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# An APRIL based chimeric antigen receptor for dual targeting of BCMA and TACI in Multiple Myeloma

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**Keypoints:**

- APRIL is a compact, self-protein that binds two MM antigens (BCMA and TACI) with high affinity. We present an APRIL based CAR.

- Dual antigen targeting increases the availability of tumour binding sites and reduces the risk of antigen negative disease escape.

## Abstract

B-cell maturation antigen (BCMA) is a promising therapeutic target for multiple myeloma (MM), but expression is variable, and early reports of BCMA targeting chimeric antigen receptors (CARs) suggest antigen down-regulation at relapse. Dual antigen targeting increases targetable tumour antigens and reduces the risk of antigen negative disease escape. 'A proliferation-inducing ligand' (APRIL) is a natural high affinity ligand for BCMA and transmembrane activator and CAML interactor (TACI). We quantified surface tumour expression of BCMA and TACI on primary MM cells (n=50). All cases tested expressed BCMA and 39(78%) of them also expressed TACI. We engineered a third generation APRIL-based CAR (ACAR), which killed targets expressing either BCMA or TACI ($p<0.01$ and $p<0.05$ respectively, cf control, E:T ratio 16:1). We confirmed cytolysis at antigen levels similar to those on primary MM, at low effector to target ratios (56.2±3.9% killing of MM.1s at 48 hours, E:T ratio 1:32, $p<0.01$) and of primary MM cells (72.9±12.2% killing at 3 days, E:T ratio 1:1, $p<0.05$, n=5). Demonstrating tumour control in the absence of BCMA, cytolysis of primary tumour expressing both BCMA and TACI was maintained in the presence of a BCMA targeting antibody. Further, using an intramedullary myeloma model, ACAR T-cells caused regression of established tumour within 2 days. Finally, in an in vivo model of tumour escape, there was complete ACAR-mediated tumour clearance of BCMA+TACI- and BCMA-TACI+ cells while a scFv CAR targeting BCMA alone resulted in outgrowth of BCMA negative tumour. These results support the clinical potential of this approach.

## Introduction

Multiple myeloma (MM) is a cancer of plasma cells (PC) which is responsible for 2% of cancer deaths[1]. Myeloma remains largely incurable, despite significant progress seen with the inclusion of proteasome inhibitors (PIs) and immunomodulatory drugs (IMiDs) into the mainstay of treatment regimens[2]. Furthermore, current therapeutic strategies fail to benefit approximately 15% of patients who have primary refractory disease, and/or adverse genetics[3]. There remains a need for new myeloma therapies with different mechanisms of action, particularly those that can induce durable remissions.

Chimeric antigen receptors (CAR) typically graft the specificity of a monoclonal antibody (mAb) onto a T-cell, redirecting T-cell cytotoxicity to tumour by a mechanism unimpeded by MHC class restriction[4]. CAR T-cells may have advantages over mAb based approaches since CAR T-cells can actively migrate to sites of disease and persist thus engendering a sustained rejection of target cells. CD19 directed CAR T-cell therapy has been effective against refractory B-cell malignancies and sustained responses are seen in the face of chemotherapy resistant disease[5-9]. Applying CAR T-cell therapy to MM however faces several challenges not least target antigen selection. CD19 is only expressed in a small proportion of tumour cells[10] and well characterized antigens expressed by myeloma such as CD38[11,12], CD56[13,14] and CD138[15] may not be suitable targets due to expression outside the lymphoid compartment.

B-cell maturation antigen (BCMA) is a member of the tumor necrosis factor (TNF) receptor superfamily, is upregulated at the terminal stages of B-cell maturation, and selectively expressed on PC[16,17]. BCMA is absent on haemopoietic stem cells[16-18] and is expressed by nearly all cases of MM, albeit at variable, and often low density[16]. Consequently, BCMA has been targeted by several immunotherapeutic strategies in MM including CAR approaches and bispecific T-cell engager (BiTE) therapies[17,19-23]. In the first reported clinical trial investigating a BCMA targeting CAR, rapid and dose dependent disease response was seen in 4 of 12 patients despite substantial tumour load and heavy pre-treatment[24]. However, relatively high T-cell doses were needed to achieve durable remissions and, possibly akin to CD19 down-regulation in CD19 CAR T-cell studies[25], loss of BCMA expression at relapse was reported[24].

Thus, while BCMA is a promising target, challenges of low target density and target escape may compromise clinical efficacy. To address this, we hypothesized that dual antigen binding would increase the level of targetable antigen on tumour cells, while potentially reducing the incidence of antigen negative escape, in this way enhancing therapeutic potential and capacity for long term disease control. Transmembrane activator and CAML

interactor (TACI) is also a TNF receptor and is involved in maturation of B-cells, including their maturation to PC[26,27]. Importantly, TACI is also expressed on MM cells[18,28,29]. A proliferation-inducing ligand (APRIL) is a natural ligand of both BCMA and TACI and is an attractive antigen binder as it is a compact, oligomerizing, single domain, self-protein, that binds both MM antigens with high, nanomolar affinity[30,31].

In this work, we describe a novel CAR construct using a truncated form of (APRIL) as the tumour targeting domain (ACAR), which recognizes both BCMA and TACI on MM cells. We establish ACAR potency at antigen levels seen in clinical samples, at low effector to target ratios (E:T), against primary cells, as well as in murine models of myeloma and tumour escape.

## Method

### BCMA/TACI quantification

Mononuclear cells (MNCs) were stained with CD138 APC (MI15) to identify tumour and either murine IgG2a PE Isotype control, rat IgG2a PE Isotype control, anti-BCMA PE (clone 19F2) or anti-TACI PE (clone 1A1). All antibodies from BioLegend. BD Fortessa was used for cell acquisition and data analysed using FlowJo_V10 (Treestar). Antibodies bound per cell (ABC) was calculated using BD QuantiBRITE™ beads and subtracting ABC of isotype control (greater than 100 ABC considered positive).

### Cloning

All plasmids were cloned in-house[32] into the oncoretroviral vector SFG[33] and RD114-pseudotyped supernatant was produced as previously described[32]. Sequence coding for residues 116 to 250 of the canonical sequence for APRIL (Uniprot 075888) was cloned between signal peptide from IgG kappa chain V-III to CAR scaffolds comprising of either IgG1 hinge spacer, CD8 alpha spacer or IgG1 Fc domain[34] co-expressed with RQR8 using an in-frame foot-and-mouth–like 2A peptide, TaV[35]. Epidermal growth factor receptor vIII (EGFRvIII) and BCMA targeting CARs were engineered using MR1-1[36] or 11-D-5-3[17] scFvs, respectively, a CD8 spacer and a CD28-OX40-CD3ζ endodomain.

### CAR T-cells

Peripheral blood mononuclear cells (PBMC) obtained by density gradient centrifugation (Ficoll Paque, GE lifesciences) were stimulated with CD3 and CD28 antibodies (0.5µg/ml, Miltenyl) and IL-2 (100IU/ml, Genescript) then transduced as before[37] to obtain CAR T-cells.

Transduction efficiency was assessed by FACS of cells stained for RQR8 (Qbend10 antibody, R&D) or APRIL (anti-APRIL biotin) and RQR8 for ACAR T-cells.

Further methods are available in Supplementary Data.

## Results

### Primary myeloma cells express BCMA and TACI

We have previously reported variable surface expression of BCMA on tumour[16]. Here, we sought to quantify expression levels of both BCMA and TACI on the cell surface of primary BM-derived MM cells.

Ficolled BM MNCs from 50 patients were stained for CD138 to identify tumour and anti-BCMA or TACI (Figure 1A; Table S1; Figure S1A) using QuantiBRITE$^{TM}$ beads for antigen quantification. We found expression of BCMA on CD138+ tumour cells from all patients tested (median: 1061, range: 105-8323 ABC) (Figure 1B). TACI was co-expressed on tumour (Figure S1B) and detected on MM cells from 39 of these patients, at generally lower levels (median:333, range: 0-21301 ABC) (Figure 1C). Thus, we calculated that concurrent targeting of both antigens compared to BCMA alone would increase levels of target antigen in 78% of patients and result in an increased mean combined targetable antigen density on tumour of 2458 ABC compared to 1623. TACI expression also exceeded BCMA in a subset of samples (16%) (Figure S1C). Notably, 7 of these 8 patients expressed less than the median level of BCMA suggesting that concurrent TACI targeting may be particularly beneficial in a proportion of BCMA$^{lo}$ tumours.

In keeping with our previous findings[16], patients with a new diagnosis of myeloma (54%) expressed lower levels of BCMA ($p<0.05$) compared to relapsed disease (46%) but there was no such correlation with TACI expression ($p=0.3$, Figure S1D). Of the 42 (84%) of patients for whom FISH was available, the 25 (60%) of patients with high risk cytogenetic lesions had higher levels of BCMA ($p<0.05$ by Mann-Whitney) but a trend to lower levels of TACI ($p=0.06$) (Figure S1E).

Thus we confirm the surface tumour expression of BCMA on tumour from all patients tested and the co-expression of BCMA and TACI in the majority (78%) of patients, supporting a therapeutic strategy for myeloma that targets both these antigens.

## Similar expression pattern of BCMA and TACI in normal tissues

The selectivity of BCMA expression to lymphoid cells[17] and more specifically PC has been previously described[16]. TACI is also a known lymphoid antigen expressed mainly on B-cells, but at an earlier stage of maturation and particularly in maturing subsets of splenic B-cells[38]. As expression of TACI on normal tissues is less well known, we performed reverse transcription-polymerase chain reaction (qRT-PCR) of BCMA and TACI in a range of normal tissues.

Transcript analysis from 72 normal tissues, each from 3 donors, revealed highest levels of BCMA and TACI expression in lymphoid tissues. Notable expression levels were also seen in gastrointestinal and in bronchial tissues which likely reflects the presence of lymphocytes at these anatomical sites. BCMA but not TACI expression was also noted in testes and gall bladder (Figure S2; Table S2). BCMA and TACI transcripts were equally high in the splenic parenchyma but it is noteworthy that in other tissues BCMA gene expression was up to 10-fold higher than TACI.

These data are consistent with TACI expression being restricted to the lymphoid compartment, with distribution broadly similar to that of BCMA.

## Optimization of APRIL based CAR constructs

APRIL is a soluble ligand that binds BCMA and TACI. Additionally, the amino-terminus of APRIL binds proteoglycans [39,40] but is not involved in the interaction with BCMA or TACI. To confirm that a truncated form of APRIL could bind BCMA and TACI when expressed on a cell surface, truncated APRIL was fused to the CD8 transmembrane domain and expressed on SUPT1 cells. Staining with recombinant soluble BCMA and TACI confirmed that truncated APRIL is both stably expressed and maintains BCMA and TACI binding when membrane bound (Figure S3A). Further, surface plasmon resonance analysis of soluble truncated APRIL binding to TACI and BCMA confirmed previously described binding kinetics (Figure S3B)[41].

Next, three APRIL-based chimeric antigen receptors (ACAR) were constructed, consisting of truncated APRIL fused to a spacer domain, a CD28 transmembrane and tripartite endodomain (CD28-OX40-CD3ζ)[42]. Spacers were either the hinge of human IgG1 (ACAR-H), the stalk of human CD8α (ACAR-CD8) or the hinge, CH2 and CH3 domains of human IgG1 modified to reduce Fc receptor binding[43] (ACAR-Fc) (Figure 2A).

PBMCs from normal donors were activated with IL-2, anti-CD28 and CD3 antibodies, retrovirally transduced with ACAR constructs, CD56 depleted, and tested against SUPT1 cells modified to express high levels of either BCMA, TACI or non-transduced (NT) targets.

Using 4 hour $^{51}$Cr release assay, T-cells transduced with ACAR-H(n=5) and ACAR-CD8(n=6) spacer variants caused cytolysis of SUPT1$^{BCMA}$ ($p<0.01$ for both ACAR constructs compared to PBMC NT at an E:T ratio 16:1, paired *t* test) and SUPT1$^{TACI}$ ($p<0.05$ for both ACAR constructs) targets. In comparison, ACAR-Fc transduced T-cells killed SUPT1$^{BCMA}$ targets (n=3, $p<0.05$) but not TACI expressing targets (Figure 2B).

After co-culture with antigen expressing target cells (1:1 with irradiated, SUPT1 cells) for 24 hours, interferon gamma (IFNG) release from ACAR-H(n=5) and ACAR-CD8(n=6) T-cells was detected. There was significant cytokine release observed on co-culture of both these ACAR constructs with SUPT1$^{BCMA}$ ($p<0.01$ for both) and SUPT1$^{TACI}$ ($p<0.05$ for both) compared to control targets. In comparison, ACAR-Fc did not result in cytokine release against TACI or BCMA expressing SUPT1 cells (n=3) (Figure 2C).

To assess proliferation of ACAR T-cells, effector T-cells were stained with Cell Trace Violet prior to 1:1 co-culture with SUPT1 targets and analysed by FACS at 4 days. Compared to control co-cultures with SUPT1$^{NT}$ targets, there was a significant increase in the percentage of proliferated ACAR-H and ACAR-CD8 transduced T-cells with SUPT1$^{BCMA}$ and SUPT1$^{TACI}$ ($p<0.001$ for both effectors with BCMA and TACI expressing targets) (Figure 2D, Figure S4).

Taken together, these data indicate that both ACAR-CD8 and ACAR-H demonstrated greater in vitro activity, compared with ACAR-Fc transduced T-cells. Both spacer variants resulted in target cytolysis, cytokine release and effector proliferation in response to SUPT1$^{BCMA}$ or SUPT1$^{TACI}$.

**APRIL CAR causes target cytolysis at low antigen densities, at a low E:T ratio, and in the presence of soluble APRIL , BCMA and TACI**

Clinical responses will likely require ACAR activity against the low levels of BCMA and TACI found on some primary MM cells, and at low E:T ratios. We thus explored the in vitro cytolytic potential of the two most promising ACAR constructs (ACAR-H and ACAR-CD8) under these conditions.

ACAR transduced PBMCs were tested against SUPT1 targets expressing a wide range of surface BCMA (421 to 1.5x10^5 ABC) and TACI (1063 to 6.3x10^4 ABC) (Figure 3A). By $^{51}$Cr release, T-cells transduced with either ACAR construct caused significant cytolysis of all BCMA and TACI expressing targets compared to control at all E:T ratios tested (32:1 to 4:1, 16:1 shown in Figure S5A). In an attempt to more closely replicate physiological conditions, co-cultures were then extended to 48 hours and the E:T ratio lowered to 1:10 and target kill assessed by FACS. In these conditions, T-cells transduced with ACAR-H and ACAR-CD8

both caused significant target cytolysis of unirradiated targets expressing even the lowest levels of BCMA and TACI (Figure 3B).

ACAR-mediated cytolysis of MM cells was confirmed in a number of human myeloma cell lines (HMCLs, Figure S5B). ACAR activity was also demonstrated at lower E:T ratios against MM.1s and U266 HMCL with significant target cytolysis down to an E:T ratio of 1:32 on co-culture with T-cells transduced with both ACAR constructs (Figure 3C). T-cells transduced with a BCMA-targeting CAR (BCMA CAR) based on the 11-D-5-3[17,24] scFv were also compared to the ACAR and despite low E:T ratios, there was no statistically significant difference in kill of MM.1s or U266 by the BCMA CAR and ACAR (Figure S5C).

Members of the TNF receptor superfamily found in the sera of MM patients may interfere with an APRIL based therapeutic strategy by blockade or inadvertent T-cell activation. We therefore quantified APRIL, BCMA and TACI in MM BM (Figure S6A), repeated cytotoxicity assays with ACAR-H against MM.1s at low E:T ratios (Figure S6B) and measured IFNG release (Figure S6C) in the presence of physiological levels of these proteins. There was no significant cytokine release and ACAR-mediated target cytolysis was unaffected by sAPRIL and sTACI but was reduced at the highest levels of sBCMA tested ($p<0.001$ at 1000ng/ml compared to media control).

Therefore in vitro, T-cells transduced with both ACAR-H and ACAR-CD8 demonstrate equivalent cytolytic activity and we consistently observed significant cytolysis of the lowest BCMA and TACI expressers even at low E:T ratios. Furthermore, ACAR killing was equivalent to that demonstrated by a scFv BCMA targeting CAR when used against BCMA expressing targets. We also observed that ACAR T-cells are not activated by soluble ligand and while tumour kill was also unaffected by physiological levels of soluble APRIL or TACI, attenuation of target kill was seen at the highest levels of sBCMA.

**APRIL CAR causes cytolysis of primary myeloma cells**

To test ACAR activity on primary tumour cells, allogeneic PBMCs transduced with ACAR-H and ACAR-CD8 variants were CD56 depleted, then co-cultured 1:1 with CD138-selected BM derived MM cells from 5 patients. Although BCMA and TACI expression varied between patient samples (BCMA 1224-7728 and TACI 563-1213 ABC, Figure 4A), tumour cytolysis and IFNG release were seen with both ACAR constructs in all samples. Survival and proliferation of ACAR T-cells was seen with 3 patient samples (#23, #17, #1 in Figure 4A).

Combining the results from the 5 patient samples, at D+3, ACAR-H and ACAR-CD8 resulted in 72.9±12.2% and 87.7±5.4% tumour death respectively (mean± SEM cytolysis relative to control). In comparison, baseline tumour cell death was 2.8±15.3% ($p<0.05$ for both ACAR

constructs by paired *t* test). There was no significant difference in target kill, cytokine release or T-cell expansion between the two ACAR spacer variants (Figure 4B).

We tested the ability of ACAR constructs to induce cytolysis by TACI alone by conducting cytotoxicity assays in the presence of high concentrations of anti-BCMA monoclonal antibody (S307118G03). We observed that the antibody blocked ACAR-mediated cytolysis of U266 (BCMA+TACI-) but not MM.1s cells (BCMA+TACI+). Encouragingly, anti-BCMA antibody did not attenuate killing of primary MM cells from 3 patients that expressed both BCMA and TACI (Figure 4C).

Taken together, these experiments confirm ACAR-mediated cell death of primary MM cells and support the assumption that, in the event of BCMA down-regulation, tumour control could be maintained by TACI expression on primary cells.

**Efficacy of APRIL CAR against myeloma *in vivo***

As these in vitro assays did not show a significant difference in efficacy of ACAR-H and ACAR-CD8, we proceeded to test the smaller and thus simpler of the two constructs, ACAR-H in an *in vivo* model.

To establish an intramedullary myeloma model, 22 NSG mice were injected intravenously with 10x10^6 HA+Fluc+MM.1s cells. Thirteen days later, there was intramedullary disease by BLI in all mice (Figure 5A) at which point 5x10^6 EGFRvIII CAR or ACAR-H T-cells (Figure 5B) were administered by tail vein injection into 8 animals. A single animal in the EGFRvIII CAR group did not recover following T-cells and a further mouse (with the lowest disease burden pre-CAR) had disease clearance. Nonetheless, by 2 days, there was less disease in ACAR than EGFRvIII CAR treated animals by BLI ($p<0.01$ by *t* test) and continued disease suppression in ACAR treated mice (Figure 5A-B). At termination of the experiment (D+12 post ACAR T-cells, D+25 post tumour cells) FACS of the BM confirmed significant tumour clearance in ACAR treated animals compared to both control cohorts ($p<0.05$ and $p<0.001$ compared to EGFRvIII CAR and untreated cohorts, Figure 5C). Tumour clearance in ACAR-H treated cohort was confirmed by immunohistochemistry (Figure 5D).

Human APRIL binds murine BCMA and TACI at similar affinities to their human isoforms[44] and ACAR causes equivalent cytolysis of SUPT1 targets expressing human or murine BCMA and TACI (Figure S7A). This provided the unique opportunity to investigate possible off target toxicity in our mouse xenograft model without modification to the ACAR construct. Numerous tissues were harvested from test mice (full list in Supplementary data) and on examination of formalin fixed and paraffin embedded (FFPE), haematoxylin and eosin (H&E)

stained tissue sections, we found there to be no treatment related histopathological findings in ACAR treated animals (Figure S7B).

**Persistent disease control in an *in vivo* escape model**

We propose dual antigen targeting as a means of reducing the risk of antigen-negative disease escape. To model the capacity for ACAR-mediated tumour control despite BCMA downregulation, NSG mice were engrafted with a mix of SUPT1$^{BCMA}$ and SUPT1$^{TACI}$ (4:1 ratio) tumour cells by tail vein before administration of ACAR or BCMA CAR.

Twenty-one NSG mice were injected with 3.5x10^6 Fluc expressing SUPT1 cells comprising SUPT1$^{BCMA}$(5807 ABC) and SUPT1$^{TACI}$(2229 ABC) (80%:20%). At 4 days mice received 5x10^6 NT T-cells, ACAR or BCMA CAR T-cells (n=7 per group) by tail vein injection. By BLI, there was continued tumour growth with NT T-cells, partial disease suppression with BCMA CAR and greatest tumour clearance in ACAR treated animals (Figure 6A-B). On termination of the experiment (D+13 and D+9 post tumour and CAR respectively), FACS of BM from animals receiving ACAR T-cells showed clearance of both SUPT1$^{BCMA}$ and SUPT1$^{TACI}$ ($p<0.001$ and $p<0.01$ compared to NT respectively), while BM from animals receiving BCMA CAR showed persistence of SUPT1$^{TACI}$ (p=ns compared to NT). There was continued engraftment of both tumour populations in animals receiving NT T-cells (Figure 6C-D) and evident T-cell persistence in all mice (Figure S8).

These data support the assumption that in comparison to targeting BCMA alone, dual antigen targeting of BCMA and TACI facilitates continued disease suppression in the event of BCMA downregulation or loss in patients who have tumour co-expression of both antigens.

## Discussion

BCMA is emerging as a lead therapeutic target in MM, as indicated by several on-going clinical studies. The NCI group have reported 12 patients treated with their BCMA targeting CD28-CD3ζ CAR[45] observing sustained responses at the highest dose level of 9x10^6 T-cells/kg[24] and a further 21 patients treated with a separate (bb2121) 4-1BB-CD3ζ CAR with consistent responses in patients administered at least 150x10^6 CAR T-cells[46]. Cohen *et al* have described their preliminary results of the first cohort treated with a 4-1BB-CD3ζ BCMA CAR[47]. In this study, 3/9 patients developed grade 3-4 cytokine release syndrome but notably, there were deep responses and evidence of CAR T-cell expansion without prior lympho-depleting chemotherapy. Alternative T-cell redirecting therapies are CD3-BCMA

bispecific molecules on a common IgG arm[48,49], or bi-specific T-cell engagers (BiTEs), where scFvs to CD3 and BCMA are joined by a small peptide linker[23,50]. Additionally, a phase I study of an antibody-drug conjugate utilising the anti-tubulin agent, monomethyl auristatin F, reported an overall response rate of 67% in their high dose groups in multiply relapsed patients[51].

While these BCMA targeted therapies show promise, this receptor is present on tumour cells at variable and often low levels[16,17]. We found the median surface BCMA expression on MM cells to be over a log less than CD19 on acute lymphoblastic leukemia (ALL)[6]. Moreover, antigen negative tumour escape is well described in B-cell malignancies, with an incidence exceeding 10% in patients with ALL treated with a CD19 CAR[6,25]. In the BCMA CAR study described by the UPenn group[47], disease progression in two patients was associated with reduction in BCMA expression, reminiscent of the report from the NCI group using their first BCMA CAR[24]. These observations prompt a re-evaluation of such therapies targeting a single antigen.

We found BCMA and TACI to be co-expressed on tumour from the majority (78%) of patients and we hypothesized that targeting two tumour antigens could overcome the challenges of low target levels and antigen escape when targeting BCMA alone. To date, there have been several approaches to creating dual targeting CARs. These strategies have included the admixing of two populations of CAR transduced T-cells[52], engineering a single CAR construct containing two separate scFvs in tandem (TanCAR)[53,54] or the co-expression of 2 CARs on T-cells using a bi-cistronic vector or double transduction (OR gate)[55,56]. In the context of low levels of target antigen, the first approach may not ensure maximal T-cell activation as only BCMA or TACI would be recognised by individual T-cells. A bi-valent TanCAR may result in lower numbers of ligated receptors per target cell in target limited conditions; finally, an OR gate requires a large, complex bi-cistronic vector or a complex double transduction.

In comparison, APRIL is compact (135aa), non-immunogenic and natively bispecific, binding either MM antigen[31] with high affinity. Using APRIL as the CAR binder, we report target cytolysis at low E:T ratios that enforce an assessment of serial kill and at low levels of target antigen such as are present on primary tumour cells. ACAR-mediated cytolysis was also achieved at low levels of TACI, when BCMA targeting was blocked thus indicating the possibility of ACAR-mediated disease control even with BCMA down-regulation or loss. Data exists demonstrating resistance of CARs to blocking by avidity effects[57,58] and we observed reduction in ACAR killing at the highest levels of sBCMA found in MM BM but not with physiological concentrations of APRIL or TACI. In confirmation of our in vitro findings, we

observed tumour regression of established disease after only 48 hours of ACAR T-cell infusion in an intramedullary murine myeloma model. Notably, using an in vivo model of tumour escape, we observed improved disease control compared to a CAR targeting BCMA alone.

BCMA and TACI are both lymphoid antigens. BCMA is vital for the survival of long-lived PC[59], is upregulated in late memory B-cells on committing to the PC lineage[60,61] and is thus present on normal and malignant PC[16,17]. In comparison, TACI expression is found primarily on maturing B-cells, particularly marginal zone B-cells, CD27+ memory B-cell subsets and PC[38,60]. Our qRT-PCR analysis of TACI transcripts indicates expression restricted to the lymphoid compartments. Furthermore, ACAR does not appear to result in tissue toxicity in an animal model. We expect that ACAR therapy would result in loss of the entire plasma cell compartment and a subset of the B-cell compartment. The subsequent hypogammaglobinaemia may be more profound than that of CD19 targeting[62] but should not be more severe than that of BCMA targeting alone.

TACI has been implicated both as a positive and a negative immune regulator[63-66], and gene disruptions are found in 8% of patients with common variable immunodeficiency[27,64]. TACI also drives PC differentiation[67] suggesting that TACI is expressed early in PC development. We describe tumour TACI expression in the majority of patients and given the ontogeny of TACI expression, speculate that in these patients at least, expression of this antigen on putative myeloma stem cells which have a role in disease relapse and drug resistance[68,69] would add a further advantage to this approach.

In summary, using a novel ligand-based approach, we have demonstrated that the ACAR can concurrently target BCMA and TACI to increase the number of targetable tumour antigens in the majority of MM patients. ACAR T-cells were able to kill targets expressing either receptor and significant killing was seen at physiological receptor levels, at low E:T ratios or with BCMA blockade. ACAR T-cells also killed primary myeloma cells in vitro and we observed rapid and complete tumour clearance in vivo in comparison to an irrelevant CAR as well as in our tumour escape model compared to a CAR targeting BCMA alone. These observations suggest that dual antigen targeting of BCMA and TACI by ACAR T-cells may improve on the initial clinical responses seen with BCMA targeting CARs, both by extending clinical applicability to those patients with low levels of tumour BCMA, and by reducing the risk of antigen negative escape.

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## Authorship

Project conception by MP. LL, KY, MP designed the study. SO, MRJ, KY, MP supervised work. LL, BD, NC, BP, MC, DGF, SO, ST, VB, RB, PM, EK, MN, DP performed work. LL, SO, JF analysed data. LL wrote paper. JF, KY, MP reviewed the paper.

## Conflicts of interest

**LL:** Bloodwise Research Funding
**KY:** Janssen Research Funding
**LL, BD, NC, PM, KY, MP:** Autolus Equity ownership
**LL, BD, NC, KY, MP:** APRIL CAR patent
**SO, ST, VB, RB, EK, JF, MP:** Autolus employee
**MP:** *Amgen:* Honoraria; *Roche:* Honoraria.

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## Figure Legends

**Figure 1. BCMA and TACI expression on primary myeloma cells (A)** Fresh bone marrow mononuclear cells (BM MNCs) were stained with CD138 APC and one of BCMA PE, TACI PE (blue) or isotype control (red). Antigen densities of BCMA and TACI on CD138+ tumour cells (gated) were then quantified using QuantiBRITE$^{TM}$ beads and subtracting antibodies bound per cell (ABC) of isotype controls. FACS plots from 4 representative patient samples with antigen densities (ABC) shown. **(B)** Stacked plot of BCMA and TACI expression on CD138+ cells. Each bar represents a separate myeloma patient. **(C)** Distribution of BCMA and TACI expression on primary CD138+ myeloma cells (n=50; medians shown. BCMA range: 105-8323, mean: 1623. TACI range:0-21301, mean:853).

**Figure 2. Optimization of APRIL-based chimeric antigen receptors (A)** Three APRIL based 3$^{rd}$ generation chimeric antigen receptors (ACAR) were constructed, consisting of a

truncated APRIL molecule, fused to a tripartite endodomain (CD28-OX40-CD3ζ) via one of three spacers: the hinge of IgG1 (ACAR-H), the stalk of human CD8α (ACAR-CD8) or modified Fc (*FcR mutations as per Hombach[43], ACAR-Fc). PBMCs were CD3/CD28/IL-2 activated, transduced with ACAR constructs using RD114-pseodotyped retrovirus and CD56 depleted before testing against SUPT1 cells expressing high levels of BCMA (8x10^4 ABC) or TACI (16.2x10^5 ABC). **(B)** Target cell death as determined by 4 hour $^{51}$Cr release assay with **(i)**SUPT1$^{NT}$ **(ii)**SUPT1$^{BCMA}$ **(iii)**SUPT1$^{TACI}$ on co-culture with PBMCs transduced with ACAR-H (n=5), ACAR-CD8 (n=6), ACAR-Fc (n=3). Significance values indicated are compared to cytolysis with PBMCs NT by paired *t* test. **(C)** ACAR transduced T-cells were also co-cultured (1:1) with SUPT1 targets and IFNG release at D+1 measured by ELISA (Same number of experiments as before). **(D)** ACAR transduced PMNCs from further donors were labelled with Cell Trace Violet prior to co-culture with SUPT1 targets (1:1) and FACS at D+4. Percentage of ACAR positive cells proliferated with antigen expressing targets was then defined relative to co-culture with SUPT1$^{NT}$ control (n=6 for ACAR-H and ACAR-CD8, n=4 for ACAR-Fc). Mean±SEM indicated, *=p<0.05, **=p<0.01, by paired *t* test.

**Figure 3. ACAR mediated cytolysis seen at low target density and low E:T ratios (A)** SUPT1 targets were engineered to express a wide range of **(i)**BCMA and **(ii)**TACI. Antigen densities of targets indicated. Dot plots depict receptor levels found on primary MM tumour cells from 50 patients (population median indicated and hashed line represents threshold for positive expression) compared to engineered SUPT1 targets. **(B)** These targets were then co-cultured with ACAR-CD8 and H spacer variants at a low E:T ratio (1:10), and target death determined at 48 hours by FACS and expressed as percentage cytolysis compared to media control. **(C)** Specific cytolysis at 48 hours of human myeloma cell lines **(i)**MM.1s and **(ii)**U266 when co-cultured with ACAR transduced T-cells at reducing E:T ratios. Inset histograms show BCMA and TACI expression by FACS (grey filled) compared to staining with isotype control (empty). Mean±SEM of number of experiments indicated *=p<0.05, **=p<0.01, ***=p<0.001 by *t* test, compared to PBMC NT.

**Figure 4. ACAR causes cytolysis of primary myeloma cells in vitro (A)** CD138 selected bone marrow derived primary myeloma cells from 5 patients were cultured in media alone (labelled 'Tumour alone'), with allogeneic NT T-cells or T-cells transduced to express ACAR-H.2A.RQR8 or ACAR-CD8.2A.RQR8. Patients identified by number allocated in Figure 1B and Table S1. Tumour antigen densities of BCMA and TACI indicated (ABC). Relative number of viable tumour cells at D+3 shown. Cytokine release was determined at D+1 by ELISA and T-cell numbers after 7 days of co-culture with or without tumour cells determined by staining for RQR8 transgene using FACS. **(B)** Summarised **(i)**tumour kill (% cytolysis

determined relative to viable tumour cells on co-culture with NT T-cells) **(ii)**cytokine release and **(iii)**T-cell expansion. **(C)** ACAR-H transduced PBMCs from 3 donors were co-cultured 1:4 with MM.1s, U266 or CD138 selected primary bone marrow derived tumour cells from 3 further patients. Effectors and targets were cultured in media alone, 150µg/ml of anti-BCMA antibody (S307118G03) or the equivalent concentration of IgG2a control. **(i)**BCMA and TACI expression on targets **(ii)**target kill at 48 hours by FACS. Mean± SEM, *=p<0.05 compared to control by paired *t* test.

**Figure 5. ACAR-H mediated tumour clearance *in vivo* (A)** Twenty-two NSG mice were injected IV with 10x10^6 HA+Fluc+MM.1s cells at D0 and monitored by BLI for tumour burden at different time points (dorsal views shown). On D+13 there was clear evidence of intramedullary tumour in all animals at which point 6 animals were left untreated, 8 animals were intravenously injected with T-cells transduced with a control EGFRvIII targeting CAR or ACAR-H (5x10^6 CAR cells/animal). **(B)** Average radiance [p/s/cm²/sr] of whole mice in the 3 groups at different timepoints. **(C)** At termination of experiment (D+25 post tumour, D+12 post CAR), by FACS there was significant reduction of tumour in the bone marrow of ACAR treated mice compared to EGFRvIII treated and untreated animals. Tumour cells were identified as live/single/muCD11b-/HA+ with numbers normalised to Flow-Check™ beads to calculate relative engraftment. Mean±SEM shown, **=p<0.01 and ***=p<0.001 by *t* test. **(D)** Eradication of CD138+ tumour cells by ACAR was confirmed in bone marrow by IHC of femur. H&E staining shown at x12.5 and x400 (left and central panels) and immuno-staining for CD138 (right panels) at x200 original magnification.

**Figure 6. ACAR-H mediated clearance of BCMA negative tumour. (A)** BCMA-3 (5807 ABC) and TACI-2 (2229 ABC) SUPT1 targets were transduced with RQR8.2A.Fluc and HA.2A.Fluc respectively and used in an in vivo tumour escape model. Twenty-one NSG mice were intravenously injected with a total of 3.5x10^6 BCMA and TACI expressing SUPT1 cells at a ratio of 4:1 respectively. At D+4, mice were intravenously injected with NT PBMCs, T-cells transduced with ACAR-H or a CAR construct targeting BCMA alone (BCMA CAR) at a dose of 5x10^6 CAR cells/animal (n=7 per cohort). Tumour burden was monitored by BLI at different time points (dorsal views shown). **(B)** Average radiance [p/s/cm²/sr] of whole mice in the 3 groups at different timepoints. **(C)** Nine days post CAR T-cells, the experiment was terminated and FACS of BM MNCs showed persistent engraftment of BCMA and TACI SUPT1 cells following NT T-cells, clearance of both cell populations by ACAR-H T-cells and eradication of BCMA expressing tumour only by BCMA CAR (single example from 3 cohorts shown). **(D)** SUPT1 cells were identified as live/single/muCD11b-/CD2-/CD4+/CD8+ and BCMA**(i)** and TACI**(ii)** expression determined by RQR8 and HA

staining respectively with numbers normalised to Flow-Check™ beads to calculate relative engraftment. Mean±SEM shown, **=p<0.01 and ***=p<0.001 by *t* test.

Figure 1

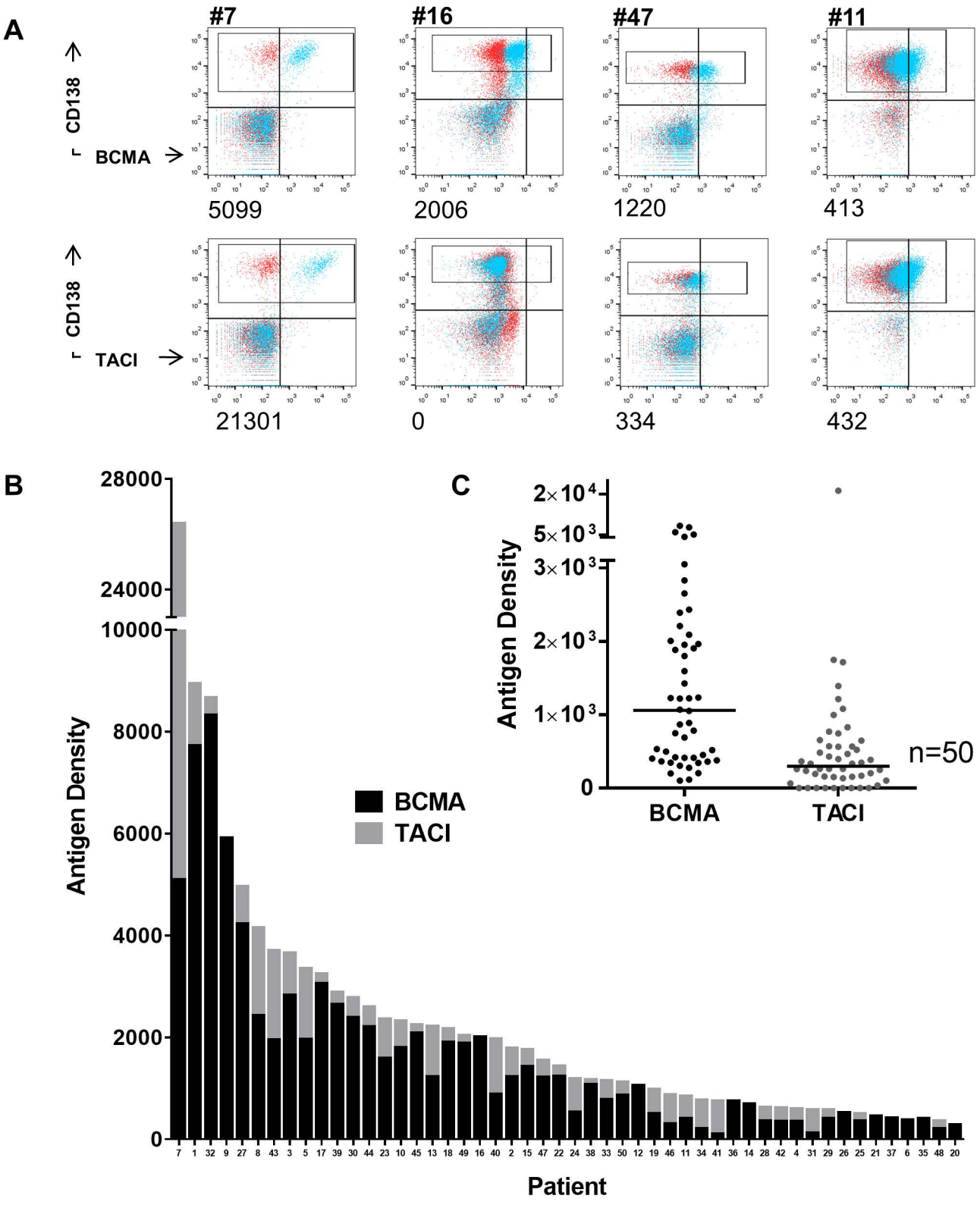
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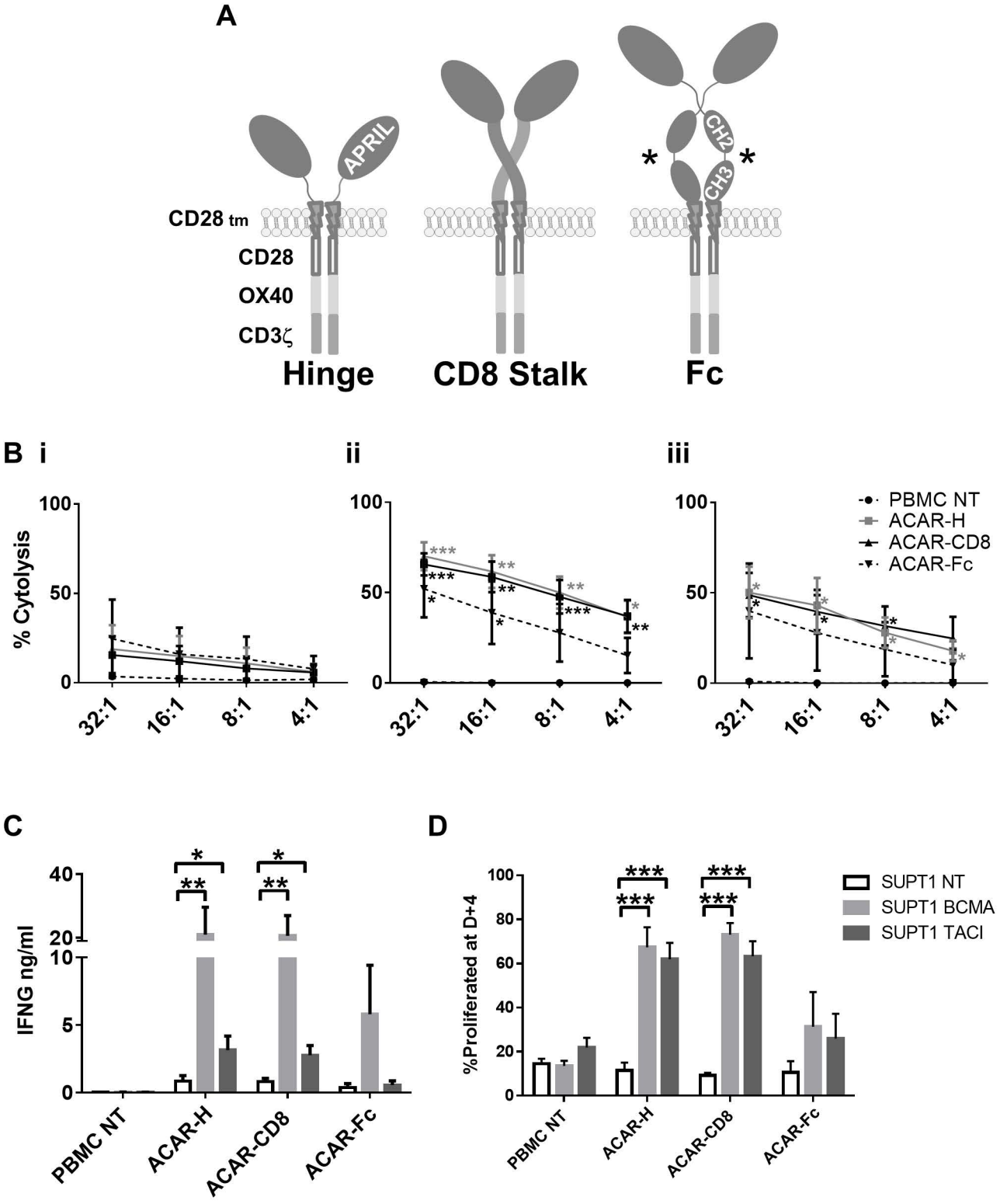
**Figure 2**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SUPT1 TACI

Figure 3

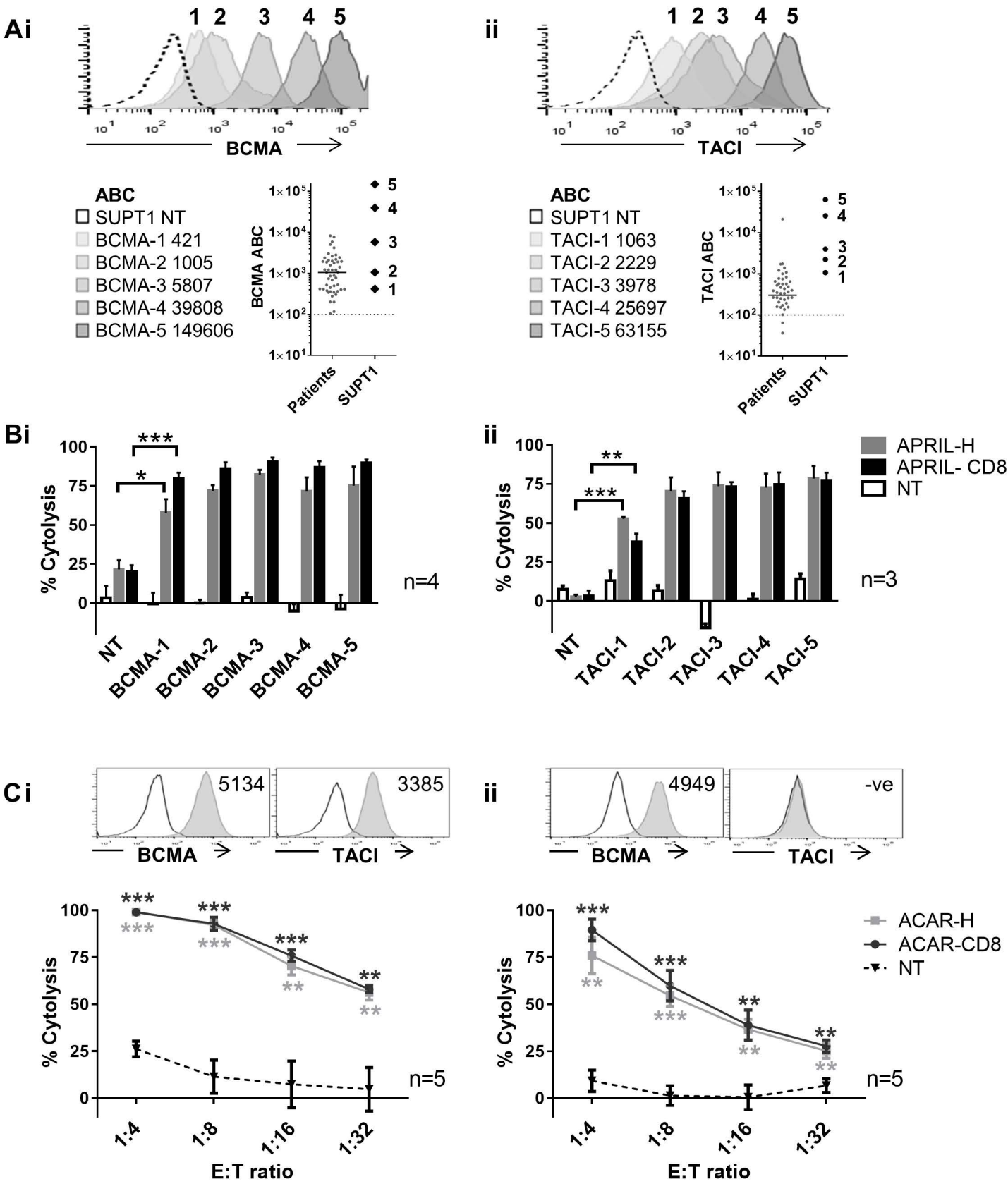
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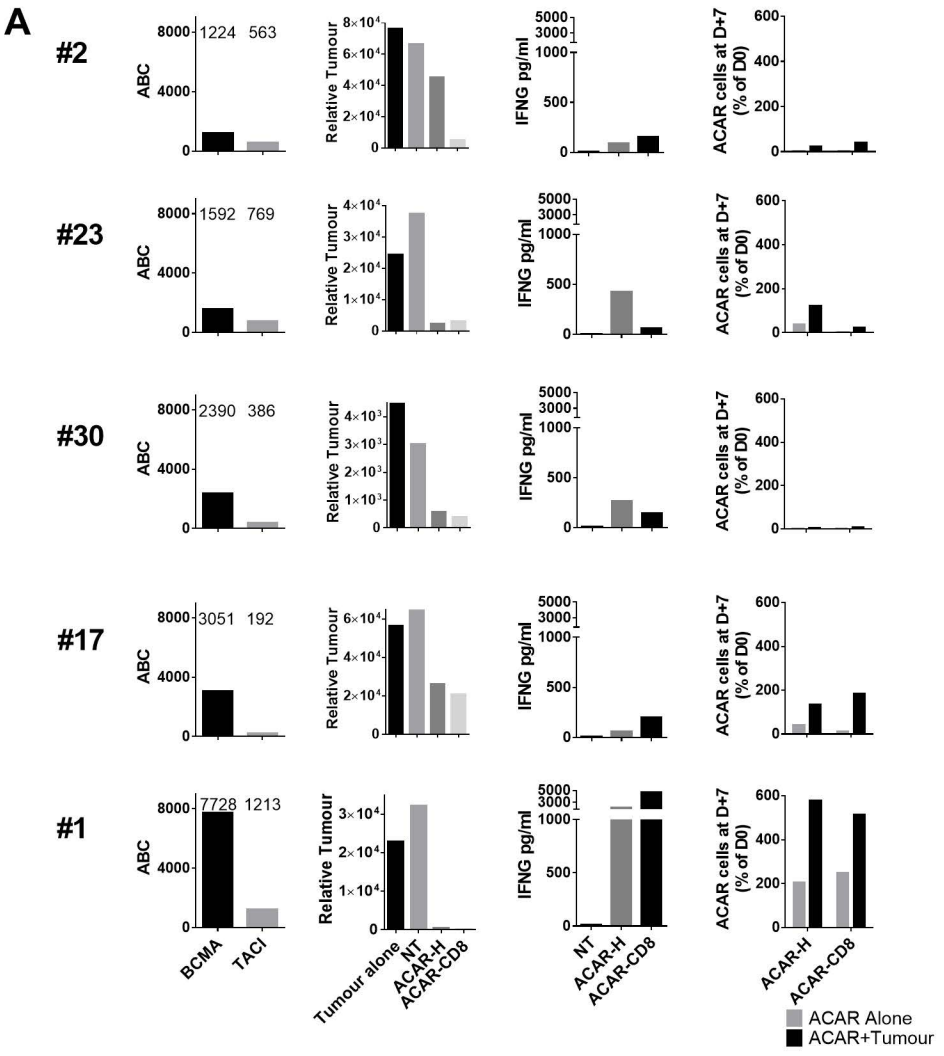
**Figure 4**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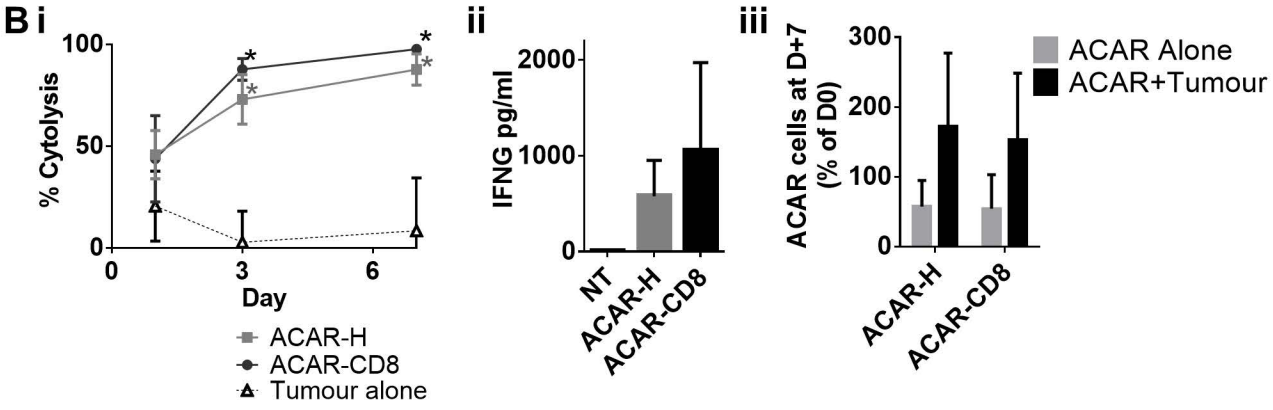
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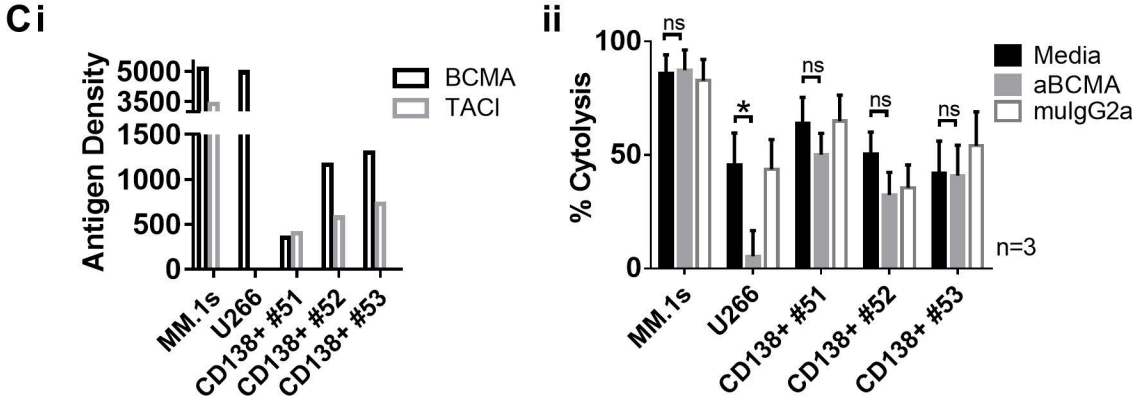
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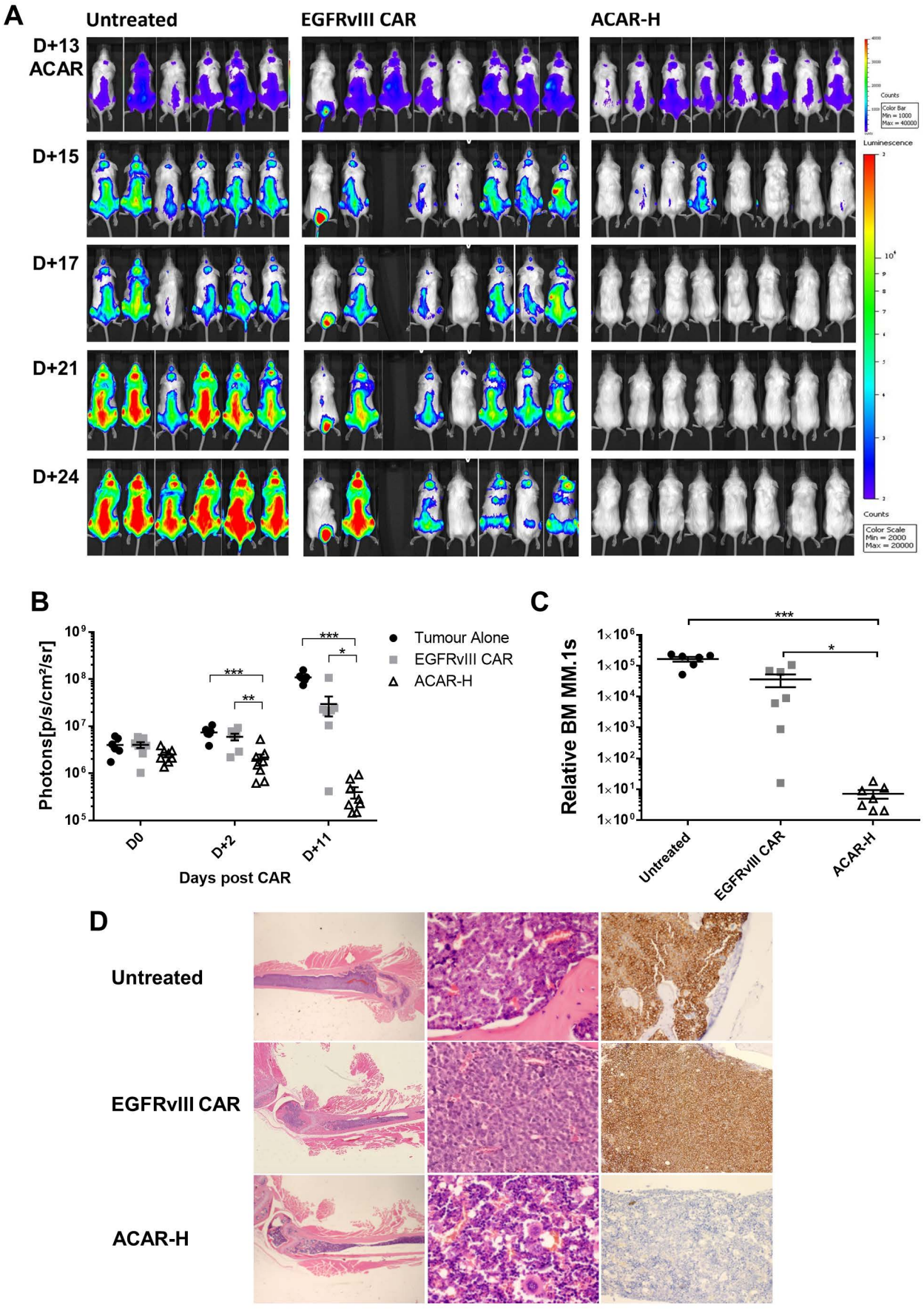
# Figure 5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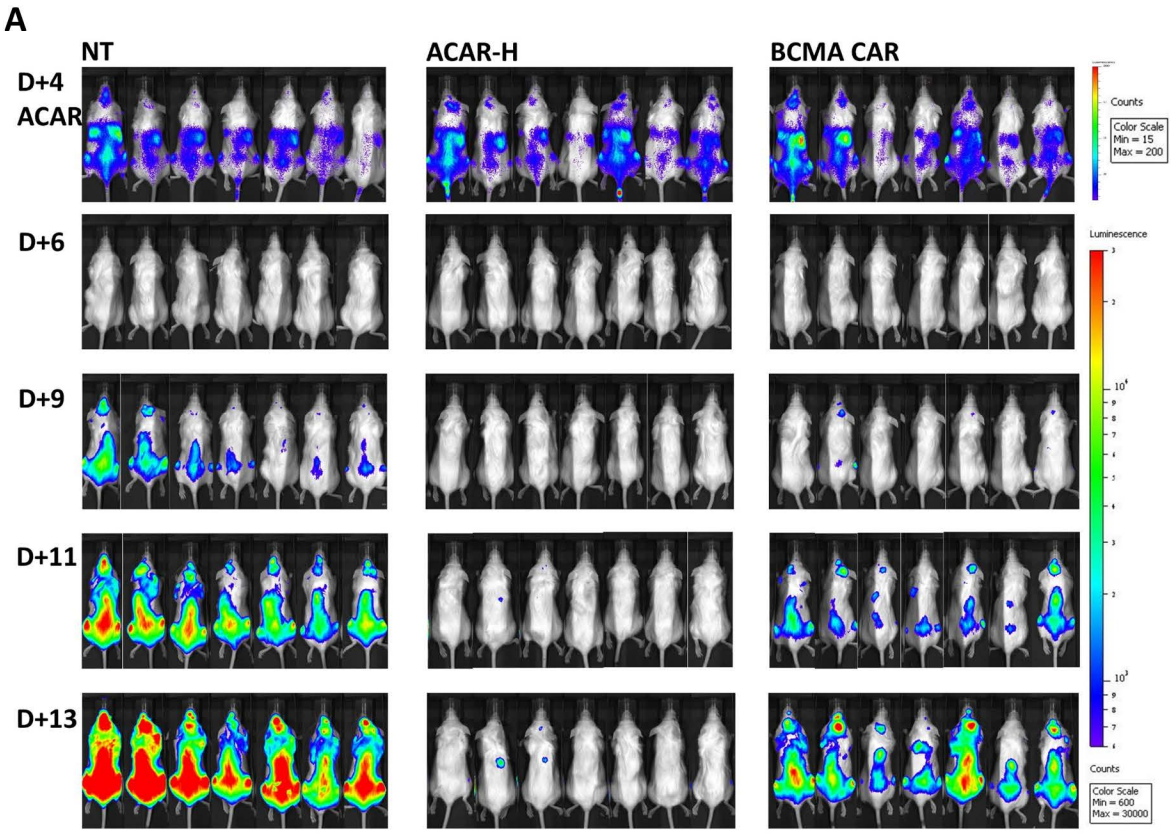
# Figure 6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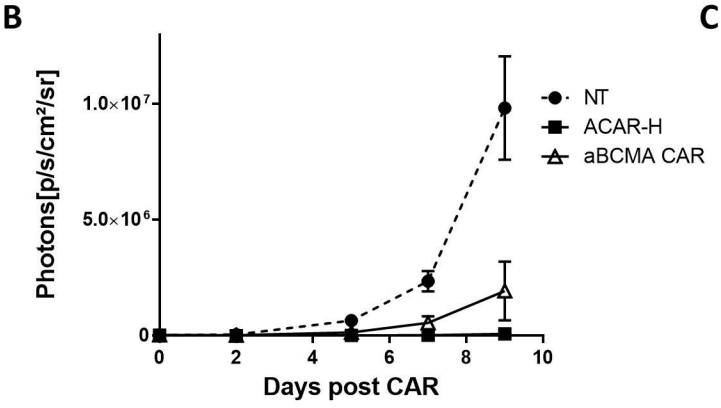
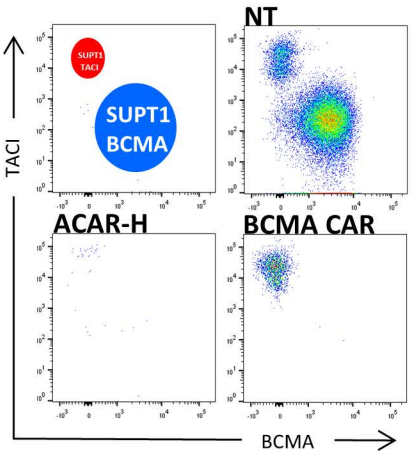
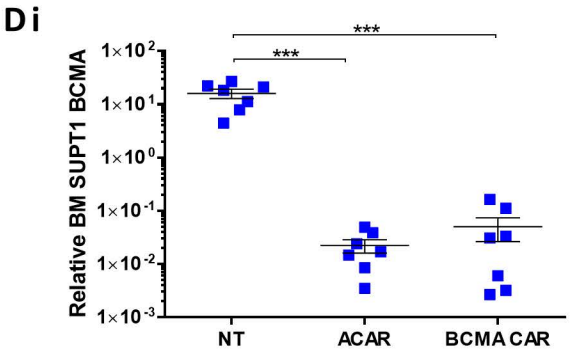
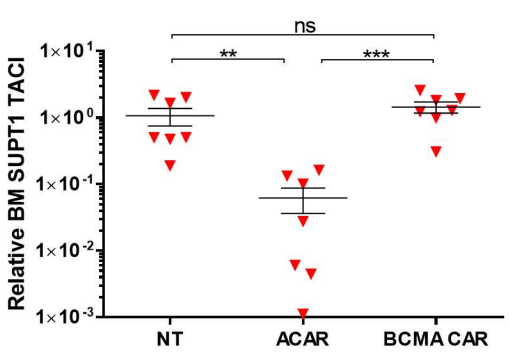
Luminescence
Counts
Color Scale
Min = 600
Max = 30000


B
Photons[p/s/cm²/sr]
1.0×10⁷
5.0×10⁶
0
0
2
4
6
8
10
Days post CAR
NT
ACAR-H
aBCMA CAR


C
NT
SUPT1
TACI
SUPT1
BCMA
ACAR-H
BCMA CAR
TACI
BCMA


Di
Relative BM SUPT1 BCMA
1×10²
1×10¹
1×10⁰
1×10⁻¹
1×10⁻²
1×10⁻³
***
***
NT
ACAR
BCMA CAR


ii
Relative BM SUPT1 TACI
1×10¹
1×10⁰
1×10⁻¹
1×10⁻²
1×10⁻³
ns
**
***
NT
ACAR
BCMA CAR

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# An APRIL based chimeric antigen receptor for dual targeting of BCMA and TACI in Multiple Myeloma

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