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Enhancing iNKT cell immunotherapy through the integration of optimized CAR endodomains and iNKT engagers

Kanagaraju Ponnusamy^{1,2*}✉, Klesti Karaxhuku^{1,2*}, Yuchao Jiang¹, Lyra Randzavola^{2,3}, Hongwei Ren², Ilia Leontari^{1,2}, Bryan Lye², Mehmood Zaidi^{1,2}, Edward J Bartlett⁴, Edward W Tate⁴, Vasileios Pardalis², Dimitrios Leonardos^{1,5}, Reza Nadafi⁶, Irene Sarkar⁶, Rogier M Reijmers⁶, Marco Bua⁷, Maria Atta⁷, Alexia Katsarou^{1,7}, Irene AG Roberts^{8,9}, Aristeidis Chaidos^{1,2,7}, Anastasios Karadimitris^{1,2,7}✉

¹Hugh & Josseline Langmuir Centre for Myeloma Research Centre for Haematology, Department of Immunology and Inflammation, Imperial College London, London, United Kingdom.

²Centre for Haematology, Department of Immunology and Inflammation, Imperial College London, London, United Kingdom.

³Centre for Inflammatory Disease, Department of Immunology and Inflammation, Imperial College London, London, United Kingdom.

⁴Department of Chemistry, Natural Sciences, Imperial College London, London, United Kingdom.

⁵University of Ioannina Medical School, Ioannina, Greece.

⁶Lumicks, Amsterdam, Netherlands.

⁷Department of Haematology, Hammersmith Hospital, Imperial College Healthcare NHS Trust, London, United Kingdom.

⁸Department of Paediatrics, University of Oxford, Oxford, United Kingdom & ⁹Molecular Haematology Unit, MRC Weatherall Institute of Molecular Medicine, University of Oxford, Oxford, United Kingdom.

* These authors contributed equally.

✉ Correspondence

Anastasios Karadimitris a.karadimitris@imperial.ac.uk

Kanagaraju Ponnusamy k.ponnusamy@imperial.ac.uk

Abstract

iNKT cells are emerging as a highly promising immunotherapy platform for the treatment of cancer. To maximise the anti-cancer activity of CAR-iNKT against the blood cancer multiple myeloma we investigated optimal CAR designs and their combination with new iNKT-specific engagers. We find that amongst five different CAR endodomains, underpinned by increased avidity and a cross talk between Plexin D1 on CAR-iNKT and Semaphorin 4A on myeloma cells, BCMA CD28z CAR-iNKT exert the highest anti-myeloma activity. Notably, CD28z CAR-iNKT outperform their CAR-T counterparts. To expand the anti-myeloma potential of CAR-iNKT, we designed and validated a high efficacy BCMA iNKT-specific engager which exerts significant anti-myeloma activity in conjunction with adoptively transferred iNKT cells. Finally, combined, dual target therapy with FCRL5 CAR-iNKT and BCMA iNKT engagers outperforms FCRL5 CAR-iNKT and limits immune escape of FCRL5-negative myeloma. Thus, optimised iNKT-based, dual-target, dual-modality immunotherapy has enhanced anti-tumor activity against multiple myeloma and potentially other malignancies.

Introduction

Cellular immunotherapies such as autologous chimeric antigen receptor (CAR)-T cells have dramatically improved the outcome of patients with some blood cancers and are potentially curative for relapsed/refractory B non-Hodgkin lymphoma¹ and B acute lymphoblastic leukemia². Cure of the B lineage malignancy multiple myeloma (MM) has proved much more difficult to achieve. However, recent results using Cilta-cel, an autologous anti-BCMA CAR-T product, showed a 15% 5-year minimal residual disease-negative state and disease-free survival in multiple relapsed disease, suggesting curative potential for some patients³. We hypothesise that cure rates for MM could be increased by employing a strategy that would harness the properties of effectors more powerful than T cells such as iNKT cells; optimise CARs, in particular their endodomain; and develop additional therapeutic modules such as iNKT-specific engagers targeting additional myeloma-associated antigens to allow broader targeting of myeloma plasma cells.

Given their ability to protect from acute graft-versus-host disease (aGVHD)^{4,5}, iNKT are emerging as an attractive allogeneic immune cell effector platform to autologous or allogeneic T cell-based products for the treatment of blood cancers and autoimmune diseases. iNKT, a rare subset of T cells, have features of both innate and adaptive immunity and possess effector as well as immunoregulatory activity. They are characterised by an invariant TCRV α 24J α 18 pairing with a TCRV β 11 chain⁶, and are restricted by the glycolipid-presenting, MHC-like molecule CD1d⁷. Their anti-tumor activity is mediated by CD1d-dependent and -independent mechanisms, including direct killing of tumor cells, antigen-presenting cell (APC) maturation and activation of tumor-specific T cells^{8,9} and depletion of immunosuppressive, CD1d-expressing tumor-associated myeloid cells^{10,11}. Moreover, iNKT can be deployed as a speedier, 'off-the-shelf' immunotherapy platform without the need for deletion of the endogenous TCR and the additional genetic

engineering this entails in allogeneic CAR-T cells, thereby reducing costs and manufacturing complexity. Additional potential advantages of allogeneic iNKT cell-over T cell-based immunotherapies include their ability to directly target CD1d-expressing tumors such as myeloma plasma cells¹² through the iTCR; the higher potency of CAR-iNKT over CAR-T as we and others have shown¹³⁻¹⁷, and a reduced risk for cytokine release syndrome and neurotoxicity¹⁸.

Efficacy of CARs is determined by several structural features including by the choice of the endodomain such as CD28z vs 41BBz. CD28z CARs achieve swifter and more robust activation of T cells while 41BBz CARs promote longer persistence of CAR-T but often fail to tackle CAR antigen low disease¹⁹. Here we describe a strategy to improve 'off-the-shelf' cellular immunotherapy for MM by engineering CAR-iNKT cells equipped with an optimal CAR endodomain that includes the critical co-stimulatory domain(s). After comparing five co-stimulatory domains we find that CD28z elicits the highest avidity against MM cells. Reflecting their enhanced avidity, CD28z CAR-iNKT outperform all other CAR-iNKT as well as CAR-T counterparts in *in vivo* models of MM. The increased avidity and *in vivo* anti-myeloma activity of CD28z CAR-iNKT is underpinned by a new mechanism that involves interaction of PLXND1 with SEMA4A, a PLXND1 ligand highly expressed on myeloma cells. To further enhance the therapeutic potential of CD28z CAR-iNKT, we developed a new iNKT cell-specific engagers which in combination with optimized CAR-iNKT achieve broader myeloma antigen targeting and higher efficacy than single CAR-iNKT or iNKT plus bispecific engager and limit CAR target-negative disease.

Results

Proliferative and avidity advantage of CD28z CAR-iNKT

We generated five different lentiviral CARs against the established anti-myeloma target BCMA using the previously described and clinically validated anti-BCMA C11d5.3 scFv²⁰, a G4S linker, CD8a-derived spacer and transmembranous domains and either 2nd generation CD28z, 41BBz, OX40z or third generation CD28-41BBz and CD28-OX40z endodomains (**Figure 1A**).

Using our previously published protocol we then generated corresponding, same donor highly pure CAR-iNKT with similarly high CAR expression levels (**Figure 1B&C**).

Using different donors we found that all five CAR-iNKT constructs were similarly cytotoxic against the BCMA high- and low-expressing H929 (CD1d+) and MM1.S (CD1d-) myeloma cells respectively (**Figure 1D**). As expected, reactivity was not observed against BCMA knock out H929 or BCMA-negative K562 cell lines (**Suppl Fig1A&B**) confirming their specificity. Similarly, there were no significant differences in production of granzyme B and interferon- γ (**Suppl Fig. 1C&D**) and, although confocal microscopy imaging showed all CAR-iNKT established more pronounced immune synapses than non-transduced (NT) iNKT cells with myeloma cells as assessed by centrosome polarisation at the immune synapse, again there were no differences between them (**Figure 1E**).

However, short term proliferation assays performed by real time imaging (**Figure 1F**) as well as assessment of long-term expansion potential in the presence of IL-15 and re-stimulation with CD1d-expressing B cell lines loaded with the glycolipid alpha-galactosylceramide (**Figure 1G**), showed that CD28z CAR-iNKT expanded significantly more than CAR-iNKT with the other four endodomains including 41BBz CAR-iNKT. In addition, six-day Incucyte® S3 (Sartorius) real time imaging-based, simultaneous assessment of effector proliferation and target cell cytotoxicity, showed that CD28z CAR-iNKT cells display a significantly greater proliferative capacity in the presence of the BCMA-expressing MM1.S myeloma cells as compared to all other counterparts (**Figure 1H**). By contrast, consistent with the 24hr cytotoxicity assays (**Figure 1D**), there was no difference in the cytotoxic activity amongst the five CAR-iNKT effectors (**Suppl Fig. 1E**).

A more detailed functional analysis focused on CD28z vs 41BBz CAR-iNKT showed that they had the same cytotoxic activity against an extended panel of myeloma cell targets (**Suppl Fig. 1F-H**) independent of the CAR expression levels (**Suppl Fig. 1I-J**). Further, in a tumor re-challenge assay, we found no significant differences in the cytotoxic activity between CD28z and 41BBz CAR-iNKT cells when repeatedly exposed to MM1.S target cells for 4 cycles (**Figure 1I**); similarly, no significant differences in the expression of the exhaustion markers PD-1, LAG-3 and TIM-3 was observed (**Suppl Fig. 1K-M**).

As *in vivo* potency of CAR-T cells is often better predicted by their avidity against their tumor targets^{17,21-23}, we employed both acoustic force- and shear stress-based avidity assays (**Figure 1J&K**) to study the strength of CAR-iNKT cell interaction with myeloma cells at steady state. We

found that in both assays and using two different myeloma cells as targets, CD28z CAR-iNKT demonstrated the highest avidity amongst all five CAR-iNKT designs (**Figure 1J&K and Suppl Fig. 1N-O**).

Therefore, higher proliferative potential and avidity distinguish CD28z from other 2nd and 3rd generation endodomains in the context of CAR-iNKT-mediated targeting of MM.

PLXND1-SEMA4A interaction determines higher avidity of CD28z CAR-iNKT

Next, we investigated the mechanism that underpins higher avidity of CD28z CAR-iNKT. Since avidity is measured within minutes of adding resting effectors to their target cells, we reasoned that differences would be mediated by proteins already expressed on the surface of the different types of CAR-iNKT cells. To identify such proteins, we compared the transcriptomes by RNA-sequencing of resting CD28z and 41BBz CAR-iNKT as well as NT NKT from three different donors. We found a small number of differentially expressed genes between CD28z or 41BBz CAR-iNKT vs iNKT cells and 28z vs 41BBz CAR-iNKT (**Figure 2A-C and Supplementary Dataset 1**). Both CD28z and 41BBz CAR-iNKT upregulate key genes related to cell adhesion and migration such as *ITGAD*, *MMP9*, *COL6A2* and also *IFNG* (**Figure 2C**). Amongst the few differentially expressed genes, we found that *PLXND1*, a semaphorin ligand family receptor was significantly upregulated in CD28z CAR-iNKT (**Figure 2B&C**). Flow cytometry analysis confirmed significantly higher levels of expression of PLXND1 in CD28z CAR-iNKT as compared to 41BBz, other CAR-iNKT designs and NT iNKT (**Figure 2D**), a finding also corroborated by quantitative immunofluorescence (**Figure 2E**). Expression of PLXND1 remained stable and did not increase upon CAR-iNKT activation in the presence of myeloma cells (**Suppl Fig. 2A**) while no significant differences of PLXND1 expression between CD4⁺ and CD4⁻ iNKT cells were observed (**Suppl Fig. 2B**). In contrast to CAR-iNKT, PLXND1 was not detected on CD28z or 41BBz CAR-T cells by flow-cytometric analysis (**Figure 2D**) although published single cell RNA-seq data shows that while naïve T cells lack or display very low expression of PLXND1, memory and particularly TEMRA CD4⁺ and CD8⁺ subsets express higher levels (**Suppl Fig. 2C-D**)⁴⁶⁻⁴⁷.

To further explore the role of PLXND1 we repeated the shear stress avidity assays in the presence of a blocking PLXND1 mAb²⁴. In relation to Ig isotype control, this resulted in a dose-dependent reduction of CD28z CAR-iNKT avidity but had no effect on the avidity status of 41BBz counterparts (**Figure 2F and Suppl Fig. 2E**). Therefore, the higher avidity of CD28z CAR-iNKT over NT and 41BBz CAR-iNKT is PLXND1-dependent.

To investigate this further we measured gene expression of *SEMA4A* and *SEMA3E*, the semaphorin family members which could potentially act as ligands for PLXND1²⁵⁻²⁷, in the two myeloma cell lines used in the avidity assays. While expression of *SEMA4A* was the highest amongst all semaphorins, *SEMA3E* was not expressed in either MM1.S or H929 cells (**Figure 2G and Suppl Fig. 2F**) with the same expression pattern seen in primary myeloma plasma cells from patients with MM (**Suppl Fig. 2G**). Of note, *SEMA4A* has been validated as a therapeutic target for CAR- and Ab-based immunotherapy of MM and similar to FCRL5^{28,29}, another clinically validated bispecific engager target, its expression is highest in the high risk chr1q-amp myeloma cells³⁰ (**Suppl Fig. 2H**).

To confirm the role of *SEMA4A* in CAR-iNKT avidity, using CRISPR/Cas9 gene editing we generated MM1.S myeloma cells lacking expression of *SEMA4A* (**Figure 2H**). Although the cytotoxic activity of CD28z and 41BBz CAR-iNKT was the same against both *SEMA4A*-expressing and gene-edited *SEMA4A*-negative MM1.S cells (**Suppl Fig. 2I**), avidity of CD28z CAR-iNKT against *SEMA4A*-negative myeloma cells was lower compared to *SEMA4A*-expressing myeloma cells, and at the same level as that of the 41BBz CAR-iNKT cells which was unaffected by abolishing *SEMA4A* expression (**Figure 2H**). These findings demonstrate that enhanced avidity of CD28z CAR-iNKT requires expression of *SEMA4A* on myeloma cells.

Furthermore, we tested whether differential cell proliferation and avidity induced by CD28z co-stimulatory domain would be independent of the CAR target. For this purpose, we synthesised 41BBz and CD28z CARs against FCRL5. The scFv of these CARs was based on the clinically active FCRL5 engager cevostomab³⁰ and was previously validated in the context of CAR-T therapy³¹. We found that as for BCMA CAR-iNKT, CD28z and 41BBz FCRL5 CAR-iNKT cells show similar cytotoxicity against FCRL5-expressing MM1.S myeloma cells (**Figure 6A and Suppl Fig. 2J**).

However, CD28z FCRL5 CAR iNKT cells showed higher proliferative potential than 41BBz FCRL5 CAR iNKT or NT iNKT cells (**Suppl Fig. 2K**) and higher expression of PLXND1 than their 41BBz counterparts commensurate with higher PLXND1-dependent avidity against FCRL5+ MM1.S cells (**Suppl Fig. 2L**).

Finally, we cloned the *PLXND1* cDNA into a lentiviral vector and exogenously over-expressed it in BCMA CAR-T and CAR-iNKT cells and the transduced CAR-iNKT and CAR-T cells were flow-sorted to purity based on GFP/PLXND1 (**Figure 2I**) followed by assessment of their avidity on MM1.S cells under shear stress (**Figure 2I and Suppl Fig. 2M**). We found that overexpression of PLXND1 increased the avidity BCMA 28z CAR-T cells and BCMA 41BBz CAR iNKT cells to the levels of BCMA CD28z CAR-iNKT cells. These data further corroborate the notion that higher PLXND1 expression underpins the higher avidity of CD28z CAR-iNKT and eventually their superior anti-myeloma activity when compared to 41BBz CAR-iNKT.

Together these findings demonstrate that the higher avidity of CD28z CAR-iNKT is mediated by its higher expression of PLXND1 and its ability to engage SEMA4A on myeloma cells and it is independent of the CAR scFv and its myeloma target.

CD28z CAR-iNKT exert a more effective anti-myeloma activity *in vivo* in a PLXND1-dependent manner

To investigate whether avidity indeed predicts anti-myeloma activity *in vivo*, we employed a systemic model of MM using luciferase- and tdTomato-expressing MM1.S (MM1.S-Luc) myeloma cells and monitored tumor burden by bioluminescence imaging (BLI). To better delineate differences between CAR constructs we used the limiting dose³² of 10^6 CAR-iNKT cells per animal for each group (**Figure 3A**). We found that CD28z CAR-iNKT followed by CD28-OX40z CAR-iNKT were the most potent in delaying tumor growth amongst the five CAR types as assessed by BLI (**Figure 3B-D and Suppl Fig. 3A&B**) while prolongation of survival was more pronounced in animals treated with CD28z CAR iNKT (**Figure 3D**).

Immunophenotypic analysis of bone marrow, spleen and peripheral blood confirmed significantly lower myeloma burden in CD28z CAR iNKT-treated mice (**Figure 3E and Suppl Fig. 3C&D**). The CAR-iNKT frequency and absolute numbers in the bone marrow at end point, although did not

reach statistical significance, were higher in CD28z and CD28-OX40z CAR-iNKT compared to other CAR-iNKT groups (**Figure 3F-H**). Together these data validate the avidity assay prediction that CD28z CAR-iNKT are the most potent amongst the five CAR-iNKT groups.

Finally, we investigated the impact of PLXND1 blockade *in vivo* by treating myeloma-bearing mice with CD28z CAR-iNKT cells plus either PLXND1 blocking Ab or isotype (Ig) control as shown in (**Figure 3I**). While both groups of CAR iNKT were effective in controlling disease progression as compared to untreated controls, survival of CAR-iNKT plus PLXND1 Ab-treated animals was significantly shorter than that of CAR-iNKT plus Ig-treated animals (**Figure 3J&K and Suppl Fig. 3E**).

Therefore, we conclude that PLXND1-dependent higher avidity accounts for the higher anti-myeloma activity of CD28z CAR-iNKT *in vivo*.

CAR-iNKT outperform CAR-T against myeloma

Next, since our previous work demonstrated that iNKT cells are a more powerful immunotherapy platform for CAR therapy of lymphoma and high risk ALL^{13,17}, we investigated whether this is also the case in MM. Indeed, CD28z BCMA CAR-iNKT from three different donors were consistently more cytotoxic towards MM1.S myeloma cells than their CAR-T counterparts (**Suppl Fig. 4A**) and exhibited higher PLXND1-dependent avidity *in vitro* than CAR-T (**Figure 4A**). In line with these observations when used at the limiting dose of 10^6 CAR+ effectors (**Figure 4B**), CAR-iNKT were significantly more potent than CAR-T counterparts *in vivo* in delaying growth of systemic myeloma (**Figure 4C&D and Suppl Fig. 4B&C**) and depleting myeloma cells from bone marrow and spleen (**Figure 4E&F**). Accordingly, survival of CAR-iNKT-treated mice was significantly longer than that of CAR-T counterparts (**Figure 4G**). Finally, in a subcutaneous model of myeloma involving the CD1d+ H929 myeloma cells, CD28z BCMA CAR-iNKT administered at the limiting dose of 10^6 CAR+ cells/mouse were more effective than CAR-T counterparts in delaying tumour growth as assessed by tumor volume *in vivo* and tumor weight at endpoint (**Figure 4H-J and suppl Fig 4D**). Importantly CD28z CAR-iNKT were also more effective than 41BBz CAR-iNKT, thus further

corroborating the findings from the systemic myeloma model (**Figure 3D**). Of note, we did not observe any clinical toxicity in either systemic or sbc myeloma models of CAR-iNKT immunotherapy. Reflecting this, there were no differences in the weight of treated animals as compared to untreated controls (**Suppl Fig. 4C&D**).

Together, these data show that CD28z BCMA CAR-iNKT outperform CAR-T counterparts against myeloma, likely in a PLXND1-dependent manner.

Development, specificity and anti-myeloma activity of iNKT cell engagers

Having identified the optimal CAR endodomain, we hypothesised that CAR-iNKT-based immunotherapy could be further enhanced by developing iNKT cell-specific engagers that would target additional myeloma-associated antigens. Such engagers would bring CAR-iNKT cells to the proximity of tumor cells facilitating their killing, as previously described for T and NK cell-specific bi- and tri-specific engagers in pre-clinical/clinical development or already licensed for clinical use³³.

To provide proof-of-concept in support of this hypothesis, we designed two bispecific iNKT engagers (biNTE). In principle, an iNKT engager could be designed to engage either the TCRV β 11 or TCRV α 24 variable, non-CDR3 segments of the iTCR or the TCRV α 24J α 18 CDR3 idotype. Here, we designed two engagers against TCRV β 11, based on the TCRV β 11 Ab clone C21⁶. Since engagement of iTCR with CD1d and lipid ligands involves primarily the TCRV α 24J α 18 CDR3 such an approach would be expected to allow iNKT activation through iTCR-CD1d interaction even in the presence of a TCRV β 11 engager.

The tumor targeting arm of the engagers was designed to bind to BCMA, and like the CAR, it was based on the C11d5.3 scFv²⁰ with modifications in the linker.

One biNTE design comprised a heterodimeric, monovalent, scFv-Fc human wild type IgG1 with Fc-silent (L234A/L235A (LALA) and a 'key-in-hole' changes (**Figure 5A**). A second biNTE design comprised a homodimeric, bivalent IgG-scFv human wild type IgG1. The anti-BCMA arm has a Fab configuration while the anti-TCRV β 11 arm has a scFv configuration (**Figure 5A**).

After cloning of the constructs into an expression vector and their transfection to 293T cells, protein was purified from the culture supernatant using Protein A columns and biNtE purity was confirmed by SDS PAGE (**Suppl Fig. 5A**).

Using the biNtE to stain purified iNKT and T cells followed by fluorescent-labelled secondary anti-human IgG1 Fc Ab, we found that compared to controls, biNtE-stained purified iNKT but not T cells in a dose-dependent manner (**Figure 5B & Suppl Fig. 5B**) thus demonstrating the specific binding of the iNKT cell arm of biNtE to the iNKT TCRV β 11 chain.

We next confirmed the ability of the tumor-binding arm of biNtE to bind their intended target BCMA showing that staining of iNKT cells occurred only in the presence of bio-sBCMA (**Suppl Fig. 5C**). To assess the potency of binding we employed surface plasmon resonance. This showed a lower K_d and thus higher affinity for bivalent over monovalent biNtE to soluble BCMA (K_d of 23.4nM vs 34.5nM respectively) (**Figure 5C and Suppl Fig. 5D-F**). In line with these findings, EC50 measured against MM1.S cells in the presence of iNKT was lower for bivalent than monovalent biNtE (2.4pM vs 6.2pM respectively) (**Suppl Fig. 5G&H**). Similarly, the bivalent biNtE demonstrated significantly higher longitudinal cytotoxic potential *in vitro* compared to the monovalent engager (**Figure 5D**). Based on its higher affinity and cytotoxic potential the bivalent engager was selected for further study.

Bivalent biNtE selectively killed myeloma cells in the presence of iNKT, but not T cells supporting the specificity of the biNtE iTCR arm (**Figure 5E**). In addition, the anti-myeloma cytotoxic activity of iNKT against CD1d-expressing BCMA⁺ (but not BCMA⁻) H929 myeloma cells were further enhanced in the presence of biNtE and aGalCer (**Figure 5F**) confirming the BCMA specificity of the tumor engaging arm of the biNtE and BCMA⁻ and iNKT cell-dependent killing of myeloma cells. These findings also validate the prediction that binding of the designed biNtE to the TCRV β 11 chain would allow the iTCR to engage with CD1d on tumor cells and to further enhance anti-myeloma activity.

We next co-cultured bone marrow cells from five patients with myeloma with iNKT cells and varying concentrations of biNTE. We found that while the frequency of myeloma PC dramatically decreased in a biNTE dose-dependent manner, that of the non-myeloma bone marrow cells was considerably less affected, suggesting specific killing of the myeloma but not of the non-myeloma bone marrow cells (**Figure 5G and Suppl Fig. 5I**).

To corroborate the *in vitro* findings, we tested the *in vivo* efficacy of the biNTE in a subcutaneous myeloma model using the BCMA-expressing MM1.S cells. After tumor engraftment, mice were left untreated or treated with either 10^7 unmodified, pre-expanded iNKT and $5\mu\text{g}/\text{mouse}$ biNTE ip 24hours post-iNKT treatment and then twice a week (**Figure 5H and Suppl Fig. 5J**). While iNKT or biNTE alone had no impact on tumor growth, the iNKT and biNTE combination significantly delayed myeloma tumour growth, first observed two days after the first engager dose (**Figure 5H**) thus providing proof-of-principle that biNTE when combined with adoptive iNKT immunotherapy has significant anti-myeloma activity.

We next compared the anti-myeloma activity of BCMA biNTE with that of BCMA CAR-iNKT cells. Myeloma MM1.S cells were co-cultured with NT iNKT and biNTE or BCMA CAR-iNKT at a 1:1 E:T ratio. Cytotoxicity against MM1.S cells showed slightly lower killing by biNTE plus iNKT compared to CAR iNKT attesting to the potency of the BCMA biNTE (**Figure 5I**). Finally, we compared the efficacy of BCMA biNTE plus iNKT vs BCMA CAR-iNKT in a systemic myeloma model using 10^7 effectors (**Figure 5J**). We found that although CAR-iNKT were overall more efficacious *in vivo*, the BCMA biNTE plus iNKT combination also significantly delayed disease progression as assessed by BLI, reduced myeloma cell frequency in bone marrow and increased overall survival as compared to iNKT only-treated and untreated controls (**Figure 5K&L and Suppl Fig. 5K-M**).

Finally, there were no differences in the longitudinally measured weight of biNTE-treated animals (**Suppl Fig. 5J&M**), suggesting lack of significant toxicity.

We conclude that biNTE are specific for iNKT and in combination with adoptive transfer of *in vitro* expanded iNKT exert a considerable anti-myeloma activity.

biNTE and CAR-iNKT for dual targeting of multiple myeloma

Having established an optimal CAR endodomain for CAR-iNKT treatment of myeloma and proof-of-principle of the anti-myeloma activity of biNTE, we next tested whether combining the two therapeutic modules would enhance the anti-myeloma activity of the iNKT cell platform by targeting two different antigens simultaneously, i.e., the clinically validated targets BCMA and FCRL5 by bivalent biNTE and 28z CAR respectively.

For this purpose, we first established that FCRL5 CD28z CAR iNKT cells are cytotoxic against FCRL5+ but not FCRL5- MM1.S myeloma cells (**Figure 6A**). We then used a systemic myeloma model generated by a 50-50 mixture of BCMA+FCRL5+ and BCMA+FCRL5- MM1.S-Luc cells, i.e., BCMA and FCRL5 are expressed by 100% and 50% respectively of tumor cells (**Figure 6B**). Such a set up would test the ability of biNTE to enhance the anti-myeloma activity of the CAR and limit immune escape even when half of the tumor cells lack expression of the CAR target.

These myeloma-bearing mice were left untreated or were treated with 10^7 FCRL5 CAR-iNKT or FCRL5 CAR iNKT plus BCMA biNTE. The combination of FCRL5 CAR-iNKT plus BCMA biNTE significantly prolonged disease progression to a greater extent than CAR-iNKT (**Figure 6C&D**). Assessment of the fraction of FCRL5- and FCRL5+ myeloma cells in bone marrow and spleen at end-point confirmed the expected ratio of ~1:1 in untreated animals while in FCRL5 CAR-iNKT plus BCMA biNTE and CAR-iNKT-treated animals this ratio was significantly higher at 10.1:1 and 5.1:1 respectively in favour of FCRL5- cells (**Figure 6E&F and Suppl Fig. 6A**), consistent with a strong selective effect of FCRL5 CAR-iNKT against FCRL5+ disease. The overall disease burden in bone marrow and spleen was also significantly lower in CAR-iNKT plus BCMA biNTE compared to CAR-iNKT treated animals (**Figure 6G and Suppl Fig. 6B**) consistent with ability of biNTE to enhance the anti-myeloma activity of the CAR even when half of the tumor cells lack expression of the CAR target. In line with this, overall survival of FCRL5 CAR-iNKT plus BCMA biNTE-treated animals was significantly longer than that of animals treated with CAR-iNKT (**Figure 6H**). Again, compared to untreated animals, no differences in the weight of treated animals were observed (**Suppl Fig. 6C**).

Together, these data provide strong proof-of-principle evidence that the combined CAR-iNKT plus biNTE modality immunotherapy enhances the therapeutic potency of the iNKT cell platform and can limit immune escape of tumor cells lacking the CAR target antigen in myeloma.

Discussion

As iNKT cells emerge as a potentially more advantageous allogeneic immunotherapy platform than autologous or allogeneic T cells for cancer, there is a need to maximise their efficacy by optimising the therapeutic modules with which they are engineered. This would be particularly pertinent to multiple myeloma, an incurable blood cancer for which conventional autologous anti-BCMA CAR-T therapy may have potential to induce durable complete remissions in a small minority of patients³.

Here we describe a strategy to improve iNKT-based ‘off-the-shelf’ cellular immunotherapy for MM first by optimising CAR design. CAR design, especially CD28z vs 41BBz endodomain choice, impacts efficacy and persistence of CAR-T cells by differential activation of downstream signalling pathways¹⁹. While CD28z supports robust T cell activation and more efficient targeting of CAR antigen low disease in part due to a lower level of trogocytosis by CAR-T cells of CAR antigen³⁴ and robust phosphorylation of CD3z by LCK³⁵, 41BBz promotes longer term persistence of CAR-T cells, in part through activation of non-canonical NFkB pathway³⁶. Here, our systematic and multilayered comparison of five 2nd and 3rd generation CAR endodomain designs provides robust evidence that in the context of CAR-iNKT immunotherapy for myeloma a CD28z design would be the most advantageous in terms of potency without evidence of reduced persistence. Our data shows that a new mechanism involving PLXND1-SEMA4A interaction determines differential avidity and thus higher anti-myeloma activity of CD28z CAR-iNKT as compared to CAR-iNKT with four different CAR endodomain designs. While engagement of PLXND1 on endothelial cells by SEMA4A has been shown to limit angiogenesis²⁷, little is known on the role of PLXND1-SEMA4A in modulating function of immune cells. Plxnd1 through its interaction with Sema3E is required for migration of positively selected thymocytes into the medulla of the thymus³⁷ while interaction of the Plxnd1-associated receptor Neuropilin 1 with Sema4A promotes transcriptional and

functional stability of Tregs³⁸. Of the potential PLXND1 ligands²⁵, only SEMA4A is expressed in myeloma cells, hence its validation as a target for Ab- and CAR-based immunotherapy of multiple myeloma²⁸. SEMA4A is also expressed in several other cancers and is required for checkpoint inhibitor-induced enhancement of anti-tumor T cell responses in lung cancer³⁹, while Sema4A-expressing dendritic cells enhance activation and differentiation of antigen specific T cells via engagement of Tim2⁴⁰. Additional work will be required to determine the signalling pathways that operate downstream of PLXND1 and underpin this advantage of CD28z CAR-iNKT.

Since both *FCRL5* and *SEMA4A* are chr1q genes and are overexpressed in myeloma cells with chr1q-amp, a critical adverse prognosis genetic feature, CD28z-based CAR-iNKT approach targeting FCRL5 in combination with BCMA could be particularly advantageous for this high-risk myeloma⁴¹. This contrasts with the fact that both clinically licensed anti-BCMA CAR-T products for MM feature a 41BBz endodomain. However, since pre-clinical comparison of CD28z vs 41BBz FCRL5 CAR-T demonstrated the superior ability of the former to control myeloma *in vivo*²⁹, whether 41BBz would be the preferred endodomain for CAR-T treatment of MM remains an open question. Our data also highlight the need for testing and identifying optimal CAR co-stimulatory domains for each innate lymphocyte subset such as NK and $\gamma\delta$ T cells.

In terms of potency, consistent with previous work by us and others showing higher anti-tumor activity of CAR-iNKT in pre-clinical B cell lymphoma, high risk B ALL, and solid tumors¹³⁻¹⁷, we demonstrate that CAR-iNKT also outperform CAR-T in the context of multiple myeloma. This advantage of iNKT over T cells could be mediated both directly by the iTCR interacting with CD1d expressed on some tumors, including myeloma cells¹², and engagement of activating NK receptors (e.g., NKG2D) by their ligands expressed by tumor cells; or indirectly- through enhancement of innate and adaptive anti-tumor immune responses, as well as targeting and depletion of CD1d-expressing immunosuppressive and tumor-promoting myeloid cells^{10,15,16,42}. The latter mechanism, although not directly addressed in this work, would also be expected to contribute to the anti-tumor activity of CAR-iNKT cells in multiple myeloma given that tumor-

associated myeloid cells have been shown to suppress effective anti-myeloma T cell responses and promote myeloma cell survival⁴³.

Guided by pre-clinical and recent early clinical work demonstrating the anti-cancer potential of bispecific engagers administered in combination with adoptive transfer of effectors such as NK cells⁴⁴ and to expand the options for multi-modular targeting of multiple myeloma we developed iNKT cell-specific engagers and demonstrated their anti-myeloma activity. Such engagers, with the exception of a $\gamma\delta$ TCR engager that cross-reacts with the TCRV α 24J α 18 CDR3 idiotype of the iTCR⁴⁵, have not been described. Our TCRV β 11-engaging bispecific antibody has two distinct properties: firstly, the option to target any tumor-associated antigen with one arm; and secondly, the availability of the TCRV α 24J α 18 CDR3 loop to engage with CD1d-presented glycolipids thus further contributing to CD1d-dependent tumor killing. Between the two bispecific engager designs, i.e. monovalent vs bivalent, the latter appears to be more potent in in vitro assays, likely reflecting its higher affinity and when tested against soluble BCMA and higher avidity when tested against myeloma cells in the presence of iNKT cells. We envisage that iNKT engagers, with single or dual anti-tumor specificity, can be used flexibly in conjunction with other therapeutic modalities such as single or dual CAR and/or TCR thus achieving dual, triple or even quadruple targeting of myeloma, other blood or solid tumor cancers. Clinical development of this modular therapy would entail infusions of the iNKT cell engagers in conjunction with transfer of autologous or allogeneic, in vitro expanded iNKT or CAR/TCR-iNKT cells.

In summary, in the context of multiple myeloma, we provide proof-of-concept that iNKT engineered with optimally selected CAR design display superior anti-myeloma activity over CAR-T cells and their efficacy can be further broadened and enhanced by addition of iNKT cell engagers.

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Author Contributions

KP established all CAR iNKT and performed *in vitro*, *in silico* and *in vivo* experiments and analysed data. KP & KK & YJ contributed to biNTE and FCRL5 CAR work. LR performed microscopy experiments and data analysis, KP & RN, IS & RR established and performed avidity assays. KP & AC performed RNA seq analysis. HR, IL, BL, MZ, VP, DL for technical support and MB, MA, AK supported with patient material and KP, EB & ET performed SPR and data analysis and IR co-wrote the manuscript. AK and KP conceived the experimental plan and wrote the manuscript.

Competing interests

AK and KP report holding options/shares in Arovella Therapeutics Ltd. The rest of the authors declare no competing interest.

References

1. Neelapu SS, Jacobson CA, Ghobadi A, et al. Five-year follow-up of ZUMA-1 supports the curative potential of axicabtagene ciloleucel in refractory large B-cell lymphoma. *Blood*. 2023;141(19):2307-2315.
2. Laetsch TW, Maude SL, Rives S, et al. Three-Year Update of Tisagenlecleucel in Pediatric and Young Adult Patients With Relapsed/Refractory Acute Lymphoblastic Leukemia in the ELIANA Trial. *J Clin Oncol*. 2023;41(9):1664-1669.
3. Jagannath S, Martin TG, Lin Y, et al. Long-Term ($\geq$ 5-Year) Remission and Survival After Treatment With Ciltacabtagene Autoleucel in CARTITUDE-1 Patients With Relapsed/Refractory Multiple Myeloma. *J Clin Oncol*. 2025:JCO2500760.
4. Chaidos A, Patterson S, Szydlo R, et al. Graft invariant natural killer T-cell dose predicts risk of acute graft-versus-host disease in allogeneic hematopoietic stem cell transplantation. *Blood*. 2012;119(21):5030-5036.
5. Mavers M, Maas-Bauer K, Negrin RS. Invariant Natural Killer T Cells As Suppressors of Graft-versus-Host Disease in Allogeneic Hematopoietic Stem Cell Transplantation. *Front Immunol*. 2017;8:900.
6. Dellabona P, Padovan E, Casorati G, Brockhaus M, Lanzavecchia A. An invariant V alpha 24-J alpha Q/V beta 11 T cell receptor is expressed in all individuals by clonally expanded CD4-8- T cells. *J Exp Med*. 1994;180(3):1171-1176.

7. Salio M, Silk JD, Jones EY, Cerundolo V. Biology of CD1- and MR1-restricted T cells. *Annu Rev Immunol*. 2014;32:323-366.
8. Fujii S, Shimizu K, Okamoto Y, et al. NKT cells as an ideal anti-tumor immunotherapeutic. *Front Immunol*. 2013;4:409.
9. Terabe M, Berzofsky JA. NKT cells in immunoregulation of tumor immunity: a new immunoregulatory axis. *Trends Immunol*. 2007;28(11):491-496.
10. Song L, Asgharzadeh S, Salo J, et al. Valpha24-invariant NKT cells mediate antitumor activity via killing of tumor-associated macrophages. *J Clin Invest*. 2009;119(6):1524-1536.
11. Cortesi F, Delfanti G, Grilli A, et al. Bimodal CD40/Fas-Dependent Crosstalk between iNKT Cells and Tumor-Associated Macrophages Impairs Prostate Cancer Progression. *Cell Rep*. 2018;22(11):3006-3020.
12. Spanoudakis E, Hu M, Naresh K, et al. Regulation of multiple myeloma survival and progression by CD1d. *Blood*. 2009;113(11):2498-2507.
13. Rotolo A, Caputo VS, Holubova M, et al. Enhanced Anti-lymphoma Activity of CAR19-iNKT Cells Underpinned by Dual CD19 and CD1d Targeting. *Cancer Cell*. 2018;34(4):596-610 e511.
14. Li YR, Zhou Y, Yu J, et al. Generation of allogeneic CAR-NKT cells from hematopoietic stem and progenitor cells using a clinically guided culture method. *Nat Biotechnol*. 2024.
15. Simonetta F, Lohmeyer JK, Hirai T, et al. Allogeneic CAR Invariant Natural Killer T Cells Exert Potent Antitumor Effects through Host CD8 T-Cell Cross-Priming. *Clin Cancer Res*. 2021;27(21):6054-6064.
16. Delfanti G, Cortesi F, Perini A, et al. TCR-engineered iNKT cells induce robust antitumor response by dual targeting cancer and suppressive myeloid cells. *Sci Immunol*. 2022;7(74):eabn6563.
17. Ren H, Elliott N, Lye B, et al. Off-the-shelf dual CAR-iNKT cell immunotherapy eradicates medullary and leptomeningeal high-risk KMT2A-rearranged leukemia. *Blood*. 2025.
18. Ramos CC, AN.; Lulla, PD.; Chimere Hill, L.; Kamble, RT.; Carrum, G.; Wang, T.; Di Pierro, EJ.; Brenner, MK.; Heslop, HE.; Metelitsa, LS.; Off-the-Shelf CD19-Specific CAR-NKT Cells in Patients with Relapsed or Refractory B-Cell Malignancies. *Transplantation and Cellular Therapy*. 2024;30(2):S41-S42.
19. Feucht J, Sadelain M. Function and evolution of the prototypic CD28zeta and 4-1BBzeta chimeric antigen receptors. *Immuno-oncol Technol*. 2020;8:2-11.
20. Carpenter RO, Evbuomwan MO, Pittaluga S, et al. B-cell maturation antigen is a promising target for adoptive T-cell therapy of multiple myeloma. *Clin Cancer Res*. 2013;19(8):2048-2060.
21. Larson RC, Kann MC, Bailey SR, et al. CAR T cell killing requires the IFNgammaR pathway in solid but not liquid tumours. *Nature*. 2022;604(7906):563-570.
22. Leick MB, Silva H, Scarfo I, et al. Non-cleavable hinge enhances avidity and expansion of CAR-T cells for acute myeloid leukemia. *Cancer Cell*. 2022;40(5):494-508 e495.
23. Fernandez de Larrea C, Staehr M, Lopez AV, et al. Defining an Optimal Dual-Targeted CAR T-cell Therapy Approach Simultaneously Targeting BCMA and GPRC5D to Prevent BCMA Escape-Driven Relapse in Multiple Myeloma. *Blood Cancer Discov*. 2020;1(2):146-154.
24. Carvalho T, Affandi AJ, Malvar-Fernandez B, et al. Induction of Inflammation and Fibrosis by Semaphorin 4A in Systemic Sclerosis. *Arthritis Rheumatol*. 2019;71(10):1711-1722.

25. Kumanogoh A, Kikutani H. Immunological functions of the neuropilins and plexins as receptors for semaphorins. *Nat Rev Immunol*. 2013;13(11):802-814.
26. Gay CM, Zygmunt T, Torres-Vazquez J. Diverse functions for the semaphorin receptor PlexinD1 in development and disease. *Dev Biol*. 2011;349(1):1-19.
27. Toyofuku T, Yabuki M, Kamei J, et al. Semaphorin-4A, an activator for T-cell-mediated immunity, suppresses angiogenesis via Plexin-D1. *EMBO J*. 2007;26(5):1373-1384.
28. Anderson GSF, Ballester-Beltran J, Giotopoulos G, et al. Unbiased cell surface proteomics identifies SEMA4A as an effective immunotherapy target for myeloma. *Blood*. 2022;139(16):2471-2482.
29. Yu Z, Li H, Lu Q, Zhang Z, Tong A, Niu T. Fc receptor-like 5 (FCRL5)-directed CAR-T cells exhibit antitumor activity against multiple myeloma. *Signal Transduct Target Ther*. 2024;9(1):16.
30. Li J, Stagg NJ, Johnston J, et al. Membrane-Proximal Epitope Facilitates Efficient T Cell Synapse Formation by Anti-FcRH5/CD3 and Is a Requirement for Myeloma Cell Killing. *Cancer Cell*. 2017;31(3):383-395.
31. Jiang D, Huang H, Qin H, et al. Chimeric antigen receptor T cells targeting FcRH5 provide robust tumour-specific responses in murine xenograft models of multiple myeloma. *Nat Commun*. 2023;14(1):3642.
32. Zhao Z, Condomines M, van der Stegen SJC, et al. Structural Design of Engineered Costimulation Determines Tumor Rejection Kinetics and Persistence of CAR T Cells. *Cancer Cell*. 2015;28(4):415-428.
33. Wei J, Yang Y, Wang G, Liu M. Current landscape and future directions of bispecific antibodies in cancer immunotherapy. *Front Immunol*. 2022;13:1035276.
34. Hamieh M, Dobrin A, Cabriolu A, et al. CAR T cell trogocytosis and cooperative killing regulate tumour antigen escape. *Nature*. 2019;568(7750):112-116.
35. Salter AI, Ivey RG, Kennedy JJ, et al. Phosphoproteomic analysis of chimeric antigen receptor signaling reveals kinetic and quantitative differences that affect cell function. *Sci Signal*. 2018;11(544).
36. Philipson BI, O'Connor RS, May MJ, June CH, Albelda SM, Milone MC. 4-1BB costimulation promotes CAR T cell survival through noncanonical NF-kappaB signaling. *Sci Signal*. 2020;13(625).
37. Choi YI, Duke-Cohan JS, Ahmed WB, et al. PlexinD1 glycoprotein controls migration of positively selected thymocytes into the medulla. *Immunity*. 2008;29(6):888-898.
38. Delgoffe GM, Woo SR, Turnis ME, et al. Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. *Nature*. 2013;501(7466):252-256.
39. Naito Y, Koyama S, Masuhiro K, et al. Tumor-derived semaphorin 4A improves PD-1-blocking antibody efficacy by enhancing CD8(+) T cell cytotoxicity and proliferation. *Sci Adv*. 2023;9(20):eade0718.
40. Kumanogoh A, Marukawa S, Suzuki K, et al. Class IV semaphorin Sema4A enhances T-cell activation and interacts with Tim-2. *Nature*. 2002;419(6907):629-633.
41. Avet-Loiseau H, Davies FE, Samur MK, et al. International Myeloma Society/International Myeloma Working Group Consensus Recommendations on the Definition of High-Risk Multiple Myeloma. *J Clin Oncol*. 2025;43(24):2739-2751.

42. Zhou X, Wang Y, Dou Z, et al. CAR-redirected natural killer T cells demonstrate superior antitumor activity to CAR-T cells through multimodal CD1d-dependent mechanisms. *Nat Cancer*. 2024;5(11):1607-1621.
43. Giannotta C, Autino F, Massaia M. The immune suppressive tumor microenvironment in multiple myeloma: The contribution of myeloid-derived suppressor cells. *Front Immunol*. 2022;13:1102471.
44. Nieto Y, Banerjee P, Kaur I, et al. Allogeneic NK cells with a bispecific innate cell engager in refractory relapsed lymphoma: a phase 1 trial. *Nat Med*. 2025;31(6):1987-1993.
45. Lameris R, Ruben JM, Iglesias-Guimaraes V, et al. A bispecific T cell engager recruits both type 1 NKT and Vgamma9Vdelta2-T cells for the treatment of CD1d-expressing hematological malignancies. *Cell Rep Med*. 2023;4(3):100961.
46. Monaco G, Lee B, Xu W, et al. RNA-Seq Signatures Normalized by mRNA Abundance Allow Absolute Deconvolution of Human Immune Cell Types. *Cell Rep*. 2019;26(6):1627-1640 e1627.
47. Schmiedel BJ, Singh D, Madrigal A, et al. Impact of Genetic Polymorphisms on Human Immune Cell Gene Expression. *Cell*. 2018;175(6):1701-1715 e1716.

Methods

Ethics statement

The peripheral blood of unidentifiable healthy donor received from NHS Blood and Transplant, UK under ethical approval (NCI no: 2758). Primary myeloma cells were collected under ethical committee approval (REC no. 11/H0308/9) from bone marrow samples derived from myeloma patients. For tumor models in animals, the study was conducted by following the licence (PPL no. PP2944557) provided by UK Home Office. The maximum tumor size exceeding 12.5mm diameter or weight loss >15% or hunching, piloerection lasting >24hrs or tumor inflammation with ulceration or signs of extensive bone marrow involvement such as reduced limb movement or when tumours obstruct normal movement set as humane end points. The tumor burden was measured by vernier calliper for subcutaneous or bioluminescence imaging for systemic models, and the maximal tumor burden was not exceeded and the experiments were terminated upon reaching the humane endpoints or scientific objectives.

iNKT isolation and expansion, Cell culture, Primary cells

iNKT cells were isolated from healthy donor-derived peripheral blood mononuclear cells (PBMCs) using anti-iNKT microbeads (cat no. 130-094-842; Miltenyi Biotec) followed by stimulation with irradiated PBMCs and anti-CD3 and anti-CD28 antibodies and cultured in R10 media (RPMI 1640 supplemented with 10% fetal bovine serum and 1% Pen/strep, 4mM L-Glutamine, 1% non-essential amino acids, 1mM sodium pyruvate, 15mM Hepes buffer, 0.05mM 2-Mercaptoethanol) supplemented with 150U IL-15 (cat. no 130-095-764; Miltenyi Biotec) and expanded as described^{13,17}. The myeloma cell lines MM1.S cells (CRL-2974, ATCC) and NCI-H929 cells (CRL-3580, ATCC) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 1% Pen/strep, 1% L-Glutamine, 1% non-essential amino acids, 1% sodium pyruvate. The myeloma cells were identified by staining the cells with anti-human CD138 (Syndecan-1) (clone: MI15) and anti-human CD38 (clone: HIT2).

Cloning, lentiviral transduction

BCMA CAR sequences were modified from Carpenter et al²³, and modified by inserting (G4S)₃ linker between light and heavy chain of scFv and appropriate costimulatory molecules and CD8a hinge and transmembrane and 7AA of cytoplasmic domain as shown in Figure 1A, and cloned into second generation plasmid containing SFFV promoter and WPRE. CAR lentiviral particles were packaged in 293T cells by transfection of 4.1µg CAR plasmid with packaging plasmids 5.4 µg psPAX2 and 2.9 µg pMD2.G plasmid for 48hrs. The viral particles were concentrated by ultracentrifugation at 23000 RPM, 4°C. The cells were transduced on 8µg/ml retronectin-coated plates by inoculating viral particles and cells at 1000g at 37°C.

CD28 BCMA CAR AA sequences:

MALPVTALLLPLALLLHAARPDIVLTQSPPSLAMSLGKRATISCRASESVTILGSHLIHWYQQKPGQPPTLLIQL
ASNVQTGVPARFSGSGSRTDFTLTIDPVEEDDVAVYYCLQSRTIPRTFGGGTKLEIKGGGSGGGGSGGGGS
QIQLVQSGPELKKPGETVKISCKASGYFTDYSINWVKRAPGKGLKWMGWINTETREPAYAYDFRGRFAFSL
ETSASTAYLQINNLYEDTATYFCALDYSYAMDYWGQGTSTVSSFPVFLPAKPTTTPAPRPPTPAPTASQP
LSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRNRSKRSRLHSDYMNMTPRR
PGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGK

PRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR* (CD28 domain sequences)

41BB domain: KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

OX40 domain: ALYLLRRDQRLPPDAHKKPPGGGSFRTPIQEEQADAHSTLAKI

CD28-41BB domain:

RSKRSRLLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

CD28-OX40 domain:

RSKRSRLLHSDYMNMTPRRPGPTRKHYPYAPPRDFAAYRSALYLLRRDQRLPPDAHKKPPGGGSFRTPIQEEQADAHSTLAKI

These costimulatory domains were replaced in CD28 BCMA CAR to create all the BCMA CARs with different costimulatory molecules.

FCRL5 CAR AA sequences:

MALPVTALLPLALLLHAARPTGEVQLVESGPGLVKPSETLSLTCTVSGFSLTRFGVHWVRQPPGKGLEWLGV
IWRGGSTDYNAAFVSRLTISKDNSKNQVSLKLSSVTAADTAVYYCSNHYYGSSDYALDNWGGQGLTVTVSSGG
GGSGGGGGSGGGGSDIQMTQSPSSLSASVGDRVTITCKASQDVRNLVWVFQKPKGKAPKLLIYSGSYRYSKV
PSRFSGSGSGTDFTLTISLQPEDFATYYCQGHYSPPYTFGQGTKEIKFVPVFLPAKPTTTPAPRPPTPAPTAS
QPLSLRPEACRPAAGGAVHTRGLDFACDIYWAPLAGTCGVLLSLVITLYCNHRNRSKRSRLLHSDYMNMT
PRRPGPTRKHYPYAPPRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDRRGRDPEMG
GKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR*
(CD28 domain sequences)

Flow cytometry and cytotoxicity assay

iNKT cells were stained with anti-human CD3 (clone: UCHT1), anti-human TCR Valpha24-Jalpha18 (iNKT cell) (clone: 6B11), anti-human TCR Vbeta11 (clone: REA559) anti-human CD45 (HI30), anti-human CD8 (clone: SK1) and anti-human CD4 (clone: OKT4) for 30min at 4°C in FACS buffer (1x PBS+0.5% HSA). The cells were stained with 7AAD after washing and analysed using flow cytometry. PlexinD1 (cat no. FAB4160P; R&D systems), anti-human CD307e (FCRL5) (clone: REA391), anti-human BCMA (clone: REA315) antibodies were used. CAR functional tests were

performed by co-culturing CAR iNKT cells with target cells expressing CAR antigen at different E:T ratio in R10 media. The target cells were stained with cell trace violet (cat. no C34557; ThermoFisher), and the effector cells were depleted of cytokines overnight. After the indicated time of co-culture, the cells were washed with FACS buffer and cell viability was determined by staining the cells with 7AAD and flow cytometry. For primary myeloma cells, the total target cells were stained with cell trace violet before the coculture with NT iNKT cells, and the cells were stained with anti-CD138 antibodies to determine frequency of myeloma plasma cells after 20hours of culture and analysed using flow cytometry. The data were normalized to target cells alone control. The MM1.S cells were incubated with recombinant BCMA protein (clone: 10620-H40H-B-SIB) to block BCMA in biNte experiment followed by incubation with iNKT cells (1:1) and biNTes at EC50 dose. For iNKT staining, the cells were stained with CD3 APC-Cy (clone: UCHT1), CD4 PE (clone: OKT4), CD8 FITC (clone: RPA-T8), anti- TCR Va24-Ja18 BV421 (clone: 6B11), anti- TCR Vb11-APC (Order no. 130-108-733).

Intracellular cytokine measurement

Non-transduced (NT) iNKT and BCMA CAR iNKT cells were co-cultured with MM1.S cells in the presence of 1x Brefeldin A overnight in R10 media without any cytokine stimulation. The cells were fixed and permeabilized by following standard manufacturer's instructions (BioLegend, cat# 420801 and 421002). The cells were stained with granzyme B (clone: GB11) and IFN γ (clone: 4S.B3) in FACS buffer for 30min, 4°C and measured in flow cytometry after washing twice with FACS buffer.

Avidity assay

The iNKT cell avidity was determined using z-Movi[®] Cell Avidity Analyzer (Lumicks/Lumicks CA BV). Briefly, 10⁶ cells/ml BCMA-expressing MM1.S or H929 cell monolayer was prepared on poly-L-lysine-coated temperature-controlled microfluidic chips (Lumicks/Lumicks CA BV) for 1hr. Simultaneously NT or CAR-modified iNKT cells were stained with cell trace far red dye (cat no. C34564; ThermoFisher) by following manufacturer's instructions and incubated on the monolayer for 5 minutes. The indicated acoustic force was applied, and the fraction of bound

cells was measured. For shear stress models, cell avidity was measured by applying shear-stress where MM1.S cell monolayer was prepared on 1 μ g/ml retronectin-coated ibid-treated slides for 1hr at 37°C. Simultaneously the effector iNKT cells were stained with cell trace green (cat no. C2925; ThermoFisher) or far-red dyes. The iNKT cells were incubated on the monolayer for 5 minutes and the indicated shear stress was applied continuously with an interval of 30 seconds between incremental shear stress levels. Total number of stained iNKT cells in the field was captured under fluorescence microscopy and the images were captured at every shear stress level. For PlexinD1 blocking, iNKT cells were incubated with either 1 μ g/ml isotype or anti-PLXND1 neutralizing antibody (cat no. PA5-47012; ThermoFisher) for 1 hr, 37°C and washed them in R10 media before applying them on the monolayer to measure the cell avidity efficiency as stated above. SEMA4A knock-out cells were generated by electroporating MM1.S cells with SEMA4A CRISPR gRNA and guide (156156422 CUCCAACAGGGGAUGAACGU – Modified) and SpCas9 (Synthego). The bound cells at all shear stress levels were counted using Image J software and normalized to bound cells at pre-shear stress application (0 dyne/cm²). For PLXND1 overexpression model, PLXND1 full cDNA was cloned into LeGO-iG2 (addgene #27341) which express EGFP as fluorescence marker and transduced into expanded BCMA CAR iNKT cells and BCMA CAR-T cells through lentiviral transduction followed by FACS- purification for pure PLXND1 expression cells. The avidity status of mock (i.e EGFP only)-transduced or PLXND1 overexpressed cells were studied using shear stress flow model as stated above.

Cell proliferation and tumor challenge in real-time Incucyte® S3 imaging

5x10³ CAR iNKT cells with different co-stimulatory molecules were incubated with MM1.S cells which express tdTomato as a fluorescence marker at 1:1 E:T ratio without IL15 cytokine supplement. The cytotoxicity potency was monitored by tdTomato fluorescence levels and normalized to hour 0. The cell proliferation of CAR iNKT cells after stimulation with target cells were calculated by total live cells (Phase object) to tdTomato+ (Red fluorescence object) target MM1.S cells and normalized to hour 0 in real time live imaging using Incucyte® S3 software. For tumor challenge assay, 1x10⁴ BCMA CAR iNKT cells and target cells tdTomato+ MM1.S cells were

incubated at 1:1 E:T ratio for 4 days without cytokine IL15 and repeated for 4 challenges by adding target cells at 1:1 E:T ratio. TdTomato fluorescence signals were measured in real time live imaging using Incucyte® S3 system. The exhaustion markers were measured in flow cytometry using PD-1 (clone: A17188A), LAG-3 (clone: 11C3C65), TIM-3 (clone: 7D3) antibodies levels before the challenge and after 4th challenge.

Systemic and subcutaneous *in vivo* models of myeloma and bioluminescence

MM1.S cells were transduced with retrovirus to express luciferase gene and tdTomato, an enhanced, tandem-dimeric, monomeric red fluorescent protein derived from the original DsRed protein as a fluorescence marker (MM1.S-Luci). To establish a systemic myeloma model, $5-7 \times 10^6$ MM1.S-luci cells were injected intravenously to engraft in NOD SCID Gamma (NSG) mice (8-12 weeks old). The animals were purchased from Charles River, UK and caged in individually ventilated cages, social housing with enriched bedding with sterile food and water and maintained at 20-24° C, 45-65% humidity, 12hr light cycle. The mice were imaged for bioluminescent signal (BLI) to track the disease burden under 2% isoflurane (Zoetis UK)/medical oxygen administration via inhalation and one single dose injection of 150 mg/Kg D-Luciferin injection, potassium salt (cas no. 115144-35-9; Merk) in PBS via intraperitoneal (IP) route for 10 min before imaging the mice. The images were captured for up to 3 minutes at auto mode and medium binning of IVIS for both ventral and dorsal side of each mouse. The region of interest (ROI) data analysis was performed using Living Image software and expressed in radiance (unit of photons/s/cm²/sr) and the total BLI was calculated by adding both dorsal and ventral side signals. For subcutaneous model, 10^7 MM1.S myeloma cells were injected with 200µl Matrigel® Basement Membrane Matrix (cat no. 354234; Corning) subcutaneously and the tumor size was measured using vernier calliper and tumor volumes were assessed using the formula (Volume = $1/2 \times \text{Length} \times \text{Width}^2$). To establish H929 cells subcutaneous model, 5×10^6 H929 cells were subcutaneously injected with 200µl Matrigel® Basement Membrane Matrix. The tumor volume was measured as stated above. For treatment, NT iNKT or CAR-iNKT cells or CAR-T cells were injected through tail vein intravenous route. For biNTE treatment, 5µg biNTE antibody per mouse was injected intraperitoneally twice a week for up to 5 weeks. For PLXND1 blocking *in vivo*, the

BCMA CAR iNKT treated group mice were injected with either 5µg Goat anti-human isotype or PLXND1 neutralizing antibody per mouse (cat no. PA5-47012; ThermoFisher) intraperitoneally for 6 weeks, twice a week. The untreated and isotype control of BCMA treated group shown in Figure 3K were served as negative and positive control respectively shown in Figure 5L as these experiments were performed at the same period and were designed to reduce animal usage and to comply with 3R's policy. Upon reaching humane end points of MM disease, the mice were sacrificed, and the organs were collected followed by cell preparation in FACS buffer and measured for tdTomato to track MM1.S cells in flow cytometry.

RNA sequencing

iNKT cells were transduced with BCMA CARs and expanded as stated above after purification of BCMA CAR iNKT cells on day 5 post-transduction, to have >95% CARs for all the BCMA CARs, by staining the cells with recombinant BCMA-biotin (cat no. 10620-H40H-B-SIB; Stratech Scientific) followed by magnetically labelled anti-streptavidin PE beads-based purification by MACS magnetic column. The CAR and iNKT purity was assessed by flow cytometry before isolation of the total RNA, and RNA sequencing was performed after enriching for poly-A mRNA and sequenced by Novogene.

Surface Plasmon Resonance (SPR)

Kinetics data were collected on Biacore S200 Biosensors (Cytiva). Soluble biotinylated BCMA were immobilised to Series S SA sensorchips, which were maintained with a continuous flow of PBS-P+ (20mM phosphate buffer with 2.7mM KCl, 137mM NaCl and 0.05% Surfactant P20). SA sensorchips were prepared as per Cytiva protocols; the sensor surface was conditioned with three consecutive one-minute injections of 1M NaCl in 50mM NaOH and washed with 70% isopropanol in 1M NaCl and 50mM NaOH after each ligand injection. The biotin tagged ligand was injected at 20ng/µL for 1800 seconds, reaching an RU of 3500. Kinetic data were collected via SCK experiments with a 4-point dilution series (0.1, 1, 10, 200nM) in PBS-P+. The diluted compound series was injected in order of ascending concentration for 400s, across a specific flow cell. Dissociation was monitored during a 400s injection of assay buffer. An equivalent series of

injections was carried out using Assay Buffer, which was used to correct flowcell-specific artefacts. Kinetics and affinity data were gathered using the Biacore Evaluation software.

Bispecific antibody development

Monovalent biNTE composed of heterodimeric iTCR (clone: C21) scFv and anti-BCMA (clone: C11D5.3) scFv linked with Fc human wild type IgG1 with Fc-silent (L234A/L235A (LALA) and a 'key-in-hole' changes. A homodimeric IgG–scFv bispecific bivalent was constructed as the heavy chain (HC) encoded an anti-BCMA (C11D5.3) IgG (VH–CH1–hinge–CH2–CH3) C-terminally fused to an anti-Vβ11 scFv (C21) via a (G₄S)₃ linker. The light chain (LC) encoded the matched anti-BCMA (C11D5.3) VK–CK. HC and LC were cloned in separate mammalian expression vectors. The biNTes were expressed by transiently co-transfecting 30μg of both plasmids of dimeric biNTE with 180μg PEI (cat no. 7854; biotechne) (PEI:DNA 3:1 w/w mixed in 2ml plain DMEM media) in 293T cells at 85% confluence grown in T175 flask. The media was replaced with 30ml fresh media after 24hours and added additional 30ml fresh media replaced with 30 mL fresh complete DMEM (10% FBS, penicillin/streptomycin, glutamine) after 24 hours and added additional 30 mL complete medium after 48hours. Cultures were maintained at 37 °C, 5% CO₂. On Day 6, culture supernatants were collected and clarified by two sequential centrifugations. The clarified supernatant was then filtered through a 0.4 μm membrane. The engagers were purified using rProtein A/Protein G GraviTrap column (cat no. GE28-9852-56; Cytiva) by passing 60ml of culture supernatant followed by washing twice with 10ml 1xPBS. The antibody was eluted in 1ml fractions of 0.1M Glycine-HCL (pH 2.7) elution buffer and neutralized by 60μl 1M Trizma hydrochloride solution pH 9.0 (cat no. T2819; Merk) and the elution was repeated for 6 times. The eluted biNTE were concentrated using Amicon Ultra-15 centrifugal filters (Diameter (Metric): 29.7 mm MWCO: 30,000 d) (cat no. UFC903024; Merk) and washed twice with 1x PBS. The purity of biNTE was assessed by SDS-PAGE under reducing and non-reducing conditions.

iTCR scFv AA sequences:

DIKMTQSPSSMYASLGERVTITCKASQDINSYLSWFQQKAGKSPKTLIYRANRLVDGVPSRFSGSGSGQDYSL
TISSLEYEDMGIYYCLQYDEFPFTFGGGTRLEIKGGGGSGGGGSGGGGSQVQLQQSGPEVVRPGVSVKISCK

GSGYRFTDSAMHWWKQSHAKSLEWIGVISSYNGNTNYNQKFKGKATMTVDKSSSTAYMELARMTSEDSAI
YYCARSRDAMDYWGQGSTVTVSS

anti-BCMA scFV AA sequences:

DIVLTQSPPSLAMSLGKRATISCRASESVTILGSHLIHWYQQKPGQPPTLLIQLASNVQTGVPARFSGSGSRTD
FTLTIDPVEEDDVAVYYCLQSRTIPRTFGGGTKLEIKGGGGSGGGGSGGGGSQIQLVQSGPELKKPGETVKISC
KASGYTFTDYSINWVKRAPGKGLKWMGWINTETREPAYAYDFRGRFAFSLETSASTAYLQINNLYEDTATY
FCALDYSYAMDYWGQGSTVTVSS

Monovalent (anti-BCMA scFV-IgG) AA sequences:

MPLLLLLPLLWAGALADIVLTQSPPSLAMSLGKRATISCRASESVTILGSHLIHWYQQKPGQPPTLLIQLASNV
QTGVPARFSGSGSRTDFTLTIDPVEEDDVAVYYCLQSRTIPRTFGGGTKLEIKGGGGSGGGGSGGGGSQIQLV
QSGPELKKPGETVKISCKASGYTFTDYSINWVKRAPGKGLKWMGWINTETREPAYAYDFRGRFAFSLETSAS
TAYLQINNLYEDTATYFCALDYSYAMDYWGQGSTVTVSSGGGGSGGGGSGGGGSDKHTCPCPAPEAA
GGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLT
VLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLWCLVKGFYPSDIAVE
WESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

Monovalent (iTCR scFV-IgG) AA sequences:

MPLLLLLPLLWAGALADIKMTQSPSSMYASLGERVTITCKASQDINSYLSWFQQKAGKSPKTLIYRANRLVDG
VPSRFSGSGSGQDYSLTISSEYEDMGIYYCLQYDEFPTFGGGTRLEIKGGGGSGGGGSGGGGSQVQLQQS
GPEVVRPGVSVKISCKGSGYRFTDSAMHWWKQSHAKSLEWIGVISSYNGNTNYNQKFKGKATMTVDKSSST
AYMELARMTSEDSAIYYCARSRDAMDYWGQGSTVTVSSGGGGSGGGGSGGGGSDKHTCPCPAPEAAAG
GPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV
LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPCRDELTKNQVSLTCLVKGFYPSDIAVEW
ESNGQPENNYKTTTPVLDSDGSFFLTSLKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPG

Anti-BCMA FAB AA sequences:

Heavy chain:

QIQLVQSGPELKKPGETVKISCKASGYTFTDYSINWVKRAPGKGLKWMGWINTETREPAYAYDFRGRFAFSL
ETSASTAYLQINNLYEDTATYFCALDYSYAMDYWGQGSTVTVSS

Light chain:

DIVLTQSPPSLAMSLGKRATISCRASESVTILGSHLIHWYQQKPGQPPTLLIQLASNVQTGVPARFSGSGSRTD
FTLTIDPVEEDDVAVYYCLQSRTIPRTFGGGTKLEIK

Fc IG AA sequences:

ASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSS
SLGTQTYICNVNHKPSNTKVDKKVEPKSCDKTHTCPPCPAPEAAGGPSVFLFPPKPKDTLMISRTPEVTCVTV
DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI
SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSK
LTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLSLS

Immunofluorescence and confocal microscopy

For immune synapse imaging, 10^6 /ml of untransduced iNKT or BCMA CAR-iNKT cells were mixed with MM1.S target cell at 1:1 ratio in serum-free RPMI and incubated for 30 to 45min at 37°C on a glass microscope slide (#PH299 BK, Hendley Essex). Cells were fixed in ice-cold methanol for 5min, blocked in 2% BSA in PBS for 40 minutes and stained overnight at 4°C with rabbit anti-actin (cat no. A2066, MilliporeSigma) and mouse anti- γ Tubulin (cat no. T6557, MilliporeSigma) primary antibodies. The next day, samples were washed and incubated with a donkey anti-rabbit (cat no. A-31572, ThermoFisher) or a goat anti-mouse (cat no. A-11001, ThermoFisher) fluorophore-conjugated secondary antibodies. For PLXND1 expression analysis, iNKT cells only were incubated on the slides for 15min at 37°C, fixed for 15 min in 4% paraformaldehyde (cat no. 15710-S, Electron Microscopy Systems) and permeabilized in 0.1% Triton X-100 (Sigma) for 5min. Samples were then blocked and stained as described above using a primary anti-Plexin D1 antibody (cat no. ab28762; Abcam) followed by a goat anti-rabbit fluorophore-conjugated secondary antibody (cat no. A-21428; ThermoFisher) and Alexa Fluor® 488 phalloidin (cat no. 8878; Cell Signaling Technology) for 45min at RT. All samples were mounted in antifade medium with DAPI (cat no. H-1500-10; Vector Laboratories) and were acquired on an LSM-780 confocal laser scanning microscope. Z-stack series of 0.62 μ m intervals were taken with a $\times 63$ oil DIC Plan-Apochromat objective (numerical aperture 1.4).

Data analysis and software

All the flow cytometry analysis was performed using Flow Jo software. The graphical data and statistics were performed using Prism 10 software and bioRender tools under Imperial academic licence. Unpaired t-test or non-parametric Mann-Whitney U test or ordinary one-way Anova used to compare independent groups and significance were calculated based on the p values. For the line graphs, the significance colour code matches the sample colour in the graph. All the RNA sequencing traces were aligned using STAR and differential analysis performed using DESeq2 R Bioconductor packages by paired analysis. Image J was used for automatic cell counting of bound cells on monolayers for cell avidity experiments. In-vivo Imaging system (IVIS) and Aura software were used for *in vivo* bioluminescence photons analysis. PLXND1 expression in immune cells was derived from the Human Protein Atlas. The original datasets from which the relevant data were curated were from Monaco et al⁴⁶, and Schmiedel et al⁴⁷.

Data availability

The RNA sequencing data has been submitted to GEO database (accession #GSE312032) <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE312032> and all the associated data has been submitted in the supplementary files and source data spread sheets shared in figshare repository <https://doi.org/10.6084/m9.figshare.32390247>. Public data sets for supplementary figure 2C-D⁴⁶⁻⁴⁷ were derived from the human protein atlas database <https://www.proteinatlas.org/ENSG00000004399-PLXND1/single+cell>. SEMA4A expression for MM1S and H929 cells (Figure 2G & Suppl Fig. 2F) derived from CCLE data sets <https://sites.broadinstitute.org/ccle/datasets>, and MMRF data sets for primary cells (Suppl Fig. 2G &H) <https://themmrf.org/for-researchers/data-access-via-virtual-lab/>.

Figure Legends

Figure 1. Higher proliferative potential and avidity of CD28z CAR-iNKT

A. Structure of CARs.

B. Left: iNKT frequency in PBMC before and after selection as identified. Right: cumulative data (n=8 healthy donors).

C. Transduction efficiency as assessed by flow-cytometry (left) and cumulative data (right).

D. Surface expression of BCMA and CD1d detected by flow-cytometry in MM1.S and H929 cells (left) and cytotoxic activity of BCMA CAR-iNKT cells as compared to non-transduced (NT) iNKT cells (n=5 donors).

E. Immune synapse formation of BCMA CAR-iNKT cells conjugated to MM1.S target cells. 3D confocal microscopy reconstruction of z-stacks is shown and used to quantitate the distance of the centrosome (green) to the synapse; distance $<3\mu\text{M}$ =polarised centrosome (n= 3 donors, each point represents a synapse point distance).

F. Increased short-term proliferative capacity of CD28z CAR-iNKT cells as compared to all other CAR- and NT- iNKT cells in the presence of IL-15 alone, or without external IL-15 assessed by real-time longitudinal Incucyte® imaging (n=3 donors). $p = 0.01, 0.0001, 0.047, <0.0001, 0.008$ for NT, 41BBz, OX40z, CD28-41BBz, CD28-OX40z as compared to CD28z at 108 hours.

G. Absolute numbers and fold expansion of CD28z CAR-iNKT after stimulation with alphaGalCer-loaded C1R-CD1d cells as compared to all other CAR-iNKT (n=3 donors).

H. CD28z CAR-iNKT proliferation capacity as compared to other co-stimulatory domain CAR-iNKT in the presence of tdTomato expressing MM1.S cells, without any cytokine supplementation (n=duplicates of 3 donors). Colour code of the p values matches the sample colour as compared to CD28z.

I. Myeloma re-challenge assay. Cytotoxic activity of CD28z and 41BBz CAR-iNKT against MM1.S cells at E:T ratio 1:1 for 4 repeated challenges as assessed by real-time Incucyte® imaging (n=duplicates of 3 donors).

J. Left: CAR- and NT-iNKT cell binding capacity on MM1.S cells under increasing acoustic force; Right: CAR-iNKT binding at rForce 1000pN (representative of n=2 donors, datapoints shown for individual runs).

K. Avidity of CAR-iNKT against MM1.S cells under shear-stress in flow-chamber assay. Representative image of cell binding at 24 dyne/cm^2 , binding efficiency and cumulative data. (n= 4 (OX40z, CD28-41BBz, CD28-OX40z) & 5 (NT, CD28z, 41BBz) donors). Two-tailed statistical analysis Mann-Whitney U test (E &K), unpaired t test (F, G, H) and ordinary one-way Anova (J) performed. All error bars show mean $\pm$ SEM; box-whiskers show minimum-to-maximum values. $p < 0.0001$ is referred as $p < 0.0001$. non significance measured by $p > 0.05$.

Figure 2. PLXND1-SEMA4A interaction determines higher avidity of CD28z CAR-iNKT

A. Venn diagram shows number of commonly shared differentially regulated genes between CD28z and 41BBz compared to NT iNKT cells at steady state (n=3 donors (d1-d3)).

B. Volcano plot for differentially regulated genes between CD28z and 41BBz CAR iNKT cells at steady state (n=3 donors). The names of top 25 significantly regulated genes are shown including PLXND1.

C. Heatmap shows the log2 count values of key genes differentially expressed in NT, CD28z and 41BBz CAR iNKT cells. ZBTB16, a marker for iNKT cells shown as control. (n=3 donors).

D. PLXND1 expression in BCMA CAR iNKT cells with different co-stimulatory domains and NT iNKT as assessed by flow cytometry (histogram, top) and in BCMA CD28z and 41BBz CAR-T cells (histogram, bottom); right: Geometric Mean of Fluorescence Intensity (GMFI) cumulative data (n= iNKT (5 donors), T (4 donors)).

E. Left: PLXND1 expression in CD28z CAR iNKT cells compared to NT and 41BBz iNKT cells as assessed by immunofluorescence; right: cumulative data of intensity of staining. Each dot represents a cell (n=3 pooled donors).

F. Cell binding efficiency of CD28z CAR iNKT cells on MM1.S cells in the presence of PLXND1-blocking antibody or isotype control under shear stress (n= CD28z (5 donors) and 41BBz (4 donors)).

G. Expression of *SEMA4A* mRNA and its family members in MM1.S cells from CCLE dataset.

H. Left: *SEMA4A* expression in *SEMA4A* in wild type and knock-out MM1.S cells. right: Avidity of NT, CD28z and 41BBz CAR iNKT cells under shear stress against *SEMA4A*-expressing and non-expressing MM1.S cells (n= NT (4 donors), CD28z and 41BBz (5 donors each)).

I. PLXND1 and GFP co-expression as assessed by flow cytometry of shown flow-sorted effectors on the basis of PLXND1 expression (left). Flow chamber avidity assay of indicated CAR iNKT cells over a range of shear stress levels (right) and representative images of indicated effectors bound to MM1.S myeloma cells at 24 dyne/cm² (middle). (n=3 donors).

Two-tailed statistical analysis Mann-Whitney U test (D, E, F, H), Unpaired t-test (I) performed. All error bars show mean±SEM, and box and whiskers show minimum to maximum values with all data points. p <0.0001 is referred as p < 0.0001. non significance measured by p > 0.05.

Figure 3. CD28z CAR-iNKT outperform other CAR designs in PLXND1-dependent manner

A. Schematic of *in vivo* experimental design associated with figure 3 (A-H).

B-C. Representative BLI images of mice from control and BCMA CAR iNKT with different co-stimulatory molecules-treated groups for before treatment (day -1) and post-treatment (day 21) and its cumulative photon strength shown for each group. (n=4 mice (control and OX40z each group), 5 mice (CD28z, 41BBz, CD28-41BBz and CD28-OX40z each group)).

D. Kaplan-Meier survival curve for all the groups treated with 1×10^6 BCMA CAR iNKT cells and control group.

E. Left: Representative flow cytometry dot plots and its cumulative data (right) for frequency of MM1.S-luci cells as measured by tdTomato fluorescence marker by flow cytometry in the bone marrow of all the groups collected at end point. (n=4 mice (control and OX40z each group), 5 mice (CD28z, 41BBz, CD28-41BBz and CD28-OX40z each group)).

F&G. Representative flow cytometry analysis plots for iNKT frequency in the bone marrow for each group (F), and its cumulative data (G). (n=4 mice (control and OX40z each group), 5 mice (CD28z, 41BBz, CD28-41BBz and CD28-OX40z each group)).

H. Absolute iNKT cell numbers in the bone marrow of each group counted based on flow cytometry analysis using counting beads. (n=4 mice (control and OX40z each group), 5 mice (CD28z, 41BBz, CD28-41BBz and CD28-OX40z each group)).

I. Schematic of *in vivo* experimental design associated with figure 3J&K.

J. Total body BLI signals of mice treated with anti-Plexin D1 antibody treated group as compared to isotype antibody-treated group and untreated control group (J).

K. Kaplan-Meier survival curve for J. (n=4 mice (control group); 5 mice (isotype group); 6 mice (Ab group)). Two-tailed statistical analysis Mann-Whitney U test (E, G, H & J) and ordinary one-way Anova (N), The Gehan-Breslow-Wilcoxon test (D & K) performed. All error bars show mean $\pm$ SEM, and box and whiskers show minimum to maximum values with all data points. $p < 0.0001$ is referred as $p < 0.0001$. non significance measured by $p > 0.05$.

Figure 4. CAR iNKT outperform CAR-T against myeloma

A. BCMA CAR iNKT cell avidity on MM1.S cells shown as compared to BCMA CAR-T cells of same donors with isotype or PLXND1 blocking antibody at 1µg/ml under shear stress (n=3 donors). p value colour matches the compared sample colour.

B. Schematic of *in vivo* experimental design associated with Figure 4A-G.

C&D. Representative BLI images of mice from control, BCMA CAR iNKT and BCMA CAR-T cell-treated group before (day -1) and after treatment (day 21) (C) and its cumulative photon emission shown for each group (D). (n=4 mice (control group); 5 mice (treatment group each)).

E&F. Left: Representative dot plots and cumulative data (right) for frequency of MM1.S-luci cells in the bone marrow (E) and spleen (F) analysed by flow cytometry using tdTomato fluorescence marker at end point. (n=4 mice (control group); 5 mice (treatment group each)).

G. Kaplan-Meier survival curve for myeloma-bearing mice treated with CAR iNKT or CAR-T as compared to untreated control as shown in B. (n=4 mice (control group); 5 mice (treatment group each)).

H. Images of CD1d+ H929 subcutaneous myeloma tumour retrieved from NSG mice treated with 1×10^6 indicated effector cells.

I. Volume of subcutaneous myeloma tumour over time in NSG mice treated with indicated effectors. (n=5 mice (control group); 6 mice (treatment group each)).

J. Weight of above subcutaneous myeloma tumours at end of experiment. (n=5 mice (control group); 6 mice (treatment group each)).

Two-tailed statistical analysis unpaired t-test (A), Mann-Whitney U test (D, E, F, I & J), The Gehan-Breslow-Wilcoxon test (G) performed. All error bars show mean±SEM, and box and whiskers show minimum to maximum values with all data points. p < 0.0001 is referred as p < 0.0001. non significance measured by p > 0.05.

Figure 5. Development and anti-myeloma activity of iNKT specific engagers

A. Design of monovalent and bivalent biNTE.

B. Left: Flow-cytometric analysis of same-donor purified iNKT and T cells after staining with increasing concentrations (0-1µg) of bivalent biNTE; (right) cumulative data is shown as GMFI values for iNKT and T cells (n=3 donors).

- C.** Representative data showing affinity to soluble BCMA of bivalent vs monovalent biNTE as determined by surface plasmon resonance (SPR) (n=3 donors).
- D.** Cytotoxic activity of NT-iNKT cells incubated with bivalent vs monovalent biNTE against MM1.S as assessed by real-time live Incucyte® imaging for 72 hours (n=3 donors).
- E.** Cytotoxic activity of same-donor iNKT and T cells incubated with bivalent biNTE against MM1.S myeloma cells (representative n=3 donors).
- F.** Cytotoxic activity of iNKT Incubated with biNTE in the presence or not of alphaGalCer against BCMA-expressing parental and BCMA knock out CD1d+ H929 myeloma cells (n=3 donors).
- G.** iNKT-biNTE-mediated cytotoxicity of patient-derived primary bone marrow myeloma cells at 2:1 E:T ratio and different biNTE antibody concentrations for 20hr. Left: representative dot plot for patient #1. Right: cumulative data normalized to target alone (n=5 patients). CD138+ cells were measured for each condition and normalized to myeloma target cells alone.
- H.** MM1.S cell subcutaneous tumor size (left) and volume (right) treated with NT-iNKT and/or biNTE and untreated mice. (n= 4 mice (control and biNTE group each), 5 mice (iNKT and iNKT+biNTE group each). p value colour codes matches the compared sample line.
- I.** Cytotoxic activity of NT-iNKT cells incubated with biNTE and BCMA CAR-iNKT cells against MM1.S cells shown for 16hr (n=3 donors).
- J.** Schematic of experimental design shown for MM systemic model for biNTE treatment related to Figure 5K&L.
- K.** Left: Representative BLI of mice with systemic myeloma treated with BCMA CAR-iNKT or NT iNKT plus biNTE before (day -1) and after treatment (day 17); cumulative data shown in Suppl Fig. 5K (n= 4 mice (control and NT iNKT group each), 5 mice (iNKT+biNTE and CAR iNKT group each).
- L.** Kaplan-Meier survival of myeloma-bearing mice treated as shown in Figure 5J.
- Two-tailed statistical analysis unpaired t test (D), Mann-Whitney U test (H), and The Gehan-Breslow-Wilcoxon test (L) performed. All error bars show mean±SEM. $p < 0.0001$ is referred as $p < 0.0001$. non significance measured by $p > 0.05$.

Figure 6. Enhanced anti-myeloma activity by dual CAR-iNKT plus biNTE targeting

- A.** Flow cytometry plots show exogenous expression of FCRL5 in MM1.S (MM1.S-FCRL5+) cells and corresponding WT (MM1.S_FCRL5-) cells (left) and cytotoxic activity of FCRL5 CAR iNKT or NT iNKT cells incubated with or without biNTE against MM1.S_FCRL5- and MM1.S-FCRL5+ cells (right). (n=3).
- B.** Schematic of *in vivo* experimental design associated with figure 6.

C&D. Representative BLI images of mice from control and FCRL5 CAR-iNKT with or without biNTE group before (day -1) and after treatment (day 28) (C) and their cumulative BLI data (D). (n=5 mice per group). Significance colour code indicates corresponding sample colour.

E&F. Flow cytometry analysis at end point of the frequency of bone marrow MM1.S₋FCRL5₋ and MM1.S-FCRL5₊ cells in treated and control mice shown as dot plot (E) and cumulative data for each group (F). Numbers indicate the ratio between FCRL5₋ and FCRL5₊ cells. (n=5 mice per group).

G. Flow cytometry analysis at end point of total MM1.S myeloma burden as determined by tdTomato fluorescence marker in the bone marrow of treated and control mice. (n=5 mice per group).

H. Kaplan-Meier survival curve of myeloma-bearing mice left untreated (control) or treated with FCRL5 CAR iNKT cells or FCRL5 CAR iNKT plus biNTE (n=5 mice per group) as shown in Figure 6B. Two-tailed statistical analysis unpaired t test (A), Mann-Whitney U test (D, F, G), and The Gehan-Breslow-Wilcoxon test (H) performed. All error bars show mean±SEM, and box and whiskers show minimum to maximum values with all data points. $p < 0.0001$ is referred as $p < 0.0001$. non significance measured by $p > 0.05$.

Figure 1

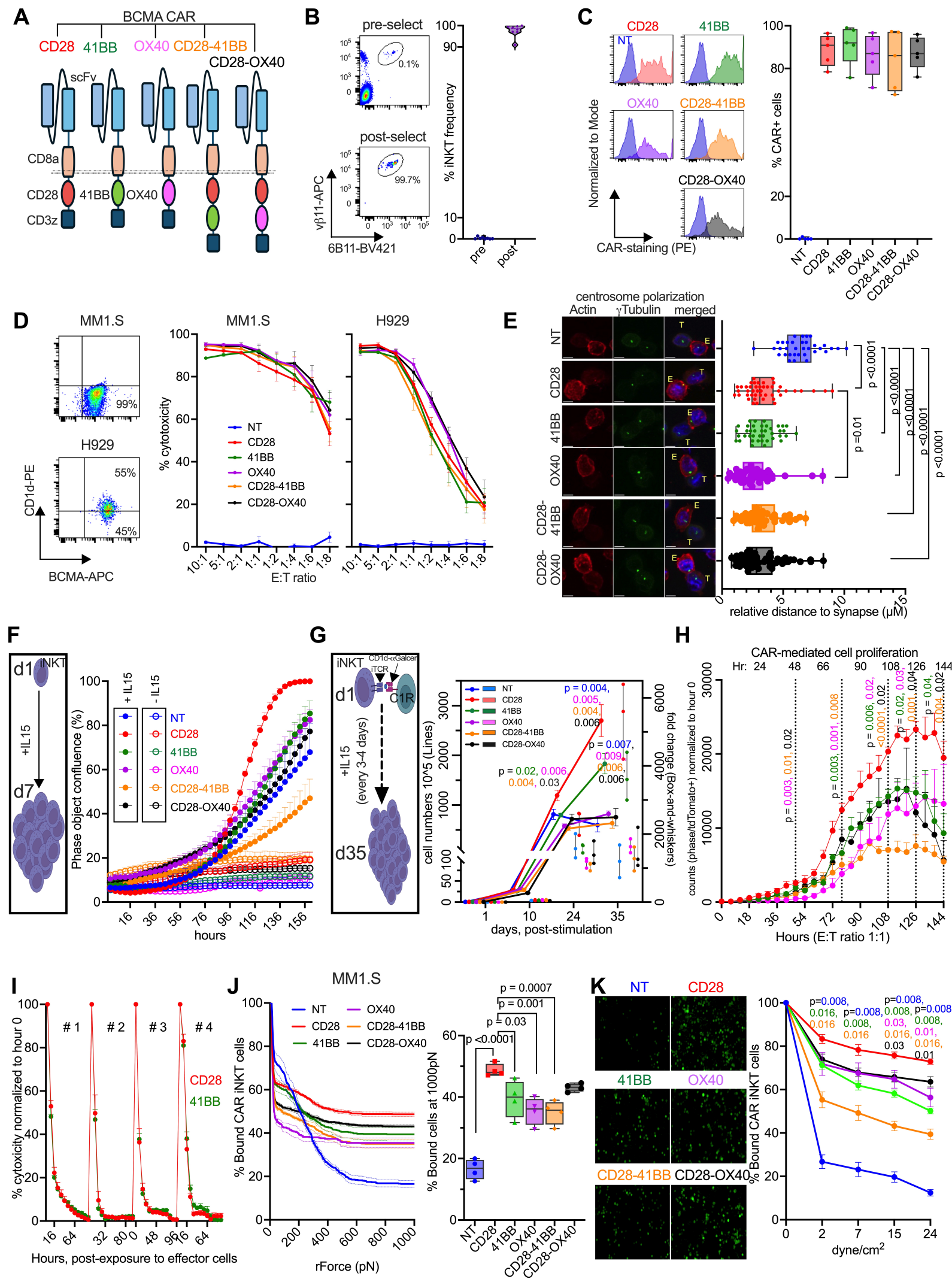


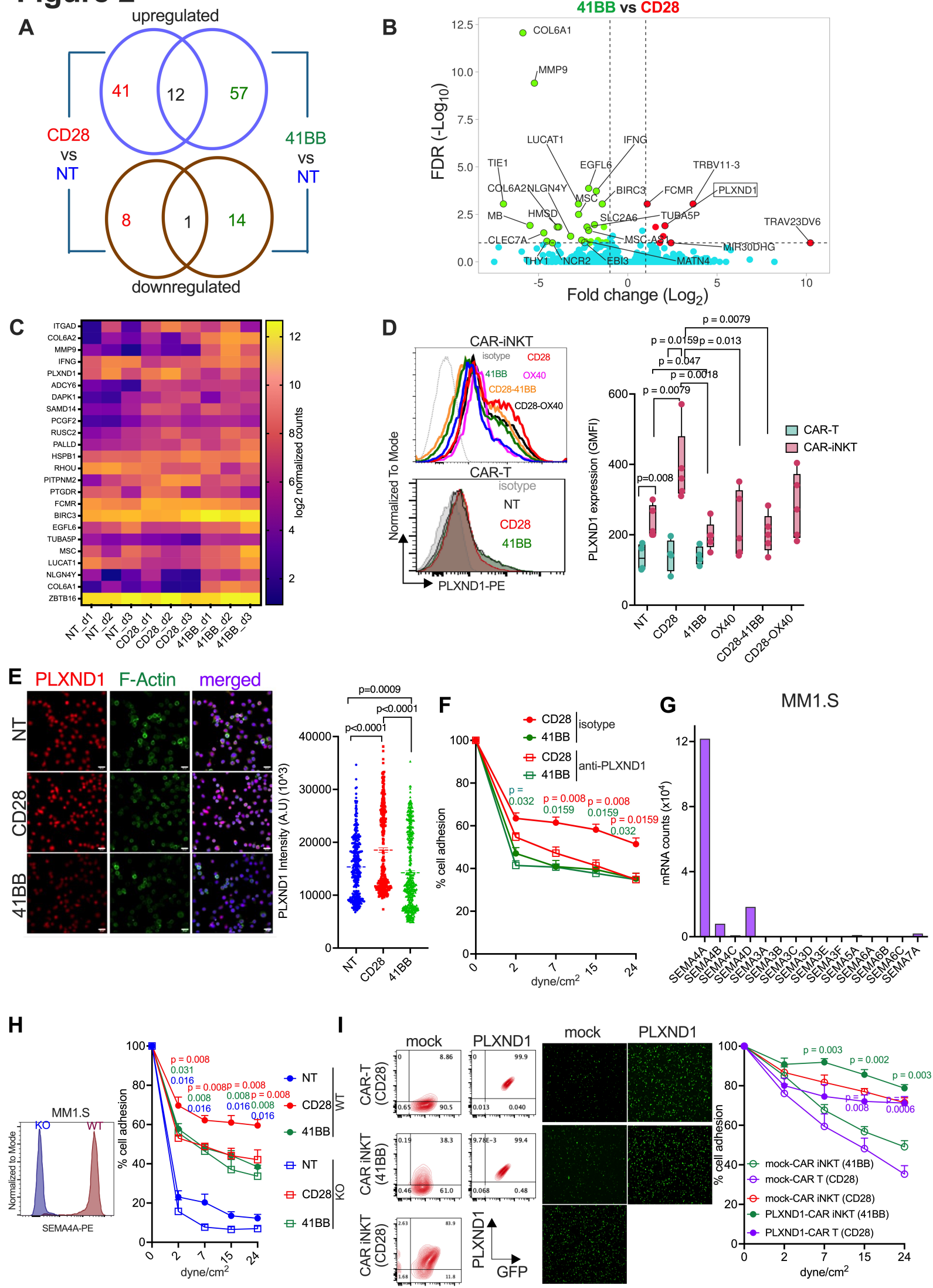
Figure 2

Figure 4

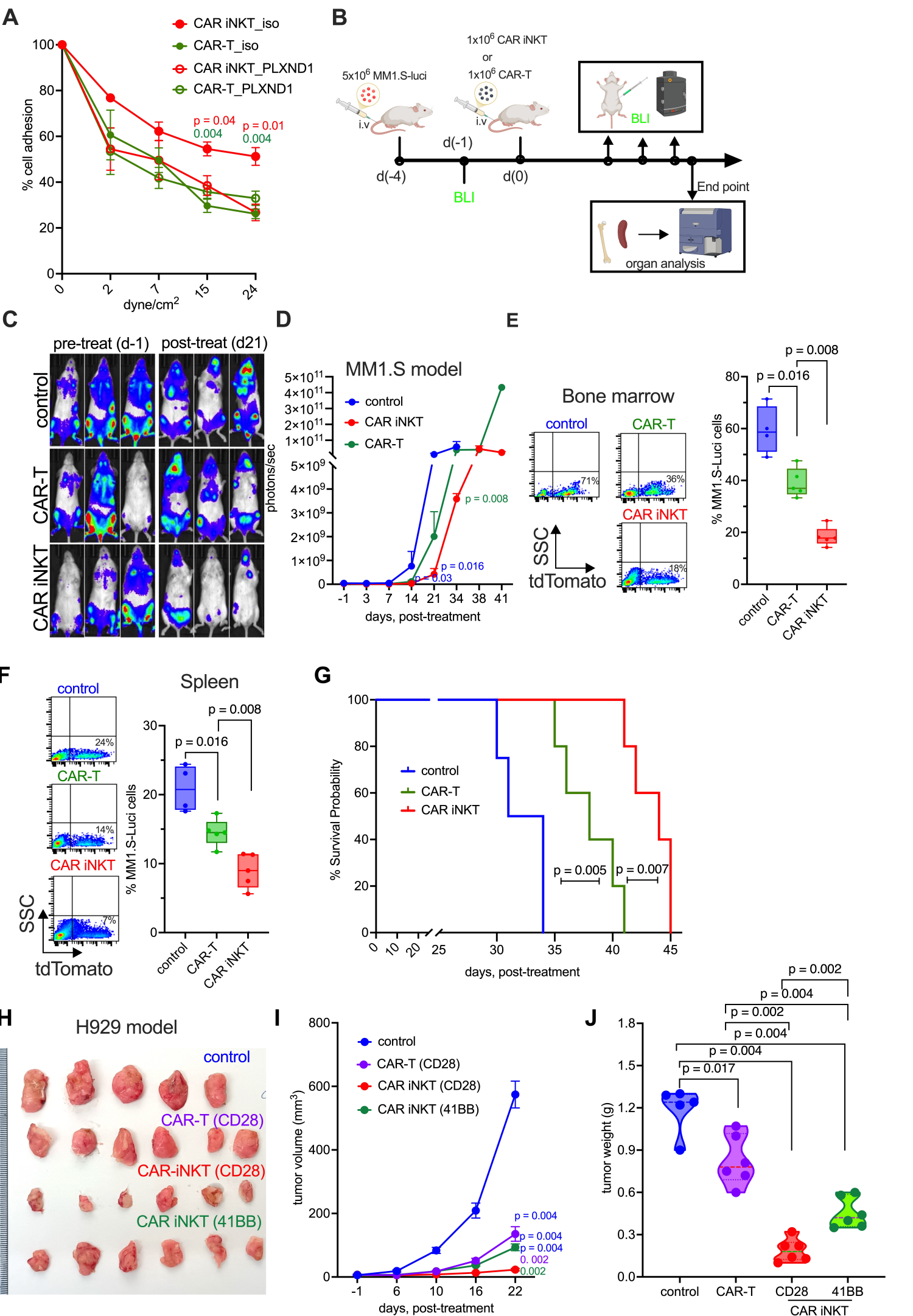


Figure 5

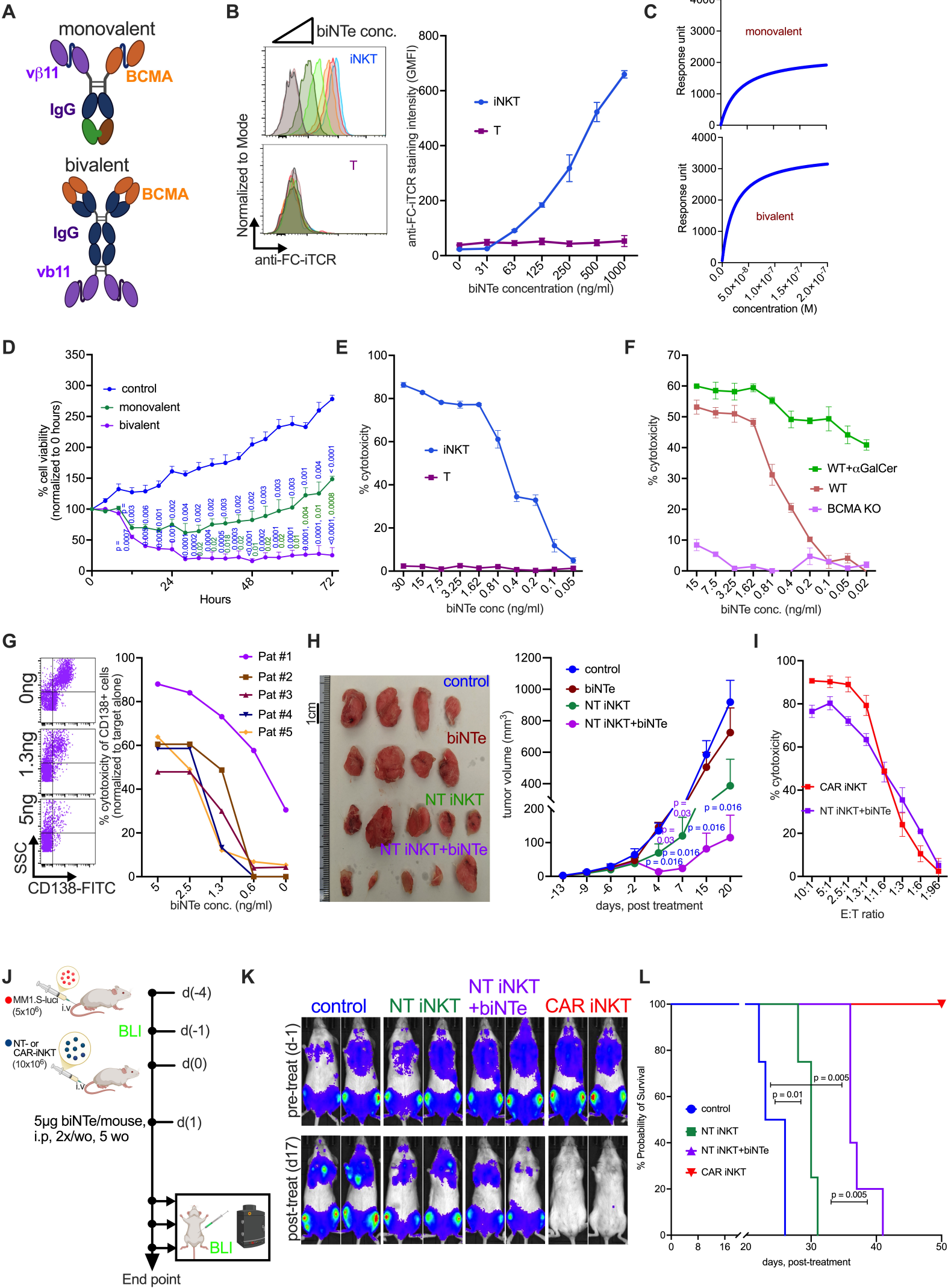


Figure 6