann-Whitney test was run to determine p values for experiments with two groups, Kruskal-Wallis one-way ANOVA is used for multiple groups. Error bars indicate s.e.m..

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References:

Barberis M, Helikar T, Verbruggen P (2018) Simulation of Stimulation: Cytokine Dosage and Cell Cycle Crosstalk Driving Timing-Dependent T Cell Differentiation. *Front Physiol* 9: 879

Hu J, Srivastava K, Wieland M, Runge A, Mogler C, Besemfelder E, Terhardt D, Vogel MJ, Cao L, Korn C *et al* (2014) Endothelial cell-derived angiopoietin-2 controls liver regeneration as a spatiotemporal rheostat. *Science* 343: 416-419

Mandikian D, Takahashi N, Lo AA, Li J, Eastham-Anderson J, Slaga D, Ho J, Hristopoulos M, Clark R, Totpal K *et al* (2018) Relative Target Affinities of T-Cell-Dependent Bispecific Antibodies Determine Biodistribution in a Solid Tumor Mouse Model. *Mol Cancer Ther* 17: 776-785

Rafidi H, Estevez A, Ferl GZ, Mandikian D, Stainton S, Sermeno L, Williams SP, Kamath AV, Koerber JT, Boswell CA (2021) Imaging Reveals Importance of Shape and Flexibility for Glomerular Filtration of Biologics. *Mol Cancer Ther* 20: 2008-2015

Ryman JT, Meibohm B (2017) Pharmacokinetics of Monoclonal Antibodies. *CPT Pharmacometrics Syst Pharmacol* 6: 576-588

Shimizu H, Mitsuhashi N, Ohtsuka M, Ito H, Kimura F, Ambiru S, Togawa A, Yoshidome H, Kato A, Miyazaki M (2005) Vascular endothelial growth factor and angiopoietins regulate sinusoidal regeneration and remodeling after partial hepatectomy in rats. *World J Gastroenterol* 11: 7254-7260

Sun LL, Ellerman D, Mathieu M, Hristopoulos M, Chen X, Li Y, Yan X, Clark R, Reyes A, Stefanich E *et al* (2015) Anti-CD20/CD3 T cell-dependent bispecific antibody for the treatment of B cell malignancies. *Sci Transl Med* 7: 287ra270

Tabrizi M, Funelas C, Suria H (2010) Application of quantitative pharmacology in development of therapeutic monoclonal antibodies. *AAPS J* 12: 592-601

Teachey DT, Lacey SF, Shaw PA, Melenhorst JJ, Maude SL, Frey N, Pequignot E, Gonzalez VE, Chen F, Finklestein J *et al* (2016) Identification of Predictive Biomarkers for Cytokine Release Syndrome after Chimeric Antigen Receptor T-cell Therapy for Acute Lymphoblastic Leukemia. *Cancer Discov* 6: 664-679

Dear Dr. Ye,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that I asked to re-evaluate your study, you will find below. As you will see, the referees now fully support the publication of your study in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised version of the manuscript:

- Patricia Himmels is not marked as co-corresponding author on the title page, but in the submission system she is indicated as such. Please check. Either add this to the title page or remove her as co-corresponding author in the submission system.

- Please provide the abstract written in present tense throughout.

- Please order the manuscript sections like this, using these names:
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials and Methods - Data availability section - Acknowledgements (including funding information) - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends.

- Please make sure that funding information is entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please remove the author contributions section from the manuscript text file. See also guide to authors:
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- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (main, EV and Appendix figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please show the data as separate datapoints without error bars and statistics. If n<5, please show single datapoints for diagrams.

- Please remove the paragraphs 'Resource availability' and 'Materials availability' from the methods part.

- Please do not provide the data availability section as table. Please also remove the referee token and make sure that the data are public latest upon online publication of the study.

- Please upload the information provided in the 'Reagent And Tools Table' and the antibodies for the flow cytometry analysis in one combined 'Reagents and Tools table' and remove both tables from the methods part. I have attached templates for that in word or excel format. Please upload the filled in table to the manuscript tracking system as a new 'Reagent Table' file. Please also adjust any callouts to this table. The example linked below shows how the table will display in the published article and includes examples of the type of information that should be provided for the different categories of reagents and tools. Please list your reagents/tools using the categories provided in the template and do not add additional subheadings to the table. Reagents/tools that do not fit in any of the specific categories can be listed under "Other":
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- Please add scale bars of similar style and thickness to the microscopic images (main and EV), using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently, some scale bars seem rather thin.

- Please check the zoom & highlight boxes in Figure EV1 E. The magnification boxes seem to be incorrect, and the images do not correlate to the marked areas.

- Please upload the 5 Appendix tables as separate datasets, naming these Dataset EVx and putting a title and a legend on the first TAB of the excel files. Finally, please change their callouts in the manuscript text to Dataset EVx and remove the Appendix table information from the manuscript text file.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text. Please use the attached file as basis for further revisions and provide your final manuscript file with

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Best,

Achim Breiling
Senior Editor
EMBO Reports

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This manuscript is ready for publications.

Among the several comments, only one point was not experimentally addressed - KO or inhibition of CD9 (which was also raised by another reviewer). However, the authors put forward compelling arguments for not performing this experiment. All the other points were either addressed by new data, or supported by logical explanation based on relevant literature.

This version of the manuscript is compelling, and will provide important reference for future studies in the field.

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My comments have been addressed satisfactorily.

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The authors have adequately addressed my concerns. I recommend publication.

The authors addressed the minor editorial issues.

Dr. Patricia Himmels
Genentech Inc
Molecular Oncology
1 DNA Way
South San Francisco, CA 94080
United States

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The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	tested for mycoplasma

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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods (p33)
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For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods (p33), Figure Legends (p42-48)

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure Legends (p42-48)
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