CAR T cells expressing a bacterial virulence factor trigger potent bystander antitumour responses in solid cancers

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Chimeric antigen receptor T cells (CAR T cells) are effective against haematologic malignancies. However, in solid tumours, their potency is hampered by local immunosuppression and by the heterogeneous expression of the antigen that the CAR targets. Here we show that CAR T cells expressing a pluripotent pro-inflammatory neutrophil-activating protein (NAP) from Helicobacter pylori trigger endogenous bystander T-cell responses against solid cancers. In mice with subcutaneous murine pancreatic ductal adenocarcinomas, neuroblastomas or colon carcinomas, CAR(NAP) T cells led to slower tumour growth and higher survival rates than conventional mouse CAR T cells, regardless of target antigen, tumour type and host haplotype. In tumours with heterogeneous antigen expression, NAP secretion induced the formation of an immunologically ‘hot’ microenvironment that supported dendritic cell maturation and bystander responses, as indicated by epitope spreading and infiltration of cytotoxic CD8+ T cells targeting tumour-associated antigens other than the CAR-targeted antigen. CAR T cells armed with NAP neither increased off-tumour toxicity nor hampered the efficacy of CAR T cells, and hence may have advantageous translational potential.

Therapies with adoptive transfer of autologous T cells engineered ex vivo with a chimeric antigen receptor (CAR) directed against CD19 are efficacious and approved for refractory and relapse B-cell leukaemia and lymphoma, while B-cell maturation antigen-directed CAR T-cell treatment is approved for multiple myeloma. In solid tumours, a particular tumour-associated antigen that CAR T cells could target is often heterogeneously expressed, which impairs the efficiency of CAR T-cell therapy. Furthermore, the immunosuppressive microenvironment in solid tumours obstructs CAR T-cell efficacy. Therefore, combating CAR target antigen heterogeneity and reducing immunosuppression is of utmost importance in improving CAR T-cell therapy of solid tumours.

Various approaches to reinforce the efficacy of CAR T cells against solid tumours have been evaluated, including engineering CAR T cells to produce endogenous enzymes, cytokines, chemokines that potentiate either CAR T cells or other host immune cells, or both. Many exogenous molecules of bacterial origin have strong immunomodulatory properties. One such protein is the neutrophil-activating protein (NAP) from Helicobacter pylori, which attracts innate immune cells, induces maturation and T helper type-1 (Th1) polarization of dendritic cells (DCs), and creates a local pro-inflammatory milieu with enhanced interleukin (IL)-12 production. We investigate and report that arming of CAR T cells with NAP can improve their activity in various solid tumour models.

Results

We use retroviral vectors (RV) (Fig. 1a) to engineer murine T cells. After validating inducible NAP expression (Supplementary Fig. 1), we evaluated the efficacy of CAR(NAP) T cells to target endogenously expressed murine CD19 and disialoganglioside (GD2) in two immunocompetent, syngeneic mouse models of cancer, A20 (lymphoma) and NXS2 (neuroblastoma) (Fig. 1b,c). Conventional CAR T cells and CAR(NAP) T cells exhibited similar cytotoxicity against both cell lines in vitro (Extended Data Figs. 1a,b and 2a–c), confirming that insertion of the NAP transgene did not compromise the cytotoxic capacity of CAR T cells. When evaluated in vivo, CAR(NAP) T cells controlled tumour growth and significantly prolonged the survival of tumour-bearing mice in both models (Fig. 1d–g, and Extended Data Figs. 1c–e and 2d–f). Approximately 75% of the mice became tumour-free when treated with CAR(NAP) T cells, while conventional CAR T-cell treatment only cured 30% of the mice with A20 lymphoma (Fig. 1d,e). Mice cured from A20 tumours were protected against rechallenge with the same tumour cell line (Fig. 1e), indicating the establishment of immunological memory. Decreased B-cell counts were observed in the blood of mice after treatment with CD19 targeting CAR(NAP) T cells but not in mice treated with conventional CAR T cells (Extended Data Fig. 1f), which is also a sign of potency of CAR(NAP) T cells. Of note, this enhanced efficacy of CAR(NAP) T cells was neither dependent on the nature of the target antigen, nor the tumour types or host haplotypes, as superior therapeutic efficacy was also observed for CAR(NAP) T cells in various tumour models, including NXS2-mCD19 neuroblastoma (Extended Data Fig. 3), Panc02-mCD19 pancreatic adenocarcinoma (Extended Data Fig. 4) and CT26-hPSCA colon carcinoma (Extended Data Fig. 5). These data confirm that CAR(NAP) T cells perform better than CAR T cells in vivo despite exhibiting similar potency in vitro.

We also investigated whether the potential immunogenicity of NAP can counteract the performance of CAR(NAP) T cells. Encouragingly, CAR(NAP) T-cell persistence was not affected, as CAR T cells were detected in the blood of approximately half of the tumour-free mice in both treatment groups (Supplementary Fig. 2). Importantly, the therapeutic efficacy of CAR(NAP) T cells was not diminished in mice with pre-existing anti-NAP antibodies.
Antigen heterogeneity is a major hurdle for CAR T-cell therapy (Supplementary Fig. 3). Additionally, repeated treatment with CAR(NAP) T cells did not result in elevated toxicity compared with conventional CAR T cells when assessing systemic cytokine release (Supplementary Fig. 4) or body weight (Supplementary Fig. 5). In conclusion, arming CAR T cells with NAP therefore constitutes a viable strategy for targeting various solid tumours.

Antigen heterogeneity is a major hurdle for CAR T-cell therapy of solid tumours\(^\text{19}\). To evaluate whether CAR(NAP) T cells can overcome antigen heterogeneity, we established a heterogeneous tumour model by subcutaneously injecting a mixture (1:1 ratio) of wild-type (antigen-negative) NXS2 cells and antigen-positive NXS2-mCD19 cells (Fig. 2a). CAR(NAP) T-cell treatment significantly reduced the growth of tumours with heterogeneous CAR-target antigen expression and prolonged mouse survival, while conventional CAR T-cell treatment failed to yield any therapeutic benefit compared with mock T-cell treatment (Fig. 2b,c and Supplementary Fig. 6). Furthermore, mice cured by CAR(NAP) T-cell treatment were protected against rechallenge with wild-type NXS2 cells, indicating establishment of protective bystander immunological memory (Fig. 2d–f). Tumour-infiltrating CD8\(^{+}\) T cells isolated from CAR(NAP) T-cell-treated mice exhibited an antigen-experienced memory-like (CD62L\(^{+}\), CD44\(^{+}\)) phenotype and were degranulated (CD107a\(^{+}\)) to a higher degree than those isolated from CAR T-cell-treated mice (Fig. 2d–f). Importantly, splenocytes isolated from CAR(NAP) T-cell-treated mice, but not those from CAR T-cell-treated mice, responded to wild-type (CAR-targeted antigen-negative) NXS2 tumour cell lysate (Fig. 2g and Supplementary Fig. 7), indicating systemic activation of an endogenous bystander T-cell response, probably explaining why cured mice were protected when rechallenged with wild-type NXS2 (Fig. 2c).

A key feature of the bystander T-cell response is epitope spreading, that is, activation of endogenous CD8\(^{+}\) T cells directed against antigens other than the antigen targeted by the CAR, which is vital for eradication of antigen-heterogeneous solid tumours\(^\text{19}\).

\(P \leq 0.05, **P \leq 0.001, ***P \leq 0.0001\). Precise \(P\) values are reported in Supplementary Table 3.
Fig. 2 | CAR(NAP) T-cell treatment induces epitope spreading and bystander CD8+ T-cell killing of tumour cells lacking the antigen targeted by the CAR. a. Treatment schedule of the mixed tumour model with 50% of tumour cells expressing the mCD19 CAR target antigen. b, c. Tumour size (mean) until the first mouse had to be killed (b) and mouse survival (Kaplan–Meier curve) after treatment and rechallenge (arrow) of cured mice with wild-type NXS2 tumour cells (c). The experiment was performed twice and all data were pooled. Tumour sizes were compared using two-way ANOVA, and survival curves were compared using log-rank test. d–f. Characteristics of tumour-infiltrating CD8+ T cells on day 18. Representative plots (d) and the percentage of memory-like (CD44+CD62L+) CD8+ T cells (e), and CD107a expression (f) on CD8+ T cells in treatment groups. Groups were compared using t-test. MFI, mean fluorescence intensity. g. Analysis of epitope spread as a consequence of treatment, with IFN-γ levels from co-cultures of splenocytes from treated mice and autologous DCs loaded with wild-type NXS2 (CD19−) tumour cell lysate. Values from co-cultures with unloaded DCs were seen as background and subtracted. Groups were compared using ANOVA with Bonferroni correction. h–j. Epitope spreading towards a non-targeted model antigen (OVA) for tumour-infiltrating CD8+ T cells after mCD19 CAR T-cell treatment of Panc02-mCD19-OVA tumours in C57BL/6 mice. Representative density plots (h) showing OVA(SIINFEKL)-specific CD8+ T cells and percentage (i) of these T cells in tumours from each group. Groups were compared using ANOVA with Bonferroni correction. j. IFN-γ production by splenocytes after mCD19 CAR T-cell treatment of NXS2-mCD19-OVA tumours in A/J mice upon rechallenge with different OVA peptides. In all panels, error bars represent s.e.m. (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001). Precise P values are reported in Supplementary Table 3.
We investigated epitope spreading using two tumour models: Panc02-mCD19-OVA and NXS2-mCD19-OVA, wherein ovalbumin (OVA) was introduced as a bystander antigen not targeted by the CAR T cells. Tumour infiltration of OVA-specific (SIINFEKL-directed) CD8+ T cells were significantly increased in mice treated with CAR(NAP) T cells compared with CAR
Finally, to test the applicability of human CAR(NAP) T cells, we performed an in vitro proof-of-concept validation in freshly isolated human peripheral blood mononuclear cells (PBMCs) (Fig. 4a). NAP was expressed by human CAR(NAP) T cells upon target-antigen recognition (Fig. 4b,c), and human CAR(NAP) T cells and conventional CAR T cells killed human cancer cells equally well in vitro (Fig. 4d), even though transduction efficacy was higher for CAR T cells than for CAR(NAP) T cells (Supplementary Fig. 8a). Phenotypic characterization of CAR(NAP) T cells and CAR T cells upon transduction revealed similar CD4 and CD8 (Supplementary Fig. 8b), memory and effector T-cell composition (Supplementary Fig. 8c), and activation/exhaustion phenotype (Supplementary Fig. 8d). However, upon target cell recognition in vitro, gene expression analysis (Fig. 4e) revealed a distinct gene expression pattern for CAR(NAP) T cells compared with conventional CAR T cells, with GO terms enriched for adaptive immune response, cellular response to IFN-γ, regulation of cytokine production, type-I IFN signalling, NK cell-mediated immunity and granulocyte migration (Fig. 4f,g). In addition, the supernatants from co-cultures of CAR(NAP) T cells and tumour cells expressing the antigen targeted by the CAR were rich in potent chemo-attractants that induced migration and activation of DCs (Fig. 4h and Extended Data Fig. 8), neutrophils (Extended Data Fig. 9) and monocytes (Extended Data Fig. 10), confirming the findings of the gene set enrichment analysis (Fig. 4f,g).

In the presence of autologous DCs, CAR(NAP) T cells exhibited higher cytotoxicity than conventional CAR T cells (Fig. 4i), with enhanced IFN-γ secretion (Fig. 4j) and degranulation (Fig. 4k). Furthermore, gene expression analysis revealed that genes representing pathways involved in cytokine-mediated signalling were upregulated (Supplementary Fig. 9), and a Th1 transcriptional profile was apparent (Fig. 4i). The levels of IL-12 (Fig. 4m) and those of various pro-inflammatory cytokines and chemokines (Fig. 4n) were increased in supernatants from the co-culture of CAR(NAP) T cells, DCs and tumour cells, verifying the observed gene expression profile. Taken together, these observations indicate that the cytotoxic and immunostimulatory capacity of human CAR(NAP) T cells are superior to those of conventional human CAR T cells, which is consistent with our findings in the various mouse models.

The above observations align with published data that NAP, provided as a recombinant protein21 or delivered as a therapeutic transgene,14,17 promotes DC maturation and Th1 polarization, and recruits and activates monocytes and neutrophils22. Accordingly, we propose that the enhanced therapeutic efficacy of CAR(NAP) T cells is associated with the ability of secreted NAP to attract and activate innate immune cells to kill tumour cells, and to activate DCs to facilitate the induction of epitope spreading, leading to CAR-target-independent CD8+ T-cell bystander immunity (Fig. 5).

Discussion

CAR T cells have been armed with several host-derived endogenous molecules (IL-12,15 IL-18,8–10, Flt3L11, IL-7/CCL1913, Heparinase14 and CD40L1) that engage host immune cells to counteract antigen

Fig. 4 | Human CAR T cells armed with NAP exhibit similar advantageous immunological characteristics as murine CAR(NAP) T cells. a, Self-inactivating (∆ΔU3) lentiviral constructs used for human T-cell engineering. b,c, Representative histogram (b) and MFI (c) of NAP expression in CAR-positive T cells upon target cell recognition. Dashed line, MFI of isotype staining. d, Relative viability of target cells after exposure to CAR-engineered T cells. e-g, Gene expression in CAR T cells and CAR(NAP) T cells after exposure to target cells. e, Schematic illustration of the experimental setting. f, GO term enrichment network of the uniquely upregulated genes in CAR(NAP) T cells according to Metascape analysis of the NanoString gene expression data. Numbers in brackets are −log10(P) values. g, Heat map of genes uniquely upregulated in CAR(NAP) T cells presented as normalized z-score of raw gene counts. h, Percentage of DCs migrating towards supernatant collected from a 5:1 co-culture of engineered T cells and Daudi target cells. i-k, Potency of CAR(NAP) T cells assisted by autologous DCs, presented as relative viability of target cells (i), IFN-γ secretion (j) and CD107a expression (k). l-m, CAR-engineered human T cells were co-cultured with autologous immature DCs and Daudi target cells (5:1) and assayed for T-cell polarization (l), and production of IL-12 (m) and other cytokines and chemokines (n). All experiments were performed three times and data were pooled. Error bars represent s.e.m. (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). Precise P values are reported in Supplementary Table 3.
**Figure a:** Diagrams showing the expression of CAR (CD19) in CAR-T cells compared to LV (mock) and LV (CD19CAR) transduced cells.

**Figure b:** Graphs illustrating the DC migration (%), NAP expression (MFI, ×10^3), and cytokine-mediated signalling (31).

**Figure c:** Graphs showing the Relative viability of target cells (%), IL2 (pg ml–1) and T-bet/GATA3 ratio.

**Figure d:** Graphs depicting the IFN-α/β (pg ml–1), T-bet/GATA3 ratio, and IL-12 (pg ml–1).

**Figure e:** Diagrams illustrating the in vitro co-culture of hCD19 CAR-T with Daudi (CD19^+) cells.

**Figure f:** Diagrams showing the regulation of cytokine production (11) and Type-I IFN signalling (13.5).

**Figure g:** Graphs illustrating the NanoString mRNA profile of the CAR-T cells.

**Figure h:** Graphs showing the IL-12 (pg ml–1) and T-bet/GATA3 ratio.

**Figure i:** Graphs depicting the relative viability of target cells (%).

**Figure j:** Graphs illustrating the DC migration (%).

**Figure k:** Graphs showing the CD3^+ CAR+ T cells.

**Figure l:** Graphs depicting the Relative viability of target cells (%).

**Figure m:** Graphs illustrating the IL-12 (pg ml–1).

**Figure n:** Graphs showing the regulation of hemopoiesis (7), adaptive immune response (13) and cytokine-mediated signalling (31).
heterogeneity in solid tumours. These factors primarily work by engaging antigen-presenting cells. Here we demonstrate that expression of a bacteria-derived virulence factor by CAR T cells can mediate bystander immunity with epitope spreading, leading to eradicating antigen-heterogeneous tumours. Our results suggest that NAP engages a broader range of immune effectors, for example, DCs, monocytes, NK cells and neutrophils to potentiate stronger antitumour immunity. NAP has been shown to substantially enhance the immunogenicity of poor immunogens. This can be favourable as our data suggest that the immunogenicity of NAP can reverse the suppressive immune landscape in solid tumours. On the other hand, NAP immunogenicity can also be unfavourable, as immune response against NAP may hamper the persistence of the CAR(NAP) T cells, similar to what has been observed for T cells expressing other foreign proteins, or cause adverse effects such as anaphylaxis. However, we show that persistence and efficacy of CAR(NAP) T cells were not altered in mice pre-immunized to have anti-NAP antibodies. It is even possible that pre-existing anti-NAP immunity can neutralize potential NAP-related systemic toxicity. However, the actual situation needs to be evaluated in a careful dose-escalation clinical study with CAR(NAP) T cells.

We used an inducible nuclear factor of activated T-cells (NFAT) promoter to control NAP expression as proof of concept. The same promoter was used in a γ-retroviral vector to control IL-12 expression in a clinical trial evaluating IL-12-armed tumour-infiltrating lymphocytes. Toxicities observed in patients in that trial were mainly attributed to basal level of systemic IL-12 expression. Therefore, other tightly regulated promoters may be worth further investigation. Whether NAP expressed from CAR(NAP) T cells would cause toxicity in a similar fashion as IL-12 in cancer patients is unknown. However, analysis of systemic cytokine levels in blood and body weight of mice during treatment did not show any CAR(NAP) T-cell-treatment-related toxicity. Furthermore, to the best of our knowledge, there are no reports describing NAP-related toxicity in patients with H. pylori-induced gastric inflammation, which further supports a safe profile of using NAP as a therapeutic transgene in CAR T cells.

Arming CAR T cells with a potent bacterial virulence factor is a unique concept in CAR T-cell design when compared with using host-derived factors. There may be other pathogen-derived factors that are as effective, therefore our study opens up possibilities for evaluating intricate multifaceted pathogen-derived immune activators as ammunition for CAR T cells. We demonstrate that arming CAR T cell with NAP improves the treatment of solid tumours with heterogenous antigen expression. This approach may be used to engineer CAR T cells regardless of the CAR target antigen and tumour types, thus expanding translatability to various human cancers.
Methods

Antibodies. Detailed information on the antibodies used in this study is provided in Supplementary Table 1.

Primary cell isolation and cell culture. The human B-cell lymphoblast cell line Dauid (ATCC CL-213), human lymphoma cell line BC-3 (ATCC CRL-2277) and murine lymphoma cell line A20 (ATCC TIB-208) were cultured in RPMI-1640 medium supplemented with 1 mM sodium pyruvate, 100 μM l-penicillin, 100 μg/ml streptomycin (1% PenSt) and 10% (v/v) heat-inactivated foetal bovine serum (FBS). The mouse neuroblastoma cell line NXS2 (GD2), a gift from Holger N. Lode, University of Greifswald, mouse pancreatic cancer cell line Panc2 (a gift from Rainer Heuchel, Karolinska Institute) and mouse colon carcinoma cell line CT26 (ATCC CRL-6388) were cultured in DMEM supplemented with 1 mM sodium pyruvate, 1% PenSt and 10% FBS.

To generate NXS2-mCD19 and Panc2-mCD19 cell lines stably expressing mouse CaI (NM_0010844.2), NXS2 cells and Panc2 cells were transduced with a lentiviral vector (LV) pBMIN(mCD19-T2A-FLuc-F2A-puro) encoding murine CaI, and selected on 5 μg/ml puromycin. The expression of mouse CaI was determined by flow cytometry (CytoFLEX LX, Beckman Coulter) using an anti-murine CaI antibody. To generate CT26-hPSCA stably expressing human prostate stem cell antigen (hPSCA), CT26 cells were transduced with a lentiviral vector pBMIN(hPSCA-T2A-FLuc-F2A-puro) encoding human PSCA, and selected on 10 ng/ml puromycin. To generate ovulamin (OVA)-expressing target cells, NXS2 and Panc2 cells were first transduced with a lentiviral vector pBMIN(OVA-T2A-FLuc-F2A-puro) encoding full length chicken ovalbumin (NM_205152.2), and selected on 5 μg/ml puromycin. The cells were then transduced with pBMIN(mCD19(T2A-FLuc-F2A-puro) as mentioned above and CaI-positive cells were sorted by flow cytometry (BD FACs Melody, BD Biosciences). All cell culture reagents were from Thermo Fisher, unless stated otherwise.

PBMCs were isolated using Ficoll–Paque (GE Healthcare) from freshbuffy coats of healthy anonymized donors at the Blood Centre at Uppsala University Hospital. PBMCs were cultured in RPMI-1640 supplemented with 10% FBS, 1 mM sodium pyruvate and 1% PenSt. CD14+ cells in the PBMC pool were isolated using specific beads (Miltenyi Biotec) and differentiated over 5 d into immature DCs (imDCs) in the presence of 30 ng/ml granulocyte-macrophage colony-stimulating factor (Gentauro) and 25 ng/ml IL-4 (Gentauro). Fresh medium with cytokines was replaced every 2 d. All cells were cultured in a humidified incubator under a 5% CO₂ atmosphere at 37 °C.

Vector design, retroviral and lentiviral production. The murine CD19-targeting CAR sequence, containing murine CD3z and CD28 signalling domains, has been described previously. The transgene sequence was placed under the control of an inducible NFAT-IL-2 promoter, designed as described previously. The orientation of the Nap promoter was opposite to that of the minimal IL-2 promoter. The transgene sequence was placed under the control of a murine IL-2 promoter31. The orientation of the scFv sequence was described previously. The CD19 scFvs in RV(CD19CAR) were expressed using the human PSCA CAR antibody clone 14G2a) was kindly provided by Dr Eric Yvon and Dr Malcolm Brenner from Baylor College of Medicine (Houston, TX). The human CaI scFv sequence was described previously. The CD19 scFvs in RV(CD19CAR) or RV(NAP-CARD19CAR) constructs were replaced with corresponding scFvs to generate RV(GD2CAR), RV(NAP-GD2CAR), RV(hPSACAR) and RV(NAP-hPSACAR). All constructs were sequenced and subcloned by Genscript. Retroviruses were produced using a packaging plasmid pCL-Eco (Inder Verma, Addgene plasmid 12371) and Gryphon retroviral packaging cell line (BioLegend). Stained cells were analysed in a CytoFLEX S flow cytometer (Beckman Coulter) and data were analysed using FlowJo v10.2.0.8.1.

Adoptive transfer of murine CAR T cells in murine tumour models. Spleens from C57Bl/6NRj mice (n = 12). For tumour microenvironment analysis, tumour tissues were collected for analysis 3–5 d after the last treatment (n = 3). NXS2-GD2 model. Female A/J mice (6–8 weeks old) were inoculated s.c. with 10⁵ NXS2 cells in 100 μl DPBS into the right hind flank. The mice were then treated with 3 × 10⁵ T cells injected into the lateral tail vein 4, 8 and 12 d after tumour cell implantation. The mice were monitored regularly and euthanized upon reaching the humane endpoint (mock T cells: n = 6, CAR T cells and CAR(NAP) T cells: n = 7). For tumour microenvironment analysis, tumour tissues were collected for analysis 3–5 d after the last treatment (n = 3).

NXS2-mCD19 model. Female A/J mice (6–8 weeks old) were inoculated s.c. with 10⁵ NXS2 cells in 100 μl DPBS into the right hind flank. The mice were then treated with 3 × 10⁵ T cells injected into the lateral tail vein 4, 8 and 12 d after tumour cell implantation. The mice were monitored regularly and euthanized upon reaching the humane endpoint. The experiment was repeated twice and all data were pooled together (mock T cells: n = 16, CAR T cells and CAR(NAP) T cells: n = 17).
NXS2 and NXS2-mCD19 mixed tumour model. Female A/J mice (6–8 weeks old) were inoculated s.c. with 1 × 10⁶ tumour cells (1:1 mixture of NXS2 and NXS2-mCD19 cells) in 100 µl DPBS into the right hind flank. The mice were then treated with 3 × 10⁹ T cells injected into the lateral tail vein 4, 8 and 12 d after tumour cell implantation. For survival analysis, the mice were monitored regularly and euthanized upon reaching the humane endpoint. The experiment was repeated twice and all data were pooled together (mock T cells: n = 17, CAR T cells: n = 17, CAR(NAP) T cells: n = 13). For tumour microenvironment analysis, tumour tissues were collected for analysis 7 d after the last treatment (n = 3). For recall assay, spleens were collected for analysis 7 d after the last treatment (n = 4).

**Survival analysis.** The animals were monitored individually for tumour growth and body weight until humane endpoints were reached or until the tumour volume exceeded the study endpoint volume (EPV, 1,000 mm³); tumour size was calculated as volume (mm³) = length × width²/2. The time until tumour growth exceeded the study endpoint volume (EPV) was calculated as TTE (log(EPV)–b)/m, where the constant b is the intercept and m is the slope of the line obtained by linear regression (time vs tumour volume) of a log-transformed tumour growth data set, which comprised the first measured tumour volume when EPV was exceeded and three consecutive measured tumour volumes immediately before the attainment of EPV. Any animal determined to have died from treatment-related causes was assigned a TTE value equal to the day of death. Any animal that died from non-treatment-related causes was excluded from the analysis. Survival curve was generated on the basis of the TTE value using the Kaplan–Meier method, and compared using the log-rank (Mantel–Cox) test.

**Rechallenge model.** Cured mice after treatment in the A20 model and the NXS2/NXS2-mCD19 mixed model, and newly purchased naive mice (as control) were inoculated s.c. with corresponding tumour cells (2 × 10⁶ A20 or 1 × 10⁶ NXS2) in 100 µl DPBS into the left hind flank. Tumour growth was monitored regularly until tumour size in the control group reached the humane endpoint (1,000 mm³).

Mouse T-cell cytotoxicity assay and IFN-γ ELISA. The cytotoxicity assay was performed using luciferase-expressing target cells. Mouse splenocytes were collected, activated and transduced with CAR-encoding retroviral vectors as described above. The CAR-engineered T cells were co-cultured with firefly luciferase-tagged target cells at indicated ratios for 5 d, in a total volume of 200 µl in a 96-well plate. Luciferase expression and activity (as an indicator of target cell viability) were determined using ONE-Glo reagent (Promega) as previously described. Specific lysis of each sample was calculated using the luminescence of co-cultured samples against target cells alone. The supernatants were collected after 18 h of co-culture, and IFN-γ levels determined by ELISA using mouse IFN-γ ELISA kit (Mabtech).

**Analysis of CD45⁺ tumour-infiltrating cells.** NanoString. Tumour samples were collected from NXS2-mCD19 animals on day 18 of the experiment. The samples were collected and enzymatically digested (Liberase, Roche) into single-cell suspensions. CD45⁺ cells were sorted using BD FACs Melody (BD Biosciences) and RNA was isolated from these cells (RNeasy Plus mini kit, Qiagen). Then, mRNA levels were directly measured using the Mouse-Pan cancer immune-oncology kit from NanoString nCounter gene expression system (NanoString). Differential expression analyses of mRNA were performed using nSolver analysis software (NanoString) and visualized by ClustVis™. Gene Ontology (GO) annotation analyses of the top 200 most upregulated with ≥2-fold enrichment (in comparison to tumour-infiltrating cells) were calculated using the Immune Epitope Database (IEDB). The top 12 peptides predicted using the Immune Epitope Database (IEDB) tool. The top 12 peptides were used for the Epitope spreading assay.

**Immunohistochemistry analyses of the tumour microenvironment.** Frozen tumour tissues were collected from the A20 model after treatment and sliced into 6 µm sections. The sections were fixed with ice-cold acetone (Sigma-Aldrich) for 15 min and dried. The slides were dehydrated with PBS for 3 min (repeated 3 times), followed by blocking with 3% BSA-PBS for 2 h at rt. The sections were stained with antibodies listed in the murine tumour immune cells staining panel (Supplementary Table 1): CD4 (1:200), GR1 (1:200) and MPO (1:100), CD4 (1:500) and FoxP3 (1:50), at 4 °C overnight, values in parentheses are antibody dilution factors in the final working solution. The sections were washed twice with PBS-T and incubated with Alexa-488 streptavidin (1:1000) for 30 min to visualize the biotin-conjugated MPO antibody. The sections were then washed twice with PBS-T, stained with Hoechst 33342 (Thermo Fisher) for 15 min at rt, and mounted with Fluoromount-G (Southern Biotech). The sections were imaged in a Zeiss AxioImager microscope (Zeiss).

**Human T-cell transduction and expansion.** Human PBMCs (5 × 10⁵) isolated as described above were stimulated with 3 µg/ml OLT-3 (100 µg/ml, BioLegend) in a culture medium containing IL-2 (100 µU/ml), Pulexulin, Novartis). Activated T cells (5 × 10⁵) were seeded in 2 × 10⁶ U/mL human serum, supplementing with 10 mg ml⁻¹ protamine sulphate (Sigma-Aldrich) and IL-2 (100 U/ml) for 24 h, and then cultured for 3 days with 10 µg/ml monolayer of 100 µl total volume, and incubated for 4 h. Then, 1 ml of fresh culture medium was added. The T cells were transduced again the following day in the same manner, and then cultured in 1 ml of culture medium containing IL-2 (100 U/ml) for 7 d. Before further experiments, T cells were expanded for 2 weeks using a rapid expansion protocol as previously described.

**Human T-cell cytotoxicity assay.** The cytotoxicity assay was performed using luciferase-expressing target cells. CAR-engineered human T cells were allowed to rest for 3 d after rapid expansion in a medium containing a low dose of IL-2 (200 U/ml) to 1% IL-2 (200 U/ml) before being used for functional assays. The T cells were co-cultured with firefly luciferase-targeted cells (Daudi, CD19⁺; at a 5:1 ratio), or with Daudi and autologous imDCs (at a 5:1:1 ratio) for 4 d, in a total volume of 200 µl in a round-bottom 96-well plate. Luciferase expression and activity (as an indicator of target cell viability) were determined using ONE-Glo reagent (Promega) as previously described. Specific lysis of each sample was calculated using the luminescence of co-cultured samples against target cells alone.

**NAP expression in human CAR(NAP) T cells.** NAP secretion by CAR(NAP) T cells was assessed after co-culture with Daudi cells (CD19⁺). The cells were mixed at a ratio of 5:1 and cultured for 24 h. NAP secretion was then blocked by 12 h incubation with BreFledin A (BD Biosciences). Before using for functional assays. The T cells were then stained for NAP expression using antibodies in the human CAR T cell panel (Supplementary Table 1): CD3, primary anti-NAP antibody (1:10) and a secondary monochromatic anti-anti-IgG antibody (1:500), rabbit anti-mouse IgG(1+H+) Fab fragment (CAR expression). Stained cells were analysed using a CytoFLEX S flow cytometer (Beckman Coulter) and data were analysed using FlowJo v10.2-v10.8.1.
CAR-engineered T-cell characterization. The CAR expression level was detected using F(ab’), fragment goat anti-mouse IgG (Jackson ImmunoResearch). Cells were also stained for surface markers CD3, CD4 and CD8 as antibodies listed in CAR expression in the human CAR T-cell panel (Supplementary Table 1). CAR-positive T cells were gated on CD3+ T-cell population. CD8 and CD4 T-cell compositions were gated on the CAR+CD3+ population.

LV-engineered human T cells were stained for CD3, CD4, CD8, CAR, CD45RA and CD62L at 7 d after viral transduction to evaluate memory effector phenotype using antibodies listed in the human T-cell memory/effector phenotype panel (Supplementary Table 1). In yet another setting, these cells were stained for CD3, CD4, CD8, CAR, PD1 and TIM3 to evaluate exhaustion phenotype using antibodies listed in the exhaustion phenotypic human T-cell panel (Supplementary Table 1).

Phenotypic analysis of immune cells in vitro. Phenotypic analysis of human DCs after exposure to the supernatant from a co-culture of engineered T cells and target cells was performed using antibodies staining: CD14, CD1a, CD83, and CD86 and target cells was performed using antibodies staining: CD14, CD1a, CD83, CD80, CD86 and CD70 (human DC markers panel in Supplementary Table 1). Engineered human T cells were co-cultured with Daudi and autologous imDCs (at a 5:1:1 ratio) for 20–24 h. The Th1/Th2 phenotype of human T cells was analysed by staining of T cells with the surface markers: CD3, CD4 and CD8 (human T-cell identification panel in Supplementary Table 1), followed by 1 h cell permeabilization with True-Nuclear transcription factor buffer set (BioLegend), and staining with antibodies for T-bet and GATA3. The T-bet/GATA3 ratio was then calculated for the CD3+CD4+ T-cell population.

Cytokine secretion and CD107a degranulation. Engineered human T cells were co-cultured with Daudi target cells (at a 5:1 ratio), or with Daudi and autologous imDCs (at a 5:1:1 ratio) for 20–24 h. The supernatant was collected, and IFN-γ or IL-12 levels were analysed by ELISA (Mabtech). CAR T cells were stained with antibodies for: CD3, goat anti-mouse IgG (H+L) Fab fragment (CAR expression) and CD107a (human T-cell degranulation panel in Supplementary Table 1) before evaluation by flow cytometry (BD Canto II, BD Biosciences). The CD107a+ T-cell population was then calculated as a percentage of the CAR+ T-cell population.

Cytokine profiling. Tumours were collected from NXS2-mCD19 animals on day 19 after tumour implantation, and frozen on dry ice. The tumours were then treated with 500 μl RIPA buffer (Thermo Fisher) and sonicated twice at 40% amplitude for 30 s, with 30 s intervals. The tumour samples were rotated (end over end) for 30 min at 4°C, and centrifuged at 1,500 r.p.m. for 10 min. The supernatants were collected and analysed using Proteome Profiler mouse cytokine array kit (R&D Systems).

Migration assay. Cell migration was assessed using a 96-well ChemoTx disposable chemotaxis system (Neuro Probe) with a polycarbonate membrane filter (3 μm pore size). Supernatant from 4 d co-cultures of the human CAR T cells with Daudi target cells and autologous imDCs (mixed at a 5:1:1 ratio) was collected, and the cytokine and chemokine levels were analysed using the Proteome Profiler human cytokine array kit (R&D Systems).

Long-term persistence analysis of CAR T cells and CAR(NAP) T cells. Blood samples were collected on day 70 from tumour-free mice in the A20 model (CAR T cells: n = 6 and CAR(NAP) T cells: n = 8), and on day 60 from mice immunized to have pre-existing anti-NAP antibodies (pre-immunized: n = 4 and non-immunized: n = 5). RNA was isolated as mentioned above and analysed using qPCR to detect the presence of CAR T cells, with primers specifically targeting the CAR molecule: pF: 5’-CTCATGGAAGCACCACAAAGGCG-3’ and pR: 5’-CCTCTTGTTCTTGCTGAGGGTGTTGAG-3’; GFPDH gene expression (detected with primers: pF: 5’-ACCACAGTCATGCCATCATC-3’ and pR: 5’-TCCACACCCTGTGCTGTGCTA-3’); set to 4-fold change) and visualization were performed as with murine samples mentioned above.

Detected cytokines in the blood. Female Balb/c mice (6–8 weeks old) were inoculated with 2 × 106 A20 tumour cells in 100 μl DPBS into the right hind flank. The mice were treated with 3 × 105 T cells injected into the lateral tail vein on days 10 and 14. For survival analysis, the mice were monitored regularly and euthanized upon reaching the humane endpoint. The experiments were repeated 2 times and data were pooled for analysis (CAR T cells: n = 14 and CAR(NAP) T cells: n = 13).

Ethical approval. The Uppsala Research Animal Ethics Committee approved all animal studies (N164/15; N185/16; 5.8.18-19434/2019). The human buffy coats obtained from healthy blood donors were anonymized.

Statistical analyses. The data are reported as mean ± s.e.m. Statistical analysis was performed using GraphPad Prism software version 6.07-9.3.1. Statistical analyses in all figures, unless otherwise specified in Supplementary Table 3, were performed using multiple comparison of parametric analysis of variance (ANOVA) (more than two data groups) with recommended post-hoc corrections, or non-parametric t-tests (only two data groups compared). Values of P < 0.05 were considered to be statistically significant and precise P values are reported in Supplementary Table 3.

Data availability. The main paper supporting the results in this study are available within the paper and its Supplementary Information. Source data for the tumour growth curves are provided with this paper. All data generated in this study are available from the corresponding authors on reasonable request. Source data are provided with this paper.

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References
1. Abramson, J. S. et al. Liscata-bagene maraleucel for patients with relapsed or refractory large B-cell lymphomas (TRANSCEND NHL 001): a multicentre seamless design study. Lancet 396, 839–852 (2020).
Extended Data Fig. 1 | Therapeutic effect of mCD19-directed murine CAR T cells and CAR(NAP) T cells against mouse lymphoma (A20) in vitro and in vivo. (a) Expression level of mouse CD19 on A20 cells cultured in vitro (C) and isolated from resected subcutaneously growing A20 tumours (T). (b) The cytotoxic effect of engineered mCD19-targeted murine CAR T cells against in vitro-cultured A20. Error bars represent SEM (**: \( P < 0.001 \)). (c-e) Tumour size of individual mice after treatment with (C) Mock T cells, (D) CAR T cell, and (E) CAR(NAP) T cell treatment. (f) B-cell (CD19+) count in peripheral blood in mice treated with engineered T cells (n = 4, *: \( P < 0.05 \)). Precise \( P \)-values are reported in Supplementary Table 3.
Extended Data Fig. 2 | Therapeutic effect of GD2-directed murine CAR T cells and CAR(NAP) T cells against mouse neuroblastoma (NXS2) in vitro and in vivo. (a) Expression level of GD2 on NXS2 cells cultured in vitro (C) and isolated from resected NXS2 subcutaneously growing tumours (T). (b) The cytotoxic effect of engineered GD2-targeted murine T cells against NXS2 cells in vitro, and (c) IFN-γ secretion by engineered T cells upon antigen encounter. Error bars represent SEM (n.s.: no statistical significance, ****: \( P < 0.0001 \)). (d-f) Tumour size of individual mice after treatment with Mock T cells (D), CAR T cells (E), and CAR(NAP) T cells (F). Precise \( P \)-values are reported in Supplementary Table 3.
Extended Data Fig. 3 | Therapeutic effect of mCD19-directed murine CAR T cells and CAR(NAP) T cells against NXS2-mCD19 in vitro and in vivo. (a) Expression level of murine CD19 on wild-type NXS2 cells and NXS2 engineered to express murine CD19 (NXS2-mCD19), both cultured in vitro (C) and isolated from resected subcutaneous tumours (T). (b) The cytotoxicity of engineered mCD19-directed murine T cells against NXS2-mCD19 cells in vitro, and (c) IFN-γ secretion by engineered T cells upon antigen encounter. Error bars represent SEM. (d) Treatment schedule for the subcutaneous tumour model. (e) Tumour size (mean) and (f) mouse survival (Kaplan-Meier curve) after treatment. Groups were compared by using the log-rank test (*: $P < 0.05$, **: $P < 0.001$, ***: $P < 0.0001$). (g-i) Tumour size of individual mice after treatment with (G) Mock T cells, (H) CAR T cells, and (I) CAR(NAP) T cells. Precise $P$-values are reported in Supplementary Table 3.
Extended Data Fig. 4 | Therapeutic effect of mCD19-directed murine CAR T cells and CAR(NAP) T cells against Panc02-mCD19 in vitro and in vivo. (a) Expression level of murine CD19 on wild-type Panc02 and Panc02 engineered to express murine CD19 (Panc02-mCD19), both cultured in vitro (C) and isolated from resected subcutaneous tumours (T). (b) The cytotoxicity of engineered murine T cells against Panc02-mCD19 in vitro. (c) Treatment schedule for the subcutaneous tumour model. (d) Tumour size (mean) and (e) mouse survival (Kaplan-Meier curve) after treatment. Groups were compared by using the log-rank test. Error bars represent SEM (n.s.: no statistical significance, *: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001). (f-h) Tumour size of individual mice after treatment with (F) Mock T cells, (G) CAR T cells, and (H) CAR(NAP) T cells. Precise P-values are reported in Supplementary Table 3.
Extended Data Fig. 5 | Therapeutic effect of hPSCA-directed murine CAR T cells and CAR(NAP) T cells against CT26-hPSCA in vitro and in vivo. (a) Expression level of human prostate stem cell antigen (hPSCA) on wild-type CT26 and CT26 engineered to express human PSCA (CT26-hPSCA) cultured in vitro. (b) The cytotoxicity of engineered hPSCA-targeted murine T cells against CT26-hPSCA tumour cells in vitro, and (c) IFN-γ secretion by engineered T cells upon antigen encounter. Error bars represent SEM. (d) Treatment schedule for the subcutaneous tumours. (e) Tumour size (mean) and (f) mouse survival (Kaplan-Meier curve) after treatment. Groups were compared by using the log-rank test (n.s.: no statistical significance, *: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001). (g–i) Tumour size of individual mice after treatment with (G) Mock T cells, (H) CAR T cells, and (I) CAR(NAP) T cells. Precise P-values are reported in Supplementary Table 3.
Extended Data Fig. 6 | Characterization the tumour-infiltrating T cells in the NXS2-mCD19-OVA model in A/J mice after treatment. (a) tSNE analysis and unsupervised clustering of pooled tumour-infiltrating CD8+ T cells revealed six populations (Pop1-6) clustered (left) based on expression of phenotypic markers and their distribution across treatment groups (right). (b) Median expression of each marker (Z-score-transformed) in Pop1-6. (c) Mean percentage of CD8+ T cells within Pop1-6. Asterisk indicating comparison to the CAR(NAP)-T group (*: P < 0.05, **: P < 0.01). (d) tSNE plots showing expression of surface markers on tumour-infiltrating CD8+ T cells. (e) Percentage of tumour-infiltrating CD8+ T cells with single, double, and triple positive expression of LAG3, PD-1, and TIM3. Precise P-values are reported in Supplementary Table 3.
Extended Data Fig. 7 | CAR(NAP) T cell treatment alters immune cell infiltration in the A20 lymphoma tumour model. (a) Experimental set-up. (b-g) Immune cell infiltration in tumours harvested on day 25 after tumour cell implantation from different treatment groups, analysed by fluorescence staining. (b) Representative images and (c) quantification of tumour-infiltrating CD8+ T cells. (d) Representative images and (e) quantification of Gr1+MPO+ neutrophils. (f) Representative images and (g) quantification of CD4+FoxP3+ T cells. Scale bars in all larger panels, 50 μm; scale bars in insets, 20 μm. Error bars represent SEM (*: P < 0.05, **: P < 0.01). Precise P-values are reported in Supplementary Table 3.
Extended Data Fig. 8 | NAP secreted by activated CAR(NAP) T cells promotes monocyte-derived DC maturation and activation. (a) Illustration of experimental design for assessment of DC maturation and activation in vitro. (b–e) Surface marker expression on DCs after 48 h culture in supernatants from co-cultures of engineered T cells and target tumour cells (5:1). All experiments were repeated and data were pooled. Error bars represent SEM (*: P < 0.05, **: P < 0.01). Precise P-values are reported in Supplementary Table 3.
Extended Data Fig. 9 | NAP secreted by activated CAR(NAP) T cells promotes neutrophil recruitment and activation. (a) Overview of experimental design for assessing neutrophil recruitment and activation in vitro. (b) Percentage of neutrophil migration towards the supernatant from different co-cultures of engineered T cells and target tumour cells (5:1). (c–e) Cytokine levels (IL-12, IL-1β, and IFN-γ) in supernatant of activated neutrophils, determined by ELISA. (f–i) Cell surface markers on neutrophils (gated as CD15⁺ cells) after 48 h co-culture, assessed by flow cytometry. All experiments were repeated and data were pooled. Error bars represent SEM (*: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001). Precise P-values are reported in Supplementary Table 3.
Extended Data Fig. 10 | NAP secreted by activated CAR(NAP) T cells promotes monocyte differentiation and activation. (a) Overview of experimental design for assessing monocyte differentiation and activation in vitro. (b) Percentage of monocyte migration towards the supernatant from different co-cultures of engineered T cells and target tumour cells. (c) Monocyte differentiation into immature DCs, presented as a percentage of CD14+ cells (monocytes) remaining after 4 d co-culture and (d) the expression of CD1a on CD14− (differentiated) cells. (e−h) Expression of DC activation markers on differentiated monocytes (gated as CD14−CD1a+). All experiments were repeated and data were pooled. Error bars represent SEM (**: P < 0.01). Precise P-values are reported in Supplementary Table 3.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- n/a | Confirmed
- □ | The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- □ | A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
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  * Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
- □ | A description of all covariates tested
- □ | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons
- □ | A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- □ | For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted
  
  * Give P values as exact values whenever suitable.*
- □ | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- □ | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- □ | Estimates of effect sizes (e.g. Cohen's d, Pearson's r), indicating how they were calculated

*Our web collection on statistics for biologists contains articles on many of the points above.*

Software and code

Policy information about availability of computer code

Data collection
- Flow cytometry: BD Diva; CytExpert; BD FACSChorus; BD LSRFortessa
- Microscopy: Zeiss AxioImager
- Protein multiplexing imaging: BioRad Imager

Data analysis
- Graphpad v6.07 - v9.3.1;
- Nanostring: nSolver 4.0
- GO analysis: Metascape
- Flow: FlowJo v10.2 - v10.8.1

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

The main data supporting the results in this study are available within the paper and its Supplementary Information. Source data for the tumour-growth curves are provided with this paper. All data generated in this study are available from the corresponding authors on reasonable request.
**Field-specific reporting**

Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [x] Life sciences
- [ ] Behavioural & social sciences
- [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/documents/nr-reporting-summary-flat.pdf

### Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | No sample-size calculations were performed. Sample size was based on previous experience and pilot studies, and each experiment was repeated at least once. |
|-------------|-------------------------------------------------------------------------------------------------------------------------------------|
| Data exclusions | Mice injected with tumour cells and that did not show tumour growth were not included. Mice that died for unknown reasons were excluded. No data were excluded from the analyses. |
| Replication | The animal studies were performed at least twice, and the data were pooled. In vitro data were generated from at least 3 donors, as detailed in the relevant figure captions. |
| Randomization | All groups of mice were age-matched. The mice were randomized prior to treatment, without knowledge of tumor burden. |
| Blinding | Blinding was irrelevant to the study. All mice experiments were carried out by researchers who also prepared the CAR-T cells before administration into mice. |

### Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

#### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| [ ] | Antibodies            |
| [x] | Eukaryotic cell lines |
| [ ] | Palaeontology and archaeology |
| [ ] | Animals and other organisms |
| [ ] | Human research participants |
| [x] | Clinical data         |
| [x] | Dual use research of concern |

#### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| [x] | ChiP-seq              |
| [ ] | Flow cytometry        |
| [x] | MRI-based neuroimaging |

#### Antibodies

- **Antibodies used**: All antibodies are listed in Supplementary Table 1.
- **Validation**: Antibody validation was performed by the relevant supplier.

#### Eukaryotic cell lines

**Policy information about cell lines**

- **Cell line source(s)**: Daudi, BC-3, A20 and CT26 cells were procured from ATCC; NXS2 was a gift from Holger N. Lode, University of Greifswald; Panc02 was a gift from Rainer Heuchel, Karolinska Institute. Human PBMCs (source of T cells) were freshly isolated from buffycoat. Mouse splenocytes (source of T cells) were freshly isolated from mouse spleens. The Gryphon retroviral packaging cell line was procured from Allele Biotechnology, San Diego, CA. The 293T lentiviral/retroviral vector packaging cell line was procured from ATCC.
- **Authentication**: The cell lines purchased from ATCC were not authenticated. Murine cell lines from C57bl/6 were confirmed with the strain, but cannot be authenticated by STR.
- **Mycoplasma contamination**: All cell lines tested negative for mycoplasma, using a Lonza kit.
Commonly misidentified lines
(See ICACC register)
No commonly misidentified cell lines were used (according to ICACC version 11; checked on 07 February 2022).

Animals and other organisms

Policy information about studies involving animals: ARRIVE guidelines recommended for reporting animal research

Laboratory animals
6–8-week-old female C57Bl/6Nj and Balb/c from Taconic Denmark, and 6–8-week old female A/J mice from Envigo, the Netherlands.

Wild animals
The study did not involve wild animals.

Field-collected samples
The study did not involve samples collected from the field.

Ethics oversight
The Uppsala Research Animal Ethics Committee approved all animal studies (N164/15; N185/16; 5.8.18-19434/2019).

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Human research participants

Policy information about studies involving human research participants

Population characteristics
The human buffy coats obtained from healthy blood donors had been anonymized.

Recruitment
Peripheral blood mononuclear cells were isolated by Ficoll-Paque (GE Healthcare Life Science, Uppsala, Sweden) from fresh buffy coats of healthy anonymized donors, collected at the Blood Centre at Uppsala University Hospital.

Ethics oversight
Because the samples had been anonymized, an ethical permit was not required.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
For the cell-culture experiments: suspension cells were harvested directly, washed with PBS and resuspended in ca. 200 µL of PBS containing the indicated antibody mixture (prepared as a master-mix solution).

For samples with intracellular-staining steps: cells were first stained with surface markers, then permeabilized with BDperm buffer (BD Biosciences) and additionally stained with antibodies targeting an intracellular marker (such as IL-2).

For samples with intra-nucleus staining steps: cells were first stained with surface markers, then permeabilized with True-Nuclear Transcription Factor Buffer set (Biolegend), then washed, and stained with additional antibodies (such as T-bet).

For NAP-expression detection: cells were treated with Brefeldin A (BD GolgiPlug, BD BioSciences) before staining.

For the in vivo experiments: The tumour samples were collected and enzymatically digested (Liberase, Roche) into single-cell suspensions. CD45+ cells were bead-isolated (Miltenyi Biotec) and stained with the appropriate antibody mixture.

Instrument
BD Canto II; BD Melody; CytoFLEX S; CytoFLEX XL.

Software
BD Diva; CytExpert; BD FACSChorus.

Cell population abundance
At least 10,000 cells were recorded for CD3+ for T cells; at least 1x10^6 for alive CD45+ cells were recorded when analysing tumour-infiltrating cells; otherwise, at least 10,000 cells in the FSC/SSC gate were recorded.

Gating strategy
Preliminary FSC-A/SSC-A gates were used on morphology and FSC-A/FSC-H for singlets, then followed by Zombie Aqua live/dead gating. Further gating for each population are detailed in Methods and in the relevant figure captions.

Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.