Materials and Methods

Cell cultures and patient sample processing

All CD138-expressing MM cell lines were grown in RPMI1640 (Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, and 100μg/mL streptomycin (Invitrogen) as previously described.[1] Patient MM and normal donor samples were obtained after informed consent was provided, in accordance with the Declaration of Helsinki and under the auspices of a Dana-Farber Cancer Institute Institutional Review Board approved protocol. Primary CD138+ plasma cells and pDC were purified from BM aspirates using anti-CD138 and -CD304 (BDCA-4/Neuropilin-1) microbeads, respectively (Miltenyi Biotech, Auburn, CA).[2,3] Residual CD138-negative BMMCs were further cultured in RPMI 1640/10% FCS for 3 to 6 weeks to derive BMSCs.

Real-time quantitative RT-PCR

Real-time PCR was performed using TaqMan gene expression assay primer sets for BCMA (Hs03045080_m1) and internal controls (18S, HS03003631_g1; GAPDH, Hs02758991_g1) on an ABI Prism 7700 sequence detection system (Applied Biosystems).

NFκB signaling MSD analysis

H929 cells were washed 3 times and resuspended in serum free medium. J6M0 or isotype control antibody (0-100μg/ml) was added to a 96 well plate along with human BAFF (0.6 μg/ml) or APRIL (0.2 μg/ml). H929 cells ($7.5x10^4$ cells/well) were then added to the plate for 30 minutes followed by the MSD Phospho-NFκB (Ser536) assay (K111ECD-1; Meso Scale Discovery, Rockville MD).

Flow cytometric analysis

To determine the amount of cell surface expression of BCMA on human cancer cell lines and primary MM tumor cells, direct and indirect immunofluorescence flow cytometric analysis was performed using a Coulter Epics XL with data acquisition software (Cytomics™ FC500-RXP) (Beckman Coulter, Miami, FL). BCMA expression for Figure 2 was generated using an anti-BCMA antibody (clone 118G03) generated by GSK conjugated directly to AlexaFluor488 on BD LSRII (BD Biosciences, San Jose, CA). Data was analyzed using Flowjo version 8.6.6 (Tree Star, Inc., Ashland, OR).

Cytotoxicity assays

The growth inhibitory and anti-survival effects of J6M0 ADCs on MM (10^4/well for cell line and 2-5 x10^5/well for patient cells) and various effector cells (1-2 x 10^5/well), alone or cocultured with BMSC or pDC, were assessed by CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI) in 96-well tissue culture plates.[1] IC_{50} values for these 2 ADCs were generated by plating cell lines in 96-well assay plates at 4,000 cells/well/90μl in triplicates for each dose ranged from19.5 ng/mL to 10 μg/mL for 144 hrs. Data analysis was performed with GraphPad Prism 5 (GraphPad Software, Inc, La Jolla, CA) software to plot curve fits and perform statistical analyses. Nonlinear variable slope curve-fits were plotted as log[ADC ng/mL] vs. Response and IC_{50} values and 95% confidence intervals were generated.

Colony formation of MM cell lines was done in methylcellulose culture (R&D Systems).[2] Cell cycle analysis was assessed by propidium iodide (PI) staining and measured by flow cytometry. Drug-induced apoptosis was determined by annexin-V/PI staining, flow cytometric data analysis, and Caspase-Glo Assays (Promega).

Preparation for NK cells, monocyte, and macrophage culture

NK (CD56+CD3-) cells and monocytes (CD14+) were enriched from peripheral blood (PB) with the RosetteSep human NK and monocyte cell enrichment cocktail (StemCell Technologies Inc. Vancouver, Canada), respectively. PB mononuclear cells (PBMC) were obtained from healthy donors or MM patients. Monocytes were further cultured with macrophage colony-stimulating factor (M-CSF, 150 ng/ml) (PeproTech, Rocky Hill, NJ) for 5d to derive macrophages (CD11b+). Cells were confirmed to be >85% pure by flow cytometry before experiments.[4]

ADCC assay

ADCC was measured by calcein-AM release assay using human NK and PBMC effectors from normal donors or MM patients [4-6] at effector: target (E:T) ratios of 10:1 (MM cell lines) and 20:1 (patient MM cells). In some experiments, effector cells were pretreated with lenalidomide (2 μM, Selleck Chemicals) for 5d before use in ADCC assays.[4,6] Results were shown as group mean of % lysis $\pm$ SD.

MM xenograft mouse model (plasmacytoma model)

All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals. NCI-H929 (5×10^6) or OPM2 (1×10^7) cells in 100 μl BD Matrigel (BD Biosciences) were subcutaneously implanted in the right flank of CB.17 SCID mice receiving a subcutaneous Plexx IMI-1000 transponder for unique identification in left flank. From around day 15 onwards, all tumors were measured thrice weekly using the Plexx DAS-6002 caliper system, and length and width of each mouse tumor recorded to calculate tumor volume (volume = length x (width^2) x 0.5) When the mean tumor volume reached ~150 mm^3, mice were randomized into dose groups intraperitoneally (IP)

twice weekly for 2 weeks. All tumors were measured, and individual mice were euthanized once their tumor reached a mean tumor measurement of 1.5 cm.

Disseminated MM model

All experimental procedures and protocols had been approved by the Institutional Animal Care and Use Committee (Dana Farber Cancer Institute). Mice were injected with MM1SLuc intravenously and imaged weekly until they reached a mean bioluminescence (BLI) of 3E$\pm$06 [1]. At that point, mice were then divided into 5 groups (n=8 per group) receiving vehicle, iso-mcMMAF, J6M0, and J6M0-mcMMAF (q3d x 9, ip.). All mice were imaged and weighed every 5-12 days for 3.5 months.

Immunohistochemistry

At sacrifice, femurs from each group of mice were grossed, fixed (10% neutral buffered formalin), decalcified (EDTA based pH 7.4, Versenate DCV0150 - American MasterTech Scientific) and embedded in paraffin. To facilitate IHC assay validation and to address bone marrow fixative penetration/epitope stability concerns, comparator pellets of BCMA negative (Ku812) and BCMA positive (H929) cells were suspended in Histogel (Richard Allen Scientific, Kalamazoo, MI) and processed in a similar manner to the bone samples. Four µm study sections were cut and stained for CD138 (0.1 µg murine MCA2459GA AbD Serotec at 37^{o}C), J6M0 (humanized J6M0 mAb for BCMA) and F4/80 (rat antibody for macrophage). IHC were done using standard protocols on the Ventana Discovery XT platform (Ventana Medical Systems, Tucson, AZ) with appropriate IgG controls. Serial sections were also stained using a standard H&E protocol.

Antibody dependent cellular phagocytosis (ADCP) assay

MM cells were labeled green with a fluorescence cell membrane dye PKH67, washed, and incubated with macrophages derived from M-CSF-stimulated monocytes in the presence of J6M0-mcMMAF or control iso-mcMMAF in triplicates at an E/T ratio of 4/1 for 2-4h. The cell mixture was then stained with APC/Cy7 anti-human CD11b (BioLegend) to identify the macrophages. Cells were fixed with 1% paraformaldehyde followed by flow cytometry to detect double fluorescence, an indication of phagocytic activity. The number of PKH67+CD11b+ cells was divided by the total number of PKH67+ cells to determine % of phagocytosis.

Statistics

In vitro experiments were done in triplicates, and repeated at least twice. A representative experiment is shown (means $\pm$ SD). Statistical significance of differences observed in ADC (or naked mAb) - versus control-treated groups was determined using the Student t test. The minimal level of significance was $p<0.05$. Survival data were plotted by the Kaplan-Meier method and analyzed by the log-rank test using Prism Version 6.0 (GraphPad Software).

Reference

1. Tai YT, Landesman Y, Acharya C, et al. CRM1 inhibition induces tumor cell cytotoxicity and impairs osteoclastogenesis in multiple myeloma: molecular mechanisms and therapeutic implications. *Leukemia*. 2013.

2. Tai YT, Chang BY, Kong SY, et al. Bruton tyrosine kinase inhibition is a novel therapeutic strategy targeting tumor in the bone marrow microenvironment in multiple myeloma. *Blood*. 2012;120(9):1877-1887.

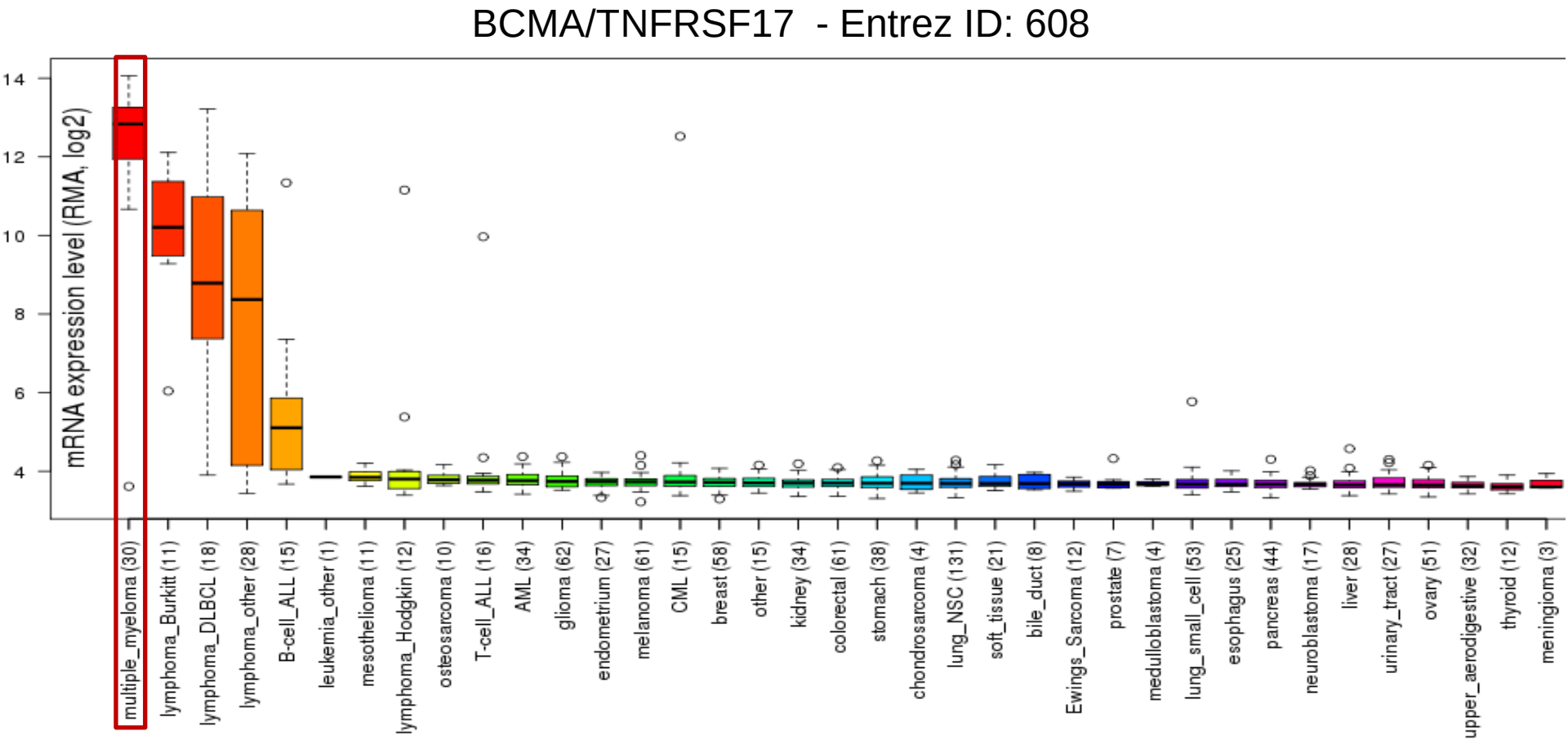
3. Chauhan D, Singh AV, Brahmandam M, et al. Functional interaction of plasmacytoid dendritic cells with multiple myeloma cells: a therapeutic target. *Cancer Cell*. 2009;16(4):309-323.

4. Tai YT, Horton HM, Kong SY, et al. Potent in vitro and in vivo activity of an Fc-engineered humanized anti-HM1.24 antibody against multiple myeloma via augmented effector function. *Blood*. 2012;119(9):2074-2082.

5. Tai YT, Dillon M, Song W, et al. Anti-CS1 humanized monoclonal antibody HuLuc63 inhibits myeloma cell adhesion and induces antibody-dependent cellular cytotoxicity in the bone marrow milieu. *Blood*. 2008;112(4):1329-1337.

6. de Weers M, Tai YT, van der Veer MS, et al. Daratumumab, a novel therapeutic human CD38 monoclonal antibody, induces killing of multiple myeloma and other hematological tumors. *J Immunol*. 2011;186(3):1840-1848.

A



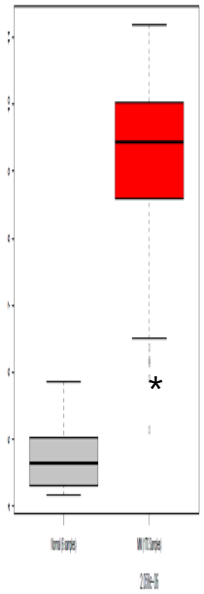
Data were analyzed from Cancer Cell Line Encyclopedia (CCLE) encompassing 36 tumor types & 1036 samples
http://www.broadinstitute.org/ccle

Fig. S1

B

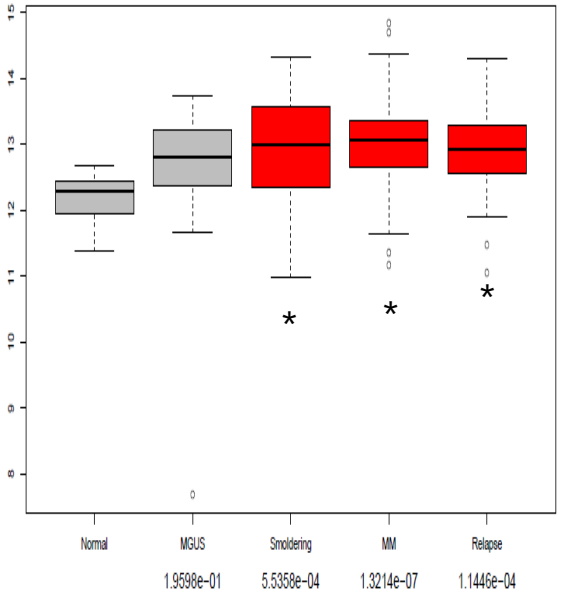
206641_at (BCMA/TNFRSF17) Signal

GSE39754



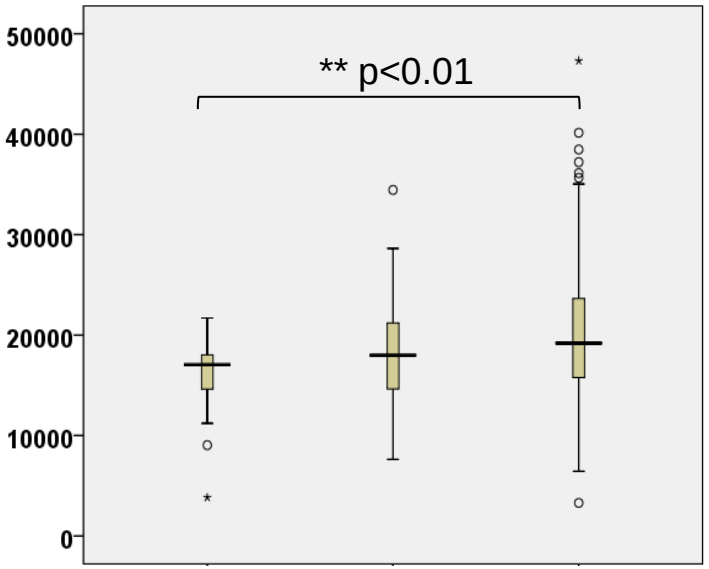
NPC (n=6) MM (n=170)

GSE6477



NPC (n=15) MGUS (n=22) SMM (n=24) MM (n=28) relapsed (n=28)

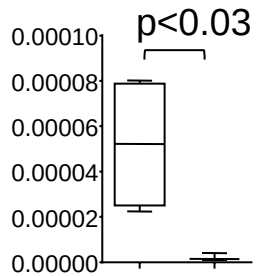
GSE2658



NPC (n=22) MGUS (n=44) MM (n=351)

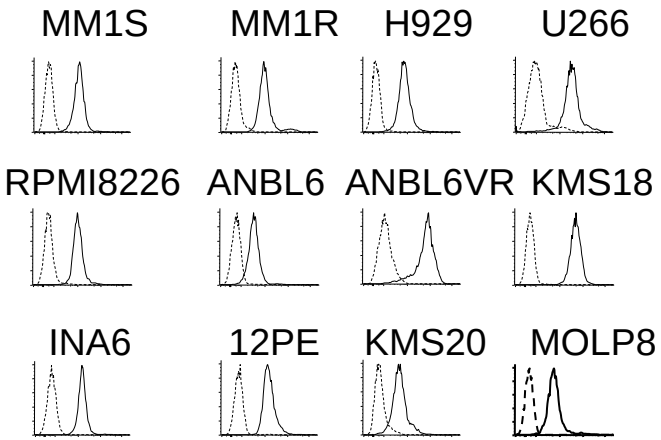
C

pDC

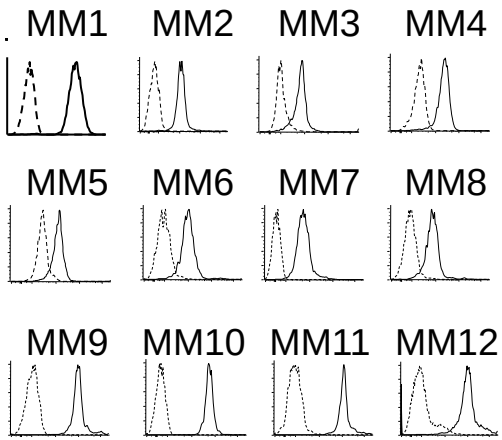


D

MM cell lines



CD138+ MM patient cells



pDC from MM patients

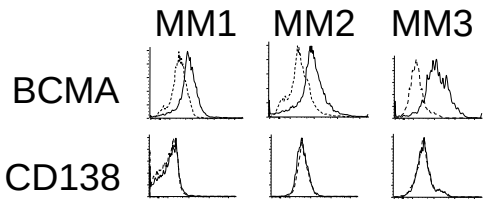
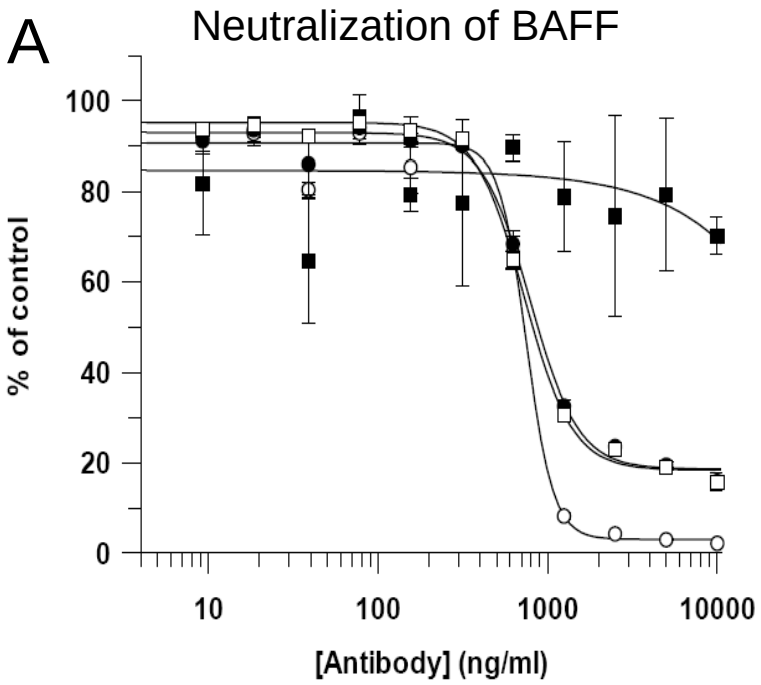
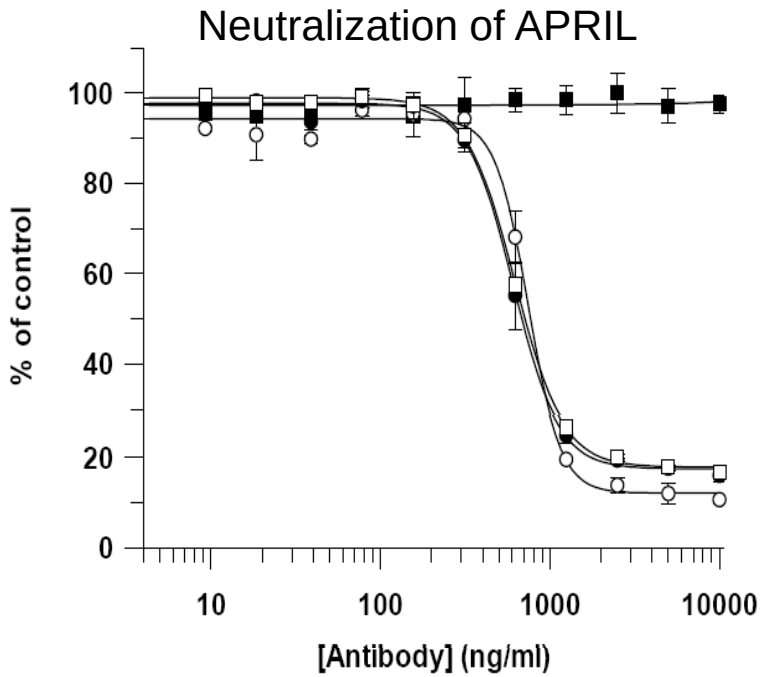


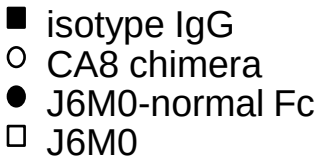
Fig. S1

Fig. S1. A novel anti-BCMA mAb 1180G binds to BCMA protein on the cell membrane of CD138+ plasma cells and pDC from MM patients. BCMA mRNA was analyzed in (**A**) Cancer Cell Line Encyclopedia (CCLE) encompassing 36 tumor types & 1036 samples (http://www.broadinstitute.org/ccle) as well as (**B**) published MM databases GSE39754, GSE6477, and GSE2658. * $p<0.05$ (in red) ** $p<0.01$ when compared with normal plasma cells (NPC). (**C**) Real-time qRT-PCR showed a significantly higher BCMA transcript in pDC from MM patients vs. normal donors. $p<0.03$ (**D**) MM cell lines (left panel), CD138+ cells from 12 MM patients (middle panel), and pDC (BDCA4+) from 3 MM patients were stained with Alexafluro647-labeled anti-BCMA mAb 1180G (——) or isotype control (------). As expected, pDC did not react with anti-CD138 mAb.

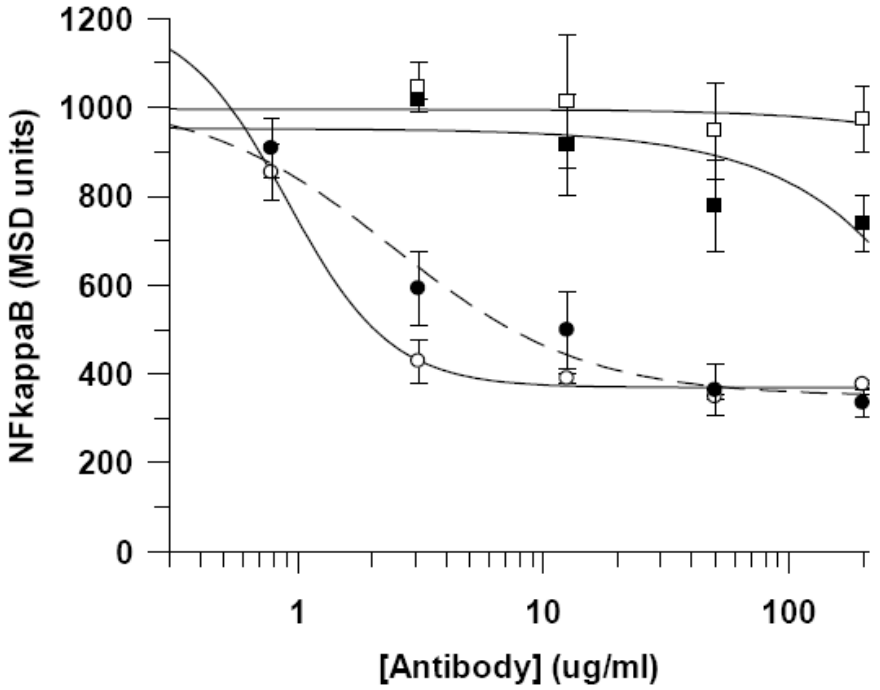
A







B



	IC_{50} (μg/ml)
BAFF	0.91
APRIL	2.43

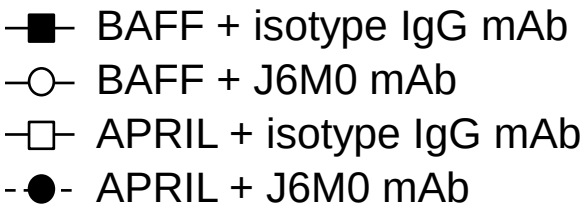


Fig. S2

Fig. S2. J6M0 mAb inhibits BAFF/APRIL ligand binding. (**A**) Various versions of this anti-BCMA mAb (chimeric CA8, humanized CA8 version J6M0 with wild type Fc/J6M0-normal Fc, and J6M0 which has afucosylated Fc) were tested in ligand neutralization assays. The ability of CA8 and J6M0 to neutralize binding of BAFF (left panel) or APRIL (right panel) to BCMA coated on ELISA plates. OD values were used to calculate the antibody mediated inhibition of the maximal signal achieved by the relevant ligand alone binding to recombinant BCMA. Data is reported as % inhibition of the maximal signal. (**B**) H929 cells were treated with J6M0 or isotype IgG control mAb in the presence of recombinant human BAFF or APRIL followed by the MSD pNFκB (ser536) assays.

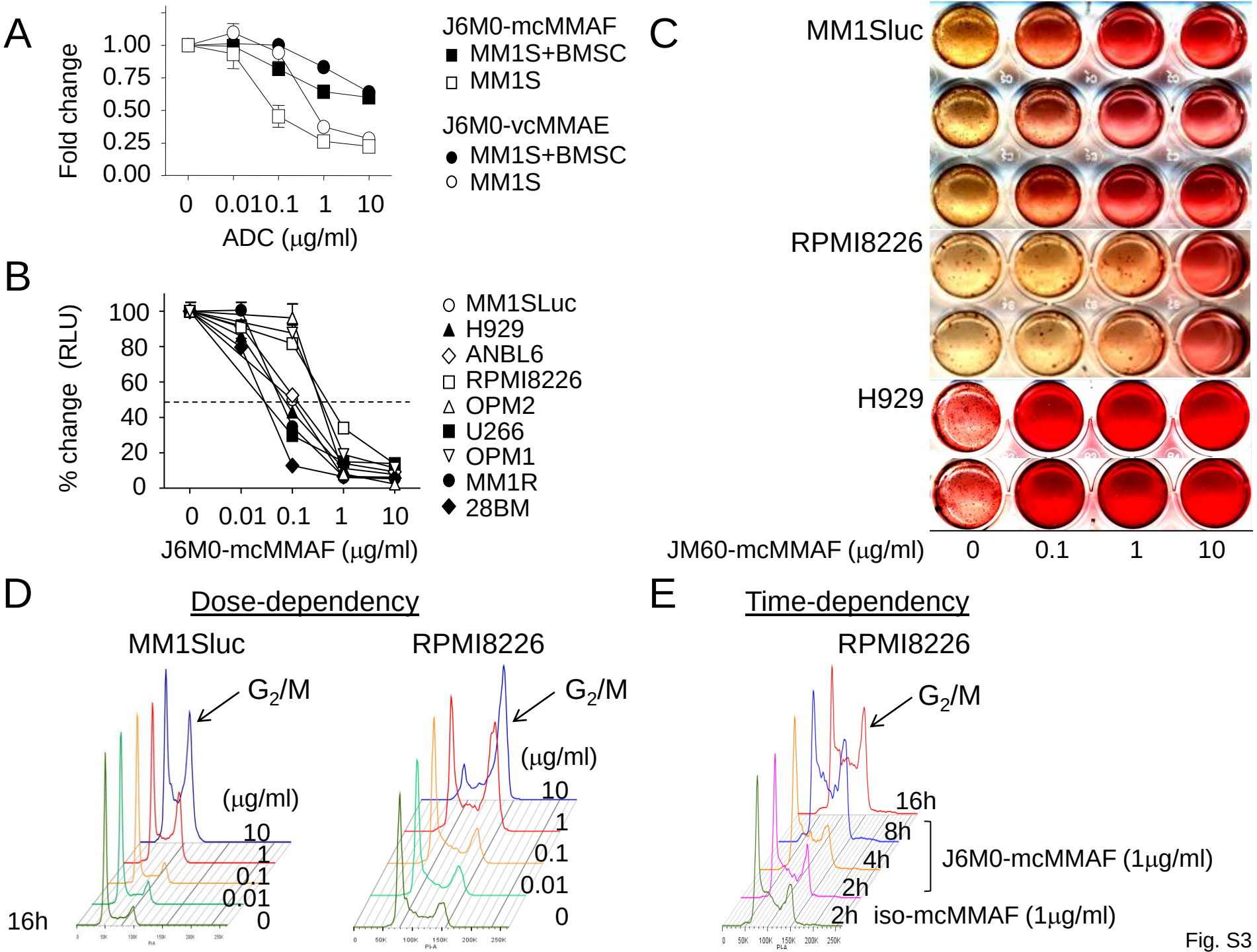

A
Fold change
1.00
0.75
0.50
0.25
0.00
0
0.01
0.1
1
10
ADC (μg/ml)
J6M0-mcMMAF
MM1S+BMSC
MM1S
J6M0-vcMMAE
MM1S+BMSC
MM1S
B
% change (RLU)
100
80
60
40
20
0
0
0.01
0.1
1
10
J6M0-mcMMAF (μg/ml)
MM1SLuc
H929
ANBL6
RPMI8226
OPM2
U266
OPM1
MM1R
28BM
C
MM1Sluc
RPMI8226
H929
JM60-mcMMAF (μg/ml)
0
0.1
1
10
D
Dose-dependency
MM1Sluc
RPMI8226
G2/M
(μg/ml)
10
1
0.1
0.01
0
16h
E
Time-dependency
RPMI8226
G2/M
16h
8h
4h
2h
J6M0-mcMMAF (1μg/ml)
2h
iso-mcMMAF (1μg/ml)

Fig. S3

Fig. S3. J6M0-mcMMAF blocked MM cell viability and colony formation via G_2/M growth arrest and apoptosis. (**A**) MM1S cells were treated with J6M0-mcMMAF or -vcMMAE, in the presence or absence of BMSC for 2d followed by a luminescent cell viability assay. (**B**) MM cell lines were incubated with serial dilutions of J6M0-mcMMAF for 3d. (**C**) J6M0-mcMMAF significantly blocked colony formation of MM cells in 21-d methylcellulose culture. (**D**) MM1Sluc and RPMI8226 (**D-E**) cells were treated with J6M0-mcMMAF for 16h (**D**) or indicated time points (**E**) followed by flow cytometric analysis for cell cycle profile.

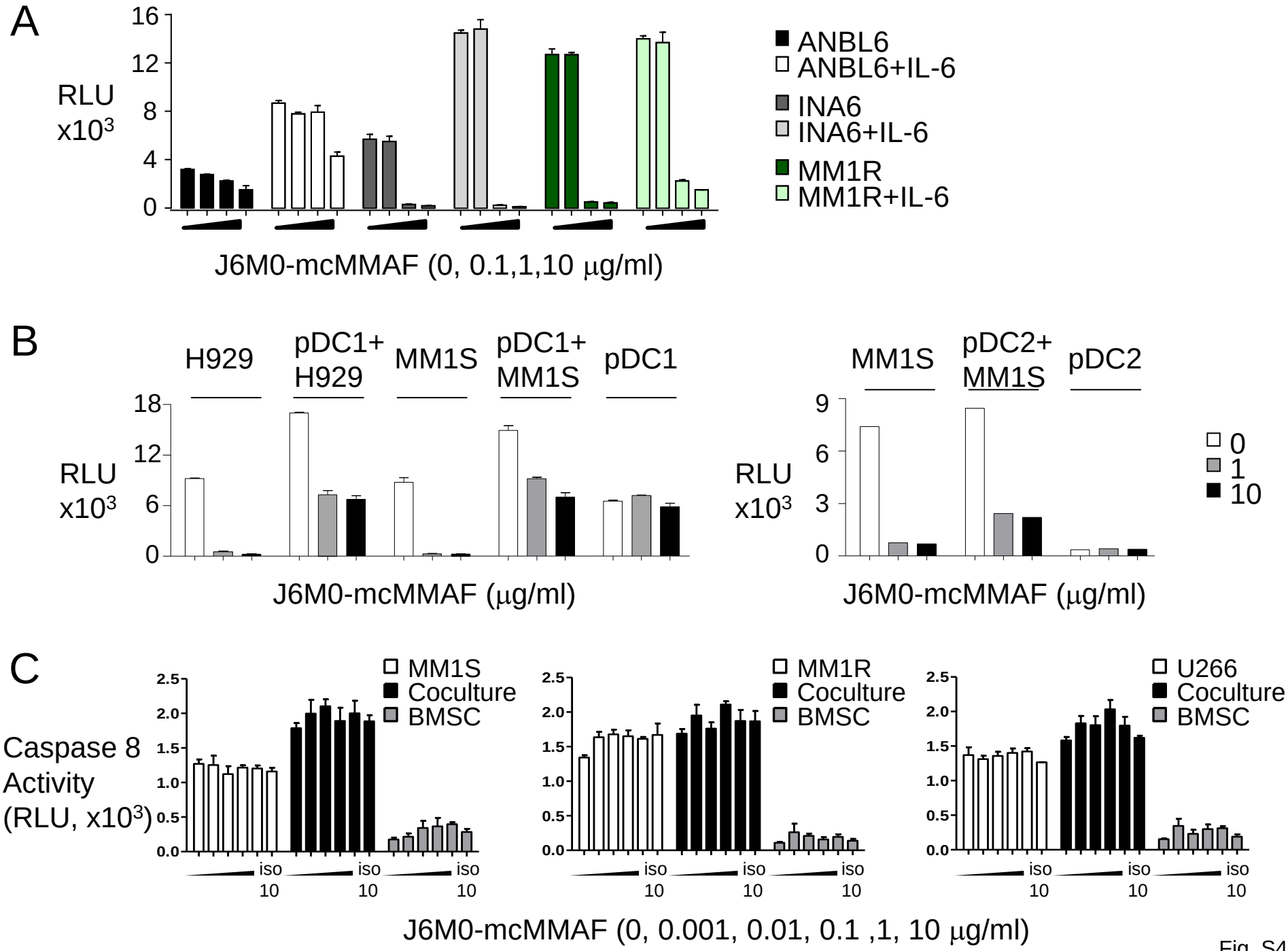
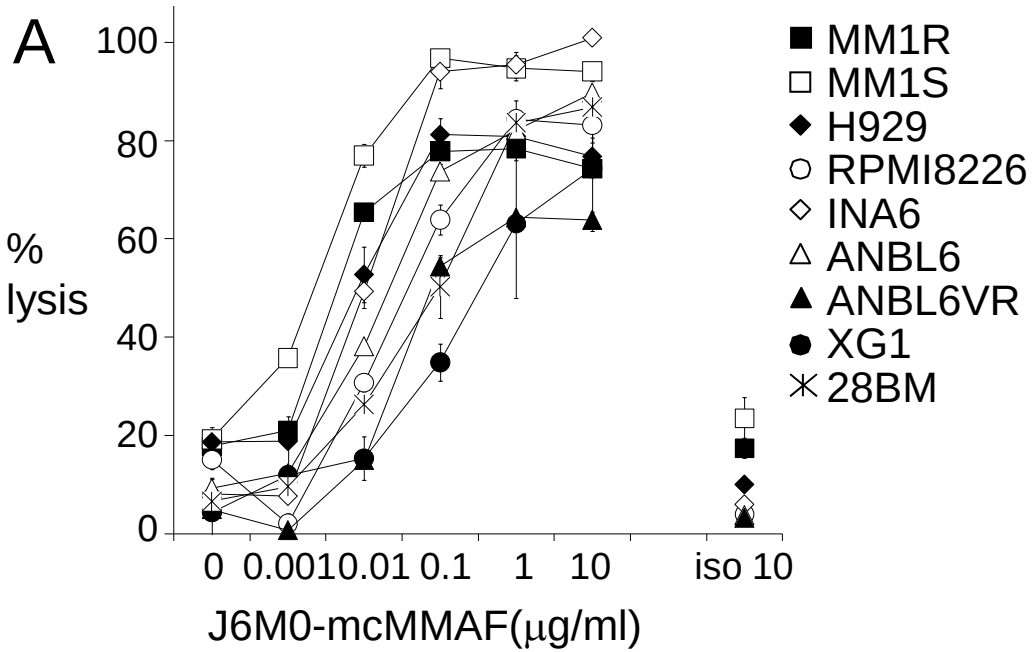
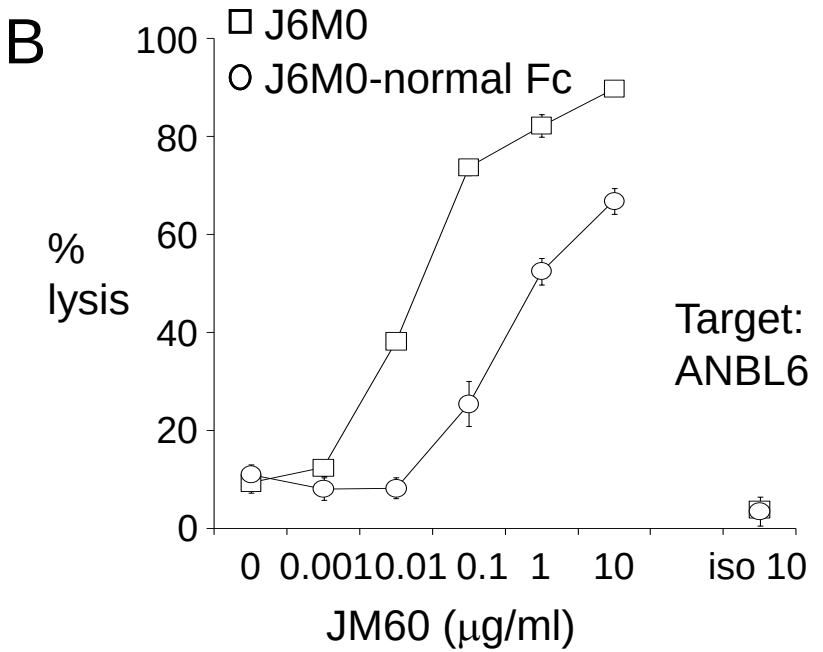

A
RLU x10³
16
12
8
4
0
ANBL6
ANBL6+IL-6
INA6
INA6+IL-6
MM1R
MM1R+IL-6
J6M0-mcMMAF (0, 0.1,1,10 μg/ml)
B
H929
pDC1+ H929
MM1S
pDC1+ MM1S
pDC1
RLU x10³
18
12
6
0
J6M0-mcMMAF (μg/ml)
MM1S
pDC2+ MM1S
pDC2
9
6
3
0
0
1
10
C
Caspase 8 Activity (RLU, x10³)
MM1S
Coculture
BMSC
MM1R
U266
2.5
2.0
1.5
1.0
0.5
0.0
iso
10
J6M0-mcMMAF (0, 0.001, 0.01, 0.1 ,1, 10 μg/ml)

Fig. S4

Fig. S4. IL-6 or pDC could not overcome J6M0-mcMMAF-blocked MM cell viability. (**A**) ANBL6, INA6 (both cells require IL-6 to grow and survive), and IL-6-independent MM1R cells were treated with J6M0-mcMMAF for 3d, in the presence or absence of IL-6. (**B**) H929 and MM1S cells were treated with J6M0-mcMMAF for 4d, in the presence or absence of pDC derived from 2 MM patients (pDC1-2). (**C**) Three J6M0-mcMMAF-treated MM cells (in Fig. 3B) were followed by caspase 8 activity assays. iso 10, iso-mcMMAF (10μg/ml)





	EC_{50} (ng/ml)
MM1R	3.67
MM1S	10.98
H929	5.89
RPMI8226	17.75
INA6	9.45
ANBL6	14.86
ANBL6VR	25.31
XG-1	85.63
28BM	62.31

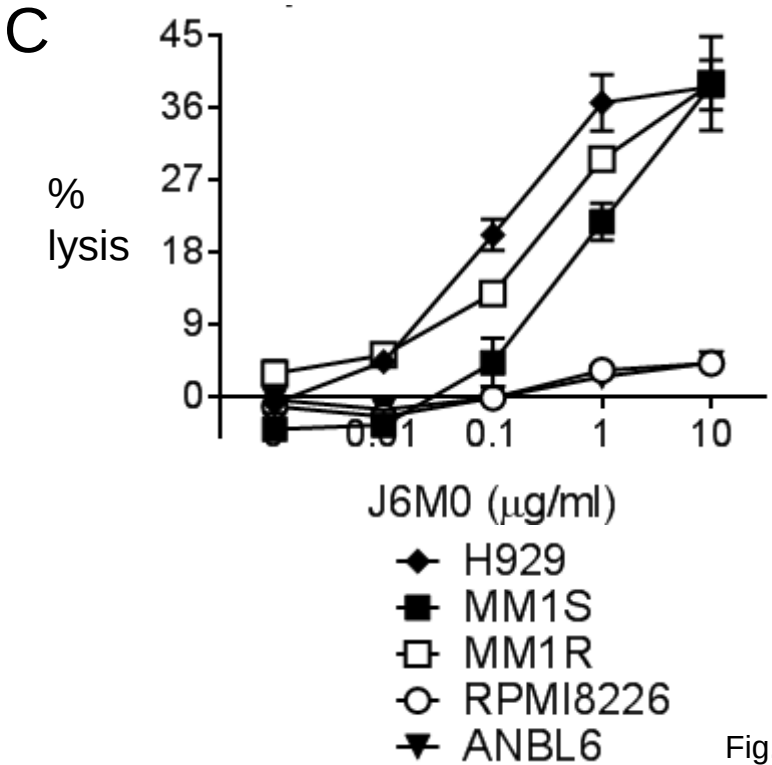


Fig. S5

Fig. S5. J6M0-mcMMAF has strong ADCC activity against various MM cell lines mediated by NK or monocytes. Target MM cell lines (**A**, n=9; **C**, n=5) and ANBL6 cells (**B**) were labeled with calcein-AM, washed, and incubated with indicated mAb and NK (**A**, **B**) or monocyte (**C**) effector cells from healthy donors. Percent lysis was calculated at the end of ADCC assay based on calcein-AM release.

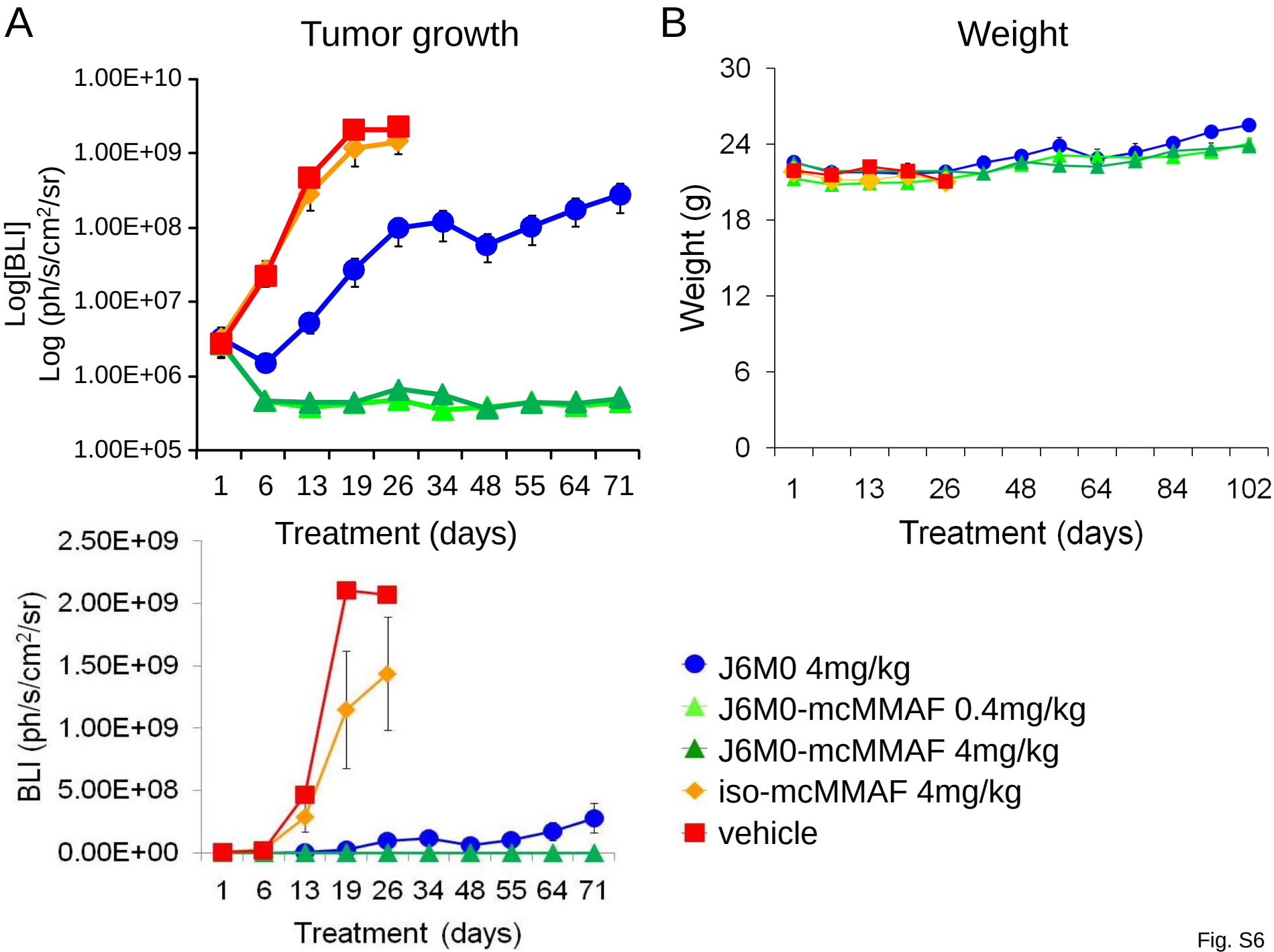

A
Tumor growth
Log[BLI]
Log (ph/s/cm2/sr)
1.00E+10
1.00E+09
1.00E+08
1.00E+07
1.00E+06
1.00E+05
1 6 13 19 26 34 48 55 64 71
Treatment (days)
B
Weight
Weight (g)
30
24
18
12
6
0
1 13 26 48 64 84 102
Treatment (days)
BLI (ph/s/cm2/sr)
2.50E+09
2.00E+09
1.50E+09
1.00E+09
5.00E+08
0.00E+00
1 6 13 19 26 34 48 55 64 71
Treatment (days)
J6M0 4mg/kg
J6M0-mcMMAF 0.4mg/kg
J6M0-mcMMAF 4mg/kg
iso-mcMMAF 4mg/kg
vehicle

Fig. S6

Fig. S6. J6M0-mcMMAF potently depletes MM1Sluc tumor *in vivo* without any weigh loss observed in mice. MM1Sluc tumor growth in bone marrow of SCID-beige mice (**A**, log[BLI] and BLI at the top and lower panel, respectively) and weights of mice (**B**) were followed following various treatments. Light and dark green lines for J6M0-mcMMAF-treated groups are superimposed and below BLI baseline of 3E+06 in all mice (n=8 each group).

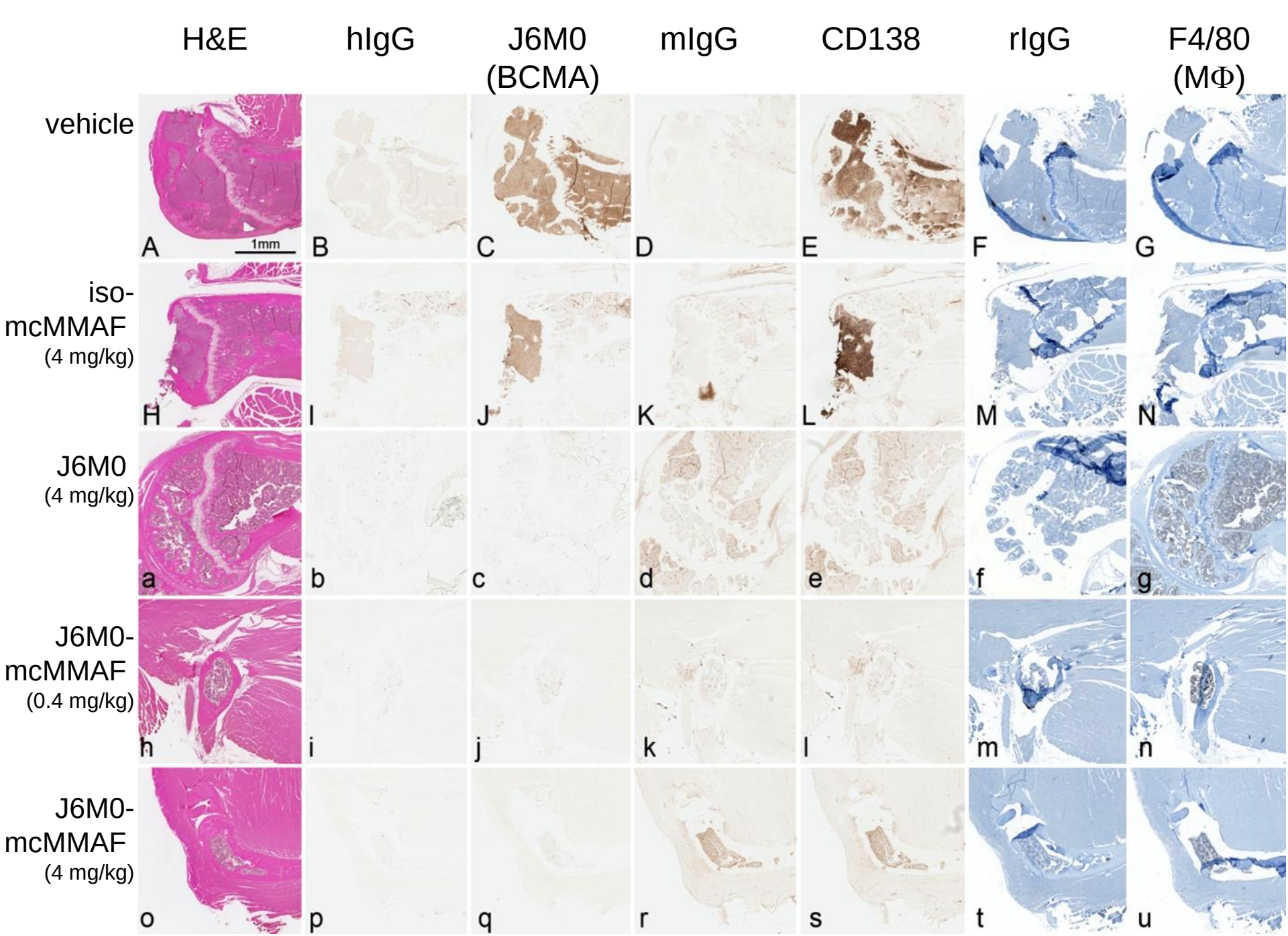

H&E
hIgG
J6M0 (BCMA)
mIgG
CD138
rIgG
F4/80 (MΦ)
vehicle
iso-mcMMAF (4 mg/kg)
J6M0 (4 mg/kg)
J6M0-mcMMAF (0.4 mg/kg)
J6M0-mcMMAF (4 mg/kg)
1mm
A
B
C
D
E
F
G
H
I
J
K
L
M
N
a
b
c
d
e
f
g
h
i
j
k
l
m
n
o
p
q
r
s
t
u

Fig. S7

Fig. S7. IHC studies for bone tissues derived from the MM1Sluc bone marrow dissemination model. Long bone tissue sections from vehicle (A-G), iso-mcMMAF (4mg/kg) (H-N), J6M0 (4mg/kg) (a-g), J6M0-mcMMAF (0.4mg/kg) (h-n), and J6M0-mcMMAF (4mg/kg) (o-u) treated animals stained by H&E (A,H,a,h,o) and by IHC with human IgG (hIgG: B,I,b,i,p), J6M0 (C,J,c,j,q for BCMA), murine IgG (mIgG: D,K,d,k,r), murine anti-human CD138 antibody (E,L,e,l,s), rat IgG & haematoxylin counterstain (rIgG: F,M,f,m,t), or F4/80 & haematoxylin counterstain (G,N,g,n,u for MΦ) - scale bar = 1mm