Title: Endogenous CD28 drives CAR T cell responses in multiple myeloma

Author List and Affiliations: Mackenzie M. Lieberman¹, Jason H. Tong¹, Nkechi U. Odukwe¹, Colin A. Chavel¹, Terence J. Purdon², Rebecca Burchett³, Bryan M. Gillard⁴, Craig M. Brackett⁵, A. J. Robert McGray¹, Jonathan L. Bramson³, Renier J. Brentjens^{1,2}, Kelvin P. Lee⁶, Scott H. Olejniczak^{1*}

¹Department of Immunology, Roswell Park Comprehensive Cancer Center, Buffalo, NY 14263, USA.

²Department of Medicine, Roswell Park Comprehensive Cancer Center, Buffalo, NY 14263, USA.

 3 Department of Pathology and Molecular Medicine, McMaster Immunology Research Centre, McMaster University, Hamilton, ON, Canada.

⁴Department of Pharmacology and Therapeutics, Roswell Park Comprehensive Cancer Center, Buffalo, NY, USA.

5 Department of Cell Stress Biology, Roswell Park Comprehensive Cancer Center, Buffalo, NY 14263, USA.

⁶Indiana University Melvin and Bren Simon Comprehensive Cancer Center, Indianapolis, IN, 46202, USA.

*Corresponding author: Scott H. Olejniczak, Department of Immunology, Roswell Park Comprehensive Cancer Center, Carlton and Elm Streets, Buffalo, NY 14203. Phone: 716-845- 8538; Email: scott.olejniczak@roswellpark.org

1 **Abstract**
2 Recer
3 myeloma
4 clinical tria Recent FDA approvals of chimeric antigen receptor (CAR) T cell therapy for multiple
myeloma (MM) have reshaped the therapeutic landscape for this incurable cancer. In pivotal
clinical trials B cell maturation antigen (BCMA myeloma (MM) have reshaped the therapeutic landscape for this incurable cancer. In pivotal
clinical trials B cell maturation antigen (BCMA) targeted, 4-1BB co-stimulated (BB ζ) CAR T cells
dramatically outperformed stan dinical trials B cell maturation antigen (BCMA) targeted, 4-1BB co-stimulated (BBζ) CAR T cells
dramatically outperformed standard-of-care chemotherapy, yet most patients experienced MM
relapse within two years of therapy dramatically outperformed standard-of-care chemotherapy, yet most patients experienced MM

relapse within two years of therapy, underscoring the need to improve CAR T cell efficacy in

MM. We set out to determine if inhibi relapse within two years of therapy, underscoring the need to improve CAR T cell efficacy in
MM. We set out to determine if inhibition of MM bone marrow microenvironment (BME) survival
signaling could increase sensitivity MM. We set out to determine if inhibition of MM bone marrow microenvironment (BME) survival
signaling could increase sensitivity to CAR T cells. In contrast to expectations, blocking the
CD28 MM survival signal with abatac signaling could increase sensitivity to CAR T cells. In contrast to expectations, blocking the
CD28 MM survival signal with abatacept (CTLA4-Ig) accelerated disease relapse following CAR
T therapy in preclinical models, po CD28 MM survival signal with abatacept (CTLA4-Ig) accelerated disease relapse following CAR

T therapy in preclinical models, potentially due to blocking CD28 signaling in CAR T cells.

Knockout studies confirmed that end T therapy in preclinical models, potentially due to blocking CD28 signaling in CAR T cells.

11 Knockout studies confirmed that endogenous CD28 expressed on BBζ CAR T cells drove *in*

12 *vivo* anti-MM activity. Mechanist 11 Knockout studies confirmed that endogenous CD28 expressed on BBζ CAR T cells drove *in*
12 vivo anti-MM activity. Mechanistically, CD28 reprogrammed mitochondrial metabolism to
13 maintain redox balance and CAR T cell *vivo* anti-MM activity. Mechanistically, CD28 reprogrammed mitochondrial metabolism to
maintain redox balance and CAR T cell proliferation in the MM BME. Transient CD28 inhibition
with abatacept restrained rapid BB_S CAR maintain redox balance and CAR T cell proliferation in the MM BME. Transient CD28 inhibition
with abatacept restrained rapid BBζ CAR T cell expansion and limited inflammatory cytokines in
the MM BME without significantly a with abatacept restrained rapid BBζ CAR T cell expansion and limited inflammatory cytokines in
the MM BME without significantly affecting long-term survival of treated mice. Overall, data
directly demonstrate a need for CD the MM BME without significantly affecting long-term survival of treated mice. Overall, data
16 directly demonstrate a need for CD28 signaling for sustained *in vivo* function of CAR T cells and
17 indicate that transient directly demonstrate a need for CD28 signaling for sustained *in vivo* function of CAR T cells and

indicate that transient CD28 blockade could reduce cytokine release and associated toxicities.

18
 Keywords: CD28, co-s

indicate that transient CD28 blockade could reduce cytokine release and associated toxicities.
18
Keywords: CD28, co-stimulation, CAR T cells, multiple myeloma, tumor microenvironmer
metabolism
21 --
19
20
21 19 **Keywords:** CD28, co-stimulation, CAR T cells, multiple myeloma, tumor microenvironment,
20 metabolism
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22 20 metabolism
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24 **Introduction**
25 Chimeric
26 extraordinarily
27 autologous T 25 Chimeric Antigen Receptor (CAR) T cells are a form of immunotherapy that has seen
26 extraordinarily success in treating hematologic malignancies¹⁻¹². Expression of a CAR in
27 autologous T cells isolated from cancer extraordinarily success in treating hematologic malignancies¹⁻¹². Expression of a CAR in extraordinarily success in treating hematologic malignancies¹⁻¹². Expression of a CAR in
autologous T cells isolated from cancer patients redirects T cell specificity toward an antigen of
interest and delivers activatio 27 autologous T cells isolated from cancer patients redirects T cell specificity toward an antigen of
28 interest and delivers activation signals upon antigen ligation¹³⁻¹⁵. Activation signals in clinically
29 relevant interest and delivers activation signals upon antigen ligation¹³⁻¹⁵ interest and delivers activation signals upon antigen ligation¹³⁻¹⁵. Activation signals in clinically

relevant second-generation CAR T cells are mediated by CD3 ζ as well as a co-stimulatory

domain, most commonly C 29 relevant second-generation CAR T cells are mediated by CD3 ζ as well as a co-stimulatory
20 domain, most commonly CD28 or 4-1BB, although others including ICOS and OX40 remain
21 under investigation ¹⁶⁻²¹. Second 30 domain, most commonly CD28 or 4-1BB, although others including ICOS and OX40 remain
31 under investigation ¹⁶⁻²¹. Second generation CAR T cells targeting the B cell antigen, CD19, are
32 FDA approved for B cell leuke under investigation ¹⁶⁻²¹ 31 under investigation $16-21$. Second generation CAR T cells targeting the B cell antigen, CD19, are
32 FDA approved for B cell leukemias and lymphomas and have resulted in potent, decade long
33 remissions for some of t 32 FDA approved for B cell leukemias and lymphomas and have resulted in potent, decade long
33 remissions for some of the initial patients who received this therapy^{7,10,22-24}. Within the last few
34 years, FDA approval remissions for some of the initial patients who received this therapy^{7,10,22-24} remissions for some of the initial patients who received this therapy^{7,10,22-24}. Within the last few
years, FDA approval for CAR T cells has expanded to include those directed against the tumor
necrosis factor receptor years, FDA approval for CAR T cells has expanded to include those directed against the tumor
necrosis factor receptor (TNF-R) superfamily molecule, B cell maturation antigen (BCMA) for
the treatment of multiple myeloma (MM 35 necrosis factor receptor (TNF-R) superfamily molecule, B cell maturation antigen (BCMA) for
36 the treatment of multiple myeloma (MM)^{25,26}. Despite robust initial response rates greatly
37 outperforming standard of c the treatment of multiple myeloma (MM) 25,26 36 the treatment of multiple myeloma $(MM)^{25,26}$. Despite robust initial response rates greatly
37 outperforming standard of care in heavily pre-treated MM patient populations, recent clinical
38 studies have shown that outperforming standard of care in heavily pre-treated MM patient populations, recent clinical
studies have shown that approximately 40% of MM patients will experience disease progression
within the first 24 months of BCMAstudies have shown that approximately 40% of MM patients will experience disease progression
within the first 24 months of BCMA-targeted CAR T cell infusion ²⁷⁻²⁹. Therefore, there is an
urgent need to understand and ove within the first 24 months of BCMA-targeted CAR T cell infusion $27-29$. Therefore, there is an within the first 24 months of BCMA-targeted CAR T cell infusion $27-29$. Therefore, there is an urgent need to understand and overcome resistance mechanisms that hinder CAR T cell success in MM patients.
At also subset tha

Figure 10 urgent need to understand and overcome resistance mechanisms that hinder CAR T cell
141 success in MM patients.
142 MM remains an incurable malignancy of plasma cells, a terminally differentiated B cell
143 subse 41 success in MM patients.
42 MM remains an incl
43 subset that typically res
44 immunity through the pr MM remains an incurable malignancy of plasma cells, a terminally differentiated B cell
subset that typically reside in the bone marrow (BM) and contribute to protective humoral
immunity through the production of immunoglob subset that typically reside in the bone marrow (BM) and contribute to protective humoral
immunity through the production of immunoglobulin. The long-term survival of plasma cells is
critically dependent upon interactions immunity through the production of immunoglobulin. The long-term survival of plasma cells is

critically dependent upon interactions occurring within the BM niche, with many soluble and

contact-dependent stromal interacti critically dependent upon interactions occurring within the BM niche, with many soluble and

contact-dependent stromal interactions also contributing to survival and disease progression of

MM³⁰. MM relies on CXCL12 chem contact-dependent stromal interactions also contributing to survival and disease progression of
MM³⁰. MM relies on CXCL12 chemokine gradients to home into the BM niche, as well as
adhesion molecules including LFA-1 to me MM^{30} AM^{30} . MM relies on CXCL12 chemokine gradients to home into the BM niche, as well as
adhesion molecules including LFA-1 to mediate attachment and retention within the
microvasculature³¹⁻³⁴. Several soluble mediators s adhesion molecules including LFA-1 to mediate attachment and retention within the
microvasculature³¹⁻³⁴. Several soluble mediators secreted by BM resident dendritic cells (DCs),
microvasculature³¹⁻³⁴. Several soluble m microvasculature³¹⁻³⁴ microvasculature³¹⁻³⁴. Several soluble mediators secreted by BM resident dendritic cells (DCs),

The microvasculature³¹⁻³⁴. Several soluble mediators secreted by BM resident dendritic cells (DCs),

macrophages, osteoblasts, and stromal cells, including IL-6 and BCMA ligands, APRIL, and

BAFF, sustain MM survival and proliferation³⁵⁻⁴⁰. Additionally contact-dependent interactions

regulate anti-apoptotic molecule ex BAFF, sustain MM survival and proliferation $35-40$ BAFF, sustain MM survival and proliferation³⁵⁻⁴⁰. Additionally contact-dependent interactions

segulate anti-apoptotic molecule expression and chemotherapeutic resistance in MM. A key

mediator of contact-dependent survi regulate anti-apoptotic molecule expression and chemotherapeutic resistance in MM. A key
mediator of contact-dependent survival in MM is the canonical T cell co-stimulatory receptor,
CD28, whose expression on MM cells is h mediator of contact-dependent survival in MM is the canonical T cell co-stimulatory receptor,

CD28, whose expression on MM cells is highly correlated with myeloma progression⁴¹.

Importantly, CD28 ligands CD80 and CD86 CD28, whose expression on MM cells is highly correlated with myeloma progression⁴¹. CD28, whose expression on MM cells is highly correlated with myeloma progression⁴¹.

Importantly, CD28 ligands CD80 and CD86 are expressed on DCs, stromal cells, and even

MM cells within the BM microenvironment^{30,42-44} Importantly, CD28 ligands CD80 and CD86 are expressed on DCs, stromal cells, and even

MM cells within the BM microenvironment^{30,42-44}. Ligation of CD28 on MM cells by CD80/CD86

transduces a PI3K/Akt pathway dependent, MM cells within the BM microenvironment $^{30,42\cdot44}$ 56 MM cells within the BM microenvironment^{30,4244}. Ligation of CD28 on MM cells by CD80/CD86 transduces a PI3K/Akt pathway dependent, pro-survival signal protecting them from chemotherapy and growth factor withdrawal-in transduces a PI3K/Akt pathway dependent, pro-survival signal protecting them from

chemotherapy and growth factor withdrawal-induced death^{42,45-47}. Importantly, CD28 interaction

with CD80/CD86 can be blocked by the CTL chemotherapy and growth factor withdrawal-induced death^{42,45-47} chemotherapy and growth factor withdrawal-induced death $42.45-47$. Importantly, CD28 interaction
with CD80/CD86 can be blocked by the CTLA4-Ig fusion protein abatacept, which is FDA
approved for the treatment of rheumatoi 59 with CD80/CD86 can be blocked by the CTLA4-Ig fusion protein abatacept, which is FDA
60 approved for the treatment of rheumatoid arthritis, psoriatic arthritis, polyarticular juvenile
61 idiopathic arthritis and acute 60 approved for the treatment of rheumatoid arthritis, psoriatic arthritis, polyarticular juvenile
61 idiopathic arthritis and acute graft versus host disease^{48,49}. In myeloma, pre-clinical studies have
62 shown that CT idiopathic arthritis and acute graft versus host disease $^{48,49}\,$ idiopathic arthritis and acute graft versus host disease^{48,49}. In myeloma, pre-clinical studies have

shown that CTLA4-Ig in combination with melphalan can significantly reduce tumor burden⁴⁵,

leading to a phase II c shown that CTLA4-Ig in combination with melphalan can significantly reduce tumor burden⁴⁵. shown that CTLA4-Ig in combination with melphalan can significantly reduce tumor burden⁴⁵,

leading to a phase II clinical trial of abatacept plus standard of care chemotherapy for treatment

of patients with relapsed/r

63 leading to a phase II clinical trial of abatacept plus standard of care chemotherapy for treatment
64 of patients with relapsed/refractory MM (NCT03457142).
65 Given the recently reported ~3-fold improvement in overall 64 of patients with relapsed/refractory MM (NCT03457142).

65 Given the recently reported ~3-fold improvement in

66 was added to standard of care therapy⁵⁰, we hypothes

67 would similarly sensitize MM to CAR T cell th 65 Given the recently reported ~3-fold improvement in overall response rate when abatacept
66 was added to standard of care therapy⁵⁰, we hypothesized that systemic blockade of CD28
67 would similarly sensitize MM to CA was added to standard of care therapy 50 was added to standard of care therapy⁵⁰, we hypothesized that systemic blockade of CD28
would similarly sensitize MM to CAR T cell therapy. We reasoned that unlike endogenous T cells
that require CD28 co-stimulation to would similarly sensitize MM to CAR T cell therapy. We reasoned that unlike endogenous T cells
that require CD28 co-stimulation to mount an anti-tumor response, second generation CAR T
cells receive co-stimulation directly that require CD28 co-stimulation to mount an anti-tumor response, second generation CAR T
cells receive co-stimulation directly from the CAR and would therefore be relatively unaffected
by blockade of endogenous CD28. FDA 69 cells receive co-stimulation directly from the CAR and would therefore be relatively unaffected

50 by blockade of endogenous CD28. FDA approved CAR T cell products for MM, idacabtagene

51 vicleucel and ciltacabtagene by blockade of endogenous CD28. FDA approved CAR T cell products for MM, idacabtagene

vicleucel and ciltacabtagene autoleucel, employ 4-1BB co-stimulatory domains, which have

been shown to transduce a weaker signal than vicleucel and ciltacabtagene autoleucel, employ 4-1BB co-stimulatory domains, which have
been shown to transduce a weaker signal than CD28 co-stimulatory domains in CD19 targeted
CAR T cells^{51,52}. In this context, the we 27 been shown to transduce a weaker signal than CD28 co-stimulatory domains in CD19 targeted

27 CAR T cells^{51,52}. In this context, the weaker 4-1BB co-stimulatory signal reduced CAR T cell

27 exhaustion and enhanced $CAR T$ cells 51,52 CAR T cells^{51,52}. In this context, the weaker 4-1BB co-stimulatory signal reduced CAR T cell
exhaustion and enhanced *in vivo* persistence when compared to CD28 driven co-
stimulation^{53,54}. However, recent evidence su z exhaustion and enhanced *in vivo* persistence when compared to CD28 driven co-
175 stimulation^{53,54}. However, recent evidence suggests that enhanced CD28 signaling in CTLA-4 stimulation^{53,54} ²⁷⁵ stimulation^{53,54}. However, recent evidence suggests that enhanced CD28 signaling in CTLA-4

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²⁷⁶

²⁷⁶

knockout 4-1BB co-stimulated (BB ζ) CAR T cells improves their anti-tumor efficacy⁵⁵. Moreover, 26 knockout 4-1BB co-stimulated (BB ζ) CAR T cells improves their anti-tumor efficacy⁵⁵. Moreover,
27 endogenous tumor-reactive cytotoxic T cells require CD28 signaling to acquire effector
28 properties in the tumor endogenous tumor-reactive cytotoxic T cells require CD28 signaling to acquire effector

Transporties in the tumor microenvironment⁵⁶, suggesting that perhaps the CAR 4-1BB co-

stimulatory domain alone is insufficient t properties in the tumor microenvironment⁵⁶

properties in the tumor microenvironment⁵⁶, suggesting that perhaps the CAR 4-1BB costimulatory domain alone is insufficient to stimulate potent anti-tumor activity.

In the current study, we employed human and mouse ort 30 stimulatory domain alone is insufficient to stimulate potent anti-tumor activity.

30 In the current study, we employed human and mouse orthotopic models of

31 (MM) to directly test whether endogenous CD28 affected tum In the current study, we employed human and mouse orthotopic models of multiple myeloma

81 (MM) to directly test whether endogenous CD28 affected tumor control by BCMA targeted, 4-

82 1BB co-stimulated CAR T cells. Somew 81 (MM) to directly test whether endogenous CD28 affected tumor control by BCMA targeted, 4-
82 1BB co-stimulated CAR T cells. Somewhat unexpectedly, we found that continuous blockade of
83 CD28 interaction with B7 protein 1BB co-stimulated CAR T cells. Somewhat unexpectedly, we found that continuous blockade of

83 CD28 interaction with B7 proteins using abatacept significantly impaired CAR T cell control of

84 MM growth, resulting in shor CD28 interaction with B7 proteins using abatacept significantly impaired CAR T cell control of

MM growth, resulting in shorter survival of MM-bearing mice. Data indicate that abatacept

primarily affected endogenous CD28 MM growth, resulting in shorter survival of MM-bearing mice. Data indicate that abatacept
primarily affected endogenous CD28 signaling on CAR T cells, as inducible deletion of CD28
from 4-1BB co-stimulated CAR T cells also primarily affected endogenous CD28 signaling on CAR T cells, as inducible deletion of CD28

from 4-1BB co-stimulated CAR T cells also reduced their *in vivo* anti-MM efficacy.

Mechanistically, we provide evidence that end from 4-1BB co-stimulated CAR T cells also reduced their *in vivo* anti-MM efficacy.

Mechanistically, we provide evidence that endogenous CD28 signaling increases 4-1BB co-

stimulated CAR T cell expansion in the MM bone m Mechanistically, we provide evidence that endogenous CD28 signaling increases 4-1BB co-
stimulated CAR T cell expansion in the MM bone marrow microenvironment (BME) by
stimulating oxidative phosphorylation and maintaining stimulated CAR T cell expansion in the MM bone marrow microenvironment (BME) by
stimulating oxidative phosphorylation and maintaining redox balance. Transient inhibition of
endogenous CD28 on 4-1BB co-stimulated CAR T cell stimulating oxidative phosphorylation and maintaining redox balance. Transient inhibition of
endogenous CD28 on 4-1BB co-stimulated CAR T cells resulted in decreased accumulation of
CD4⁺ CAR T cells and release of inflam endogenous CD28 on 4-1BB co-stimulated CAR T cells resulted in decreased accumulation of
CD4⁺ CAR T cells and release of inflammatory cytokines in the MM TME, without significantly
impairing anti-MM activity. Collectivel CD4⁺ CAR T cells and release of inflammatory cytokines in the MM TME, without significantly 91 CD4⁺ CAR T cells and release of inflammatory cytokines in the MM TME, without significantly
92 impairing anti-MM activity. Collectively, our findings reveal that CAR T cell function is affected by
93 endogenous CD28, impairing anti-MM activity. Collectively, our findings reveal that CAR T cell function is affected by
endogenous CD28, which can potentially be transiently blocked to reduce toxic pro-
inflammatory cytokine release while m endogenous CD28, which can potentially be transiently blocked to reduce toxic pro-
inflammatory cytokine release while maintaining anti-tumor activity.
95
Materials and Methods
97 Cell lines: Perental ETCM1 cells appenai

inflammatory cytokine release while maintaining anti-tumor activity.
95
Materials and Methods
97 Cell lines: Parental 5TGM1 cells generously provided by G. David R
98 Nunce lastivizally transduced to express truncated by --
96
97
98 96 **Materials and Methods**
97 *Cell lines:* Parental 5TGM
98 were lentivirally transduce
99 be used as target cells. Gell lines: Parental 5TGM1 cells generously provided by G. David Roodman (Indiana University)
were lentivirally transduced to express truncated human BCMA (hBCMA) and firefly luciferase to
be used as target cells. Pure pop were lentivirally transduced to express truncated human BCMA (hBCMA) and firefly luciferase to
be used as target cells. Pure populations were achieved following fluorescence-activated cell
sorting. MM.1S and U266 luciferas 99 be used as target cells. Pure populations were achieved following fluorescence-activated cell

90 sorting. MM.1S and U266 luciferase expressing clones were generated and supplied by Kelvin

91 Lee (Indiana University). 100 sorting. MM.1S and U266 luciferase expressing clones were generated and supplied by Kelvin
101 Lee (Indiana University). MM cell lines were maintained in RPMI 1640 (Gibco) supplemented
101 Lee (Indiana University). MM 101 Lee (Indiana University). MM cell lines were maintained in RPMI 1640 (Gibco) supplemented

with 10% heat-inactivated FBS (R&D Systems), 1% nonessential amino acids (Gibco), 1mM
sodium pyruvate (Gibco), 10 mM HEPES (Gibco), 2 mM L-glutamine (Gibco) and 1%
penicillin/streptomycin (Gibco). Cell lines were maintaine sodium pyruvate (Gibco), 10 mM HEPES (Gibco), 2 mM L-glutamine (Gibco) and 1%

penicillin/streptomycin (Gibco). Cell lines were maintained in culture for 2-3 months at a time.

293T packaging cell lines were purchased from penicillin/streptomycin (Gibco). Cell lines were maintained in culture for 2-3 months at a time.

105 293T packaging cell lines were purchased from ATCC and maintained in DMEM, 10% FBS and

106 1% L-glutamine for 2-3 weeks 293T packaging cell lines were purchased from ATCC and maintained in DMEM, 10% FBS and
106 1% L-glutamine for 2-3 weeks prior to transient transfection. Stably expressing 293 Galv9 cells
107 were kindly provided by Renier 106 1% L-glutamine for 2-3 weeks prior to transient transfection. Stably expressing 293 Galv9 cells

107 were kindly provided by Renier Brentjens. All cell lines were routinely tested for mycoplasma

108 using the Lonza My were kindly provided by Renier Brentjens. All cell lines were routinely tested for mycoplasma
108 using the Lonza MycoAlert Detection Kit.
109 Construct generation: Second generation CAR constructs were generously provided

using the Lonza MycoAlert Detection Kit.
109
110 Construct generation: Second generat
111 Jonathan Bramson (McMaster University) 110
111
112
112 Jonathan Bramson (McMaster University) and Renier Brentjens (Roswell Park) ⁵⁷

Construct generation: Second generation CAR constructs were generously provided by
111 Jonathan Bramson (McMaster University) and Renier Brentjens (Roswell Park) ⁵⁷.
112 *Mouse ahBCMA CAR (hBCMAmBBmζ) :* CAR encoding DNA Jonathan Bramson (McMaster University) and Renier Brentjens (Roswell Park)⁵⁷.
112 *Mouse ahBCMA CAR (hBCMAmBBmζ) :* CAR encoding DNA was subcloned int
113 cloning site of the pRV2011 retroviral vector which also contains *Mouse αhBCMA CAR (hBCMAmBBmζ) :* CAR encoding DNA was subcloned into the multiple
cloning site of the pRV2011 retroviral vector which also contains an internal ribosome entry site
(IRES) and Thy1.1. Murine CAR constructs cloning site of the pRV2011 retroviral vector which also contains an internal ribosome entry site

114 (IRES) and Thy1.1. Murine CAR constructs consisted of an anti-human BCMA single chain

115 variable fragment (scFv) (C1 (IRES) and Thy1.1. Murine CAR constructs consisted of an anti-human BCMA single chain
115 variable fragment (scFv) (C11D5.3), CD8 hinge, CD28 transmembrane, 4-1BB signaling domain
116 and CD3ζ activation domain as describe and CD3^{ζ} activation domain as described previously ⁵⁸.

variable fragment (scFv) (C11D5.3), CD8 hinge, CD28 transmembrane, 4-1BB signaling domain

and CD3ζ activation domain as described previously ⁵⁸.
 Mouse ahCD19 CAR (hCD19m28mζ) : Off-target CAR constructs consisted of and CD3ζ activation domain as described previously ⁵⁸.
117 *Mouse ahCD19 CAR (hCD19m28mζ) :* Off-target CAR
118 CD19 scFv (FMC63), CD28 transmembrane, CD28
119 domain.
120 *Human αhBCMA CAR (hBCMABB^z)* : Antigen recep *Mouse ahCD19 CAR (hCD19m28mζ) :* Off-target CAR constructs consisted of an anti-human
118 CD19 scFv (FMC63), CD28 transmembrane, CD28 signaling domain and CD3ζ activation
119 *Human αhBCMA CAR (hBCMABBζ)* : Antigen recog

118 CD19 scFv (FMC63), CD28 transmembrane, CD28 signaling domain and CD3ζ activation
119 domain.
120 Human αhBCMA CAR (hBCMABBζ) : Antigen recognition was defined by an anti-human BCMA
121 scFv previously reported. Human 119 domain.
120 *Human*
121 scFv pr
122 transme *Human αhBCMA CAR (hBCMABBζ)* : Antigen recognition was defined by an anti-human BCMA
121 scFv previously reported. Human CAR constructs consisted of an CD8a hinge, CD8a
122 transmembrane, CD28 or 4-1BB signaling domain a transmembrane, CD28 *or* 4-1BB signaling domain and CD3ζ activation domain 57,59

scFv previously reported. Human CAR constructs consisted of an CD8a hinge, CD8a
122 transmembrane, CD28 or 4-1BB signaling domain and CD3ζ activation domain ^{57,59}.
123 *hBCMA-tNGFR:* Lentiviral expression plasmid, LeGO-L transmembrane, CD28 or 4-1BB signaling domain and CD3ζ activation domain ^{57,59}.
123 *hBCMA-tNGFR:* Lentiviral expression plasmid, LeGO-Luc2, was a gift from E
124 (Addgene plasmid #154006) which was further modified to *hBCMA-tNGFR:* Lentiviral expression plasmid, LeGO-Luc2, was a gift from Boris Fehse

124 (Addgene plasmid #154006) which was further modified to co-express hBCMA-tNGFR. Murine

125 5TGM1 cells were transduced with lentivi (Addgene plasmid #154006) which was further modified to co-express hBCMA-tNGFR. Murine
125 5TGM1 cells were transduced with lentiviral supernatant to drive expression of the target
126 antigen, hBCMA, and permit *in vivo* 125 5TGM1 cells were transduced with lentiviral supernatant to drive expression of the target
126 antigen, hBCMA, and permit *in vivo* imaging of tumor bearing animals.
127 All constructs were verified by sanger sequencing 126 antigen, hBCMA, and permit *in vivo* imaging of tumor bearing animals.
127 All constructs were verified by sanger sequencing.

127 All constructs were verified by sanger sequencing.

127 All constructs were verified by sanger sequencing.

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131 *Mouse CAR T cell production:* Mouse CAR T cell production was adapted from previous reports

⁶⁰. Briefly, pan CD3⁺ murine T cells were isolated from single cell splenocyte suspensions of 6 –

121 12-week-old mice thr 60 . Briefly, pan CD3⁺ murine T cells were isolated from single cell splenocyte suspensions of 6 – ⁶⁰. Briefly, pan CD3⁺ murine T cells were isolated from single cell splenocyte suspensions of 6 –
131 12-week-old mice through a negative selection process (STEMCELL Technologies). T cells were
132 activated with α 131 12-week-old mice through a negative selection process (STEMCELL Technologies). T cells were

activated with αCD3/αCD28 Dynabeads as specified by manufacturer's instructions in the

presence of 100 IU/mL recombinant mo activated with αCD3/αCD28 Dynabeads as specified by manufacturer's instructions in the
presence of 100 IU/mL recombinant mouse (rm) IL-2 and 10 ng/mL rmIL-7 (BioLegend) and
cultured in RPMI1640 supplemented with 10% FBS, presence of 100 IU/mL recombinant mouse (rm) IL-2 and 10 ng/mL rmIL-7 (BioLegend) and
134 cultured in RPMI1640 supplemented with 10% FBS, 2 mM L-glutamine, 10mM HEPES, 0.5% 2-
135 mercaptoethanol, and 1% penicillin/strepto cultured in RPMI1640 supplemented with 10% FBS, 2 mM L-glutamine, 10mM HEPES, 0.5% 2-
135 mercaptoethanol, and 1% penicillin/streptomycin. Retroviral transduction was achieved by
136 spinoculation of 3 x 10⁶ mouse T cel 135 mercaptoethanol, and 1% penicillin/streptomycin. Retroviral transduction was achieved by

136 spinoculation of 3 x 10⁶ mouse T cells on retronectin-coated plates (Takara Bio) with neat

137 retroviral supernatant ha spinoculation of 3 x 10^6 mouse T cells on retronectin-coated plates (Takara Bio) with neat 136 spinoculation of 3 x 10° mouse T cells on retronectin-coated plates (Takara Bio) with neat
137 retroviral supernatant harvested from 293T packaging cells (2000xg, 60 min., 30 °C) at 24 and
138 48 hr. post activation. 137 retroviral supernatant harvested from 293T packaging cells (2000xg, 60 min., 30 °C) at 24 and

138 48 hr. post activation. CAR T cells were maintained at 1 x 10⁶ cells/mL for 7-10 days *in vitro* in

140

141 Human 48 hr. post activation. CAR T cells were maintained at 1 x 10 6 138 48 hr. post activation. CAR T cells were maintained at 1 x 10⁶ cells/mL for 7-10 days *in vitro* in

139 the presence of rmlL-2 and rmlL-7 ⁶⁰.

140 Human CAR T cell production: Human CAR T cell production was adapt the presence of rmIL-2 and rmIL-7 60 .

the presence of rmIL-2 and rmIL-7 $\rm{^{60}}$.

140

141 Human CAR T cell production: Hum

142 published protocols $\rm{^{57}}$. De-identified, h

143 were obtained through the Beauell 141
142
143 Human CAR T cell production: Human CAR T cell production was adapted from previously

published protocols ⁵⁷. De-identified, healthy donor peripheral blood mononuclear cells (PBMCs)

were obtained through the Roswell Par published protocols ⁵⁷. De-identified, healthy donor peripheral blood mononuclear cells (PBMCs) published protocols ⁵⁷. De-identified, healthy donor peripheral blood mononuclear cells (PBMCs)
were obtained through the Roswell Park donor center under the approved protocol BDR
144 115919. PBMCs were isolated from who were obtained through the Roswell Park donor center under the approved protocol BDR
144 115919. PBMCs were isolated from whole blood through density gradient centrifugation. PBMCs
145 were activated with T cell TransAct po 144 115919. PBMCs were isolated from whole blood through density gradient centrifugation. PBMCs
145 were activated with T cell TransAct polymeric nanomatrix (Miltenyi Biotec) according to
146 manufacturer's specifications were activated with T cell TransAct polymeric nanomatrix (Miltenyi Biotec) according to

146 manufacturer's specifications in the presence of 100 IU/mL recombinant human (rh) IL-2 and 10

147 ng/mL rhIL-7 (Peprotech). Spin manufacturer's specifications in the presence of 100 IU/mL recombinant human (rh) IL-2 and 10
147 mg/mL rhIL-7 (Peprotech). Spinoculation with neat 293 Galv9 retroviral supernatant was
148 performed at 48, 72 and 96 hr. po ng/mL rhIL-7 (Peprotech). Spinoculation with neat 293 Galv9 retroviral supernatant was
148 performed at 48, 72 and 96 hr. post activation (3200 rpm, 60 min., 30 °C). Human CAR T cells
149 were expanded for 14 days and subs performed at 48, 72 and 96 hr. post activation (3200 rpm, 60 min., 30 °C). Human CAR T cells

149 were expanded for 14 days and subsequently cryopreserved in 90% FBS, 10% DMSO.

150 Cytotoxicity assays: In vitro CAR T cell

were expanded for 14 days and subsequently cryopreserved in 90% FBS, 10% DMSO.
150
151 Cytotoxicity assays: In vitro CAR T cell killing assays were performed using firefly lu
152 expressing target cells. Briefly, 2 x 10⁴ ---
151
152
153 151 *Cytotoxicity assays: In vitro* CAR T cell killing assays were performed using firefly luciferase
152 expressing target cells. Briefly, 2 x 10⁴ target cells were seeded in 96 well plates, varying
153 numbers of CAR T expressing target cells. Briefly, 2 x 10^4 expressing target cells. Briefly, 2 x 10⁴ target cells were seeded in 96 well plates, varying
153 numbers of CAR T cells were added to assess CAR T cell mediated killing within the linear
153 numbers of CAR T cells were 153 numbers of CAR T cells were added to assess CAR T cell mediated killing within the linear

range. Target cell viability following co-culture incubation was determined using the ONE-glo
155 luciferase reporter assay (Promega). For cytokine secretion assays, supernatants were
156 collected 24 hrs. after co-culture Interactional assay (Promega). For cytokine secretion assays, supernatants were
156 collected 24 hrs. after co-culture and analyzed on a Luminex xMAP INTELLIFLEX system.
157 Flow cytometry: Data was acquired on either a LS

collected 24 hrs. after co-culture and analyzed on a Luminex xMAP INTELLIFLEX system.
157
158 Flow cytometry: Data was acquired on either a LSR Fortessa (BD Biosciences) or Cytek A
159 full spectrum analyzer (Cytek Bioscie ---
158
159
160 *Flow cytometry:* Data was acquired on either a LSR Fortessa (BD Biosciences) or Cytek Aurora

159 **full spectrum analyzer (Cytek Biosciences)**. Analysis was performed using FlowJo (Tree Star

160 Inc.) or FCS Express soft full spectrum analyzer (Cytek Biosciences). Analysis was performed using FlowJo (Tree Star
160 Inc.) or FCS Express software (De novo Software). Briefly, cell suspensions were harvested,
161 washed, and stained with fixabl Inc.) or FCS Express software (De novo Software). Briefly, cell suspensions were harvested,
161 washed, and stained with fixable live/dead blue (Invitrogen) in PBS followed by surface antibody
162 staining in FACS buffer (washed, and stained with fixable live/dead blue (Invitrogen) in PBS followed by surface antibody
162 staining in FACS buffer (1% BSA, 0.1% sodium azide in PBS). Antibodies were titrated for
163 optimal staining for 20 min. staining in FACS buffer (1% BSA, 0.1% sodium azide in PBS). Antibodies were titrated for

optimal staining for 20 min. at 4°C. Intracellular cytokine staining was conducted following

fixation and permeabilization accordin optimal staining for 20 min. at 4°C. Intracellular cytokine staining was conducted following
164 fixation and permeabilization according to manufacturer's instructions (BioLegend). Antibodies
165 used in phenotypic analysi fixation and permeabilization according to manufacturer's instructions (BioLegend). Antibodies
165 used in phenotypic analysis are included in Supplemental Table 1.
166 Cytokine analyses (Luminex and Isoplexis): Co-culture

used in phenotypic analysis are included in Supplemental Table 1.
166
167 Cytokine analyses (Luminex and Isoplexis): Co-culture supernat
168 frozen and stored at -80 °C until Luminex assays were run. De ---
167
168
170 Cytokine analyses (Luminex and Isoplexis): Co-culture supernatants were immediately snap
168 frozen and stored at -80 °C until Luminex assays were run. Detection of mouse CAR T cell
169 cytokine production was performed us frozen and stored at -80 °C until Luminex assays were run. Detection of mouse CAR T cell
169 cytokine production was performed using the MILLIPLEX Th17 premixed panel and acquired on
170 a Luminex xMAP INTELLIFLEX system. cytokine production was performed using the MILLIPLEX Th17 premixed panel and acquired on
170 a Luminex xMAP INTELLIFLEX system. Data was analyzed using the Belysa® Immunoassay
171 Curve Fitting Software (Millipore Sigma). a Luminex xMAP INTELLIFLEX system. Data was analyzed using the Belysa® Immunoassay a Luminex xMAP INTELLIFLEX system. Data was analyzed using the Belysa[®] Immunoassay
171 Curve Fitting Software (Millipore Sigma). Cytokine production within the BM TME was evaluated
172 using Isoplexis' CodePlex secretome Curve Fitting Software (Millipore Sigma). Cytokine production within the BM TME was evaluated
172 using Isoplexis' CodePlex secretome chips. Briefly, bilateral, tumor-bearing hind limbs (femur
173 and tibia) were harveste using Isoplexis' CodePlex secretome chips. Briefly, bilateral, tumor-bearing hind limbs (femur

and tibia) were harvested, and BM was collected into 30 μL PBS. Soluble phase fractions were

stored at -80 °C in low-bind Ep and tibia) were harvested, and BM was collected into 30 µL PBS. Soluble phase fractions were
174 stored at -80 °C in low-bind Eppendorf tubes until loaded onto a CodePlex secretome chip and
175 analyzed in an IsoLight inst stored at -80 °C in low-bind Eppendorf tubes until loaded onto a CodePlex secretome chip and

175 analyzed in an IsoLight instrument.

176 Seahorse: The day prior to assay CAR T cells were stimulated overnight at an E:T ra

analyzed in an IsoLight instrument.
176
177 Seahorse: The day prior to assay (
178 Cell**¤Tak¤Coated XF96 microplat** 177
178 177 *Seahorse:* The day prior to assay CAR T cells were stimulated overnight at an E:T ratio of 2:1.
178 Cell**ZTakZCoated XF96** microplate was prepared to support testing of cells grown in 178 Cell**Tak**ZCoated XF96 microplate was prepared to support testing of cells grown in

179 suspension. Sensor cartridge was hydrated in XF Calibrant and incubated overnight at 37 °C in

180 a non-CO₂ incubator.

181 On the day of the experiment Seahorse XF DMEM Medium pH 7.4 (Agilent Technologies) was

18 180 a non-CO₂ incubator.
181 On the day of the ex
182 supplemented with
183 consumption rate (C 181 On the day of the experiment Seahorse XF DMEM Medium pH 7.4 (Agilent Technologies) was
182 supplemented with 10 mM glucose, 1 mM pyruvate and 2 mM glutamine for oxygen
183 consumption rate (OCR) examination and 2mM glu 182 supplemented with 10 mM glucose, 1 mM pyruvate and 2 mM glutamine for oxygen
183 consumption rate (OCR) examination and 2mM glutamine for extracellular acidification rate
184 (ECAR) examination and pre-warmed to 37 °C 183 consumption rate (OCR) examination and 2mM glutamine for extracellular acidification rate

184 (ECAR) examination and pre-warmed to 37 °C. Suspension cells were harvested, washed in 1X

185 PBS, resuspended in the pre 184 (ECAR) examination and pre-warmed to 37 °C. Suspension cells were harvested, washed in 1X
185 PBS, resuspended in the prepared assay media, and gently seeded on the Cell^o Tak^o Coated
186 plate at 2 x 10⁵ cells/w 185 PBS, resuspended in the prepared assay media, and gently seeded on the CellTakTCoated
186 plate at 2 x 10⁵ cells/well. The seeded plate was incubated in a 37 °C non-CO₂ incubator for 1
187 hour prior to the assay. plate at 2 x 10⁵ cells/well. The seeded plate was incubated in a 37 °C non-CO₂ incubator for 1 plate at 2 x 10° cells/well. The seeded plate was incubated in a 37 °C non-CO₂ incubator for 1
187 bour prior to the assay. During the incubation, test compounds specific to the assay type were
188 prepared and added to hour prior to the assay. During the incubation, test compounds specific to the assay type were
188 prepared and added to the ports of the hydrated cartridge. The loaded cartridge was moved to
189 the XFe96 Analyzer and ini prepared and added to the ports of the hydrated cartridge. The loaded cartridge was moved to

189 the XFe96 Analyzer and initial calibration was performed. Following the 1-hour incubation the

190 Cell-Tak plate was transf the XFe96 Analyzer and initial calibration was performed. Following the 1-hour incubation the

190 Cell-Tak plate was transferred to the Xfe96 Analyzer and the assay was initiated according to

191 manufacturer's recommend Cell-Tak plate was transferred to the Xfe96 Analyzer and the assay was initiated according to

191 manufacturer's recommendations.

192 All concentrations shown represent final well concentrations:

193 Mito Stress Test (O

-
- manufacturer's recommendations.

192 All concentrations shown represent

193 Mito Stress Test (OCR examination

194 Oligomycin (2.0 µM), FCCP (1.0 µM

195 Check Fisses Test (FCAR examin
- All concentrations shown represent final well concentrations:

193 Mito Stress Test (OCR examination)

194 Oligomycin (2.0 μM), FCCP (1.0 μM) and Rotenone/Antimycia

<u>Glycolysis Stress Test (ECAR examination)</u>

195 Clycol Mito Stress Test (OCR examination)
194 Oligomycin (2.0 µM), FCCP (1.0 µM
195 Glycolysis Stress Test (ECAR examination)
196 Glucose (10 mM), Oligomycin (1.0 µ Oligomycin (2.0 μM), FCCP (1.0 μM) and Rotenone/Antimycin A (0.5 μM)

195 Glycolysis Stress Test (ECAR examination)

196 Glucose (10 mM), Oligomycin (1.0 μM) and 2DG (50 mM)

197 Tell Metabolic Fitness Test

198 First inj
-
- Solycolysis Stress Test (ECAR examination)
196 Glucose (10 mM), Oligomycin (1.0 µM) and
197 T Cell Metabolic Fitness Test
198 First injection of this assay included substra
198 Fitemovis (4.0 uM), Jang shain fattus
-
- 196 Glucose (10 mM), Oligomycin (1.0 µM) and 2DG (50 mM)
197 T Cell Metabolic Fitness Test
198 First injection of this assay included substrate pathway spe
199 Etomoxir (4.0 µM), long chain fatty acid oxidation First injection of this assay included substrate pathway specific inhibitors:

199 Etomoxir (4.0 µM), long chain fatty acid oxidation

200 UK5099 (2.0 µM), glucose/pyruvate oxidation

201 EPTES (3.0 µM) glutamine oxidation
- 197 T Cell Metabolic Fitness Test
198 First injection of this assay in
199 Etomoxir (4.0 µM), lor
200 UK5099 (2.0 µM), glu
-
-
- Etomoxir (4.0 µM), long chain fatty acid oxidation

200 UK5099 (2.0 µM), glucose/pyruvate oxidation

201 BPTES (3.0 µM) glutamine oxidation

202 For controls, assay medium was used in port A ins
- 200 UK5099 (2.0 μM), glucose/pyruvate oxidation
201 BPTES (3.0 μM) glutamine oxidation
202 For controls, assay medium was used in port /
203 Following inhibitor injections Oligomycin (1.5 μM), Β, 201 BPTES (3.0 μ M) glutamine oxidation

202 For controls, assay medium was used

203 Following inhibitor injections Oligomycin (1.4

204 (0.5 μ M) were added to all wells. 202 For controls, assay medium was used in port A instead of inhibitors.

203 Following inhibitor injections Oligomycin (1.5 μ M), BAM15 (2.5 μ M) and R

204 (0.5 μ M) were added to all wells. 203 Following inhibitor injections Oligomycin (1.5 µM), BAM15 (2.5 µM) and Rotenone/Antimycin A
204 (0.5 µM) were added to all wells.
 (0.5 µM) were added to all wells.
- 204 (0.5 μ M) were added to all wells.

The last injection of each assay included Hoechst 33342 Nuclear Stain to facilitate

206 Normalization via fluorescent imaging and cell counting supported by the BioTek Cytation 5 Cell

207 Imaging Multimode Reader. Data w Normalization via fluorescent imaging and cell counting supported by the BioTek Cytation 5 Cell
207 Imaging Multimode Reader. Data was analyzed using the Wave 2.6.1 software and
208 the Seahorse Analytics cloud-based resou Imaging Multimode Reader. Data was analyzed using the Wave 2.6.1 software and
208 the Seahorse Analytics cloud-based resource.
209
210 qRT-PCR: RNA was isolated using the miRNeasy Mini Kit (Qiagen) and cDNA was synthesized

the Seahorse Analytics cloud-based resource.
209
210 *qRT-PCR*: RNA was isolated using the miRNe
211 using SuperScript IV Reverse Transcriptase. 210
211
212 *qRT-PCR*: RNA was isolated using the miRNeasy Mini Kit (Qiagen) and cDNA was synthesized
211 using SuperScript IV Reverse Transcriptase. Contaminating DNA was removed using Rnase-
212 Free Dnase (Qiagen) and qPCR was perf 211 using SuperScript IV Reverse Transcriptase. Contaminating DNA was removed using Rnase-
212 Free Dnase (Qiagen) and qPCR was performed using the QuantStudio 6 Flex Real-Time PCR
213 System with SYBR green (ThermoFisher Free Dnase (Qiagen) and qPCR was performed using the QuantStudio 6 Flex Real-Time PCR
213 System with SYBR green (ThermoFisher Scientific). Expression was normalized to TBP, and
214 relative expression was calculated usin System with SYBR green (ThermoFisher Scientific). Expression was normalized to TBP, and
214 relative expression was calculated using the ΔΔCT formula. Primer sequences are listed in
215 Supplemental Table 2.
216 MAD⁺ MA relative expression was calculated using the ΔΔCT formula. Primer sequences are listed in
215 Supplemental Table 2.
216 *NAD⁺/NADH Quantitation*: NADH:NAD⁺ ratios were determined using the NAD/NADH-Glo assay
218 (Dram

215 Supplemental Table 2.
216
217 NAD⁺/NADH Quantitati
218 (Promega) according to 217
218
219
228 *NAD+ /NADH Quantitation*: NADH:NAD+ *NAD⁺/NADH Quantitation*: NADH:NAD⁺ ratios were determined using the NAD/NADH-Glo assay

(Promega) according to manufacturer's instructions. Briefly, 1 x 10⁵ stimulated CAR T cells were

washed with 1x PBS prior to c (Promega) according to manufacturer's instructions. Briefly, 1 \times 10⁵ 218 (Promega) according to manufacturer's instructions. Briefly, 1 x 10⁵ stimulated CAR T cells were
219 washed with 1x PBS prior to cell lysis. NAD⁺ and NADH levels were quantified independently
220 using acid/base tr washed with 1x PBS prior to cell lysis. NAD⁺ and NADH levels were quantified independently washed with 1x PBS prior to cell lysis. NAD⁺ and NADH levels were quantified independently
220 using acid/base treatment. Luminescence values were read on a BioTek Synergy H1 plate
221 reader (Agilent).
222 using acid/base treatment. Luminescence values were read on a BioTek Synergy H1 plate

221 reader (Agilent).

222 Mice and in vivo models:

224 Syntomia Tumar Madela: NOD acid gamma (NSC) mice (NOD Ca Brkdo^{scid} Ilarg^{im}

221 reader (Agilent).
222
223 Mice and in vivo
224 Systemic Tumor 223
224
225 223 *Mice and in vivo models:*
224 *Systemic Tumor Models:*
225 ages 6-12 weeks were p
226 mouse colony at Roswel *Systemic Tumor Models: NOD scid gamma (NSG) mice (NOD.CgPrkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice* Systemic Tumor Models: NOD scid gamma (NSG) mice (NOD.Cg*Prkdc^{scid} Il2rg^{im1WI}*/SzJ) mice
ages 6-12 weeks were purchased from the Comparative Oncology Shared Resource in-house
mouse colony at Roswell Park. RAG2^{-/-} (B 225 ages 6-12 weeks were purchased from the Comparative Oncology Shared Resource in-house

226 mouse colony at Roswell Park. RAG2^{-/-} (B6.Cg-*Rag2<sup>tm1.1Cgn*/J) mice were purchased from the

227 Jackson Laboratory and sub</sup> mouse colony at Roswell Park. RAG2-/- (B6.Cg-*Rag2tm1.1Cgn* 226 mouse colony at Roswell Park. RAG2^{-/-} (B6.Cg-*Rag2^{tm1.1Ggn*/J) mice were purchased from the
227 Jackson Laboratory and subsequently bred in our facility under the approved protocol 1425M.
228 NSG mice were intraven} 227 Jackson Laboratory and subsequently bred in our facility under the approved protocol 1425M.

228 NSG mice were intravenously injected with 1 x 10⁶ MM.1S-Luc at week –4 and 3 x 10⁶ CAR T

229 cells at week 0. RAG2 NSG mice were intravenously injected with 1 x 10⁶ MM.1S-Luc at week –4 and 3 x 10⁶ NSG mice were intravenously injected with 1×10^6 MM.1S-Luc at week -4 and 3×10^6 CAR T
cells at week 0. RAG2^{-/-} mice were injected with 2×10^6 5TGM1^{hBCMA}-Luc at week -2 to and $3 \times$
 10^6 CAR T cells cells at week 0. RAG2^{-/-} mice were injected with 2 x 10⁶ 5TGM1^{hBCMA} 229 cells at week 0. RAG2^{-/-} mice were injected with 2 x 10[°] 5TGM1^{nBCMA}-Luc at week –2 to and 3 x
230 10^{⁶ CAR T cells at week 0 to compensate for differences in tumor engraftment rate amongst the} $10⁶$ 230 10^6 CAR T cells at week 0 to compensate for differences in tumor engraftment rate amongst the

two models. Bioluminescence was measured 2x/week using an IVIS® Spectrum In Vivo Imaging two models. Bioluminescence was measured 2x/week using an IVIS® Spectrum In Vivo Imaging
232 System (PerkinElmer) to assess tumor burden. Mice were injected with 150 mg Luciferin/kg of
233 body weight and briefly anestheti System (PerkinElmer) to assess tumor burden. Mice were injected with 150 mg Luciferin/kg of

233 body weight and briefly anesthetized through isoflurane inhalation during image acquisition.

234 Data was analyzed on the Li body weight and briefly anesthetized through isoflurane inhalation during image acquisition.

234 Data was analyzed on the Living Image analysis software (PerkinElmer). In some settings,

235 retroorbital blood collection Data was analyzed on the Living Image analysis software (PerkinElmer). In some settings,

235 retroorbital blood collection was performed 1 week after CAR T cell infusion to examine CAR T

236 cell frequency in circulation retroorbital blood collection was performed 1 week after CAR T cell infusion to examine CAR T

cell frequency in circulation. Mice were monitored daily for signs of deteriorating condition or

disease progression including cell frequency in circulation. Mice were monitored daily for signs of deteriorating condition or

237 disease progression including decreased activity, hunched posture, ruffled coat, or hind limb

238 paralysis and euthani

disease progression including decreased activity, hunched posture, ruffled coat, or hind limb

paralysis and euthanized upon veterinary recommendation.

All animal studies were performed in accordance with the Roswell Park paralysis and euthanized upon veterinary recommendation.

239 All animal studies were performed in accordance with the R

240 Center Institutional Animal Care and Use Committee guid

242 CO29^{KO}: CD29^{KO} mise were genera 239 All animal studies were performed in accordance with the Roswell Park Comprehensive Cancer
240 Center Institutional Animal Care and Use Committee guidelines under the approved protocol
242 CD28^{iKO}: CD28^{iKO} mice wer

Center Institutional Animal Care and Use Committee guidelines under the approved protocol

241 1094M.

242 CD28^{jKO}: CD28^{jKO} mice were generated by Ozgene (Australia). LoxP sites flanking exon 2 and 3

243 of the CD28 g 241 1094M.

242 *CD28^{iKO}*

243 of the C

244 were ge *CD28^{<i>iKO}*: CD28^{*iKO*} mice were generated by Ozgene (Australia). LoxP sites flanking exon 2 and 3</sup> $CD28^{14}$. $CD28^{14}$ mice were generated by Ozgene (Australia). LoxP sites flanking exon 2 and 3 of the CD28 gene were introduced to allow for Cre-mediated deletion of the CD28 gene. Mice were generously provided by Kelvi of the CD28 gene were introduced to allow for Cre-mediated deletion of the CD28 gene. Mice

244 were generously provided by Kelvin Lee (Indiana University) and subsequently bred in-house

245 under protocol 1425M. Splenocy were generously provided by Kelvin Lee (Indiana University) and subsequently bred in-house

under protocol 1425M. Splenocytes were isolated as previously described and CAR T cells were

expanded in the presence of 250 nM 4 under protocol 1425M. Splenocytes were isolated as previously described and CAR T cells were

246 expanded in the presence of 250 nM 4-hydroxytamoxifen (Sigma-Aldrich) for 4 days to induce

247 CD28 deletion.

248 T-lux: T

expanded in the presence of 250 nM 4-hydroxytamoxifen (Sigma-Aldrich) for 4 days to induce

247 CD28 deletion.

248 T-lux: Transgenic T-lux mice generated by Casey Weaver at the University of Alabama at

249 Birmingham wer 247 CD28 deletion.
248 T-lux: Transger
249 Birmingham we
250 agreement (MT *T-lux:* Transgenic T-lux mice generated by Casey Weaver at the University of Alabama at
249 Birmingham were acquired by Robert McGray (Roswell Park) under a material transfer
250 agreement (MTA). Mice were utilized as spl Eirmingham were acquired by Robert McGray (Roswell Park) under a material transfer
250 agreement (MTA). Mice were utilized as splenocyte donors for CAR T cell manufacturing for *in*
251 *vivo* imaging of CAR T cell traffic agreement (MTA). Mice were utilized as splenocyte donors for CAR T cell manufacturing for *in*
251 *vivo* imaging of CAR T cell trafficking and expansion.
252 Statistical analyses: All statistical analyses were performed u

vivo imaging of CAR T cell trafficking and expansion.
252 **Statistical analyses:** All statistical analyses were pe
254 **Data points represent independent biological replicate** ---
253
254
255 253 *Statistical analyses:* All statistical analyses were performed using GraphPad Prism software.
254 Data points represent independent biological replicates. Error bars represent standard deviation
255 unless otherwise s Data points represent independent biological replicates. Error bars represent standard deviation
255 unless otherwise stated. Statistical significance between groups was determined by paired or
256 unpaired Student's t tes 255 unless otherwise stated. Statistical significance between groups was determined by paired or
256 unpaired Student's t test, one-way or two-way ANOVA. Survival analysis was performed using a
256 unpaired Student's t tes 256 unpaired Student's t test, one-way or two-way ANOVA. Survival analysis was performed using a

257 log-rank (Mantel-Cox) test. A p value ≤0.05 is considered significant: *p<0.05, **p<0.01,

258 ***p<0.001, ****p<0.0001.
 **Results
** *Results*
 Blockade of endogenous CD28 impairs hBCMABBζ CAR T cell anti-MM activi

258 $***$ p<0.001, $***$ p<0.0001.

259 **Results

260** *Blockade of endogenou*

261 Since inhibition of the 259 **Results**
260 *Blockad*
262 to chemo **Blockade of endogenous CD28 impairs hBCMABBZ CAR T cell anti-MM activity.**
261 Since inhibition of the CD28 survival signal in multiple myeloma (MM) cells sensi
262 to chemotherapy^{45,46}, we sought to determine whether Since inhibition of the CD28 survival signal in multiple myeloma (MM) cells sensitizes them

262 to chemotherapy^{45,46}, we sought to determine whether CD28 inhibition similarly sensitized MM

263 cells to killing by CAR T to chemotherapy^{45,46}, we sought to determine whether CD28 inhibition similarly sensitized MM 262 to chemotherapy^{45,46}, we sought to determine whether CD28 inhibition similarly sensitized MM
263 cells to killing by CAR T cells. Human CAR T cells targeting BCMA and containing a 4-
264 1BB/CD3 ζ intracellular s 263 cells to killing by CAR T cells. Human CAR T cells targeting BCMA and containing a 4-
264 1BB/CD3ζ intracellular signaling domain (hBCMABBζ) similar to FDA approved CAR products
265 for MM (Fig. 1A) were generated fro 1BB/CD3ζ intracellular signaling domain (hBCMABBζ) similar to FDA approved CAR products
265 for MM (Fig. 1A) were generated from healthy donor peripheral blood mononuclear cells
266 transduced with a previously described 265 for MM (Fig. 1A) were generated from healthy donor peripheral blood mononuclear cells

266 transduced with a previously described retroviral vector 57 (Supplemental Fig. 1A – 1E). Co-

267 culture of hBCMABB ζ transduced with a previously described retroviral vector 57 (Supplemental Fig. 1A – 1E). Co-266 transduced with a previously described retroviral vector \degree' (Supplemental Fig. 1A – 1E). Co-
267 culture of hBCMABB ζ CAR T cells with human MM cell lines MM.1S or U266, which differ in
268 their expression prof culture of hBCMABBζ CAR T cells with human MM cell lines MM.1S or U266, which differ in
268 their expression profiles of CD28 and B7 ligands (MM.1S = CD28⁺, CD86⁺, CD80⁻ & U266 =
269 CD28⁺, CD86⁻, CD80⁻; Suppl their expression profiles of CD28 and B7 ligands (MM.1S = CD28⁺, CD86⁺, CD80⁻ their expression profiles of CD28 and B7 ligands (MM.1S = CD28⁺, CD86⁺, CD80⁻ & U266 =
269 CD28⁺, CD86⁻,CD80⁻; Supplemental Fig. 1F), resulted in cytotoxicity across a range of effector
270 to target ratios (Fi CD28⁺ , CD86- ,CD80- CD28⁺, CD86⁻, CD80⁻; Supplemental Fig. 1F), resulted in cytotoxicity across a range of effector
270 to target ratios (Fig. 1B). Intriguingly, addition of abatacept to co-cultures mildly enhanced
271 sensitivity of C to target ratios (Fig. 1B). Intriguingly, addition of abatacept to co-cultures mildly enhanced

271 sensitivity of CD86⁺ MM.1S, but not CD86⁻ U266, to hBCMABBZ CAR T cell killing, indicating

272 that blocking CD28-CD8 sensitivity of CD86⁺ MM.1S, but not CD86⁻ 271 sensitivity of CD86⁺ MM.1S, but not CD86⁻ U266, to hBCMABBζ CAR T cell killing, indicating
272 that blocking CD28-CD86 interactions on MM cells may sensitize them to CAR T cell therapy
273 (Fig. 1B). In agreement that blocking CD28-CD86 interactions on MM cells may sensitize them to CAR T cell therapy

(Fig. 1B). In agreement with potential MM sensitization to CAR T killing, abatacept did not alter

hBCMABBζ CAR T cell production (Fig. 1B). In agreement with potential MM sensitization to CAR T killing, abatacept did not alter

hBCMABBζ CAR T cell production of effector cytokines or proinflammatory molecules including

interferon-gamma (IFN-γ), tum hBCMABBζ CAR T cell production of effector cytokines or proinflammatory molecules including

interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNFα) or granulocyte-macrophage

colony-stimulating factor (GM-CSF) in co

275 interferon-gamma (IFN-γ), tumor necrosis factor alpha (TNFα) or granulocyte-macrophage
276 colony-stimulating factor (GM-CSF) in co-culture assays (Supplemental Fig. 1G).
277 Due to the modest capacity of abatacept to colony-stimulating factor (GM-CSF) in co-culture assays (Supplemental Fig. 1G).

Due to the modest capacity of abatacept to enhance CAR T cell-mediated

vitro, we evaluated the ability of abatacept to enhance hBCMABBZ CAR Due to the modest capacity of abatacept to enhance CAR T cell-mediated cytotoxicity *in*

278 vitro, we evaluated the ability of abatacept to enhance hBCMABBζ CAR T cell control of

279 orthotopic CD28⁺, CD86⁺ myeloma *vitro*, we evaluated the ability of abatacept to enhance hBCMABBζ CAR T cell control of
279 orthotopic CD28⁺, CD86⁺ myeloma. Luciferase tagged MM.1S (MM.1S-Luc) cells were
280 implanted i.v. into NSG hosts followed f orthotopic CD28⁺, CD86⁺ orthotopic CD28⁺, CD86⁺ myeloma. Luciferase tagged MM.1S (MM.1S-Luc) cells were

280 implanted i.v. into NSG hosts followed four weeks later by infusion of 3 x 10⁶ hBCMABB_S CAR T

281 cells ± 3x/weekly injections o implanted i.v. into NSG hosts followed four weeks later by infusion of 3 x 10 6 280 implanted i.v. into NSG hosts followed four weeks later by infusion of 3×10^6 hBCMABBζ CAR T

281 cells ± 3x/weekly injections of abatacept continued until endpoint (Fig. 1C). Bioluminescence

282 imaging was use cells ± 3x/weekly injections of abatacept continued until endpoint (Fig. 1C). Bioluminescence
282 imaging was used to confirm bone marrow engraftment and to normalize average tumor burden
182 282 imaging was used to confirm bone marrow engraftment and to normalize average tumor burden

imaging was used to confirm bone marrow engraftment and to normalize average tumor burden 283 across groups immediately prior to therapy. Following hBCMABBζ CAR T infusion, MM.1S
284 burden was assessed by serial bioluminescence imaging. MM regression was observed in all
285 hBCMABBζ CAR T cell treated mice, w burden was assessed by serial bioluminescence imaging. MM regression was observed in all

285 hBCMABB ζ CAR T cell treated mice, with most mice apparently tumor free 2 to 3 weeks

286 following infusion (Fig. 1D). Unexp 285 hBCMABBζ CAR T cell treated mice, with most mice apparently tumor free 2 to 3 weeks
286 following infusion (Fig. 1D). Unexpectedly, MM relapse was more rapidly seen in mice receiving
287 abatacept + hBCMABBζ CAR T cel following infusion (Fig. 1D). Unexpectedly, MM relapse was more rapidly seen in mice receiving
abatacept + hBCMABBZ CAR T cells compared to those receiving single agent hBCMABBZ
CAR T cells (Fig. 1E), resulting in signific 287 abatacept + hBCMABBζ CAR T cells compared to those receiving single agent hBCMABBζ
288 CAR T cells (Fig. 1E), resulting in significantly shorter survival of MM.1S bearing mice in the
289 abatacept + hBCMABBζ CAR T gro

CAR T cells (Fig. 1E), resulting in significantly shorter survival of MM.1S bearing mice in the

abatacept + hBCMABBζ CAR T group (Fig. 1F).

Prior work has demonstrated that CD28 can contribute to an immunosuppressive MM 289 abatacept + hBCMABBζ CAR T group (Fig. 1F).
290 Prior work has demonstrated that CD28 can
291 by interacting with CD80/CD86 on bone marrov
292 and the tryptophan metabolizing enzyme, inde Prior work has demonstrated that CD28 can contribute to an immunosuppressive MM BME
291 by interacting with CD80/CD86 on bone marrow resident DCs and inducing production of IL-6
292 and the tryptophan metabolizing enzyme, by interacting with CD80/CD86 on bone marrow resident DCs and inducing production of IL-6

292 and the tryptophan metabolizing enzyme, indoleamine 2,3-dioxygenase (IDO)⁴⁴. In the low

293 tumor burden setting, abatacept and the tryptophan metabolizing enzyme, indoleamine 2,3-dioxygenase (IDO)⁴⁴. In the low and the tryptophan metabolizing enzyme, indoleamine 2,3-dioxygenase $(IDO)^{44}$. In the low
tumor burden setting, abatacept may function through ligation of B7 family proteins on BMDCs
to create an immunosuppressive MM BME 293 tumor burden setting, abatacept may function through ligation of B7 family proteins on BMDCs
294 to create an immunosuppressive MM BME. We therefore repeated CAR T cell \pm abatacept
295 treatment regimen in a high M 294 to create an immunosuppressive MM BME. We therefore repeated CAR T cell \pm abatacept
295 treatment regimen in a high MM.1S tumor burden setting in which an immunosuppressive MM
296 BME should already be established. treatment regimen in a high MM.1S tumor burden setting in which an immunosuppressive MM
296 BME should already be established. We found that abatacept similarly accelerated relapse
297 following hBCMABBC CAR T cell infusio 296 BME should already be established. We found that abatacept similarly accelerated relapse
297 following hBCMABBζ CAR T cell infusion in the high tumor burden setting (Fig. 1G) and
298 shortened overall survival (Fig. 1 following hBCMABBζ CAR T cell infusion in the high tumor burden setting (Fig. 1G) and
298 shortened overall survival (Fig. 1H) suggesting that induction of immunosuppression in the MM
299 bone marrow microenvironment was 298 shortened overall survival (Fig. 1H) suggesting that induction of immunosuppression in the MM
299 bone marrow microenvironment was likely not the primary effect of abatacept exposure.
200 **Endogenous CD28 enhances 4-1B**

bone marrow microenvironment was likely not the primary effect of abatacept exposure.

200
 Endogenous CD28 enhances 4-1BB co-stimulated CAR T cell efficacy.

202 Despite the very clear reduction to *in vivo* CAR T cell 301
302
303 **Endogenous CD28 enhances 4-1BB co-stimulated CAR T cell efficacy.**

Despite the very clear reduction to *in vivo* CAR T cell efficacy imparted by c

treatment, data shown in Fig. 1 does not differentiate effects of abatac 302 Despite the very clear reduction to *in vivo* CAR T cell efficacy imparted by continuous abatacept treatment, data shown in Fig. 1 does not differentiate effects of abatacept on cells in the MM BME versus effects of bl 303 treatment, data shown in Fig. 1 does not differentiate effects of abatacept on cells in the MM
304 BME versus effects of blocking endogenous CD28 on CAR T cells. To examine CAR T cell
305 intrinsic effects of endogenou 304 BME versus effects of blocking endogenous CD28 on CAR T cells. To examine CAR T cell
305 intrinsic effects of endogenous CD28, we generated a tamoxifen inducible CD28 knockout
306 mouse model (CD28^{iKO}) by crossing C intrinsic effects of endogenous CD28, we generated a tamoxifen inducible CD28 knockout

306 mouse model (CD28^{iKO}) by crossing CD28-floxed mice to mice expressing CreERT2 from the

307 ROSA26 locus⁶¹. Following hBCMAmBB mouse model (CD28^{iKO} 306 mouse model (CD28^{IKO}) by crossing CD28-floxed mice to mice expressing CreERT2 from the
307 ROSA26 locus⁶¹. Following hBCMAmBBmζ CAR transduction of CD28^{IKO} or littermate control T
308 cells lacking CreERT2 expre ROSA26 locus⁶¹. Following hBCMAmBBmζ CAR transduction of CD28^{iKO} ROSA26 locus⁵¹. Following hBCMAmBBmζ CAR transduction of CD28^{ikO} or littermate control T
308 cells lacking CreERT2 expression, 4-hydroxytamoxifen was introduced into culture media for 4
308 cells lacking CreERT2 expres 308 cells lacking CreERT2 expression, 4-hydroxytamoxifen was introduced into culture media for 4

cells lacking CreERT2 expression, 4-hydroxytamoxifen was introduced into culture media for 4

days to induce CD28 deletion (Fig. 2A). Surface protein expression was evaluated by flow
310 cytometry over the course of CAR T cell expansion and immediately prior to functional
311 assessment. Importantly, CD28 surface e 310 cytometry over the course of CAR T cell expansion and immediately prior to functional
311 assessment. Importantly, CD28 surface expression was reduced to near background levels in
312 CD28^{iKO} CAR T cells by 4-OHT ex 311 assessment. Importantly, CD28 surface expression was reduced to near background levels in
312 CD28^{iKO} CAR T cells by 4-OHT exposure (Fig. 2B,2C), while CD4 : CD8 ratio and CAR
313 expression was unaffected (Suppleme $\mathsf{CD28}^\mathsf{iKO}$ 312 CD28^{INO} CAR T cells by 4-OHT exposure (Fig. 2B,2C), while CD4 : CD8 ratio and CAR
313 expression was unaffected (Supplemental Fig. 2A – 2C). To interrogate functionality of murine
314 CAR T cells we engineered synge 313 expression was unaffected (Supplemental Fig. $2A - 2C$). To interrogate functionality of murine
314 CAR T cells we engineered syngeneic 5TGM1 MM cells to express a chimeric hBCMA-tNGFR
315 target antigen (5TGM1^{hBCMA}) 314 CAR T cells we engineered syngeneic 5TGM1 MM cells to express a chimeric hBCMA-tNGFR
315 target antigen (5TGM1^{hBCMA}) ⁶²⁻⁶⁵ (Supplemental Fig. 2D, 2E). Coupling the extracellular
316 domains of hBCMA to a signaling target antigen (5TGM1^{hBCMA}) ⁶²⁻⁶⁵ 315 target antigen (5TGM1^{nBCMA}) ⁶²⁻⁶⁵ (Supplemental Fig. 2D, 2E). Coupling the extracellular
316 domains of hBCMA to a signaling deficient NGFR transmembrane domain allowed us to
317 uncouple the target function of BC domains of hBCMA to a signaling deficient NGFR transmembrane domain allowed us to
317 uncouple the target function of BCMA from its survival signal. In co-culture assays, CD28^{iko}
318 CAR T cells were nearly as effective uncouple the target function of BCMA from its survival signal. In co-culture assays, CD28^{iKO} ---
318
319
320 CAR T cells were nearly as effective as control $CD28^{\frac{f}{f}}$ CAR T cells at killing 5TGM1^{hBCMA} ---
319
320
321 319 myeloma cells, indicating that endogenous CD28 does not directly impact CAR T cell
320 cytotoxicity (Fig. 2D). CD28^{ft/fl} and CD28^{iKO} CAR T cells also produced comparable amounts of
321 proinflammatory cytokines wh cytotoxicity (Fig. 2D). CD28^{fl/fl} and CD28^{iKO} proinflammatory cytokines when stimulated by 5TGM1^{hBCMA} in vitro (Fig. 2E).

space cytotoxicity (Fig. 2D). CD28tM and CD28^{tKO} CAR T cells also produced comparable amounts of
321 proinflammatory cytokines when stimulated by 5TGM1^{hBCMA} *in vitro* (Fig. 2E).
322 In contrast to *in vitro* findin proinflammatory cytokines when stimulated by 5TGM1^{nBCMA} in vitro (Fig. 2E).

In contrast to *in vitro* findings, CD28^{iKO} hBCMAmBBmζ CAR T cells dif

hBCMAmBBmζ CAR T cells generated from littermate controls in their ab In contrast to *in vitro* findings, CD28^{iKO} In contrast to *in vitro* findings, CD28^{INO} hBCMAmBBmζ CAR T cells differed greatly from

hBCMAmBBmζ CAR T cells generated from littermate controls in their ability to control *in vivo*

myeloma growth. CD28^{iKO} hBCMA hBCMAmBBmζ CAR T cells generated from littermate controls in their ability to control *in vivo*
324 myeloma growth. CD28^{iKO} hBCMAmBBmζ CAR T cells transiently controlled systemic growth of
325 luciferase labeled 5TGM1^h myeloma growth. CD28^{iKO} hBCMAmBBm(CAR T cells transiently controlled systemic growth of 324 myeloma growth. CD28^{IKO} hBCMAmBBmζ CAR T cells transiently controlled systemic growth of
325 luciferase labeled 5TGM1^{hBCMA} myeloma in RAG2^{-/-} mice while CD28^{fI/f|} littermate control
326 hBCMAmBBmζ CAR T cells luciferase labeled 5TGM1^{hBCMA} myeloma in RAG2^{-/-} mice while CD28^{fl/fl} Iuciferase labeled 5TGM1^{nBCMA} myeloma in RAG2^{-/-} mice while CD28tM littermate control

1326 hBCMAmBBmζ CAR T cells demonstrated extended myeloma control (Fig. 2F, 2G). As a result,

1327 the median survival of 5TGM1 hBCMAmBBmζ CAR T cells demonstrated extended myeloma control (Fig. 2F, 2G). As a result,
327 the median survival of 5TGM1^{hBCMA} myeloma bearing mice treated with CD28^{f/ff} hBCMAmBBmζ
28 CAR T cells was nearly twice as l the median survival of 5TGM1^{hBCMA} myeloma bearing mice treated with CD28^{fl/fl} the median survival of 5TGM1^{nBCMA} myeloma bearing mice treated with CD28^{ft/1} hBCMAmBBmζ
CAR T cells was nearly twice as long as those treated with CD28^{iKO} hBCMAmBBmζ CAR T cells
(Fig. 2G, 2H), mirroring effects of a CAR T cells was nearly twice as long as those treated with CD28^{iKO} hBCMAmBBm(CAR T cells CAR T cells was nearly twice as long as those treated with $CD28^{\text{hO}}$ hBCMAmBBmζ CAR T cells

(Fig. 2G, 2H), mirroring effects of abatacept blockade of endogenous CD28 signaling (Fig. 1E,

1F). Moreover, pro-inflammator (Fig. 2G, 2H), mirroring effects of abatacept blockade of endogenous CD28 signaling (Fig. 1E,
330 1F). Moreover, pro-inflammatory cytokines in the MM BME of CD28^{iKO} hBCMAmBBmζ CAR T
331 cell treated mice were substantial 1F). Moreover, pro-inflammatory cytokines in the MM BME of CD28^{iKO} 1F). Moreover, pro-inflammatory cytokines in the MM BME of CD28^{INO} hBCMAmBBmζ CAR T
cell treated mice were substantially reduced (Fig. 2I). These data indicate that CD28^{IKO} CAR T
cells did not induce a proinflammatory cell treated mice were substantially reduced (Fig. 2I). These data indicate that CD28^{iKO} cell treated mice were substantially reduced (Fig. 2l). These data indicate that CD28^{IKO} CAR T
232 cells did not induce a proinflammatory MM BME despite being able to readily produce pro-
233 inflammatory cytokines in re cells did not induce a proinflammatory MM BME despite being able to readily produce pro-
inflammatory cytokines in response to CAR ligation (Fig. 2E).
334 333 inflammatory cytokines in response to CAR ligation (Fig. 2E).
334
Alla

Endogenous CD28 supports CAR T cell oxidative metabolism.
336 **CD28** controls metabolic reprogramming of activated T cells to
inflammatory cytokines and anti-tumor immunity⁶⁶⁻⁶⁹. Since CAR T
metabolic state of infused 336 CD28 controls metabolic reprogramming of activated T cells to enhance production of pro-
337 inflammatory cytokines and anti-tumor immunity⁶⁶⁻⁶⁹. Since CAR T cell efficacy is linked to the
338 metabolic state of inf inflammatory cytokines and anti-tumor immunity⁶⁶⁻⁶⁹ inflammatory cytokines and anti-tumor immunity⁶⁶⁻⁶⁹. Since CAR T cell efficacy is linked to the
metabolic state of infused cells⁷⁰, we evaluated glycolytic and mitochondrial metabolism of
unstimulated and 5TGM1^{hBCMA} metabolic state of infused cells⁷⁰, we evaluated glycolytic and mitochondrial metabolism of metabolic state of infused cells⁷⁰, we evaluated glycolytic and mitochondrial metabolism of
339 unstimulated and 5TGM1^{hBCMA} stimulated CD28^{ft/f1} and CD28^{ikO} hBCMAmBBmζ CAR T cells
340 using Seahorse assays. Change unstimulated and 5TGM1^{hBCMA} stimulated CD28^{f/fl} and CD28^{iKO} unstimulated and 5TGM1^{nBCMA} stimulated CD28^{ft/ff} and CD28^{kO} hBCMAmBBm ζ CAR T cells
340 using Seahorse assays. Changes in extracellular acidification rate (ECAR) in response to
341 glucose addition or inhibition using Seahorse assays. Changes in extracellular acidification rate (ECAR) in response to
341 glucose addition or inhibition of mitochondrial ATP synthesis were equivalent between CD28^{f//f}
342 and CD28^{iKO} CAR T cells (glucose addition or inhibition of mitochondrial ATP synthesis were equivalent between CD28^{f/fl} 342
343
344 and CD28^{iKO} CAR T cells (Fig. 3A, 3B), indicating that mBBm⁷ CAR signaling was sufficient to 342 and CD28^{KO} CAR T cells (Fig. 3A, 3B), indicating that mBBmζ CAR signaling was sufficient to
343 induce glycolytic metabolism. In contrast, CD28^{KO} hBCMAmBBmζ CAR T cells displayed
344 reduced oxygen consumption rat induce glycolytic metabolism. In contrast, CD28^{iKO} induce glycolytic metabolism. In contrast, $CD28^{\text{hO}}$ hBCMAmBBm ζ CAR T cells displayed

344 reduced oxygen consumption rate (OCR) and this pattern differed based on stimulation (Fig.

345 3C,3D). Basal and uncoupled 344 reduced oxygen consumption rate (OCR) and this pattern differed based on stimulation (Fig.
365 3C,3D). Basal and uncoupled OCR were decreased in unstimulated CD28^{iKO} CAR T cells while
346 uncoupled OCR and spare resp 3C,3D). Basal and uncoupled OCR were decreased in unstimulated CD28^{iKO} CAR T cells while 345 3C,3D). Basal and uncoupled OCR were decreased in unstimulated CD28^{kO} CAR T cells while
346 uncoupled OCR and spare respiratory capacity (SRC) were decreased in stimulated CD28^{ikO}
347 CAR T cells. Reduced OCR in C uncoupled OCR and spare respiratory capacity (SRC) were decreased in stimulated CD28^{iKO} 347
348
349 CAR T cells. Reduced OCR in CD28^{iKO} CAR T cells is consistent with the established role of CAR T cells. Reduced OCR in CD28^{kO} CAR T cells is consistent with the established role of

CD28 in priming mitochondria to support a robust recall response in of memory CD8 T cells⁶⁷.

Yet in contrast to memory CD8 CD28 in priming mitochondria to support a robust recall response in of memory CD8 T cells⁶⁷. CD28 in priming mitochondria to support a robust recall response in of memory CD8 T cells⁹⁷.
349 Yet in contrast to memory CD8 T cells, reduced mitochondrial OCR in CD28^{iKO} CAR T cells did
350 not result from diminishe Yet in contrast to memory CD8 T cells, reduced mitochondrial OCR in CD28^{iKO} CAR T cells did 349 Yet in contrast to memory CD8 T cells, reduced mitochondrial OCR in CD28^{IKO} CAR T cells did
350 not result from diminished fatty acid oxidation nor from an inability of CD28^{IKO} CAR T cells to
351 oxidize other maj not result from diminished fatty acid oxidation nor from an inability of $CD28¹⁶ CAR$ T cells to not result from diminished fatty acid oxidation nor from an inability of CD28^{IKO} CAR T cells to
351 oxidize other major anapleurotic substrates glucose and glutamine (Supplemental Fig. 3A).
352 Moreover, no difference in oxidize other major anapleurotic substrates glucose and glutamine (Supplemental Fig. 3A).
352 Moreover, no difference in mitochondria content was observed when comparing CD28^{iKO} and
353 CD28^{ft/fl} hBCMAmBBmζ CAR T cells Moreover, no difference in mitochondria content was observed when comparing CD28^{iKO} Moreover, no difference in mitochondria content was observed when comparing CD28^{thO} and
353 CD28^{ft/f1} hBCMAmBBmζ CAR T cells (Supplemental Fig. 3B), further suggesting that
354 endogenous CD28 signaling regulates mitoc $CD28^{fl/fl}$ hBCMAmBBm ζ CAR T cells (Supplemental Fig. 3B), further suggesting that 253 CD28^{tim} hBCMAmBBmζ CAR T cells (Supplemental Fig. 3B), further suggesting that
354 endogenous CD28 signaling regulates mitochondrial oxidative phosphorylation in 4-1BB co-
355 stimulated CAR T cells.
256 Mitochondr

endogenous CD28 signaling regulates mitochondrial oxidative phosphorylation in 4-1BB co-
355 stimulated CAR T cells.
Mitochondrial oxidative phosphorylation relies on the electron carriers NADH to donate
357 electrons to c 355 stimulated CAR T cells.
356 Mitochondrial oxidat
357 electrons to complex I a
358 chain (ETC), driving mito Mitochondrial oxidative phosphorylation relies on the electron carriers NADH to donate

357 electrons to complex I and FADH2 to donate electrons to complex II of the electron transport

358 chain (ETC), driving mitochondri electrons to complex I and FADH2 to donate electrons to complex II of the electron transport

chain (ETC), driving mitochondrial oxygen consumption and creating a proton gradient across

the inner mitochondria membrane to 358 chain (ETC), driving mitochondrial oxygen consumption and creating a proton gradient across
359 the inner mitochondria membrane to fuel ATP synthase. Deletion of endogenous CD28 did not
360 alter the contribution of co 359 the inner mitochondria membrane to fuel ATP synthase. Deletion of endogenous CD28 did not
360 alter the contribution of complex I nor complex II to mitochondrial oxygen consumption by 360 alter the contribution of complex I nor complex II to mitochondrial oxygen consumption by

360 361 hBCMAmBBmζ CAR T cells (Supplemental Fig. 3C). Interestingly however, the ratio of NADH to
362 NAD⁺ was increased in target cell stimulated CD28^{ft/ff} when compared to CD28^{ikO}
363 hBCMAmBBmζ CAR T cells (Fig. 3E) NAD⁺ was increased in target cell stimulated CD28^{fl/fl} when compared to CD28^{iKO} 363
364
365 363 hBCMAmBBmζ CAR T cells (Fig. 3E), indicating that endogenous CD28 signaling increases
364 ETC substrate availability in 4-1BB co-stimulated CAR T cells. Several crucial metabolic
365 enzymes reduce NAD⁺ to NADH or o 364 ETC substrate availability in 4-1BB co-stimulated CAR T cells. Several crucial metabolic
365 enzymes reduce NAD⁺ to NADH or oxidize NADH to NAD⁺ (Fig. 3F), thereby maintaining redox
366 balance. Quantitative RT-PCR enzymes reduce NAD⁺ to NADH or oxidize NADH to NAD⁺ enzymes reduce NAD⁺ to NADH or oxidize NADH to NAD⁺ (Fig. 3F), thereby maintaining redox
366 balance. Quantitative RT-PCR revealed that among these enzymes, only *Gapdh* gene
367 expression was altered in CD28^{ikO} hB balance. Quantitative RT-PCR revealed that among these enzymes, only *Gapdh* gene
367 expression was altered in CD28^{iKO} hBCMAmBBmζ CAR T cells (Fig. 3g). Expression of the
368 NADP⁺ reducing enzymes *Idh1* and *Idh2* expression was altered in CD28^{iKO} expression was altered in CD28^{KO} hBCMAmBBmζ CAR T cells (Fig. 3g). Expression of the
368 NADP⁺ reducing enzymes *ldh1* and *ldh2* were also slightly increased in CD28^{KO}
369 hBCMAmBBmζ CAR T cells (Supplemental Fig. NADP⁺ reducing enzymes *Idh1* and *Idh*2 were also slightly increased in CD28^{iKO} 369
370
371 hBCMAmBBmζ CAR T cells (Supplemental Fig. 3D). Differences in gene expression, if reflected
in functional enzyme changes, occur in the opposite direction of what would be expected based
on differences in NADH to NAD⁺ ra in functional enzyme changes, occur in the opposite direction of what would be expected based

on differences in NADH to NAD⁺ ratio between CD28^{ft/fl} and CD28^{iKO} CAR T cells, indicating that

gene expression changes on differences in NADH to NAD⁺ ratio between CD28^{ft/fl} and CD28^{iKO} on differences in NADH to NAD⁺ ratio between CD28^{IM} and CD28^{INO} CAR T cells, indicating that
372 gene expression changes are unlikely to explain the difference in redox state when CD28 is
373 knocked out of hBCMAmBBm gene expression changes are unlikely to explain the difference in redox state when CD28 is

373 knocked out of hBCMAmBBmζ CAR T cells.

374 **Endogenous CD28 enhances CAR T cell expansion in the MM BME**

375 **Endogenous CD2**

873 knocked out of hBCMAmBBmζ CAR T cells.
374
375 **Endogenous CD28 enhances CAR T cell ε**
376 Due to the known influence of mitochol 375
376
377 **Endogenous CD28 enhances CAR T cell expansion in the MM BME**
376 Due to the known influence of mitochondrial respiration and red
377 proliferation^{71,72}, we evaluated proliferation of CD28^{ft/fl} and CD28^{ikO} CAR
378 pr 376 Due to the known influence of mitochondrial respiration and redox balance on T cell
377 proliferation^{71,72}, we evaluated proliferation of CD28^{ft/fl} and CD28^{ft/fl} and CD28^{ft/fl} and CD28^{ft/fl} and CD28^{ft/fl} a proliferation^{71,72}, we evaluated proliferation of CD28^{f/fl} and CD28^{iKO} CAR T cells. No difference in proliferation^{71,72}, we evaluated proliferation of CD28^{n/m} and CD28^{n/c} CAR T cells. No difference in

proliferation of CD28^{ft/ft} and CD28^{iKO} CAR T cells was observed over the course of *ex vivo* CAR

manufacture proliferation of CD28^{fl/fl} and CD28^{iKO} proliferation of CD28^{ti/fi} and CD28^{KO} CAR T cells was observed over the course of *ex vivo* CAR
379 manufacture (Fig. 4A). Similarly, expression of the proliferation marker Ki67 induced by co-
380 culture of CD28^{ti/fi} manufacture (Fig. 4A). Similarly, expression of the proliferation marker Ki67 induced by co-

380 culture of CD28^{iM} or CD28^{iKO} hBCMAmBBmζ CAR T cells with 5TGM1^{hBCMA} target cells was

381 similar (Fig. 4B). However, culture of CD28^{f//fl} or CD28^{iKO} hBCMAmBBmζ CAR T cells with 5TGM1^{hBCMA} salar similar (Fig. 4B). However, when hBCMAmBBm ζ CAR T cells with 5TGM1^{nBCMA} target cells was
381 similar (Fig. 4B). However, when hBCMAmBBm ζ CAR T cells in the MM BME or peripheral
382 blood were enumerated 7 381 similar (Fig. 4B). However, when hBCMAmBBmζ CAR T cells in the MM BME or peripheral
382 blood were enumerated 7 days after adoptive transfer into 5TGM1^{hBCMA} bearing mice (Fig. 4C),
383 a large decrease in CD4⁺ CD2 blood were enumerated 7 days after adoptive transfer into 5TGM1^{hBCMA} blood were enumerated 7 days after adoptive transfer into 5TGM1^{nBCMA} bearing mice (Fig. 4C),

383 a large decrease in CD4⁺ CD28^{iKO} CAR T cells was observed (Fig. 4D, 4E, Supplemental Fig.

384 4A). Importantly, CD28 a large decrease in CD4⁺ CD28^{iKO} a large decrease in CD4⁺ CD28^{IRO} CAR T cells was observed (Fig. 4D, 4E, Supplemental Fig.
384 4A). Importantly, CD28^{IRO} hBCMAmBBmζ CAR T cells maintained low/negative CD28 surface
385 expression in the MM BME (Fig. 4A). Importantly, CD28^{iKO} 384 4A). Importantly, CD28^{KO} hBCMAmBBmζ CAR T cells maintained low/negative CD28 surface
385 expression in the MM BME (Fig. 4F). Abatacept similarly reduced the frequency of human CD4⁺
386 hBCMABBζ CAR T cells in the expression in the MM BME (Fig. 4F). Abatacept similarly reduced the frequency of human CD4⁺ 385 386 hBCMABBζ CAR T cells in the MM BME of MM.1S bearing mice (Fig. 4G), while it had no effect
https://www.thendelt.com/
District of MM.1S bearing mice (Fig. 4G), while it had no effect
fig. 18.18.19.19.19.19.19.19.19.19.

on human CAR T cells in the peripheral blood (Fig. 4H). The frequency of CD8⁺ CAR T cells in on human CAR T cells in the peripheral blood (Fig. 4H). The frequency of $CD8^+$ CAR T cells in
388 the MM BME or peripheral blood was unaffected by CD28 knockout or abatacept treatment (Fig.
 $4D - 4H$).
390 The observed re

388 the MM BME or peripheral blood was unaffected by CD28 knockout or abatacept treatment (Fig.
389 $4D - 4H$).
390 The observed reduction in CD4⁺ CAR T cells in the MM BME upon deletion or blockade of
391 endogenous CD2 389 4D – 4H).
390 The obser
391 endogeno
392 Approvime The observed reduction in CD4⁺ CAR T cells in the MM BME upon deletion or blockade of 390 The observed reduction in CD4⁺ CAR T cells in the MM BME upon deletion or blockade of
391 endogenous CD28 led us to test whether endogenous CD28 signaling contributes to *in vivo*
392 expansion of luciferase express endogenous CD28 led us to test whether endogenous CD28 signaling contributes to *in vivo*

expansion of luciferase expressing hBCMABB_{ζ} CAR T cells generated from T-lux mice⁷³.

Approximately one week after infusion expansion of luciferase expressing hBCMABB ζ CAR T cells generated from T-lux mice⁷³. expansion of luciferase expressing hBCMABBZ CAR T cells generated from T-lux mice³.
393 Approximately one week after infusion, which aligns with the kinetics of tumor regression, T-lux
394 hBCMAmBBmZ CAR T cell luminesce Approximately one week after infusion, which aligns with the kinetics of tumor regression, T-lux

1994 hBCMAmBBmζ CAR T cell luminescence within the hind limbs of 5TGM1^{hBCMA} myeloma bearing

1995 mice rapidly increased hBCMAmBBmC CAR T cell luminescence within the hind limbs of 5TGM1^{hBCMA} myeloma bearing hBCMAmBBmζ CAR T cell luminescence within the hind limbs of 5TGM1^{nBCMA} myeloma bearing
395 mice rapidly increased with a signal plateau observed approximately 1 week later (Fig. 4l, 4J).
396 Abatacept treatment signific mice rapidly increased with a signal plateau observed approximately 1 week later (Fig. 4I, 4J).

396 Abatacept treatment significantly blunted *in vivo* expansion of T-lux hBCMAmBBmζ CAR T cells

in the MM BME (Fig. 4I), s 396 Abatacept treatment significantly blunted *in vivo* expansion of T-lux hBCMAmBBmζ CAR T cells
397 in the MM BME (Fig. 4I), suggesting that CD28 signaling supports *in vivo* expansion of 4-1BB
398 co-stimulated CAR T c 397 in the MM BME (Fig. 4I), suggesting that CD28 signaling supports *in vivo* expansion of 4-1BB
398 co-stimulated CAR T cells.
399 **Transient CD28 blockade reduces inflammatory cytokines in the MM BME**
393 **Cince explora**

398 co-stimulated CAR T cells.
399
400 **Transient CD28 blockade**
401 Since endogenous CD28 400
401
402 **Transient CD28 blockade reduces inflammatory cytokines in the MM BME**
401 Since endogenous CD28 promoted *in vivo* hBCMAmBBmζ CAR T cell
402 inflammatory cytokine production in the MM BME, we sought to test wh
blockade of Since endogenous CD28 promoted *in vivo* hBCMAmBBmζ CAR T cell expansion and
402 inflammatory cytokine production in the MM BME, we sought to test whether abatacept
403 blockade of CD28 ligation could lessen the severity o inflammatory cytokine production in the MM BME, we sought to test whether abatacept
blockade of CD28 ligation could lessen the severity of CAR T associated cytokine release. To
this end, myeloma bearing mice were treated w blockade of CD28 ligation could lessen the severity of CAR T associated cytokine release. To
this end, myeloma bearing mice were treated with abatacept for 1 week following CAR T cell
infusion (Fig. 5A). At this early time this end, myeloma bearing mice were treated with abatacept for 1 week following CAR T cell
405 infusion (Fig. 5A). At this early timepoint, abatacept had no effect on anti-tumor activity of BCMA
406 targeted human or mouse infusion (Fig. 5A). At this early timepoint, abatacept had no effect on anti-tumor activity of BCMA
406 targeted human or mouse CAR T cells (Fig. 5B, Supplemental Fig. 4B) and only a very minor
407 effect on MM BME levels targeted human or mouse CAR T cells (Fig. 5B, Supplemental Fig. 4B) and only a very minor
407 effect on MM BME levels of human inflammatory cytokines in the MM BME (Fig. 5C). Since
408 human cytokines could only come from effect on MM BME levels of human inflammatory cytokines in the MM BME (Fig. 5C). Since

human cytokines could only come from CAR T cells or MM.1S cells, and most cytokines

measured are not known to be made by MM cells, we human cytokines could only come from CAR T cells or MM.1S cells, and most cytokines
measured are not known to be made by MM cells, we concluded that CD28 blockade with
abatacept did not affect *in vivo* CAR T cell cytokine measured are not known to be made by MM cells, we concluded that CD28 blockade with
abatacept did not affect *in vivo* CAR T cell cytokine secretion nor anti-tumor activity in the first
week following infusion. 410 abatacept did not affect *in vivo* CAR T cell cytokine secretion nor anti-tumor activity in the first 411 week following infusion.

Somewhat surprisingly, inflammatory cytokine levels in the MM BME were dramatically

reduced by CD28 deletion from mouse hBCMAmBBmζ CAR T cells (Fig. 2l) yet unaffected by

CD28 blockade using abatacept in human hBCMABBζ C reduced by CD28 deletion from mouse hBCMAmBBmζ CAR T cells (Fig. 2I) yet unaffected by

CD28 blockade using abatacept in human hBCMABBζ CAR T cell treated mice (Fig. 5C). Such

divergent findings may be due to contributio 414 CD28 blockade using abatacept in human hBCMABBζ CAR T cell treated mice (Fig. 5C). Such
415 divergent findings may be due to contributions of cells other than CAR T cells to the mouse MM
416 BME cytokine milieu or dif divergent findings may be due to contributions of cells other than CAR T cells to the mouse MM
416 BME cytokine milieu or differences between how blockade of CD28 with abatacept and deletion
417 of CD28 affect 4-1BB co-sti BME cytokine milieu or differences between how blockade of CD28 with abatacept and deletion
of CD28 affect 4-1BB co-stimulated CAR T cells. To address these possibilities, we measured
murine inflammatory cytokine levels in of CD28 affect 4-1BB co-stimulated CAR T cells. To address these possibilities, we measured
murine inflammatory cytokine levels in the MM BME of mouse hBCMAmBBmζ CAR T cell
treated 5TGM1^{hBCMA} bearing mice. Abatacept dimi murine inflammatory cytokine levels in the MM BME of mouse hBCMAmBBmζ CAR T cell
treated 5TGM1^{hBCMA} bearing mice. Abatacept diminished levels of murine inflammatory
cytokines in the MM BME of hBCMAmBBmζ CAR T cell treat treated 5TGM1^{hBCMA} bearing mice. Abatacept diminished levels of murine inflammatory treated 5TGM1^{nBCMA} bearing mice. Abatacept diminished levels of murine inflammatory

420 cytokines in the MM BME of hBCMAmBBmζ CAR T cell treated mice (Fig. 5D), yet not to the

421 extent of CD28 deletion.

422 Notable

420 cytokines in the MM BME of hBCMAmBBmζ CAR T cell treated mice (Fig. 5D), yet not to the
421 extent of CD28 deletion.
422 Notable among inflammatory cytokines affected by both abatacept treatment and CD28
423 deletion 421 extent of CD28 deletion.
422 Notable among infla
423 deletion were IP-10, which
424 and IL-12, which is mai Notable among inflammatory cytokines affected by both abatacept treatment and CD28
deletion were IP-10, which is secreted by monocytes and stromal cells in response to IFN- γ^{74} ,
and IL-12, which is mainly secreted by deletion were IP-10, which is secreted by monocytes and stromal cells in response to IFN- $γ^{74}$ deletion were IP-10, which is secreted by monocytes and stromal cells in response to IFN- γ'^4 ,
and IL-12, which is mainly secreted by monocytes, macrophages, neutrophils, and dendritic
cells⁷⁵. It is therefore likely and IL-12, which is mainly secreted by monocytes, macrophages, neutrophils, and dendritic
dells⁷⁵. It is therefore likely that other cells contribute to murine inflammatory cytokine production
in the MM BME of hBCMAmBBmζ cells 75 cells⁷⁵. It is therefore likely that other cells contribute to murine inflammatory cytokine production
in the MM BME of hBCMAmBBmζ CAR T cell treated mice and given the magnitude of cytokine
changes, that there is also in the MM BME of hBCMAmBBmζ CAR T cell treated mice and given the magnitude of cytokine

changes, that there is also a difference between how abatacept treatment and deletion of CD28

affects cytokine production.

Transla changes, that there is also a difference between how abatacept treatment and deletion of CD28

428 affects cytokine production.

429 **Transient CD28 blockade does not inhibit hBCMABBC anti-MM activity.**

And a Bessed on ou

428 affects cytokine production.
429 **Transient CD28 blockade**
431 Based on our observation 430
431
432 **Transient CD28 blockade does not inhibit hBCMABBζ anti-MM activity.**

Based on our observation that abatacept could limit pro-inflammatory c

not early anti-tumor activity of hBCMABBζ CAR T cells, we predicted that

expo Based on our observation that abatacept could limit pro-inflammatory cytokine release but
not early anti-tumor activity of hBCMABB ζ CAR T cells, we predicted that transient abatacept
exposure would not impair survival 1432 not early anti-tumor activity of hBCMABBζ CAR T cells, we predicted that transient abatacept
1433 exposure would not impair survival of myeloma bearing mice. To test this, abatacept was
1434 administered to MM.1S tum exposure would not impair survival of myeloma bearing mice. To test this, abatacept was
administered to MM.1S tumor bearing NSG mice from day -1 to day 7 post hBCMABBZ CAR T
cell infusion (Fig. 5F). Transient abatacept exp 434 administered to MM.1S tumor bearing NSG mice from day -1 to day 7 post hBCMABBζ CAR T
6435 cell infusion (Fig. 5F). Transient abatacept exposure resulted in a slight reduction in tumor
436 regression induced by hBCMAB ell infusion (Fig. 5F). Transient abatacept exposure resulted in a slight reduction in tumor
436 regression induced by hBCMABBZ CAR T cells (Supplemental Fig. 4C) but did not affect the
437 long-term survival of hBCMABBZ C regression induced by hBCMABBζ CAR T cells (Supplemental Fig. 4C) but did not affect the

1437 Iong-term survival of hBCMABBζ CAR T cell treated myeloma bearing mice (Fig. 5H).

1437 Iong-term survival of hBCMABBζ CAR T c 437 long-term survival of hBCMABBζ CAR T cell treated myeloma bearing mice (Fig. 5H).

438

439
441
442 439 **Discussion**
440 **CAR T**
441 relapsed/refr
442 remains an CAR T cell therapies targeting BCMA have shown curative potential in patients with

441 relapsed/refractory multiple myeloma (MM)^{25,27}. However, achieving long-term remissions

442 remains an ongoing challenge, with onerelapsed/refractory multiple myeloma (MM)^{25,27} relapsed/refractory multiple myeloma $(MM)^{25,27}$. However, achieving long-term remissions

remains an ongoing challenge, with one-quarter to greater than one-half of patients

experiencing myeloma relapse within one year remains an ongoing challenge, with one-quarter to greater than one-half of patients
experiencing myeloma relapse within one year of CAR T infusion. In this study we set out to
determine whether CD28 blockade using abatacep experiencing myeloma relapse within one year of CAR T infusion. In this study we set out to
determine whether CD28 blockade using abatacept could sensitize MM cells to CAR T cell
therapy in a manner analogous to standard c determine whether CD28 blockade using abatacept could sensitize MM cells to CAR T cell
therapy in a manner analogous to standard chemotherapy^{45,46,50}. In contrast to expectations, we
found that abatacept limited efficacy therapy in a manner analogous to standard chemotherapy 45,46,50 therapy in a manner analogous to standard chemotherapy^{45,46,50}. In contrast to expectations, we
found that abatacept limited efficacy of clinically relevant BCMA targeted, 4-1BB co-stimulated
CAR T cells in an establish found that abatacept limited efficacy of clinically relevant BCMA targeted, 4-1BB co-stimulated

447 CAR T cells in an established human xenograft myeloma mouse model. Using a novel CD28

448 inducible knockout mouse model CAR T cells in an established human xenograft myeloma mouse model. Using a novel CD28
inducible knockout mouse model to generate CD28-deficient (CD28^{iKO}) CAR T cells, we further
revealed a previously unrecognized role fo inducible knockout mouse model to generate CD28-deficient (CD28^{kO}) CAR T cells, we further inducible knockout mouse model to generate CD28-deficient (CD28^{ko}) CAR T cells, we further
revealed a previously unrecognized role for the endogenous CD28 receptor on 4-1BB co-
stimulated CAR T cells. CD28 deletion did n revealed a previously unrecognized role for the endogenous CD28 receptor on 4-1BB co-
stimulated CAR T cells. CD28 deletion did not alter BCMA targeted, 4-1BB co-stimulated CAR T
cell cytotoxic capabilities nor alter infla stimulated CAR T cells. CD28 deletion did not alter BCMA targeted, 4-1BB co-stimulated CAR T
cell cytotoxic capabilities nor alter inflammatory cytokine production *in vitro*, but rather resulted
in diminished mitochondria cell cytotoxic capabilities nor alter inflammatory cytokine production *in vitro*, but rather resulted
452 in diminished mitochondrial metabolism and a lower ratio of the oxidized form of nicotinamide
453 adenine dinucleot in diminished mitochondrial metabolism and a lower ratio of the oxidized form of nicotinamide

adenine dinucleotide (NADH) to its reduced form (NAD⁺). These metabolic changes were

associated with limited *in vivo* expa adenine dinucleotide (NADH) to its reduced form (NAD⁺). These metabolic changes were adenine dinucleotide (NADH) to its reduced form (NAD⁺). These metabolic changes were
associated with limited *in vivo* expansion of $CD28^{i}$ ⁶⁰ 4-1BB co-stimulated CAR T cells and a
reduction in CAR T cell induced infl associated with limited *in vivo* expansion of CD28iKO associated with limited *in vivo* expansion of CD28^{KO} 4-1BB co-stimulated CAR T cells and a
reduction in CAR T cell induced inflammatory cytokine production in the multiple myeloma bone
marrow microenvironment (MM BME). reduction in CAR T cell induced inflammatory cytokine production in the multiple myeloma bone

456 marrow microenvironment (MM BME). Abatacept treatment similarly reduced *in vivo* 4-1BB co-

457 stimulated CAR T cell expa marrow microenvironment (MM BME). Abatacept treatment similarly reduced *in vivo* 4-1BB costimulated CAR T cell expansion and inflammatory cytokine production. Importantly however, short-term blockade of endogenous CD28 us stimulated CAR T cell expansion and inflammatory cytokine production. Importantly however,

short-term blockade of endogenous CD28 using abatacept during the first week following CAR T

cell infusion reduced inflammatory c short-term blockade of endogenous CD28 using abatacept during the first week following CAR T
cell infusion reduced inflammatory cytokine levels in the MM BME without altering long-term
survival of BCMA targeted, 4-1BB co-s

cell infusion reduced inflammatory cytokine levels in the MM BME without altering long-term

460 survival of BCMA targeted, 4-1BB co-stimulated CAR T cell treated myeloma bearing mice.

461 Robust CAR T cell activation and survival of BCMA targeted, 4-1BB co-stimulated CAR T cell treated myeloma bearing mice.

Robust CAR T cell activation and expansion can induce systemic toxicities, inclu

cytokine release syndrome (CRS), immune effector ce Robust CAR T cell activation and expansion can induce systemic toxicities, including
cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome
(ICANS), and immune effector cell-associated hema cytokine release syndrome (CRS), immune effector cell-associated neurotoxicity syndrome

(ICANS), and immune effector cell-associated hematologic toxicity (ICAHT)⁷⁶⁻⁷⁸. In pivotal CAR

(ICANS), and immune effector cell-a (ICANS), and immune effector cell-associated hematologic toxicity (ICAHT)⁷⁶⁻⁷⁸ (ICANS), and immune effector cell-associated hematologic toxicity (ICAHT)⁷⁶⁻⁷⁸. In pivotal CAR

The pivotal CAR

The pixotal CAR

The p

T trials in $MM^{25,27}$, 76 – 84% of patients experienced CRS, 18 – 21% experienced ICANS, and T trials in MM^{25,27}, 76 – 84% of patients experienced CRS, 18 – 21% experienced ICANS, and

1465 nearly all patients experienced hematologic toxicity, although these were generally transient.

1466 Current treatment opt nearly all patients experienced hematologic toxicity, although these were generally transient.
466 Current treatment options for CRS and ICANS include IL-6 receptor blockade with tocilizumab,
467 corticosteroids for tocili Current treatment options for CRS and ICANS include IL-6 receptor blockade with tocilizumab,

corticosteroids for tocilizumab refractory cases, and the anti-IL-6 antibody siltuximab for

tocilizumab and corticosteroid refr 167 corticosteroids for tocilizumab refractory cases, and the anti-IL-6 antibody siltuximab for
168 tocilizumab and corticosteroid refractory toxicities⁷⁹. Additionally, the IL-1 receptor antagonist
169 anakinra is bein tocilizumab and corticosteroid refractory toxicities⁷⁹. Additionally, the IL-1 receptor antagonist
anakinra is being explored as a potential prophylactic treatment to prevent CRS and ICANS⁸⁰.
Data presented here raise anakinra is being explored as a potential prophylactic treatment to prevent CRS and $ICANS⁸⁰$. anakinra is being explored as a potential prophylactic treatment to prevent CRS and ICANS⁹⁰.
470 Data presented here raise the possibility that abatacept (CTLA4-Ig), which is FDA approved for
471 the treatment of rheuma Data presented here raise the possibility that abatacept (CTLA4-Ig), which is FDA approved for
the treatment of rheumatoid arthritis, juvenile idiopathic arthritis, and psoriatic arthritis along with
prevention of acute g the treatment of rheumatoid arthritis, juvenile idiopathic arthritis, and psoriatic arthritis along with
prevention of acute graft versus host disease^{81,82}, may also be useful as prophylactic treatment
to prevent toxici prevention of acute graft versus host disease 81,82 prevention of acute graft versus host disease^{81,82}, may also be useful as prophylactic treatment
to prevent toxicities brought on by 4-1BB co-stimulated CAR T cells. Whether abatacept could
have similar utility in preve to prevent toxicities brought on by 4-1BB co-stimulated CAR T cells. Whether abatacept could

have similar utility in preventing CD28 co-stimulated CAR T cell toxicities is an open question

currently lacking clinical rele have similar utility in preventing CD28 co-stimulated CAR T cell toxicities is an open question

currently lacking clinical relevance in the setting of multiple myeloma, where both FDA approved

CAR designs contain a 4-1BB

currently lacking clinical relevance in the setting of multiple myeloma, where both FDA approved

476 CAR designs contain a 4-1BB co-stimulatory domain.

477 Co-stimulation has long been known to be critical for anti-tumor CAR designs contain a 4-1BB co-stimulatory domain.

Co-stimulation has long been known to be critica

478 with different CAR-encoded co-stimulatory domains

properties¹⁷. Clinically available CARs contain either a

489 C Co-stimulation has long been known to be critical for anti-tumor effects of CAR T cells^{83,84} Co-stimulation has long been known to be critical for anti-tumor effects of CAR T cells^{83,84},
with different CAR-encoded co-stimulatory domains having distinct effects on CAR T cell
properties¹⁷. Clinically available C with different CAR-encoded co-stimulatory domains having distinct effects on CAR T cell
properties¹⁷. Clinically available CARs contain either a CD28 or a 4-1BB co-stimulatory domain.
CD28 co-stimulated CAR T cells exhib properties¹⁷. Clinically available CARs contain either a CD28 or a 4-1BB co-stimulatory domain. properties¹⁷. Clinically available CARs contain either a CD28 or a 4-1BB co-stimulatory domain.
480 CD28 co-stimulated CAR T cells exhibit rapid anti-tumor effector function but lack functional
481 persistence associated CD28 co-stimulated CAR T cells exhibit rapid anti-tumor effector function but lack functional

481 persistence associated with 4-1BB co-stimulated CAR T cells. Modulation of CAR-encoded

482 CD28 signaling has resulted in persistence associated with 4-1BB co-stimulated CAR T cells. Modulation of CAR-encoded

482 CD28 signaling has resulted in improved functional persistence and reduced CAR T cell

483 exhaustion in pre-clinical models^{85,86} 282 CD28 signaling has resulted in improved functional persistence and reduced CAR T cell

283 exhaustion in pre-clinical models^{85,86}. Recent studies have hinted at a role for endogenous

284 CD28 in determining CAR T c exhaustion in pre-clinical models $85,86$. Recent studies have hinted at a role for endogenous exhaustion in pre-clinical models^{85,86}. Recent studies have hinted at a role for endogenous

484 CD28 in determining CAR T cell efficacy. However, evidence for endogenous CD28 modulation

485 of CAR T cell function was 484 CD28 in determining CAR T cell efficacy. However, evidence for endogenous CD28 modulation
485 of CAR T cell function was either indirect, in the case of CTLA4 knockout⁵⁵, or complicated by
486 co-expression of IL-12 of CAR T cell function was either indirect, in the case of CTLA4 knockout⁵⁵, or complicated by of CAR T cell function was either indirect, in the case of CTLA4 knockout⁵⁵, or complicated by
co-expression of IL-12 from a fourth-generation armored CAR construct⁸⁷. Data presented here
provide the first direct evid co -expression of IL-12 from a fourth-generation armored CAR construct⁸⁷. Data presented here co-expression of IL-12 from a fourth-generation armored CAR construct⁸⁷. Data presented here
provide the first direct evidence that endogenous CD28 affects efficacy of second-generation, 4-
1BB co-stimulated CAR T cells provide the first direct evidence that endogenous CD28 affects efficacy of second-generation, 4-
188 1BB co-stimulated CAR T cells comparable to those used to treat myeloma patients. These data
188 189 1991 488 1BB co-stimulated CAR T cells comparable to those used to treat myeloma patients. These data

raise important questions about how signaling from CAR co-stimulatory domains interfaces with

490 signaling from endogenous co-stimulatory, and/or co-inhibitory receptors.

491 In light of recent evidence that CD28 co-sti signaling from endogenous co-stimulatory, and/or co-inhibitory receptors.

491 In light of recent evidence that CD28 co-stimulation in the tumor micro

492 for effector differentiation and anti-tumor function of cytotoxic In light of recent evidence that CD28 co-stimulation in the tumor microenvironment is critical
492 for effector differentiation and anti-tumor function of cytotoxic T cells^{56,88}, the context in which
493 endogenous CD28 for effector differentiation and anti-tumor function of cytotoxic T cells^{56,88} for effector differentiation and anti-tumor function of cytotoxic T cells^{56,88}, the context in which

endogenous CD28 expressed on 4-1BB co-stimulated CAR T cells encounters ligands may

influence anti-tumor efficacy and endogenous CD28 expressed on 4-1BB co-stimulated CAR T cells encounters ligands may
influence anti-tumor efficacy and/or inflammatory cytokine production. B lineage tumors targeted
clinically by CAR T cells are characteriz influence anti-tumor efficacy and/or inflammatory cytokine production. B lineage tumors targeted

dinically by CAR T cells are characterized by high levels of CD28 ligand expression, with CD80,

CD86, or both expressed on clinically by CAR T cells are characterized by high levels of CD28 ligand expression, with CD80,

496 CD86, or both expressed on tumor cells in more than half of myeloma, non-Hodgkin's

497 lymphoma, and B-ALL paitents⁸⁹⁻ CD86, or both expressed on tumor cells in more than half of myeloma, non-Hodgkin's
497 lymphoma, and B-ALL paitents⁸⁹⁻⁹¹. CD80 and CD86 are also expressed on antigen presenting
498 cells throughout the body of cancer pat lymphoma, and B-ALL paitents⁸⁹⁻⁹¹ lymphoma, and B-ALL paitents⁸⁹⁻⁹¹. CD80 and CD86 are also expressed on antigen presenting
cells throughout the body of cancer patients and may have similar or disparate effects on CAR T
cells, likely based on whether CAR cells throughout the body of cancer patients and may have similar or disparate effects on CAR T
cells, likely based on whether CAR engagement occurs concurrently with CD28 engagement. If
future studies find that the contex cells, likely based on whether CAR engagement occurs concurrently with CD28 engagement. If
future studies find that the context in which endogenous CD28 is engaged matters, CD80 and/or
CD86 expression patterns may become u future studies find that the context in which endogenous CD28 is engaged matters, CD80 and/or

501 CD86 expression patterns may become useful in determining whether CD28 or 4-1BB co-

502 stimulated CAR T cells are used to

501 CD86 expression patterns may become useful in determining whether CD28 or 4-1BB co-
502 stimulated CAR T cells are used to treat particular patients.
503 Overall, results presented here provide the first direct evidenc so stimulated CAR T cells are used to treat particular patients.

503 Overall, results presented here provide the first direct

504 important for driving anti-tumor function of 4-1BB co-stimular

505 many interesting and i 503 Overall, results presented here provide the first direct evidence that endogenous CD28 is
504 important for driving anti-tumor function of 4-1BB co-stimulated CAR T cells. These results raise
505 many interesting and i important for driving anti-tumor function of 4-1BB co-stimulated CAR T cells. These results raise
505 many interesting and important biological questions about co-stimulation and signaling in CAR T
506 cells and, perhaps m many interesting and important biological questions about co-stimulation and signaling in CAR T
cells and, perhaps more importantly raise the possibility that blocking endogenous CD28
signaling may abrogate some of the tox cells and, perhaps more importantly raise the possibility that blocking endogenous CD28
507 signaling may abrogate some of the toxic side effects associated with CAR T therapy. Future
508 studies aimed at optimizing method signaling may abrogate some of the toxic side effects associated with CAR T therapy. Future

studies aimed at optimizing methods and timing of CD28 blockade have the potential to lead to

improved clinical strategies for l studies aimed at optimizing methods and timing of CD28 blockade have the potential to lead to

improved clinical strategies for limit toxicities while maintaining CAR T cell efficacy.
 Acknowledgements
 Acknowledgements

improved clinical strategies for limit toxicities while maintaining CAR T cell efficacy.
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513 Imaging Shared Res
514 imaging studies as 512 The authors would like to thank Steven Turowski and Joseph Spernyak of the Translational
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520 **Author Contributions**
523 **Concention and decisy.** M. Lieberman, K. Lee & Okiniazak

- 519 agreement IM101-902.
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521 **Author Contributions
522 Conception and designal** 521
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- **Author Contributions

522 Conception and desig

523 Development of metho

524 Acquisition of data (process) Conception and design:** M. Lieberman, K. Lee, S. Olejniczak
 Development of methodology: M. Lieberman, K. Lee, S. Ole
 **Acquisition of data (provided animals, managed studies, p

Lieberman, J. Bramson, R. Brentjens, K. Development of methodology:** M. Lieberman, K. Lee, S. Olejniczak
 Acquisition of data (provided animals, managed studies, provide

Lieberman, J. Bramson, R. Brentjens, K. Lee, S. Olejniczak
 Analysis and interpretation
-
-
- **Acquisition of data (provided animals, managed studies, provided facilities, etc.):** M.

Lieberman, J. Bramson, R. Brentjens, K. Lee, S. Olejniczak
 Analysis and interpretation of data: M. Lieberman, K. Lee, S. Olejnicz
- Lieberman, J. Bramson, R. Brentjens, K. Lee, S. Olejniczak
526 **Analysis and interpretation of data:** M. Lieberman, K. Lee
527 **Writing, review, and/or revision of the manuscript:** M. L
**Administrative, technical, or mater Analysis and interpretation of data: M. Lieberman, K. Lee, S. Olejniczak
Writing, review, and/or revision of the manuscript: M. Lieberman, K. Le
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Purdon, R. Burchett, A.J.R. McGray, B. Gill **Administrative, technical, or material support:** M. Lieberman, J. Tong, N. Odukwe, T.
529 Purdon, R. Burchett, A.J.R. McGray, B. Gillard, C. Brackett, C. Chavel
Study supervision: S. Olejniczak
All authors have read and
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- 929 Purdon, R. Burchett, A.J.R. McGray, B. Gillard, C. Brackett, C. Chavel
530 **Study supervision:** S. Olejniczak
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534 Services
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536 Resource 534 Services and resources were provided by the Flow and Image Cytometry Shared Resource, the
535 Translational Imaging Shared Resource (TISR), and the Experimental Tumor Model Shared
536 Resource at Roswell Park. All Rosw Translational Imaging Shared Resource (TISR), and the Experimental Tumor Model Shared
536 Resource at Roswell Park. All Roswell Park shared resources were supported through NIH
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Training Grant
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544 **Institutional Review Board Statement**
543 This study involves de-identified healthy
544 reviewed by the Roswell Park Institut
545 experiments were performed in accom This study involves de-identified healthy donor samples collected under an approved protocol

sta eviewed by the Roswell Park Institutional Review Board (IRB): BDR 115919. All animal

experiments were performed in accordan 544 reviewed by the Roswell Park Institutional Review Board (IRB): BDR 115919. All animal
545 experiments were performed in accordance with the Institutional Animal Care and Use
546 Committee (IACUC) guidelines and were ap experiments were performed in accordance with the Institutional Animal Care and Use
546 Committee (IACUC) guidelines and were approved under experimental IACUC protocol: 1094M.
547 **Data Availability Statement**

546 Committee (IACUC) guidelines and were approved under experimental IACUC protocol: 1094M.
547 **Data Availability Statement**
549 All data associated with this paper are included in the manuscript and supplementary
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554 **Data Availability Statement**
549 All data associated with the
550 materials. Requests for resou
551 corresponding author, Scott H 549 All data associated with this paper are included in the manuscript and supplementary
550 materials. Requests for resources and reagents should be directed to and will be fulfilled by the
551 corresponding author, Scott

materials. Requests for resources and reagents should be directed to and will be fulfilled by the
551 corresponding author, Scott H. Olejniczak (<u>scott.olejniczak@roswellpark.org</u>)
552 **Conflicts of Interest**
553 **Conflict**

corresponding author, Scott H. Olejniczak (scott.olejniczak@roswellpark.org)
552
553 **Conflicts of Interest**
554 R.J.B. has licensed intellectual property to and collects royalties from Bri ---
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555 **Conflicts of Interest**
554 R.J.B. has licensed i
555 (BMS), Caribou, and 3
556 to BMS, Atara Biothe R.J.B. has licensed intellectual property to and collects royalties from Bristol Myers Squibb

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556 to BMS, Atara Biotherapeutics Inc, and Triumvira. R.J.B is a member of the scientific advisory
557 board for Triumvira. 556 to BMS, Atara Biotherapeutics Inc, and Triumvira. R.J.B is a member of the scientific advisory
557 board for Triumvira.
557 board for Triumvira. 557 board for Triumvira.

558 **Figure Legends:**
559 **Figure 1: Endo**
560 **efficacy.**
561 **(A)** Schematic of

- **Figure 1: Endogenous CD28 blockade impairs hBCMABBζ CAR T cell anti-myeloma

efficacy.**
 (A) Schematic of second-generation retroviral CAR construct used to generate BCMA targeted

human CAR T cells.
 (B) CAR T cell **efficacy.**

561 **(A)** Sche

562 huma

563 **(B)** CAR
- (A) Schematic of second-generation retroviral CAR construct used to generate BCMA targeted

1562 human CAR T cells.
 (B) CAR T cell cytotoxic activity during a 24-hr. co-culture with luciferase-tagged MM.1S (left) or

15 962 human CAR T cells.

563 **(B)** CAR T cell cytotoxic

564 U266 (right) myelom

565 of hBCMABBζ CAR (B) CAR T cell cytotoxic activity during a 24-hr. co-culture with luciferase-tagged MM.1S (left) or

U266 (right) myeloma cells ± abatacept. Data are shown as mean ± SD and representative

of hBCMABBC CAR T cells generated U266 (right) myeloma cells ± abatacept. Data are shown as mean ± SD and representative

of hBCMABB ζ CAR T cells generated from 3 healthy donors. *p<0.05 by two-way analysis

of variance (ANOVA) with Tukey's multiple c
- 565 of hBCMABB ζ CAR T cells generated from 3 healthy donors. *p<0.05 by two-way analysis
566 of variance (ANOVA) with Tukey's multiple comparison test.
567 **(C)** Diagram of experimental setup used to evaluate hBCMABB566 of variance (ANOVA) with Tukey's multiple comparison test.

567 **(C)** Diagram of experimental setup used to evaluate hBCMABBa

568 a human MM xenograft model. NSG mice were intraver

569 MM.1S-luc myeloma cells on day **(C)** Diagram of experimental setup used to evaluate hBCMABBζ CAR T + abatacept therapy in

a human MM xenograft model. NSG mice were intravenously inoculated with 1 x 10⁶

MM.1S-luc myeloma cells on day -28 and treated a human MM xenograft model. NSG mice were intravenously inoculated with 1 x 10^6 ---
569
571
571 MM.1S-luc myeloma cells on day -28 and treated with CAR T cells on day 0. Mice received

200 µg abatacept 3x/week beginning the day before infusion and continuing through

endpoint. Tumor burden was monitored by IVIS biolu 200 µg abatacept 3x/week beginning the day before infusion and continuing through

1571 endpoint. Tumor burden was monitored by IVIS bioluminescent imaging (BLI) 2x/week.

1572 **(D)** Representative bioluminescent images of
- endpoint. Tumor burden was monitored by IVIS bioluminescent imaging (BLI) 2x/week.
 (D) Representative bioluminescent images of MM.1S bearing mice on specified days follo

CAR T cell infusion.
 (E) Tumor burden express
- **(D)** Representative bioluminescent images of MM.1S bearing mice on specified days following
CAR T cell infusion.
(E) Tumor burden expressed as relative photon flux measured by BLI from MM.1S-luc bearing
mice treated wit 573 CAR T cell infusion.

574 **(E)** Tumor burden expre

575 mice treated with hB

576 line represents an in **(E)** Tumor burden expressed as relative photon flux measured by BLI from MM.1S-luc bearing
mice treated with hBCMABB ζ CAR T or control T cells \pm abatacept (200 µg, 3x/week). Each
line represents an individual mous
- 575 mice treated with hBCMABB ζ CAR T or control T cells \pm abatacept (200 µg, 3x/week). Each

576 line represents an individual mouse (n = 7 mice per CAR T cell treated group).

577 **(F)** Kaplan Meier analysis of 576 line represents an individual mouse (n = 7 mice per CAR T cell treated group).

577 **(F)** Kaplan – Meier analysis of survival of hBCMABB ζ CAR T or control T cells \pm at

578 µg, 3x/week) treated MM.1S-luc bearin **(F)** Kaplan – Meier analysis of survival of hBCMABBζ CAR T or control T cells ± abatacept (200 µg, 3x/week) treated MM.1S-luc bearing mice (n = 8 - 12 mice per CAR T cell treated group). Median survival of hBCMABBζ CAR T 9578 µg, 3x/week) treated MM.1S-luc bearing mice (n = 8 - 12 mice per CAR T cell treated
group). Median survival of hBCMABB ζ CAR T treated mice was >100 days post CAR T cell
infusion vs. 55 days for hBCMABB ζ CAR T group). Median survival of hBCMABBζ CAR T treated mice was >100 days post CAR T cell
580 infusion vs. 55 days for hBCMABBζ CAR T + abatacept treated mice. ****p<0.0001 by log-
581 **(G)** Tumor burden expressed as relative
- infusion vs. 55 days for hBCMABBζ CAR T + abatacept treated mice. ****p<0.0001 by log-

rank Mantel-Cox test.

(G) Tumor burden expressed as relative photon flux measured by BLI from MM.1S high tumor

burden mice (inocula 581 rank Mantel-Cox test.
582 **(G)** Tumor burden express
burden mice (inoculat **(G)** Tumor burden expressed as relative photon flux measured by BLI from MM.1S high tumor
burden mice (inoculated on day -35) treated with hBCMABBζ CAR T or control T cells ±
burden mice (inoculated on day -35) treated w burden mice (inoculated on day -35) treated with hBCMABBζ CAR T or control T cells ±

burden mice (inoculated on day -35) treated with hBCMABBζ CAR T or control T cells ±

584 abatacept (200 µg, 3x/week). Each line represents an individual mouse (n = 4 mice per

585 CAR T cell treated group).

586 **(H)** Kaplan – Meier analysis of survival of hBCMABB ζ CAR T or control T cells \pm abatac CAR T cell treated group).

586 **(H)** Kaplan – Meier analysis of

587 **µg, 3x/week) treated MM.

588 survival of hBCMABBZ CA (H)** Kaplan – Meier analysis of survival of hBCMABBζ CAR T or control T cells ± abatacept (200 µg, 3x/week) treated MM.1S-luc high tumor burden mice (n = 4 mice per group). Median survival of hBCMABBζ CAR T treated mice 958 µg, 3x/week) treated MM.1S-luc high tumor burden mice (n = 4 mice per group). Median
588 survival of hBCMABB ζ CAR T treated mice was 80 days vs. 45 days for hBCMABB ζ CAR
589 T + abatacept treated mice. ***p<0. 588 survival of hBCMABBζ CAR T treated mice was 80 days vs. 45 days for hBCMABBζ CAR
589 T + abatacept treated mice. ***p<0.0001 by log-rank Mantel-Cox test.
590 **Figure 2: CD28^{iKO} hBCMAmBBmζ CAR T cells are functionall**

Figure 2: CD28^{*iKO*} hBCMAmBBmζ CAR T cells are functionally impaired *in vivo*.

T + abatacept treated mice. ***p<0.0001 by log-rank Mantel-Cox test.
590 **Figure 2: CD28^{iKO} hBCMAmBBmζ CAR T cells are functionally impaire**
592 **(A)** Schematic depicting the process of manufacturing mouse CD28 591
592
593 **Figure 2: CD28^{IKO} hBCMAmBBmζ CAR T cells are functionally impaired** *in vivo***.

(A)** Schematic depicting the process of manufacturing mouse CD28 knockout

hBCMAmBBmζ CAR T cell.
 (B) Surface CD28 protein expression o **(A)** Schematic depicting the process of manufacturing mouse CD28 knockout (CD28^{iKO} (A) Schematic depicting the process of manufacturing mouse CD28 knockout (CD28^{INC)}
593 hBCMAmBBmζ CAR T cell.
594 **(B)** Surface CD28 protein expression on CD28^{f/fl} versus CD28^{iKO} mouse T cells during CAR T
595 cell

593 hBCMAmBBmζ CAR T cell.
594 **(B)** Surface CD28 protein expr
595 cell manufacture.
596 **(C)** Median fluorescent intensity **(B)** Surface CD28 protein expression on CD28fl/fl versus CD28iKO

(B) Surface CD28 protein expression on CD28^{n/n} versus CD28^{n/o} mouse T cells during CAR T
cell manufacture.
(C) Median fluorescent intensity (MFI) of CD28 measured by flow cytometry at the conclusion of
CD28^{ft/fl} ver s95 cell manufacture.
596 **(C)** Median fluorescer
597 CD28^{fl/fl} versus Cl
598 independent expe **(C)** Median fluorescent intensity (MFI) of CD28 measured by flow cytometry at the conclusion of

CD28^{ft/fl} versus CD28^{ikO} mouse CAR T manufacture. Data shown as mean ± SD from 10+

independent experiments. *p<0.05, ** CD28^{fl/fl} versus CD28^{iKO}

597 CD28tM versus CD28^{tKO} mouse CAR T manufacture. Data shown as mean \pm SD from 10+
598 independent experiments. *p<0.05, ****p<0.0001 by one-way ANOVA.
599 **(D)** CD28^{fM} versus CD28^{iKO} hBCMAmBBmζ CAR T cell cyt 598 independent experiments. *p<0.05, ****p<0.0001 by one-way ANOVA.

599 **(D)** CD28^{ft/fl} versus CD28^{iKO} hBCMAmBBmζ CAR T cell cytotoxic activity d

500 with luciferase-tagged 5TGM1^{hBCMA} mouse myeloma cells. Cell vi **(D)** CD28^{f/fl} versus CD28^{iKO} hBCMAmBBm(CAR T cell cytotoxic activity during 24 hr. co-culture (D) CD28tM versus CD28^{tKO} hBCMAmBBmζ CAR T cell cytotoxic activity during 24 hr. co-culture

with luciferase-tagged 5TGM1^{hBCMA} mouse myeloma cells. Cell viability was assessed by

luciferase assay. Data are shown as with luciferase-tagged 5TGM1^{hBCMA} with luciferase-tagged 5TGM1^{nBCMA} mouse myeloma cells. Cell viability was assessed by

luciferase assay. Data are shown as mean \pm SD and representative of at least 3

independent experiments. *p<0.05, **p<0.01 by two For an independent experiments. *p<0.05, **p<0.01 by two-way ANOVA with Tukey's multiple
603 independent experiments. *p<0.05, **p<0.01 by two-way ANOVA with Tukey's multiple
603 comparison test.
(E) Heatmap representat

602 independent experiments. *p<0.05, **p<0.01 by two-way ANOVA with Tukey's multiple
603 comparison test.
(E) Heatmap representation of culture supernatant mouse cytokine concentrations measured by
605 multiplexed Lumi 603 comparison test.
604 **(E)** Heatmap represe
605 multiplexed Lum
606 CD28^{f//fl} or CD28ⁱ **(E)** Heatmap representation of culture supernatant mouse cytokine concentrations measured by

multiplexed Luminex assays at the conclusion of a 24-hr. co-culture of hBCMAmBBmζ

CD28^{ft/f1} or CD28^{iKO} CAR T cells with 5 multiplexed Luminex assays at the conclusion of a 24-hr. co-culture of hBCMAmBBm_{ζ} CD28^{ft/fl} or CD28^{iKO} CAR T cells with 5TGM1^{hBCMA} myeloma cells. Log₂ transformed cytokine concentrations represent the mean o $CD28^{\text{fl/fl}}$ or $CD28^{\text{iKO}}$ CAR T cells with 5TGM1^{hBCMA}

 $CD28th$ or $CD28th$ CAR T cells with 5TGM1^{nBCMA} myeloma cells. Log₂ transformed cytokine

concentrations represent the mean of 4 independent experiments.
 (F) Diagram of experimental setup used to evaluate 607 concentrations represent the mean of 4 independent experiments.
 (F) Diagram of experimental setup used to evaluate $CD28^{f|f|}$ versus

CAR T cell therapy in a mouse MM xenograft model. RAG2 **(F)** Diagram of experimental setup used to evaluate CD28^{fl/fl} versus CD28^{iKO} 608 **(F)** Diagram of experimental setup used to evaluate CD28tM versus CD28^{tMO} hBCMAmBBm_{ζ} CAR T cell therapy in a mouse MM xenograft model. RAG2^{-/-} mice were inoculated CD28^{tMO} hBCMAmBBm_{ζ} CAR T cell therapy in a mouse MM xenograft model. $RAG2^{-/-}$ mice were inoculated CAR T cell therapy in a mouse MM xenograft model. RAG2^{-/-} mice were inoculated

Solomontary in a mouse MM xenograft model. RAG2^{-/-} mice were inoculated

Solomontary in a mouse MM xenograft model. RAG2^{-/-}

mice were i

intravenously with 2 x 10⁶ 5TGM1^{hBCMA}-luc cells on day -14 and treated with CD28^{fl/fl} intravenously with 2 x 10⁶ 5TGM1^{hBCMA}-luc cells on day -14 and treated with CD28tM or
611 CD28^{iKO} CAR T cells on day 0. Tumor burden was monitored by IVIS bioluminescent
612 imaging (BLI) 2x/week through endpoint. CD28^{iKO} CAR T cells on day 0. Tumor burden was monitored by IVIS bioluminescent

- 611 CD28^{IKO} CAR T cells on day 0. Tumor burden was monitored by IVIS bioluminescent
612 imaging (BLI) 2x/week through endpoint.
613 **(G)** Tumor burden expressed as relative photon flux measured by BLI from 5TGM1^{hBCMA}-612 imaging (BLI) 2x/week through endpoint.
613 **(G)** Tumor burden expressed as relative ph
614 bearing mice treated with CD28^{fl/fl} or CD2
615 line represents an individual mouse (n = 6
616 **(H)** Kenlop Moier applyie of **(G)** Tumor burden expressed as relative photon flux measured by BLI from 5TGM1^{hBCMA}-luc 613 **(G)** Tumor burden expressed as relative photon flux measured by BLI from 5TGM1^{nBCMA}-luc
614 bearing mice treated with CD28^{ft/fl} or CD28^{iKO} hBCMAmBBmζ CAR T or control T cells. Each
615 line represents an indivi bearing mice treated with CD28^{fl/fl} or CD28^{iKO}
- 614 bearing mice treated with CD28tM or CD28tM hBCMAmBBmζ CAR T or control T cells. Each
615 line represents an individual mouse (n = 6 mice per CAR T cell treated group).
616 **(H)** Kaplan Meier analysis of survival 615 line represents an individual mouse (n = 6 mice per CAR T cell treated group).
616 **(H)** Kaplan – Meier analysis of survival of CD28^{ft/ft} or CD28^{ikO} hBCMAmBBmζ (control T cell treated 5TGM1^{hBCMA}-luc bearing mice **(H)** Kaplan – Meier analysis of survival of CD28^{f//fl} or CD28^{iKO} 616 **(H)** Kaplan – Meier analysis of survival of CD28^{t/m} or CD28^{t/m} bBCMAmBBm ζ CAR T cell or control T cell treated 5TGM1^{hBCMA}-luc bearing mice (n = 6 mice per CAR T cell treated group). Median survival of CD28 control T cell treated 5TGM1^{hBCMA}-luc bearing mice ($n = 6$ mice per CAR T cell treated 617 control T cell treated 5TGM1^{nBCMA}-luc bearing mice (n = 6 mice per CAR T cell treated
618 group). Median survival of CD28^{ft/fl} hBCMAmBBmζ CAR T treated mice was 38 days post-
620 CAR T cell infusion vs. 24 days fo group). Median survival of CD28^{fl/fl} hBCMAmBBm(CAR T treated mice was 38 davs postgroup). Median survival of CD28tM hBCMAmBBmζ CAR T treated mice was 38 days post-
619 **CAR T cell infusion vs. 24 days for CD28^{tKO} hBCMAmBBmζ CAR T treated mice. *p<0.05,
^{**}p<0.01, ***p<0.001 by log-rank Mantel-Cox** CAR T cell infusion vs. 24 days for CD28^{iKO}
- 619 **CAR T** cell infusion vs. 24 days for CD28^{KO} hBCMAmBBmζ CAR T treated mice. *p<0.05,
620 **p<0.01, ***p<0.001 by log-rank Mantel-Cox test.
(I) Heatmap representation of cytokine levels in the MM BME 7 days followi $^{**}p<0.01$, $^{**}p<0.001$ by log-rank Mantel-Cox test.

621 **(I)** Heatmap representation of cytokine levels in the

622 **CD28^{f/fl}** or CD28^{iKO} hBCMAmBBmζ CAR T cells in

623 hind limbs were harvested, and BM was flush 621 **(I)** Heatmap representation of cytokine levels in the MM BME 7 days following infusion of CD28^{f/f} or CD28^{KO} hBCMAmBBm ζ CAR T cells into 5TGM1^{hBCMA}-luc bearing mice. Bilateral hind limbs were harvested, and CD28^{fl/fl} or CD28^{iKO} hBCMAmBBmζ CAR T cells into 5TGM1^{hBCMA} $CD28th$ or $CD28th$ hBCMAmBBm ζ CAR T cells into 5TGM1^{nBCMA}-luc bearing mice. Bilateral

hind limbs were harvested, and BM was flushed into 15 µL PBS for multiplexed cytokine

analysis. Log₂ transformed cy hind limbs were harvested, and BM was flushed into 15 μ L PBS for multiplexed cytokine

analysis. Log₂ transformed cytokine concentrations represent the mean of 3 mice per group.

625 **Figure 3: Perturbation of oxidat**
-

analysis. Log₂ transformed cytokine concentrations represent the mean of 3 mice per group.
625 **Figure 3: Perturbation of oxidative metabolism and redox homeostasis in stimulated CD28^{iKO} hBCMAmBBmζ CAR T cells.** 626
627
628 CD28^{*iKO*} hBCMAmBBmζ CAR T cells.

- **Figure 3: Perturbation of oxidative metabolism and redox homeostasis in stimulated

CD28^{iKO} hBCMAmBBmζ CAR T cells.

(A)** Representative Seahorse Glycolysis Stress Test performed 24 hr. after stimulation of

CD28^{ft/fl} **CD28^{IKO} hBCMAmBBmζ CAR T cells.**
628 **(A)** Representative Seahorse Glycolys
629 **CD28^{f/fl} or CD28^{iKO} hBCMAmBB**
630 **Connected data points represent m (A)** Representative Seahorse Glycolysis Stress Test performed 24 hr. after stimulation of
 CD28^{ft/fl} or CD28^{ikO} hBCMAmBBmζ CAR T cells with 5TGM1^{hBCMA} myeloma cells.

Connected data points represent mean extracel $\mathsf{CD28}^\mathsf{f\hspace{-.1em}l\hspace{-.1em}f\hspace{-.1em}l}$ or $\mathsf{CD28}^\mathsf{IKO}$ hBCMAmBBm ζ CAR T cells with $\mathsf{5TGM1}^\mathsf{hBCMA}$ $CD28^{1/11}$ or $CD28^{18/10}$ hBCMAmBBm ζ CAR T cells with 5TGM1^{nBCMA} myeloma cells.

Connected data points represent mean extracellular acidification rate (ECAR) \pm SD of four

technical replicates at indicated time 630 Connected data points represent mean extracellular acidification rate (ECAR) \pm SD of four
631 technical replicates at indicated time points during a representative experiment that was
632 repeated at least 3 times. technical replicates at indicated time points during a representative experiment that was

repeated at least 3 times. Dashes indicate the timing of glucose, oligomycin (Oligo) and 2-

deoxyglucose (2-DG) injection.
 (B)
- For the timing of glucose, oligomycin (Oligo) and 2-

deoxyglucose (2-DG) injection.
 (B) Quantified rates of glycolysis (left), glycolytic capacity (middle), and glycolytic reserve (left)

in CD28^{ft/fl} or CD28^{iKO} h deoxyglucose (2-DG) injection.
634 **(B)** Quantified rates of glycolysis (I
635 in CD28^{fl/fl} or CD28^{iKO} hBCMA **(B)** Quantified rates of glycolysis (left), glycolytic capacity (middle), and glycolytic reserve (left)
in CD28^{ft/fl} or CD28^{ikO} hBCMAmBBmζ CAR T cells ± 24-hr. stimulation with 5TGM1^{hBCMA}
in CD28^{ft/fl} or CD28^{ikO} in CD28^{f/fl} or CD28^{iKO} hBCMAmBBmζ CAR T cells \pm 24-hr. stimulation with 5TGM1^{hBCMA}

myeloma cells. Bars represent mean \pm SD, dots represent independent experiments. ns =

not significant by one-way ANOVA.
 (C) Representative Seahorse Mito Stress Test performed 24 hr. after stimulation of CD28^{f/fl} not significant by one-way ANOVA.

638 **(C)** Representative Seahorse Mito Stre

639 **CD28^{iKO} hBCMAmBBmζ CAR T** c

640 points represent mean oxygen cons **(C)** Representative Seahorse Mito Stress Test performed 24 hr. after stimulation of CD28^{f/fl} or (C) Representative Seahorse Mito Stress Test performed 24 hr. after stimulation of CD28tM or
639 CD28^{tKO} hBCMAmBBmζ CAR T cells with 5TGM1^{hBCMA} myeloma cells. Connected data
640 points represent mean oxygen consumpt $CD28^{\text{i} \text{KO}}$ hBCMAmBBm ζ CAR T cells with 5TGM1^{hBCMA} $CD28^{\text{hO}}$ hBCMAmBBm ζ CAR T cells with 5TGM1^{nBCMA} myeloma cells. Connected data

points represent mean oxygen consumption rate (OCR) \pm SD of four technical replicates at

indicated time points during a represen 640 points represent mean oxygen consumption rate (OCR) \pm SD of four technical replicates at indicated time points during a representative experiment that was repeated at least 3 times.

Dashes indicate the timing of o indicated time points during a representative experiment that was repeated at least 3 times.

Dashes indicate the timing of oligomycin (Oligo), FCCP, and rotenone (Rot) + antimycin A

(Ant. A) injection.
 (D) Quantified

642 Dashes indicate the timing of oligomycin (Oligo), FCCP, and rotenone (Rot) + antimycin A

643 (Ant. A) injection.

644 **(D)** Quantified basal OCR (right), uncoupled maximal respiration (middle), and spare respiratory
 643 (Ant. A) injection.
644 **(D)** Quantified basal (
645 capacity (SRC)
646 hBCMAmBBmζ C 644 **(D)** Quantified basal OCR (right), uncoupled maximal respiration (middle), and spare respiratory

645 capacity (SRC) expressed as percent of basal OCR (left) in CD28^{ft/fl} or CD28^{ikO}

646 hBCMAmBBmζ CAR T cells $\$ capacity (SRC) expressed as percent of basal OCR (left) in CD28^{fl/fl} or CD28^{iKO} 646
 647
 648 hBCMAmBBmζ CAR T cells \pm 24 hr. stimulation with 5TGM1^{hBCMA} myeloma cells. Bars 646 hBCMAmBBmζ CAR T cells \pm 24 hr. stimulation with 5TGM1^{nBCMA} myeloma cells. Bars
647 represent mean \pm SD, dots represent independent experiments. *p<0.05, **p<0.01 by one-
648 **(E)** Ratio of NADH to NAD⁺ rati

For the same of the series independent experiments. *p<0.05, **p<0.01 by one-
way ANOVA.
 (E) Ratio of NADH to NAD⁺ ratios in CD28^{f/fl} or CD28^{iKO} hBCMAmBBmζ CAR T cells prior to 4-

OHT mediated CD28 deletion or 648 way ANOVA.
649 **(E)** Ratio of NAD
650 OHT mediate
651 represent me **(E)** Ratio of NADH to NAD⁺ ratios in CD28^{fl/fl} or CD28^{iKO} **(E)** Ratio of NADH to NAD⁺ ratios in CD28tM or CD28tM hBCMAmBBmζ CAR T cells prior to 4-

OHT mediated CD28 deletion or \pm 24 hr. stimulation with 5TGM1^{hBCMA} myeloma cells. Bars

represent mean \pm SD from 3 i OHT mediated CD28 deletion or \pm 24 hr. stimulation with 5TGM1^{hBCMA} 650 OHT mediated CD28 deletion or \pm 24 hr. stimulation with 5TGM1^{nBCMA} myeloma cells. Bars
651 represent mean \pm SD from 3 independent experiments. *p<0.05 by paired Student's t test.
(F) Diagrams depicting enzym

651 represent mean ± SD from 3 independent experiments. *p<0.05 by paired Student's t test.
 (F) Diagrams depicting enzymes of central carbon metabolism that interconvert NADH an

NAD⁺.
 (G) Relative expression of m NAD⁺.

(F) Diagrams depicting enzymes of central carbon metabolism that interconvert NADH and

NAD⁺.
 (G) Relative expression of mRNAs coding enzymes depicted in (F) in CD28^{iKO} versus CD28^{f/fl}

hBCMAmBBmζ CAR T cells fo 653 . **(G)** Relative expression of mRNAs coding enzymes depicted in (F) in CD28^{iKO} versus CD28^{fl/fl} ---
655
656
657 hBCMAmBBm ζ CAR T cells following 24 hr. stimulation with 5TGM1^{hBCMA} myeloma cells. 655 hBCMAmBBm ζ CAR T cells following 24 hr. stimulation with 5TGM1^{nBCMA} myeloma cells.
656 Bars represent mean log₂ transformed $\triangle \triangle C$ t values ± SD, dots technical replicates pooled
657 from 3 independent experim 656 Bars represent mean log₂ transformed $ΔΔCt$ values $±$ SD, dots technical replicates pooled
from 3 independent experiments. *Tbp* and *Actb* were used as endogenous controls.
 $***$ p<0.0001 by two-way ANOVA.
659 from 3 independent experiments. *Tbp* and *Actb* were used as endogenous controls.
658 ****p<0.0001 by two-way ANOVA.
659 **Figure 4: Diminished** *in vivo* **expansion of 4-BB co-stimulated CD28^{iKO} CAR T cells.**

658 *****p<0.0001 by two-way ANOVA.
659
660 Figure 4: Diminished *in vivo* expans 660
|
| **Figure 4: Diminished** *in vivo* **expansion of 4-BB co-stimulated CD28iKO** 660 Figure 4: Diminished *in vivo* expansion of 4-BB co-stimulated CD28^{INO} CAR T cells.
Accord to the CAR T cells.

(A) Expansion of CD28^{fl/fl} versus CD28^{iKO} hBMCAmBBmZ CAR T cells over the course of **(A)** Expansion of CD28^{ft/11} versus CD28^{ft/0} hBMCAmBBmζ CAR T cells over the course of
662 manufacturing. ns = not significant by two-way ANOVA.
(B) Expression of the proliferation marker Ki-67 in CD28^{ft/fl} versus

- 662 manufacturing. ns = not significant by two-way ANOVA.
 (B) Expression of the proliferation marker Ki-67 in CD28^{fl/fl} v

664 T cells following 24 hr. stimulation with 5TGM1 target ce

665 analysis. ns = not signifi **(B)** Expression of the proliferation marker Ki-67 in CD28^{f//fl} versus CD28^{iKO}
- (B) Expression of the proliferation marker Ki-67 in CD28^{t/m} versus CD28^{t/C} hBCMAmBBm ζ CAR
664 T cells following 24 hr. stimulation with 5TGM1 target cells as assessed by flow cytometric
665 analysis. ns = not sign
-
- **(C)** Diagram of experimental setup. 5TGM1^{hBCMA} bearing RAG2^{-/-} mice were treated with CD28^{fl/fl}
- T cells following 24 hr. stimulation with 5TGM1 target cells as assessed by flow cytometric
665 analysis. ns = not significant by one-way ANOVA.
666 **(C)** Diagram of experimental setup. 5TGM1^{hBCMA} bearing RAG2^{-/-} mice analysis. ns = not significant by one-way ANOVA.

666 **(C)** Diagram of experimental setup. 5TGM1^{hBCMA} bear

667 or CD28^{iKO} hBCMAmBBmζ CAR T cells (4D and

668 treated with hBCMABBζ CAR T cells ± 200µg a ---
667
668
669 or CD28^{iKO} hBCMAmBBm(CAR T cells (4D and 4E) or MM.1S bearing NSG mice were 667 or CD28^{IKO} hBCMAmBBmζ CAR T cells (4D and 4E) or MM.1S bearing NSG mice were
668 treated with hBCMABBζ CAR T cells \pm 200µg abatacept on days -1, 1, 3, 5, 7 (4F) and
669 euthanized 7 days post adoptive transfer fo
- treated with hBCMABB ζ CAR T cells \pm 200µg abatacept on days -1, 1, 3, 5, 7 (4F) and

euthanized 7 days post adoptive transfer for blood collection and hind limb BM harvest.
 (D) CAR T cell frequency assessed by f 669 euthanized 7 days post adoptive transfer for blood collection and hind limb BM harvest.
670 **(D)** CAR T cell frequency assessed by flow cytometry in bone marrow (BM, left) and perip
671 blood (right) one week after ad 670 **(D)** CAR T cell frequency assessed by flow cytometry in bone marrow (BM, left) and peripheral
671 blood (right) one week after adoptive transfer of CD28^{ft/f}l or CD28^{ft/C} hBCMAmBBmζ CAR T
672 cells into 5TGM1^{hBCM} blood (right) one week after adoptive transfer of CD28^{f//fl} or CD28^{iKO} blood (right) one week after adoptive transfer of CD28^{th or} CD28^{th or} BCMAmBBmζ CAR T
cells into 5TGM1^{hBCMA} myeloma bearing mice. Bars represent mean ± SD, dots indicate
individual mice (n = 3-6 mice per group). **p cells into 5TGM1^{hBCMA} 672 cells into 5TGM1^{nBCMA} myeloma bearing mice. Bars represent mean \pm SD, dots indicate
673 individual mice (n = 3-6 mice per group). **p<0.01, ****p<0.0001 by two-way ANOVA with
674 Tukey's multiple comparison test.

individual mice (n = 3-6 mice per group). **p<0.01, ****p<0.0001 by two-way ANOVA with

Tukey's multiple comparison test.
 (E) CD28 surface protein expression on BM-infiltrating CD28^{f/fl} or CD28^{iKO} hBCMAmBBmζ CAR

T Tukey's multiple comparison test.
675 **(E)** CD28 surface protein expression
676 **T** cells one week after adoptive tra
677 **(F)** CAR T cell frequency assessed b **(E)** CD28 surface protein expression on BM-infiltrating CD28^{f//fl} or CD28^{iKO} T cells one week after adoptive transfer into 5TGM1^{hBCMA} myeloma bearing mice.

- (E) CD28 surface protein expression on BM-infiltrating CD28tM or CD28tM hBCMAmBBmζ CAR

676 T cells one week after adoptive transfer into 5TGM1^{hBCMA} myeloma bearing mice.

677 (F) CAR T cell frequency assessed by fl σ T cells one week after adoptive transfer into 5TGM1^{nBCMA} myeloma bearing mice.
 (F) CAR T cell frequency assessed by flow cytometry in bone marrow (BM, left) and

blood (right) one week after adoptive transfer of **(F)** CAR T cell frequency assessed by flow cytometry in bone marrow (BM, left) and peripheral
blood (right) one week after adoptive transfer of hBCMABB ζ CAR T cells into MM.1S
myeloma bearing mice \pm abatacept. CAR blood (right) one week after adoptive transfer of hBCMABB ζ CAR T cells into MM.1S
myeloma bearing mice \pm abatacept. CAR T population identified by surface staining and
analyzed by flow cytometry. Bars represent mea 679 myeloma bearing mice ± abatacept. CAR T population identified by surface staining and
680 analyzed by flow cytometry. Bars represent mean ± SD, dots indicate individual mice (n = 4-
681 6 5 mice per group). ***p<0.001
- 680 analyzed by flow cytometry. Bars represent mean \pm SD, dots indicate individual mice (n = 4-
681 681 682 682 682 682 682 682 682 683 684 692 693 693 694 693 694 693 694 693 694 693 694 693 694 693 694 693 694 693 69 681 5 mice per group). ***p<0.001 by two-way ANOVA with Tukey's multiple comparison test.
682 **(G)** Representative IVIS bioluminescence images of T-lux luciferase expressing hBCMAmBE
683 CAR T cells ± abatacept (200µg, 3x
- **(G)** Representative IVIS bioluminescence images of T-lux luciferase expressing hBCMAmBBmζ
CAR T cells ± abatacept (200μg, 3x/week) on day 7 or day 15 after infusion.
(H) Quantification of photon flux by IVIS imaging wi CAR T cells ± abatacept (200µg, 3x/week) on day 7 or day 15 after infusion.

684 **(H)** Quantification of photon flux by IVIS imaging within the hind limb region cover a four-week period following T-lux hBCMAmBBmζ CAR T ce **(H)** Quantification of photon flux by IVIS imaging within the hind limb region of interest (ROI) over a four-week period following T-lux hBCMAmBBmζ CAR T cell infusion \pm abatacept (200µg, 3x/week) into 5TGM1^{hBCMA} my over a four-week period following T-lux hBCMAmBBmζ CAR T cell infusion \pm abatacept

686 (200μg, 3x/week) into 5TGM1^{hBCMA} myeloma bearing mice. Dots represent individual mice (n

686 (200μg, 3x/week) into 5TGM1^{hBCMA} (200μg, 3x/week) into 5TGM1^{hBCMA} 686 (200µg, 3x/week) into 5TGM1^{nBCMA} myeloma bearing mice. Dots represent individual mice (n

(n expansive to the original mice of the control o

-
-

$= 3$ mice per group), lines are best-fit sigmoidal curves, and significance was determined by
 688 mixed effects modeling.
 689 Figure 5: Transient CD28 blockade limits inflammatory cytokines in the MM BME without
 688 mixed effects modeling.
689 **Figure 5: Transient CD28** l
691 **affecting survival of hBCM** 690
691
692 **Figure 5: Transient CD28 blockade limits inflammatory cytokines in the MM BME without**
 691 affecting survival of hBCMAmBBmζ CAR T cell treated mice.
 (A) Diagram of experimental setup. MM.1S bearing NSG mice were

-
- CAR T cells \pm 200µg abatacept on days -1, 1, 3, 5, 7 (5B and 5C) or 5TGM1^{hBCMA} bearing

affecting survival of hBCMAmBBmζ CAR T cell treated mice.
 (A) Diagram of experimental setup. MM.1S bearing NSG mice we

CAR T cells ± 200μg abatacept on days -1, 1, 3, 5, 7 (5B and

RAG2^{-/-} mice were treated with C 692 **(A)** Diagram of experimental setup. MM.1S bearing NSG mice were treated with hBCMABB_{ζ} CAR T cells \pm 200 μ g abatacept on days -1, 1, 3, 5, 7 (5B and 5C) or 5TGM1^{hBCMA} bearin RAG2^{-/-} mice were treated wi CAR T cells \pm 200µg abatacept on days -1, 1, 3, 5, 7 (5B and 5C) or 5TGM1^{nBCMA} bearing

RAG2^{-/-} mice were treated with CD28^{ft/f1} or CD28^{ikO} hBCMAmBBmζ CAR T cells (5D and 5E

and euthanized 7 days post adoptive RAG2^{-/-} mice were treated with CD28^{fl/fl} or CD28^{iKO}

-
- RAG2^{-/-} mice were treated with CD28tM or CD28tM hBCMAmBBmζ CAR T cells (5D and 5E)
and euthanized 7 days post adoptive transfer for hind limb BM harvest.
(B) Myeloma burden assessed by flow cytometry for human CD1 695 and euthanized 7 days post adoptive transfer for hind limb BM harvest.
696 **(B)** Myeloma burden assessed by flow cytometry for human CD138⁺ cells in
697 MM.1S bearing mice treated as described in 5A. Bars represent **(B)** Myeloma burden assessed by flow cytometry for human CD138⁺ cells in the bone marrow of
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(B) Myeloma burden assessed by flow cytometry for human CD138⁺ cells in the bone marrow of

697 MM.1S bearing mice treated as described in 5A. Bars represent mean \pm SD, dots indicate

698 individual mice. **p<0.01 697 MM.1S bearing mice treated as described in 5A. Bars represent mean \pm SD, dots indicate
698 individual mice. **p<0.01 by one-way ANOVA, ns = not significant.
(C) Heatmap representation of human cytokine levels in 698 individual mice. **p<0.01 by one-way ANOVA, ns = not significant.
 (C) Heatmap representation of human cytokine levels in the MM BME

MM.1S bearing mice with hBCMABB ζ CAR T cells ± abatacept. Bil

harvested, and

699 **(C)** Heatmap representation of human cytokine levels in the MM BME 7 days after treatment of

700 MM.1S bearing mice with hBCMABB ζ CAR T cells \pm abatacept. Bilateral hind limbs were

701 harvested, and BM was

MM.1S bearing mice with hBCMABB ζ CAR T cells \pm abatacept. Bilateral hind limbs were

701 harvested, and BM was flushed into 15 µL PBS for multiplexed cytokine analysis. Log₂

702 transformed cytokine concentratio Frame present the mean of 3-6 mice per group.
 702 transformed cytokine concentrations represent the mean of 3-6 mice per group.
 703 (D) Heatmap representation of murine cytokine levels in the MM BME 7 days after t

5TGM1^{hBCMA} bearing mice with hBCMAmBBm ζ CAR T cells \pm abatacept. Bilateral hind limbs

transformed cytokine concentrations represent the mean of 3-6 mice per group.
 (D) Heatmap representation of murine cytokine levels in the MM BME 7 days after tr

5TGM1^{hBCMA} bearing mice with hBCMAmBBmζ CAR T cells ± a 703 **(D)** Heatmap representation of murine cytokine levels in the MM BME 7 days after treatment of

704 5TGM1^{hBCMA} bearing mice with hBCMAmBBmζ CAR T cells ± abatacept. Bilateral hind limbs

705 were harvested, and BM w 5TGM1^{nBCMA} bearing mice with hBCMAmBBmζ CAR T cells ± abatacept. Bilateral hind limbs

were harvested, and BM was flushed into 15 μL PBS for multiplexed cytokine analysis. Log₂

transformed cytokine concentrations re

- The transformed cytokine concentrations represent the mean of 2-3 mice per group.

707 **(E)** Bar graphs showing concentrations of murine IP-10 (left) and IL-12 (right) in the

708 days after treatment of 5TGM1^{hBCMA} bear
- were harvested, and BM was flushed into 15 μ L PBS for multiplexed cytokine analysis. Log₂

transformed cytokine concentrations represent the mean of 2-3 mice per group.
 (E) Bar graphs showing concentrations of mur **(E)** Bar graphs showing concentrations of murine IP-10 (left) and IL-12 (right) in the MM BME 7

days after treatment of 5TGM1^{hBCMA} bearing mice with CD28^{f//ff} hBCMAmBBmζ CAR T cells
 \pm abatacept versus CD28^{iKO} days after treatment of 5TGM1^{hBCMA} bearing mice with CD28^{fl/fl}
- days after treatment of 5TGM1^{nBCMA} bearing mice with CD28^{n/n} hBCMAmBBmζ CAR T cells
 \pm abatacept versus CD28^{iKO} hBCMAmBBmζ CAR T cells. Bars represent mean \pm SD of 2-3

biological replicates. **p<0.01, ***p<0. \pm abatacept versus CD28 $^{\sf iKO}$ \pm abatacept versus CD28^{IKO} hBCMAmBBmζ CAR T cells. Bars represent mean \pm SD of 2-3

biological replicates. **p<0.01, ***p<0.001 by one-way ANOVA.
- 710 biological replicates. **p<0.01, ***p<0.001 by one-way ANOVA.

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- **(F)** Diagram of experimental setup. MM.1S bearing NSG mice were treated with hBCMABBζ

CAR T cells ± 200μg abatacept on days -1, 1, 3, 5, 7 and tumor burden was monitored by

bioluminescent imaging 2x/week until endpoint
- CAR T cells \pm 200µg abatacept on days -1, 1, 3, 5, 7 and tumor burden was monitored by

bioluminescent imaging 2x/week until endpoint.
 (G) Kaplan Meier analysis of survival of hBCMABB ζ CAR T \pm transient aba
- bioluminescent imaging 2x/week until endpoint.
714 **(G)** Kaplan Meier analysis of survival of hBCMA
715 transduced T cell treated MM.1S-luc bearing r
716 group). Median survival of hBCMABBZ CAR T t **(G)** Kaplan – Meier analysis of survival of hBCMABB ζ CAR T \pm transient abatacept or mock

transduced T cell treated MM.1S-luc bearing mice (n = 4-5 mice per CAR T cell treated

group). Median survival of hBCMABB $\$ transduced T cell treated MM.1S-luc bearing mice (n = 4-5 mice per CAR T cell treated
group). Median survival of hBCMABB ζ CAR T treated mice was >100 days post CAR T cell
infusion vs. 97 days for hBCMABB ζ CAR T + t
- group). Median survival of hBCMABBζ CAR T treated mice was >100 days post CAR T cell
infusion vs. 97 days for hBCMABBζ CAR T + transient abatacept treated mice. Statistical
significance was determined by log-rank Mantel-C 717 infusion vs. 97 days for hBCMABBζ CAR T + transient abatacept treated mice. Statistical
718 significance was determined by log-rank Mantel-Cox test.
- 718 significance was determined by log-rank Mantel-Cox test.

**Supplemental Figure Legends:

720 Supplemental Fig. 1: Characte

721 target cells.

722 (A) CD4:CD8 ratio in CAR T ce

733 Palative fracusses of CD4⁺ Supplemental Fig. 1: Characterization of human hBCMABBζ CAR T cells and myeloma

target cells.**
 (A) CD4:CD8 ratio in CAR T cell infusion products generated from PBMCs of healthy donors.

Relative frequency of CD4⁺ a

-
- 721 **target cells.**

722 **(A)** CD4:CD8

723 Relative

724 point rep (A) CD4:CD8 ratio in CAR T cell infusion products generated from PBMCs of healthy donors.

Relative frequency of CD4⁺ and CD8⁺ CAR T cells assessed by flow cytometry. Each data

point represents an independent donor (n Relative frequency of CD4⁺ and CD8⁺ Relative frequency of $CD4^+$ and $CD8^+$ CAR T cells assessed by flow cytometry. Each data

point represents an independent donor (n = 8).
 (B) Representative flow cytometry dot plot depicting the frequency of CAR transd
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- 9724 point represents an independent donor (n = 8).

725 **(B)** Representative flow cytometry dot plot depicting

726 **(C)** Transduction efficiency (left) and mean fluore

727 CAR expression on human CD3⁺ T cells deter **(B)** Representative flow cytometry dot plot depicting the frequency of CAR transduced cells.
 (C) Transduction efficiency (left) and mean fluorescence intensity MFI; right) of hBCMAE

CAR expression on human CD3⁺ T ce **(C)** Transduction efficiency (left) and mean fluorescence intensity MFI; right) of hBCMABBζ

CAR expression on human CD3⁺ T cells determined using αG4S linker antibody and flow

cytometry. Dots represent independent d CAR expression on human $CD3⁺$ T cells determined using α G4S linker antibody and flow
-
- (D) Surface CD28 protein expression on CD4⁺ and CD8⁺
- CAR expression on human CD3⁺ T cells determined using αG4S linker antibody and flow
cytometry. Dots represent independent donors (n = 10).
(D) Surface CD28 protein expression on CD4⁺ and CD8⁺ hBCMABBζ CAR T cells cytometry. Dots represent independent donors (n = 10).

729 **(D)** Surface CD28 protein expression on CD4⁺ and CD8⁺ l

8730 by MFI. Each data point represents an independent c

731 Student's t test **p<0.01. (D) Surface CD28 protein expression on CD4⁺ and CD8⁺ hBCMABB ζ CAR T cells determined

by MFI. Each data point represents an independent donor (n = 6). **p<0.01 by unpaired

Student's t test **p<0.01.

(E) Flow cyt
- by MFI. Each data point represents an independent donor (n = 6). **p<0.01 by unpaired

Student's t test **p<0.01.
 (E) Flow cytometry histograms depicting surface expression of MM defining phenotypic

markers CD138 and 731 Student's t test **p<0.01.

732 **(E)** Flow cytometry histogra

733 markers CD138 and BCM

734 cell lines, MM.1S and U26 **(E)** Flow cytometry histograms depicting surface expression of MM defining phenotypic

markers CD138 and BCMA along with co-receptors CD28, CD80, and CD86 on human MM

cell lines, MM.1S and U266.
 (F) Heatmap representa
- markers CD138 and BCMA along with co-receptors CD28, CD80, and CD86 on human MM
cell lines, MM.1S and U266.
(F) Heatmap representation of culture supernatant human cytokine concentrations measured
by multiplexed Luminex cell lines, MM.1S and U266.

735 **(F)** Heatmap representation of a

736 by multiplexed Luminex assa

737 T cells ± abatacept with MM (F) Heatmap representation of culture supernatant human cytokine concentrations measured
by multiplexed Luminex assays at the conclusion of a 24-hr. co-culture of hBCMABB ζ CAR
T cells ± abatacept with MM.1S myeloma cel by multiplexed Luminex assays at the conclusion of a 24-hr. co-culture of hBCMABB ζ CAR

T cells \pm abatacept with MM.1S myeloma cells. Log₂ transformed cytokine concentrations

represent the mean of 5 independent T cells \pm abatacept with MM.1S myeloma cells. Log₂ transformed cytokine concentrations

represent the mean of 5 independent experiments using CAR T cells generated from 5

healthy donors.

The concentration of the st represent the mean of 5 independent experiments using CAR T cells generated from 5

healthy donors.

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 **Supplemental Fig. 2: Characterization of mouse CD28^{iKO} hBCMAmBBmζ CAR T cells and

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739 healthy donors.
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741 **Supplemental Fig.**
742 **myeloma target cel** 741
742 **Supplemental Fig. 2: Characterization of mouse CD28iKO Tandal Supplemental Fig. 2: Characterization of mouse CD28^{IKO} hBCMAmBBmζ CAR T cells and any myeloma target cells.

Tandal myeloma target cells.

The** *myeloma target cells***.** 742 **myeloma target cells.**

1. Mouse and human antibodies used for cell phenotyping analyses performed by flow

cytometry.

2. Mouse primer sequences used for qRT-PCR.

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Supplemental Table Captions:
 **1. Mouse and human antibo

771** cytometry.
 2. Mouse primer sequences
 772 2. Mouse primer sequences 771 cytometry.
772 2. Mouse prin
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774 2. Mouse primer sequences used for qRT-PCR.
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- 775 **References**
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