Systemic Delivery of Cadherin 17-specific CAR-T Cells Allows Effective and Safe Targeting of Colorectal Cancer Liver Metastases

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One Sentence Summary: Systemic cadherin-17-specific CAR-T cell therapy is efficacious against colorectal cancer liver metastases in multiple preclinical models.

ABSTRACT

Liver metastases represent the leading cause of death in patients with colorectal cancer (CRC). Chimeric antigen receptor (CAR) T cell therapy holds promise in this context, but any effort to bring it to the bedside requires careful antigen selection and testing in clinically-relevant models. Here, we identified Cadherin-17 (CDH17) as a candidate antigen for CAR-T cell therapy of CRC liver metastases. We hence designed human CDH17 CARs differing in antigen binding and extracellular spacer regions and compared the different constructs in preclinical models of antitumor efficacy, cytokine release syndrome, and on-target off-tumor toxicity. Whereas the binding domains differed in efficacy in vitro, the spacer region shaped the kinetics of the CAR T cells in vivo. When employed in a CRC liver xenograft model, CDH17 CAR-T cells efficiently suppressed tumor growth upon either systemic or locoregional administration. However, when tested in mice reconstituted with a human immune system, CAR-T cells injected locally caused a particularly harsh cytokine release syndrome. Confocal microscopy revealed that CDH17 is exposed on the entire surface of tumor cells,

whereas its expression in healthy colon is restricted to lateral junctions between epithelial cells. Accordingly, CDH17 CAR-T cells showed dose-dependent cytokine release in response to CRC tissues slices, while displaying no reaction against healthy colon tissue samples. Overall, these findings support systemic delivery of CDH17 CAR-T cells as a safe and effective approach to treat CRC liver metastases and pave the way for a phase 1/2 clinical trial.

INTRODUCTION

Colorectal cancer (CRC) is the third most common tumor and the second leading cause of all cancer-related deaths, accounting for 1.9 million cases and 916.000 deaths globally as of 2020*(1)*. The prognosis of CRC with liver metastases (LM) is poor. About 15-25% of patients present with LMs at the time of diagnosis and 18-25% of patients will develop LMs within 5 years from diagnosis*(2)*. Curative resection preceded by neo-adjuvant chemotherapy is the standard treatment for metastatic patients. However, based on the number, size and location of metastases, surgery is only applicable in 10-20% of cases. Most patients receive systemic chemotherapy with a 5-year survival rate of around 30% *(3, 4)*.

T cells engineered to express chimeric antigen receptors (CARs) targeting CD19 and B-cell maturation antigen (BCMA) have demonstrated remarkable efficacy in patients with B cell tumors and multiple myeloma *(5–8)*. However, in the case of solid tumors, clinical experience is constrained by poor efficacy and toxicity concerns, partly owing to the paucity of target antigens with suitable expression profiles. Antigen distribution and expression are key attributes defining the success of CAR-T therapies*(9)*. CAR-T cells require higher degrees of antigen expression than endogenous T cells to achieve activation and exert cytotoxic functions. Accordingly, CAR targets should be highly and homogenously expressed by the tumor and shared by most patients to broaden treatment applicability. However, identification of tumor antigens with such features is challenging, as tumor heterogeneity and variable disease histology, both within and between patients, characterize solid tumors and favor resistance to therapy and relapses. Additionally, the optimal target should be absent from normal cells to avert undesirable damage of normal tissues*(10)*. This requirement is not easy to meet, as the surface proteome of solid tumors shares high similarities to that of their healthy counterparts. Not surprisingly, on-target off-tumor CAR-T cell reactions have been observed clinically with different degrees of severity. For example, CAR-T cells targeting carbonic anhydrase IX (CAIX) caused grade 2-4 liver toxicities due to the recognition of normal bile duct epithelium*(11)*

and human epidermal growth factor receptor 2 (HER2)-specific CAR-T cells induced fatal respiratory distress and multi-organ dysfunction due to the targeting of normal lung cells*(12)*. Additionally, patients receiving anti-claudin 18.2 (CLDN18.2) CAR-T cells developed mucosal toxicity due to antigen expression in gastric mucosal cells, although this was deemed acceptable *(13)*. Rational selection of the CAR target should therefore be informed by distribution in the tumor but ultimately driven by minimal expression in normal tissues.

In addition to antigen choice, CAR design plays a crucial role in determining performance of CAR-T cells. A CAR is a synthetic receptor that converts extracellular stimuli, namely the recognition of the antigen, to intracellular signal transduction. This process is initiated by the CAR extracellular moiety, which includes the binding and spacer domains. Far from being a simple docking device, the extracellular region is essential to CAR functioning. Most commonly, CARs bind antigens through single-chain fragment variables (scFv) generated from monoclonal antibodies (mAbs) with defined affinity and epitope. Both characteristics may impact CAR activity in ways that can be hard to predict. For example, low affinity scFvs may fail in triggering the response, whereas excessive activation can lead to exhaustion and bystander toxicities*(14)*. Additionally, long extracellular spacers can provide enhanced flexibility and allow for CAR-T cell tracking*(15)*, whereas short spacers may foster CAR phosphorylation by excluding large phosphatases from the immunological synapse *(16)*. Depending on features inherent to the target antigen, such as accessibility, density, and off-tumor expression, the optimal scFv and extracellular spacer may vary. One enabling characteristic of the CAR technology is the possibility of swapping its modular domains, which should allow tailoring CAR design to antigen's characteristics and build the most effective construct.

Here, we aimed at identifying a CAR antigen for effective and safe treatment of CRC-LMs. To this aim, we profiled RNA expression of target candidates in LMs from a cohort of patients with CRC and used expression data in normal tissues to drive antigen selection. We have since designed a panel of CARs differing in the extracellular moiety and leveraged models of antitumor efficacy, on-target

off-tumor toxicity and cytokine release syndrome (CRS) to devise a preclinical workflow for the evaluation of CAR-T cell products and delivery route holding the most favorable safety/efficacy profile. These results constitute the foundation for a phase 1/2 clinical trial in patients with CRC-LMs.

RESULTS

Cadherin 17 (CDH17) is the lead target for CAR T-cell therapy of CRC-LMs

To select targets for CAR therapy of CRC-LMs, we applied a rational workflow integrating data from the literature, online databases, and transcriptomic profiling of tumor samples obtained in our institution (Fig. 1A). We started by curating a list of 258 genes with features suitable for CAR targeting based on their reported expressions in CRC or pancreatic ductal adenocarcinoma (PDAC) and relative normal tissues. To meet the need of selecting surface proteins, the gene list was designed by selecting manuscripts reporting proteomic data *(17–22)* and refined using the Human Protein Atlas*(23)* and UniProt*(24)*, thus restricting the list to 133 candidates. To have internal controls, we also included 47 antigens already in clinical development for carcinomas, such as the carcinoembryonic antigen-related cell adhesion molecule 5 (CEACAM5)*(25)*, and B cell tumors, such as CD19*(26)* (Fig. 1B).

We then evaluated the expression of the 133 genes in tumors from 17 patients who underwent surgical resection of CRC-LMs showing more than 70% tumor cellularity from histopathological analysis *(27)* (table S1). To select highly expressed targets in the tumor, gene transcription expression deciles were calculated based on the expression ranking. The reliability of this method was confirmed by the high ranking of known epithelial CAR antigens, such as CEACAM5, and low ranking of hematological ones, such as CD19 (fig. S1). Among the 86 new candidates, we selected 30 genes showing high expression (deciles 8^{th}-10^{th}) in more than 70% of patients (Fig. 1C).

To avert on-target off-tumor toxicities, we mapped top 30 genes to the Genotype-Tissue Expression (GTEx) RNA dataset with the aim of identifying their expression profile in healthy tissues. Means and standard deviations were calculated for all genes in each tissue to identify four expression classes, ranging from 1 (not detected) to 4 (high expression), to which genes were allocated. To identify safe targets, we excluded genes showing high average expression (4) in any normal tissue except for the intestinal tract, as well as genes showing medium average expression (>3) across all normal tissues,

according to previously described criteria*(19)*. Among all analyzed genes, the only candidate satisfying safety criteria was CDH17, which showed an off-tumor expression limited to colon and small intestine (Fig. 1D).

Analysis of the Gene Expression Profiling Interactive Analysis (GEPIA) database*(28)* confirmed that CDH17 is frequently upregulated in gastrointestinal cancers compared with corresponding healthy tissues, including carcinomas arising from pancreas and stomach (fig. S2). Focusing on CRC, we observed CDH17 upregulation in malignant versus healthy tissues, and such high expression was maintained across all stages of tumor progression (Fig. 1, E and F). Immunohistochemistry staining of 20 primary CRC tumors and 10 LMs confirmed high CDH17 expression in all cases (Table 1). Moreover, CDH17 showed homogeneous expression in almost all malignant cells (Fig. 1, G and H and fig. S3, A and B). In contrast, CDH17 expression was less frequent and more heterogeneous in other tumors tested. Consistent with previous reports*(29)*, 3/10 primary PDAC showed CDH17 expression and 8/10 and 3/10 neuroendocrine tumors (NETs) derived from the ileum and from the pancreas, respectively, were CDH17 positive (fig. S3, C to E). Collectively, these data support CDH17 as the lead target for CAR therapy of metastatic CRC.

CARs including the A4_4R scFv efficiently redirect T cells against CDH17^{+} tumors

Having identified CDH17 as a suitable target for CAR-T cells, we generated different CAR constructs with the aim of applying a functional screening process to identify optimal CAR design and delivery route for the treatment of CRC-LM (Fig. 2A). To minimize the potential for immunogenicity, we generated CDH17 scFvs by exploiting the variable heavy and light chain sequences of two humanized mAbs, namely A4_4R and Lic3. To confer ranging degrees of flexibility, we leveraged three extracellular spacers. The short hinge spacer was derived from IgG1 antibody (H), whereas both the intermediate and long spacers were derived from low-affinity nerve growth factor receptor (LNGFR), either mutated to abrogate potential binding to NGF (NMS) or wild-type (NWL). LNGFR-based CAR spacers carry the benefit of serving both as flexibility modules and selection markers*(15)*. The six

extracellular domains were tethered to second generation CAR backbones containing signaling domains from CD28 and CD3ζ and cloned into bidirectional lentiviral vectors (LV) carrying CD20 as selection marker (Fig. 2B).

Functionality screening was initiated by exploiting a triple-parameter-reporter (TPR) Jurkat cellular model*(30)*. TPR Jurkat cells were transduced to express the CAR variants and stimulated with tumor cells. The specificity of CAR targeting was confirmed by the lack of signal against antigen-negative tumors, either CDH17 knock-out LoVo CRC cells or $CDH17^-$ BxPC-3 PDAC cells (fig. S4, A to C). In contrast, $CDH17^+$ LoVo cells induced a clear upregulation of calcineurin-nuclear factor of activated T cells (NFAT) and nuclear factor κB (NF-κB) signals (Fig. 2C and fig. S4, D and E). This effect was stronger for CARs bearing the A4_4R scFv over the Lic3 scFv. These results were confirmed using primary T cells, showing increased antitumor potency of constructs including A4_4R over Lic3 scFv, despite comparable integrations of CAR vector as measured by analysis of vector copy number (Fig. 2D and fig. S5A). Functionality and specificity of CAR-T cells carrying the A4_4R scFv was confirmed in killing experiments against $CDH17^-$ and $CDH17^+$ tumor cells, and by swapping the CAR costimulatory domain from CD28 to 4-1BB (fig. S5B and fig. S6, A and B). Based on these results, the Lic3 scFv was abandoned and only CDH17 CAR constructs carrying the A4_4R scFv were brought forward.

To identify the CDH17 region wherein A4_4R binding site resides, we designed CDH17 mutants carrying individual deletions of the seven extracellular domains (EC) of the protein (Fig. 2E). All CDH17 mutant-encoding sequences, alongside wild-type CDH17, were cloned into bidirectional LVs carrying the truncated LNGFR (ΔLNGFR) selection marker and transduced into $CDH17^-$ SW620 CRC cells. Tumor cells were efficiently transduced, as confirmed by ΔLNGFR staining, and comparably expressed truncated mutants, as measured by CDH17 staining (Fig. 2F). Since the antibody used for flow cytometry analysis binds to an epitope located within EC7, the ΔEC7 mutant showed no detectable CDH17 signal. Transduced cells were then co-cultured with A4_4R CAR-T cells carrying the three extracellular spacers. Only the ΔEC1 mutant was spared by CAR-T cells,

similarly to untransduced CDH17$^-$ SW620 cells, thus placing the epitope recognized by the A4_4R scFv within the EC1 domain (Fig. 2G and fig. S7).

Because cellular avidity predicts the degree of antitumor activities in vivo*(31)*, we sought to measure the interaction between tumor cells and CDH17 CAR-T cells carrying different extracellular spacers. To this aim, we exploited the z-Movi Cell Avidity Analyzer, which quantifies binding avidity by applying acoustic forces of increasing magnitude in the z-plane between the tumor cell layer and T cells. Both CDH17-H and CDH17-NWL CAR-T cells required the application of higher acoustic forces to detach from tumor cells compared with CDH17-NMS CAR-T cells (Fig. 2, H and I), suggesting stronger functional engagement between CDH17-H or CDH-NWL CAR-T cells and tumor cells. Altogether, in vitro screening identified the A4_4R scFv as the lead binding domain and pointed to NMS spacer design as the least effective among the three remaining CAR-T cell products.

CARs including H and NWL spacers exert potent antitumor activity in vivo

To confirm the superiority of the H and NWL designs, we tested CAR-T cell performances in xenograft mouse models of PDAC and CRC. NSG mice were injected subcutaneously with CDH17$^+$ AsPC-1 (Fig. 3, A to E) or LoVo (Fig. 3, F to J) cells marked with a secreted luciferase *(32)*, and then infused intravenously with CAR$^+$ or untransduced T cells (Fig. 3, A and F). AspC-1 and LoVo cells represent models for high and low CDH17 expression, respectively (fig. S8A). In the AsPC-1 model, CDH17-H and CDH17-NWL CAR-T cells proved superior to CDH17-NMS CAR-T cells, both in terms of tumor growth (Fig. 3B) and survival rates (Fig. 3C), possibly owing to defective T cell expansion (Fig. 3D) and activation (Fig. 3E) in the latter. Similarly, CDH17-NMS CAR-T cells failed to efficiently control tumor growth (Fig. 3G) and survival (Fig. 3H), and were less expanded (Fig. 3I), and activated (Fig. 3J) in mice engrafted with LoVo cells. Despite higher activation, the persisting progeny of CDH17-H and CDH17-NWL CAR-T cells retained high percentage of stem memory (CD62L$^+$CD45RA$^+$, T_{SCM}) and central memory (CD62L$^+$CD45RA$^-$ T_{CM}) T cells (fig. S8, B and C) in both xenograft mouse models. Moreover, ex vivo analysis of tumor masses revealed higher

frequencies of tumor-infiltrating lymphocytes in mice treated with CDH17-H and CDH17-NWL CAR-T cells compared with those that received CDH17-NMS CAR-T cells, together with a higher enrichment in the CD8 compartment (fig. S8, D and E).

To investigate cellular cues for defective tumor control by CDH17-NMS CAR-T cells, we measured CAR expression by LNGFR staining. Despite similar degrees of the transduction marker CD20 (fig. S8F), CDH17-NMS CAR-T cells displayed a 4-fold lower surface expression of the CAR compared with CDH17-NWL CAR-T cells (fig. S8G). This effect was also evident in the pre-infusion product (fig. S8H) and may possibly be attributed to cytosolic retention of the CAR construct, as demonstrated by intracellular staining (fig. S8I). Hence, based on the inadequate tumor control possibly ascribed to defective surface expression of the CAR, CDH17-NMS CAR-T cells were excluded from further evaluations.

Systemic CDH17 CAR-T cell delivery proves equally effective as intra-hepatic administration while curtailing CRS

Locoregional delivery of CAR-T cells has been recently applied as a strategy to counteract inefficient CAR-T cell trafficking to the tumor site*(33, 34)*. To test if this approach would improve tumor control in the context of CRC-LMs, we set up a xenograft mouse model by injecting LoVo cells in the liver of NSG mice that were subsequently treated with CDH17-H or CDH17-NWL CAR-T cells, infused either intravenously or intrahepatically (Fig. 4A). No improved functionality could be ascribed to either of the two delivery routes, which proved equally potent in controlling tumor growth (Fig. 4, B and C). Both CDH17-H and CDH17-NWL CAR-T cells expanded after locoregional and systemic infusion (Fig. 4D), with an initial raise of the $CD8^+$ population followed by a contraction phase in favor of $CD4^+$ cells (fig. S9, A and B). CAR-T cell activation and interferon (IFN)-γ release were also similar, regardless of the delivery route (Fig. 4, E and F), and all conditions were enriched in T_{SCM} and T_{CM} (Fig. 4G).

238 Overall, these results highlighted no efficacy advantage in administering CDH17 CAR-T cells
239 locoregionally rather than systemically.
240 Besides offsetting the spatiotemporal barriers, locoregional delivery should benefit CAR-T cells by
241 igniting a rapid and strong local immune reaction*(34)*. NSG mice, although appropriate to assess basic
242 antitumor efficacy of engineered T cells, cannot account for the complex crosstalk with other human
243 immune cells. Additionally, as recently demonstrated in preclinical models of hematological
244 malignancies*(35–37)*, the absence of myeloid cells rules out the possibility of profiling CRS
245 development. To overcome these limitations, we exploited a model of tumor xenograft in the liver of
246 immunodeficient mice reconstituted with a human immune system. To this aim, we transplanted
247 human hematopoietic stem and progenitor cells into triple transgenic NSG mice (SGM3) expressing
248 human interleukin (IL)-3, granulocyte-macrophage colony stimulating factor (GM-CSF) and stem
249 cell factor (SFC). Upon human reconstitution, SGM3 mice (HuSGM3) were injected intrahepatically
250 with LoVo cells and treated at high tumor burden either systemically or locoregionally with CAR-T
251 cells (Fig. 5A). Locoregional delivery of CAR-T cells accelerated tumor debulking by CDH17-H
252 CAR-T cells (Fig. 5B), possibly as consequence of faster and synchronous T-cell activation (Fig. 5C).
253 This effect was more apparent in HuSGM3 mice than in classical NSG, possibly due to the adjuvant
254 role of innate immune cells. Yet, the two delivery routes were equally effective in the long-term and
255 initial differences were smoothed for both CAR-T cell products (Fig. 5D). Moreover, similar IFN-γ
256 production was measured in mice treated with CAR-T cells systemically and locoregionally,
257 supporting an overall comparable efficacy profile among the two delivery routes (Fig. 5E). However,
258 locoregional treatment was far more toxic than systemic delivery, as mice injected with CAR-T cells
259 in the liver developed irreversible weight loss (Fig. 5F), which resulted in 100% mortality with both
260 CAR-T cell products (Fig. 5G). The effect was seemingly related to the antigen-specific activation of
261 CAR-T cells, as mice infused with control untransduced T cells did not develop any toxicity signs
262 but died from tumor outgrowth. Because rapid weight loss and fatal events have been recognized as
263 clinical signs of CRS in preclinical models of hematological malignancies*(35–37)*, we reasoned that

264 the toxicity observed might be ascribed to CRS. Licensing of myeloid cells is responsible for the
265 systemic release of cytokines which causes the syndrome *(35, 38)*. We therefore measured serum
266 amount of myeloid cytokines, and observed higher concentrations of IL-6, IFN-γ-induced protein 10
267 (IP-10), and monocyte chemoattractant protein-1 (MCP-1) after locoregional infusion of CDH17-H
268 CAR-T cells as compared with systemic delivery. Higher concentrations of IL-6 were also measured
269 in mice treated with systemic rather than locoregional CDH17-NWL CAR-T cells (Fig. 5, H to J). To
270 rule out that the systemic toxicity observed was due to recognition of the hematopoietic compartment
271 by CAR-T cells rather than to CRS development, we analyzed CDH17 expression in hematopoietic
272 cells. In line with transcriptomic data, we could not detect CDH17 expression in any of the
273 populations analyzed, including B cells, CD8 and CD4 T cells, myeloid cells, and NK cells (Fig. 5K
274 and fig. S10, A to C). Accordingly, none of these populations were recognized in killing experiments
275 by either CDH17-H or CDH17-NWL CAR-T cells (Fig. 5L). To confirm these data, we analyzed
276 hematopoietic reconstitution in tumor-bearing humanized mice treated with CDH17 CAR-T cells.
277 CDH17 CAR-T cells efficiently controlled tumor growth without causing severe weight loss (Fig. 5,
278 M and N). Most importantly, despite a clear expansion of CAR-T cells, no signs of reactivity against
279 hematopoietic cells were observed in these mice, which showed unaltered kinetics of human
280 $CD45^{+}NGFR^{-}$ cells (non-CAR-T cells), including B cells or monocytes (Fig. 5, O to R). Taken
281 together, these results point to the onset of severe CRS as the underlying cause of toxicity manifested
282 in mice treated locoregionally and support systemic infusions as the delivery route of choice to
283 increase the safety profile of CDH17 CAR-T cells in the context of CRC-LMs.
284

285 **Patient-derived CDH17 CAR-T cells are effective against primary tumors**

286 Moving to a more relevant clinical setting, we tested the two leading CDH17 CAR-T cell products
287 using patient-derived peripheral blood (PB) T cells and tumor organoids generated from patients with
288 CRC-LMs (PDOs, Fig. 6A). Analysis of T cell composition in patients with CRC-LM revealed

289 comparable frequencies of $CD4^+$ and $CD8^+$ cells (fig. S11A). As expected, however, patient T cells
290 included a high frequency of terminal effectors ($CD62L^-CD45RA^+$, T_{EMRA}) and lower frequencies of
291 $T_{N/SCM}$ and T_{CM} (fig. S11B), which are usually higher in healthy controls*(39)*. We then analyzed the
292 expression of inhibitory markers commonly expressed by exhausted T cells retrieved from patients
293 with cancer or chronic infections. A fraction of T cells co-expressed the inhibitory markers PD-1 and
294 2B4, whereas TIM-3 and LAG-3 showed minimal expression (fig. S11C).
295 To generate CAR-T cells, PB T lymphocytes from patients or healthy donors (HD) were stimulated
296 with αCD3/CD28 beads, transduced with LVs and expanded with IL-7 and IL-15, according to a
297 protocol that preserves T cell fitness*(39)*. No prominent phenotypical differences were observed in
298 the manufacturing of patients T cells compared to HDs. The procedure yielded a cellular product with
299 good activation and poor exhaustion phenotype (fig. S11, D to G), overall comparable expansion
300 kinetics (Fig. 6B) and cellular compositions, measured as CD4/CD8 ratio (Fig. 6C and fig. S11H),
301 and memory T cells compartments (Fig. 6D and fig. S11I). Transduction efficiency was analyzed by
302 staining the CD20 marker for CDH17-H CAR-T cells and the LNGFR spacer for CDH17-NWL
303 CAR-T cells. Although similar frequency of $CD20^+$ cells were observed between CDH17-H CAR-T
304 cells derived from CRC-LMs or HDs (Fig. 6E), lower percentages of $LNGFR^+$ cells were detected in
305 CDH17-NWL CAR-T cells manufactured from CRC-LMs compared with controls (Fig. 6F).
306 Nonetheless, both CAR-T cell products proved effective at killing autologous or allogeneic PDOs
307 (Fig. 6G), despite the lower cytotoxicity of CDH17-NWL CAR-T cells manufactured from CRC-
308 LMs possibly arising from lower transduction efficiency.
309 To test reactivity against PDOs in vivo, we set up a mouse model by injecting CRC-LM PDOs into
310 the livers of NSG mice, which were then treated with two systemic infusions of CDH17 CAR-T cells,
311 either CDH17-H or CDH17-NWL.28ζ (Fig. 6H). Both cellular products provided a potent control of
312 tumor growth in treated mice (Fig. 6I and fig. S11J), as opposed to mice treated with untransduced T
313 cells, which suffered from multiple tumor lesions in the liver, as confirmed by echography scans (Fig.
314 6, J and K). Eventually, treatment with CDH17 CAR-T cells significantly prolonged the survival of

PDO-bearing mice (Ut versus CDH17-H.28ζ $P = 0.0002$; Ut versus CDH17-NWL.28ζ $P < 0.0001$; Fig. 6L). In accordance with tumor shrinkage, both CDH17-H and CDH17-NWL.28ζ cells showed peripheral expansions (Fig. 6M) and, despite a clear activation, retained a high percentage of T_{SCM} cells and good representations of both $CD4^+$ and $CD8^+$ subpopulations (Fig. 6, N to P). These data, besides highlighting the potential of manufacturing procedures to overcome initial T cell defects, support the clinical exploitation of CDH17 CAR-T cells as therapeutic treatment for patients with CRC-LMs.

CDH17 CAR-T cells display poor on-target off-tumor reactivity toward healthy intestine

CDH17 is a cell-adhesion glycoprotein with a polarized expression at the cell-to-cell junctions between normal epithelial cells of small intestine and colon*(40, 41)*. To address the safety profile of CDH17 CAR-T cells, we first sought to verify the localization of the antigen in healthy and tumoral intestinal epithelial cells. To this aim, we performed a dual immunofluorescence staining of human healthy colon tissues using CDH17 and occludin, which is a marker for apical tight junctions in colonic epithelial cells*(42)*. In normal colon, CDH17 stained positive at the lateral junctions between epithelial cells but negative at the basal side, which is the one accessible to CAR-T cells (Fig. 7A). On the contrary, primary CRC or CRC-LMs showed marked disruption of tissue architecture and continuous high expression of CDH17 all over cell surface (Fig. 7, B and C).

To functionally test the on-target off-tumor potential of CDH17 CAR-T cells toward tissues with native structural integrity, we exploited the tissue slice assay previously reported as a tool to measure potential CAR-T cell-mediated lung toxicities*(43)*. Briefly, CDH17 CAR-T cells were added for 24 hours onto fresh colonic tissue slices retrieved from patients with CRC undergoing surgery and CAR-T cells reactivity was evaluated by measuring cytokine release toward tumoral or adjacent healthy tissues. Both CDH17 CAR-T cells products showed robust and dose-dependent release of IFN-γ and tumor necrosis factor (TNF)-α toward CRC tissue slices, whereas no reactivity was evident against

healthy colon tissue slices, even at the highest CAR-T cell dose (Fig. 7, D and E). As additional proof of the specificity of targeting by CAR-T cells and the suitability of the tissue slice assay, we observed no cytokine release when CDH17 CAR-T cells were challenged against antigen-negative healthy liver tissue slices (fig. S12, A and B). Altogether these results, which rule out on-target off-tumor toxicity, further support the clinical transability of CDH17 CAR-T cells for the treatment of CRC-LMs.

DISCUSSION

In this study, we designated CDH17 as the lead CAR target in CRC-LMs by applying a rational workflow integrating evidence from the literature, healthy tissue-specific RNA datasets and patient-derived transcriptomic outputs. To address the challenge of developing an effective and safe anti-CDH17 CAR-T cell therapy, we used a preclinical pipeline to screen a panel of CARs in models of antitumor efficacy, CRS, and on-target off-tumor toxicity. Our data reveal that systemic delivery of CDH17 CAR-T cells holds the most favorable toxicity profile for application in CRC-LMs, enabling efficient tumor targeting while mitigating CRS potential.

High and homogeneous antigen expression in the tumor are key attributes to increase the chances of achieving complete responses after CAR-T cell therapy. CDH17 was found to be highly and consistently expressed in almost all malignant cells of primary and metastatic CRC lesions, pointing to this tumor type as the primary indication for CDH17 CAR-T cell therapy. In line with published data*(40, 44, 45)*, PDAC, NETs and stomach adenocarcinomas also stained positive for CDH17, despite scattered signals and inter-patient variability, suggesting the possibility of widening CDH17 CAR-T cell treatment to other gastrointestinal cancers, possibly in combination with strategies fostering the clearance of antigen-negative tumor clones.

Antigen involvement in tumor biology can reduce risk of relapse due to the emergence of antigen-loss tumor cell variants. CDH17 is a cell adhesion glycoprotein responsible for maintaining intestinal integrity through homophilic interactions between adjacent epithelial cells*(41)*. In CRC, CDH17 overexpression increases tumor cell proliferation and metastatic colonization of the liver thanks to an RGD-mediated interaction with α2β1 integrin*(46)*. Given its malignant relevance, CDH17 has been targeted in CRC with mAbs inhibiting its binding to the α2β1 integrin*(47)* or with bispecific antibodies restricting TRAILR2 activation to $CDH17^{+}$ tumor cells*(48)* (NCT04137289, NCT05087992).

Recently, a CAR therapy targeting CDH17 has also been described*(49)*, which is currently under investigation in patients with gastrointestinal cancers (NCT06055439). Differently from published CAR, which comprises a CDH17 llama-derived nanobody coupled to a third-generation scaffold, in this study we employed mouse-derived humanized scFvs mounted on a second-generation CAR. In particular, we designed six CDH17 CAR constructs varying in antigen-binding domain and extracellular spacer to select the optimal CAR candidate for clinical translation. For this, we leveraged in vitro assays to screen CAR-T cell functionality, including the measure of transcriptional activation, cytotoxic activity, and binding avidity. Interestingly, this last assay was uniquely able to predict differences among spacer variants in vivo, aligning with recent reports supporting the measurement of cellular avidity as the most accurate in vitro method to predict in vivo efficacy*(31, 50)*.

In the setting of solid tumors, regional delivery of CAR-T cells has reinforced therapeutic promise. Unlike leukemic cells, which naturally circulate in the peripheral compartment, solid tumors are difficult to reach by CAR-T cells and shielded by stromal barriers and inhibitory signals. Locoregional therapy offers direct access to the tumor, which favors early activation kinetics and tumor debulking and fosters the development of robust endogenous responses*(51–53)*. As such, this delivery route is under investigation for several solid malignancies, including central nervous system tumors, peritoneal carcinomas, and CRC-LMs*(34)*. In accordance with reported data, in our models locoregional therapy resulted in faster kinetics of tumor debulking and CAR-T cell triggering compared with systemic infusion. This effect was particularly marked in mice reconstituted with a human immune system, supporting the notion that the host immune compartment reinforces antitumor responses. Despite an initial delay, however, at later time points systemic therapy resulted in comparable therapeutic efficacy as locoregional infusion, possibly owning to elevated liver vascularization. Of notice, locoregional treatment proved toxic, suggesting that the rapid, substantial and synchronous activation of the CAR-T cells might have licensed local myeloid cells to release high amounts of inflammatory mediators responsible to worsen CRS manifestations. As such, our

results clearly point to systemic delivery of CDH17 CAR-T cells as the best choice to mitigate CRS potential without compromising efficacy. Conveniently, this approach brings specific advantages compared to the challenges of locoregional therapy. The infusion of CAR-T cells in the resected tumor cavity might exacerbate postoperative sequelae due to CAR-T cell-induced cytokine storm, whereas regional delivery might be technically challenging due to the need for safe access and the volume of infusion required. In case of LMs, delivery of CEACAM5 CAR-T cells by hepatic artery infusion has been applied, but required technical optimizations to improve CAR-T cell infiltration, such as the use of Pressure-Enabled Drug Delivery*(54)*. Far from claiming that our result may fit all solid tumor applications, our findings emphasize that the effects of delivery route may be more diversified than initially expected and should be carefully evaluated for each CAR-T cell product and disease indication in preclinical models of efficacy and toxicity.

Clinical experience in solid tumors indicates that standalone CAR-T cells therapies are commonly poised to fail in achieving long-term remissions, supporting the need of developing improved strategies in this context. Recently, CD39 emerged as a key inhibitory pathway in CRC and CD39-disrupted T cell receptor-engineered T cells showed improved potency in xenograft mouse models of CRC*(27)*, offering a promising strategy to increase the functionality of CDH17 CAR-T cells as well. Additionally, applying lymphodepleting regimens enhanced by chemotherapy or biological agents might also improve the therapeutic outcome by reprogramming the tumor microenvironment and increasing CAR-T cell infiltration*(55, 56)*. Alternatively, dual targeting of a CAR and a chimeric costimulatory receptor might increase binding avidity and targeting of clones with low antigen densities*(57)*. Finally, because malignant branched N-glycans interfere with immunological synapse formation and foster inhibitory signals*(58)*, the combination of CDH17 CAR-T cells with de-glycosylating agents could allow counteracting multiple layers of tumor resistance and improve both immediate and long-term therapeutic benefit.

On-target off-tumor reactions against healthy tissues expressing the target antigen are a major concern for CAR therapy in solid tumors. Although CDH17 has been selected by applying stringent safety criteria, its expression by intestinal epithelia would make those cells immediate CAR targets. Interestingly, however, as reported for other cell-adhesion antigens of the gastrointestinal tract, such as Claudin-18, the highly polarized expression at the cell-to-cell junctions between epithelial cells grants a favorable safety profile due to the limited exposure of the CAR-binding domain to peripheral CAR-T cells*(13, 40, 41)*. In a previous work, the on-target off-tumor potential of CDH17 CAR-T cells was assessed by using a syngeneic mouse model engrafted with murine CAR-T cells. In this model, the authors underscored no toxicity toward healthy intestine. Since our anti-CDH17 scFv is not cross-reactive toward the murine antigen, in this work we measured the reactivity of CDH17 CAR-T cells towards fresh tissue slices generated from surgical material of patients with CRC *(59)*. Remarkably, whereas CDH17 CAR-T cells released Th1 cytokines in response to tumor tissues, such activity was not measured towards autologous intestinal healthy tissues, supporting the safety of devising a CAR-T cell therapy toward the CDH17 antigen. These results further highlight antigen accessibility as a key element that needs to be acknowledged and contribute to expanding the traditional view of safety studies, which have long been based mostly on antigen expression. Although CDH17 CAR-T cells proved safe in our preclinical models, we cannot exclude beyond reasonable doubt the emergence of clinical toxicities. In a translational effort toward a safe clinical application, it would be valuable to couple the CDH17 CAR construct to a safety switch for the ablation of engineered T cells at the occurrence of toxic manifestations. Suicide genes deriving from the Thymidine Kinase*(60, 61)*, inducible caspase 9*(62)* and truncated EGFR*(63)* have been used to this purpose and might offer valid solutions.

The definition of therapeutic product, patient population, and treatment schedule are crucial parameters for clinical translation. In this study, we sought of designing a CAR-T cell product featuring both immediate effector functions and long-lasting persistence capacities. CD28 co-

443 stimulated CARs typically release higher quantities of cytokines and achieve faster tumor debulking,
444 which is a crucial requisite when tackling aggressive solid tumors. Additionally, this CAR design
445 might be particularly suited in hypoxic conditions, which are characteristic of solid tumors, due to its
446 propensity to promote anaerobic glycolysis. To balance out the effector function of CD28 and build
447 a therapeutic product showcasing both early antitumor activities and long-lasting persistence, we
448 sought of using manufacturing procedures favoring the enrichment of early-memory T cells. The
449 screening process of the different CARs has identified CDH17-H.28ζ and CDH17-NWL.28ζ as the
450 most promising constructs. Both CARs share the same binding domain but differ in the extracellular
451 spacer, which is either derived from IgG1 or LNGFR, respectively. Although the two CARs
452 performed similarly, at a closer look some differences become evident. From a development
453 standpoint, the limited size of the IgG1 spacer reduces the dimension of the CAR and could improve
454 vector production and yield. Nevertheless, the inclusion of a LNGFR-based spacer enables the in vivo
455 tracking of CAR-T cells and potentially enrichment with clinical-grade immuno-magnetic beads*(15)*.
456 Moreover, systemic delivery of CDH17-NWL.28ζ product emerged as the treatment condition
457 holding the most favorable balance between antitumor activity and inflammatory reactions, according
458 to results from the humanized mouse model and in vivo reactivity against PDOs. Importantly, both
459 CDH17-H.28ζ and CDH17-NWL.28ζ cells were efficiently manufactured from patient-derived
460 PBMCs and showcased an enrichment of T_{SCM} cells*(39)*. Recently, data from the ZUMA-7 clinical
461 trial testing the use of axi-cel as second-line treatment in patients with B cell lymphoma showed that
462 high frequencies of T_{SCM} CAR-T cells in the infusion product was associated with the most durable
463 responses*(64)*. Interestingly, higher proportion of differentiated T cell subsets correlated with more
464 severe CRS and neurotoxic manifestations in both preclinical and clinical studies*(36, 64)*. Therefore,
465 the adoption of early apheresis coupled with manufacturing procedures that favor the enrichment of
466 early-memory T cells should grant better clinical outcomes and mitigate toxicities.

We acknowledge some potential limitations of our present study. First, we recognize the lack of functional results on de novo CRC or other anatomical metastatic sites. However, CDH17 staining was positive in all 20 primary CRC tumors tested, which envisions therapeutic benefit also in this setting. Second, we did not perform a direct comparison between this therapeutic approach and others already employed in the clinic for treating CRC-LMs, such as CEA CAR-T cells, but this will be the object of future studies. Lastly, although mice reconstituted with a human immune system allowed us to evaluate the reactivity of CDH17 CAR-T cells against hematopoietic cells, we didn't profile on-target off-tumor toxicity in mice transgenic for human CDH17. However, we value the tissue-slice assay to study on-target off-tumor reactions in a fully-human ex vivo system.

In summary, we have selected CDH17 as a promising target for potent and safe immunotherapy of CRC-LMs with CAR-T cells. By leveraging on multiple preclinical models, we identified the CDH17 CAR-T cell product and delivery route holding the most favorable efficacy/toxicity profile, streamlining the initiation of a phase 1/2 clinical trial in patients with CRC-LMs.

MATERIALS AND METHODS

Study design

The objective of this study was to identify a CAR antigen for the treatment of CRC-LM and devise a CAR-T cell therapy with optimal safety and efficacy profiles. To this aim, we generated CAR constructs varying the extracellular domain and screened them in preclinical models of efficacy and safety, in vitro and in vivo. All in vitro experiments were performed with at least three different healthy donors. In vivo studies were conducted using three to ten mice per group based on previous experiments indicating that this sample size ensures reliable reproducibility and the potential for statistically significant differences. The sample size was not determined through statistical methods. Tumor-bearing mice were randomized into treatment groups before T cell infusion to have similar tumor burdens in all experimental groups. Treatments were administered by an operator who was blinded to the group assignments. All in vitro and in vivo analyses were based on objectively measurable data, and the exact number of animals and experimental replicates are specified in the figure legends.

CAR constructs. CAR constructs were generated with scFvs derived either from Lic3 or A4_4R mAb, whose sequences are described in patents WO2017120557A1 and WO_2012054084A2, respectively. Both scFvs were synthetized by GeneArt (Thermo Fisher Scientific) and cloned into a CAR incorporating a CD28 trans-membrane and costimulatory domain and a CD3ζ endodomain*(65)*. To generate CDH17.BBζ, the CD28 transmembrane and co-stimulatory domains were substituted with CD8 and 4-1BB sequences, respectively. As extracellular spacers, we incorporated either an IgG1 hinge or LNGFR-derived NMS and NWL sequences previously described*(15)*. All CAR complementary DNAs were cloned into bidirectional lentiviral vectors including the human phosphoglycerate kinase promoter and provided by L. Naldini (San Raffaele-Telethon Institute for Gene Therapy)*(66)*. CAR constructs were placed in sense orientation, whereas the marker gene CD20 was cloned in antisense orientation.

Mouse experiments. All experiments were approved by the Institutional Animal Care and Use Committee of IRCCS San Raffaele Scientific Institute and by the Italian Governmental Health Institute. Female or male 6- to 9-week-old NOD.Cg-Prkdcscid Il2rgtm1Wjl (NSG) (Charles River Laboratories) and NSGTgCMV-IL3, CSF2, KITLG1Eav/MloySzJ (SGM3) mice (Charles River Laboratories) were kept in a specific pathogen-free facility within individually ventilated cages. For initial experiments addressing antitumor efficacy, NSG mice were injected subcutaneously with $1.5x10^6$ LoVo $LUCIA^+\Delta LNGFR^+$ cells or $4x10^6$ AsPC-1 $LUCIA^+\Delta LNGFR^+$ cells and treated with 10^7 CAR-T cells administered intravenously. For experiments validating the CAR-T cell delivery route, NSG mice were infused intrahepatically with 10^5 LoVo $LUCIA^+\Delta LNGFR^+$ cells and treated with 10^7 CAR-T cells delivered either intrahepatically or intravenously. For experiments in humanized mice, SGM3 mice were sub-lethally irradiated and infused intravenously with 10^5 human $CD34^+$ cells purified from umbilical cord-blood samples collected at the Gynecology Unit at IRCCS Ospedale San Raffaele under written informed consent approved by IRCCS Ospedale San Raffaele Ethics Committee (Protocol 34CB, Milan, Italy). Upon reconstitution, mice were infused intrahepatically with 10^5 LoVo $LUCIA^+\Delta LNGFR^+$ cells and then treated with $5x10^6$ CAR-T cells delivered either intrahepatically or intravenously. To evaluate CRS development, weight loss was monitored daily and the concentration of serum human cytokines was assessed using the LEGENDplex bead-based cytokine immunoassay (BioLegend, 740724). In all experiments, tumor growth was monitored by bioluminescence assay using the QUANTI-Luc detection reagent (InvivoGen, rep-qlc1) and expressed as relative light units (RLUs). Mice were euthanized at RLU $\geq$ 10^5 or when showing signs of discomfort . When specified, at euthanasia, subcutaneous tumor masses were retrieved, dissociated using gentleMACS (Miltenyi Biotec, 130-093-235, 130-095-929) and analyzed by flow cytometry. For experiments addressing the efficacy towards CRC-LM PDOs, NSG mice were infused intrahepatically with 40 LUC^+ PDOs and treated twice with 10^7 CAR-T cells

delivered intravenous on day 3 and 7. Tumor growth was monitored by bioluminescence and mice were euthanized at signals ≥ 10^9. Tumor growth was expressed as tumor fold increase over day 0 from treatment with CAR-T cells.

Statistical Analysis. Individual-level data are presented in data file S1. Statistical analysis was performed using GraphPad Prism 10.0.2. Unless otherwise specified, data are presented as mean ± SEM. Datasets were analyzed with paired or unpaired Student's t test, one-way or two-way analysis of variance (ANOVA), and the log-rank Mantel-Cox tests, depending on the experimental design. Appropriate statistical tests, and corrections for multiple testing, were applied as described in the figure legends. Biological replicates are indicated in figure legends as "independent donors," technical replicates are indicated as "independent samples." Differences with a P value < 0.05 were considered statistically significant.

Supplementary Materials

Materials and Methods

Figs. S1 to S12

Table S1

MDAR Reproducibility Checklist

Data File S1

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Competing interests: M.C. has received research support from Kite/Gilead and is a consultant of Alia Therapeutics. C. Bonini has been a member of advisory boards and a consultant for Intellia Therapeutics, TxCell, Novartis, GSK, Allogene, Kite/Gilead, Miltenyi, Kiadis and Janssen, and received research support from Intellia Therapeutics. B.G., R.E.K., F.D.G., L.F., B.C., F.C., C. Bonini and M.C. are inventors on different patents on cancer immunotherapy and genetic engineering. F.D.G. is currently an employee of Novartis Pharma AG. Her contribution to this work relates to the period 2020-2021 when she was an employee of San Raffaele Hospital (OSR). The present data are object of patent application owned by San Raffaele Hospital (OSR) and Università Vita-Salute San Raffaele and published as PCT/EP2023/081532. All other authors declare no competing interests.

Data and materials availability: All data associated with this study are present in the paper or the Supplementary Materials. All reagents will be made available to members of the research community upon reasonable request after completion of a material transfer agreement. Reagent requests should be directed to M.C. (casucci.monica@hsr.it).

Figures legends.

Fig. 1. CDH17 emerges as a lead CAR target for treatment of CRC-LM. (A and B) Schematic representation (A) and analytical workflow (B) of steps followed to identify cell surface CAR antigens overexpressed in patients with colorectal cancer liver metastases (CRC-LM) with minimal expression in healthy tissues. Candidate antigens remaining after each analytical step are shown in right boxes in (B). Schematics in (A) were created with BioRender.com. PDAC, pancreatic ductal adenocarcinoma. **(C)** Gene expression profile from CRC-LM patients of CAR target candidates is shown (n = 17). Data is expressed as decile matrix and top 30 genes expressed at highest deciles in more than 70% of patients are shown. **(D)** Expression profile of top 30 genes is shown in healthy tissues plotted using GTEx data. *CDH17* is denoted with an arrow. **(E)** Expression in transcripts per million (TPM) of *CDH17* in tumor (T, red) and adjacent normal tissue (N, gray) is shown from patients with colon adenocarcinoma (COAD, left) and rectum adenocarcinoma (READ, right). **(F)** Expression (TPM) of *CDH17* is shown during COAD progression from stage I to stage IV. In (E) and (F), expression data were retrieved using TCGA and GTEx databases using Gepia2. **(G and H)** Representative staining of tumor masses from patients with primary CRC (G, n = 20) and CRC-LM (H, n = 10) with CDH17 antibody. Original magnification 100X. P values (*P < 0.05) were determined by one-way ANOVA (E). Data are presented as quartiles and median values.

Fig. 2. CDH17 CARs carrying the A4_4R scFv perform better in vitro. (A) Schematic representation of steps followed to identify the optimal CAR construct and delivery route to target CDH17 in CRC-LM. IgG1h, IgG1 hinge; NMS, NGFR mutated short; NWL, NGFR wild-type long; TF, transcription factor; NFAT, Nuclear factor of activated T cells; GFP, green fluorescent protein; POC, proof-of-concept; CRS, cytokine release syndrome; OTOT, on-target off-tumor. **(B)** Schematics of second-generation anti-CDH17 CARs with different humanized single chain fragment variables (scFv; A4_4R and Lic3) and spacers (IgG1h, 15 amino acids; LNGFR-derived NMS, 140 amino acids.; NWL, 222 amino acids). All CARs contain a CD28 transmembrane and costimulatory domain and a CD3ζ signaling domain. **(C)** Frequency of $NFAT^+$ (GFP^+) cells among the indicated CDH17.28ζ$^+$ CAR-expressing Jurkat TPR cells after stimulation with LoVo ($CDH17^+$) cells (n = 2 independent experiments in technical replicates). **(D)** Killing of LoVo cells was quantified after coculture with the indicated healthy donor-derived CDH17.28ζ CAR-T cells at different effector-to-target (E:T) ratios (n = 3 donors). **(E)** Schematic representation of CDH17 protein and mutants. Mutants were designed by deleting each EC domain individually. MW, molecular weight; SP, signal peptide; TM, transmembrane; EC, extracellular cadherin; IC, intracellular domain. Schematics in panels (A, B, and E) were created with BioRender.com. **(F)** Flow cytometric representative plots showing NGFR and CDH17 in wild-type $CDH17^-$ SW620 cells transduced to express CDH17 mutants. Negative control is shown in black. PECY7, phycoerythrin-cyanine7; FITC, fluorescein-5-isothiocyanate. **(G)** Killing of SW620 cells expressing CDH17 mutants was quantified after coculture with CDH17-NWL.28ζ CAR-T cells at 1:5 E:T ratio (n = 3 donors). In (D) and (G), killing is expressed as elimination index (see Supplemental Materials and Methods). **(H)** Frequency of CDH17.28ζ CAR-T cells bound to AsPC-1 targets of total CDH17.28ζ CAR-T cells measured with acoustic force microfluidic microscopy (n = 3 donors from 3 separate microfluidic chips over separate days). Ut, untransduced. **(I)** Frequency of CDH17.28ζ CAR-T cells of total CDH17.28ζ CAR-T cells bound to AsPC-1 targets at 1000 pN acoustic force. P values (*P < 0.05; **P < 0.01; ***P < 0.001;

902 ****P < 0.0001) were determined by two-tailed t test (C), two-way ANOVA with Šidák's correction
903 for multiple comparisons (D), or one-way ANOVA with Dunnett's correction for multiple
904 comparisons (G and I). Data are presented as means ± SEM.

Fig. 3. The CDH17 CAR including the NMS spacer variant shows suboptimal activity in mice. **(A to J)** Efficacy of CDH17 CAR-T cells were tested against the AsPC-1 PDAC model (A to E) or the LoVo CRC model (F to J). **(A and F)** Timelines of PDAC (A) and CRC (F) in vivo experiments are shown. In the PDAC model, mice bearing subcutaneous (s.c.) AsPC-1 tumors expressing a secreted luciferase (Lucia) were treated intravenously (i.v.) with 10×10^6 T cells on day 4 (Ut, $n = 3$; CDH17-H.28ζ, $n = 8$; CDH17-NMS.28ζ, $n = 8$; CDH17-NWL.28ζ, $n = 7$). In the CRC model, mice bearing s.c. LoVo tumors expressing a secreted luciferase (Lucia) were treated i.v. with 10×10^6 T cells on day 6 (Ut, $n = 4$; CDH17-H.28ζ, $n = 8$; CDH17-NMS.28ζ, $n = 8$; CDH17-NWL.28ζ, $n = 7$). **(B and G)** Tumor growth of AsPC-1 (B) and LoVo (G) tumors was measured by bioluminescent analysis of blood samples. RLU, relative light unit. Gray regions denote basal RLU. **(C and H)** Kaplan-Meier survival plots are shown for mice bearing AsPC-1 (C) and LoVo (H) tumors. **(D and I)** Kinetics of circulating human CD45 (hCD45) cells retrieved from the blood of mice bearing AsPC-1 (D) or LoVo (I) tumors are shown. **(E and J)** Relative fluorescence intensity (RFI) is shown for HLA-DR CDH17-H.28ζ CAR-T cells retrieved from mice bearing AsPC-1 (E) or LoVo (J) tumors. P values (*$P < 0.05$; ***$P < 0.001$) were determined by log-rank Mantel-Cox test (C, H) and one-way ANOVA with Tukey's correction for multiple comparisons (E, J). Data are presented as means ± SEM.

Fig. 4. Systemic and loco-regional delivery of CDH17 CAR-T cells are equally effective. (A) Timeline of locoregional delivery in a model of tumor xenograft in the liver is shown. Briefly, mice were injected intrahepatic (i.h.) with LoVo tumor cells expressing a secreted luciferase (Lucia) and treated either intravenous (i.v.) or i.h. with 10×10^6 T cells on day 10 (Ut, $n = 4$; CDH17-H.28ζ i.v., $n = 5$; CDH17-H.28ζ i.h., $n = 5$; CDH17-NWL.28ζ i.v., $n = 5$; CDH17-NWL.28ζ i.h., $n = 5$). **(B)** Tumor burden was measured at day 11 after T cell infusion. **(C)** Kinetics of tumor growth was measured in mice treated with CDH17-H.28ζ (red, left) and with CDH17-NWL.28 ζ (blue, right) CAR-T cells. In (B) and (C), tumor growth was measured by bioluminescent analysis of blood samples. RLU, relative light unit. In (C), gray regions denote basal RLU. **(D)** Kinetics of circulating hCD45 cells retrieved from the blood of mice treated with CDH17-H.28ζ (red, left) or with CDH17-NWL.28ζ (blue, right) CAR-T cells. **(E)** Frequency of HLA-DR^+ cells among CDH17-H.28ζ (red, left) and in CDH17-NWL.28ζ (blue, right) CAR-T cells was evaluated at day 11 after infusion by flow cytometry. **(F)** Concentrations of serum IFN-γ was measured in mice treated with CDH17-H.28ζ (red, left) or CDH17-NWL.28 ζ (blue, right) CAR-T cells at day 4 after infusion. **(G)** Memory phenotypes of circulating CDH17 CAR-T cells were evaluated from the blood of mice at day 11 after infusion by flow cytometry. P values (****$P < 0.0001$) were determined by one-way ANOVA with Tukey's correction for multiple comparisons (B). Data are presented as means ± SEM.

Fig. 5. Loco-regional delivery of CDH17 CAR-T cells exacerbates CRS toxicity. (A) Timeline of the humanized model of tumor xenograft in the liver is shown. Briefly, SGM3 mice were infused with human cord blood derived hematopoietic stem and progenitor cells (HSPCs), injected intrahepatic (i.h.) with LoVo tumor cells expressing a secreted luciferase (Lucia) and treated either intravenous (i.v.) or i.h. with 5×10^6 T cells (Ut, $n = 4$; CDH17-H.28ζ i.v., $n = 7$; CDH17-H.28ζ i.h., $n = 7$; CDH17-NWL.28ζ i.v., $n = 7$; CDH17-NWL.28ζ i.h., $n = 7$). **(B)** Tumor growth was measured at day 7 after infusion. **(C)** Frequency of HLA-DR$^+$ T cells among CDH17-H.28ζ and CDH17-NWL.28ζ CAR-T cells was evaluated at day 15 after infusion. **(D)** Kinetics of tumor growth was measured in mice treated with CDH17-H.28ζ (red, left) and with CDH17-NWL.28ζ (blue, right) CAR-T cells. **(E)** Concentrations of serum IFN-γ from mice treated with CDH17-H.28ζ or CDH17-NWL.28ζ CAR-T cells were measured at day 10 after infusion. **(F and G)** Weight change (F) and Kaplan-Meier survival plot (G) is shown for mice following treatment with CDH17-H.28ζ (red, left) or with CDH17-NWL.28ζ (blue, right) CAR-T cells. **(H to J)** Concentrations of serum IL-6 (H), IP-10 (I), and MCP-1 (J) from mice treated with CDH17-H.28ζ or CDH17-NWL.28ζ CAR-T cells were measured at day 10 after infusion. **(K)** Frequency of CDH17$^+$ cells among hematopoietic cells is shown ($n = 3$ donors). **(L)** Killing of hematopoietic cells was quantified after coculture with either autologous or allogeneic CDH17-H.28ζ or CDH17-NWL.28ζ CAR-T cells at 1:10 effector-to-target (E:T) ratio ($n = 3$ donors of target cells, $n = 2$ donors of CAR-T cells). Dashed gray line denotes basal killing. **(M)** Kinetics of tumor growth was measured in mice treated with 5×10^6 T cells i.v. (Ut, $n = 2$; CDH17-NWL.28ζ, $n = 7$). In (B), (D) and (M), tumor growth was measured by bioluminescent analysis of blood samples. RLU, relative light unit. In (D) and (M), gray regions denote basal RLU. **(N)** Weight change is shown following treatment with T cells. In (F) and (N), dashed lines denote endpoint threshold for severe weight loss (≥ 25%). **(O to R)** Kinetics of circulating human NGFR$^+$ (O), CD19$^+$ (P), CD33$^+$ (Q) and CD14$^+$ (R) cells retrieved from the blood of mice treated with CDH17-NWL.28ζ CAR-T cells. NGFR$^+$ denote CAR-T cells. P values (*$P < 0.05$; **$P < 0.01$) were

964 determined by two-tailed t test (B, C, and H to J) and log-rank Mantel-Cox test (G). Data are presented
965 as means ± SEM. In violin plots, data are presented as quartiles and median values.

Fig. 6. CDH17 CAR-T cells control the growth of patient-derived tumor organoids in mice. (A) Schematics of functional testing of primary samples from patients with CRC-LM. Briefly, patient-derived CDH17.28ζ CAR-T cells were challenged in co-culture against autologous or allogeneic tumor organoids generated from surgically resected tumor samples. Schematics in panel were created with BioRender.com. **(B)** Expansion (fold increase) of Ut T cells, CDH17-H.28ζ CAR-T cells and CDH17-NWL.28ζ CAR-T cells manufactured from healthy donors or CRC-LM patients are shown **(C and D)** Frequencies of CD4 and CD8 T cells (C) and of memory T cells (D) at the end of manufacturing are shown. **(E)** Frequency of $CD20^+$ cells of total cells at day 9 from αCD3/CD28 bead stimulation is shown for CDH17-H.28ζ CAR-T cells. **(F)** Frequency of $NGFR^+$ cells of total cells at day 9 from αCD3/CD28 bead stimulation is shown for CDH17-NWL.28ζ CAR-T cells. From (B) to (F), Ut HD, n = 10; CDH17-H.28ζ HD, n = 10; CDH17-NWL.28ζ HD, ζ, n = 10; Ut CRC-LM, n = 3; CDH17-H.28ζ CRC-LM, n = 7; CDH17-NWL.28ζ CRC-LM, n = 7. **(G)** Target cell killing was measured against tumor organoids generated from surgically resected CRC-LM samples cocultured with autologous or allogeneic CDH17-H.28ζ and CDH17-NWL.28ζ CAR-T cells at 1:1 effector-to-target (E:T) ratio. Killing is expressed as elimination index (HD: n = 1 T cell donor in technical triplicate, n = 1 PDO. CRC-LM: n = 3 T cell donors in technical duplicate, n = 2 PDOs). **(H)** Schematics of the patient-derived xenograft (PDX) model is shown. Briefly, mice bearing intrahepatic CRC-LM PDOs expressing luciferase were treated intravenous (i.v.) twice with 10×10^6 T cells on day 3 and 7 (Ut, n = 7; CDH17-H.28ζ, n = 9; CDH17-NWL.28ζ, n = 10). **(I)** Tumor growth was measured by bioluminescent imaging and expressed as tumor fold increase. **(J and K)** Representative radiographic images (J) and tumor diameters (K) are shown at day 26 following treatment with either Ut T cells or with CDH17-H.28ζ or CDH17-NWL.28ζ CAR-T cells. In (J), red lines denote tumor lesions. **(L)** Kaplan-Meier survival plot is shown for mice in (H). **(M)** Kinetics of circulating hCD45 cells retrieved from the blood of mice in (H) are shown. **(N to P)** Frequency of $HLA\text{-}DR^+$ cells among CAR-T cells (N), frequency of each memory phenotype among CAR-T cells

(O), and CD4/CD8 ratio (P) of circulating CDH17-H.28ζ and CDH17-NWL.28ζ CAR-T cells was evaluated from the blood of mice at day 21 after infusion. P values (*P < 0.05; **P < 0.01) were determined by two-way ANOVA with Šidák's correction for multiple comparisons (C, O), two-tailed t test (F, G), or log-rank Mantel-Cox test (L). Data are presented as means ± SEM.

Fig. 7. CDH17 CAR-T cells do not react against healthy colon tissue slices. (A to C) Confocal microscopy images showing localization of CDH17 and Occludin in healthy colon (A), CRC (B), and CRC-LM (C) tissue. Original magnification 630X. **(D and E)** IFN-γ and TNF-α production were measured after 24 hours exposure of titrated CDH17-H.28ζ (D) or CDH17-NWL.28ζ (E) CAR-T cells to tissue slices derived either from CRC or autologous adjacent non-tumoral colon (n = 4 donors of primary tissues and n = 3 to 4 donors of CAR-T cells, in replicates). Cytokines were analyzed using LEGENDplex bead–based cytokine immunoassay. Data are presented as means ± SEM.

TABLE 1 | List of patient samples analyzed for CDH17 immunohistochemistry

Patient[#]	Tumor	CDH17 Expression (%)*
1	Primary CRC	50
2	Primary CRC	100
3	Primary CRC	100
4	Primary CRC	95
5	Primary CRC	80
6	Primary CRC	60
7	Primary CRC	95
8	Primary CRC	90
9	Primary CRC	95
10	Primary CRC	100
11	Primary CRC	100
12	Primary CRC	90
13	Primary CRC	95
14	Primary CRC	100
15	Primary CRC	100
16	Primary CRC	80
17	Primary CRC	95
18	Primary CRC	100
19	Primary CRC	70
20	Primary CRC	100
21	CRC-LM	100
22	CRC-LM	95
23	CRC-LM	90
24	CRC-LM	90
25	CRC-LM	95
26	CRC-LM	100
27	CRC-LM	95
28	CRC-LM	90
29	CRC-LM	50
30	CRC-LM	95

**calculated on neoplastic cells;*

CRC, colorectal cancer; CRC-LM, liver metastases from CRC