SUPPLEMENTARY APPENDIX

Due to interleukin-6 type cytokine redundancy only glycoprotein 130 receptor blockade efficiently inhibits myeloma growth

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SUPPLEMENTARY METHODS

Cell lines and culture

Cell lines INA-6, INA-6.Tu1 and B9 were cultivated in RPMI-1640 with GlutaMax™-I, 25 mM HEPES (Gibco®/Life Technologies GmbH, Darmstadt, Germany), 10% (v/v) heat-inactivated fetal bovine serum (FBS) (HyClone; Perbio Science, Erembodegen, Belgium), and antibiotics ($R10^+$ medium) supplemented with 2.5 ng/ml recombinant huIL-6 (Gibco®/Life Technologies GmbH, Darmstadt, Germany). The cell lines are routinely confirmed to be negative for mycoplasma contamination (Venor™GeM Mycoplasma Detection Kit, Sigma-Aldrich, St. Louis, MO).

Cytokines and other reagents

Recombinant huIL-6 was purchased from Gibco®/Life Technologies (Darmstadt, Germany), huLIF was from Reliatech (Wolfenbüttel, Germany). Recombinant muIL-6 was obtained from Peprotech (Rocky Hill, NJ), and soluble muIL-6R was from R&D Systems (Minneapolis, MN). The pan-JAK inhibitor-I (pyridone 6) was purchased from Calbiochem®/Merck Biosciences (Schwalbach/Ts, Germany). Mouse mAb TH69 against the T cell marker CD7 was generated in our laboratory and is of IgG1 isotype.[1]

Cytogenetic and FISH analysis

Metaphases were R-banded according to standard methods and karyotypes analysed at the approximately 300-band level. Karyotypes were described according to the International System for Human Cytogenomic Nomenclature (ISCN 2016) and revised using the "CyDAS Online Analysis Site".[2] Interphase FISH was performed as described previously[3] using

commercially available LSI 1p36/1q25 and LSI 19q13/19p13 Dual-Color Probe, LSI MYC Dual Color Break Apart Rearrangement Probe, IGH/CCND1 XT Dual Color Dual Fusion Translocation Probes, LSI IGH/FGFR3 Dual Color Dual Fusion Probes (all from Abbott-Vysis, Downers Grove, IL), PN13 (13q14), and PN21 p53 (17p13) / MPO (17q22) "ISO 17q" (all from Kreatech, Amsterdam, The Netherlands) as well as with assays for the detection of 1q23.1 (*IRTA1/2* gene) copy number changes.[3] For each hybridization, 100 interphase cells were analysed. Digital image acquisition, processing, and evaluation were performed using ISIS digital image analysis version 5.0 (MetaSystems, Altussheim, Germany). For the assessment of the karyotype complexity score (CS), numerical and structural chromosomal aberrations were counted only once irrespective if the same aberration was present more than once (e.g. del(1)(p22)x3).

Flow cytometry

Cell surface cytokine receptor expression was analysed by flow cytometry using the QIFIKIT according to the manufacturer's instructions (Dako, Glostrup, Denmark). Briefly, 2 x 10^5 cells were stained with antibodies B-B4 (CD138/syndecan-1; BeckmanCoulter), B-R6 (CD126/IL-6R), B-R3 (CD130/gp130) (both from Diaclone), and an antibody against the LIFR/CD118 (clone 32943; R&D Systems). Samples were analysed with a NAVIOS™ flow cytometer and data assessed using the Kaluza 1.1 software (BeckmanCoulter).

Cell growth assay

Briefly, 1-3 x 10^4 cells were cultivated in 96-well flat-bottom plates in a final volume of 200 µl medium for 72 h. MTS reagent was added for the last 3-4 h of culture time and absorbance measured at 490 nm. Values represent the mean of triplicate or quadruplicate cultures.

Cell lysis and Western blotting

Approx. 5 x 10^6 cells were resuspended in 100 µl lysis buffer containing 50 mM Tris-HCl pH 7.5, 120 mM NaCl, 20 mM NaF, 1 mM EDTA, 6 mM EGTA, 15 mM sodium pyrophosphate, 1 mM benzamidine, 1% Triton X-100, 0.5 mM sodium orthovanadate, 0.1 mM phenylmethyl-sulphonylfluoride and 15 mM para-nitrophenyl phosphate. After incubation on ice for 30 min, lysates were zentrifuged at 13,000 rpm for 10 min at 4°C and supernatants recovered. Equal amounts of protein were mixed with Laemmli buffer, heated up to 95°C for 5 min and resolved by standard SDS-polyacrylamide gel electrophoresis. Proteins were transferred onto Immuno-Blot PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking in 5% non-fat dry milk in TBS containing 0.1% Tween-20 (TBS-T), membranes were stained with antibodies (against Tyr705-phosphorylated STAT3 (3E2), Thr202/Tyr204-phosphorylated ERK1/2 (E10) and Ser235/236-phosphorylated S6 ribosomal protein (D57.2.2E). After stripping, membranes were reprobed with antibodies against total STAT3 (79D7), ERK1/2 (9102), and S6 protein (54D2). Staining for ß-tubulin (9F3) served as loading control. All antibodies were from Cell Signaling Technology (Danvers, MA) and applied overnight at 4°C. Peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG antibodies (Jackson ImmunoResearch Lab., West Grove, PA) and SuperSignal® West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL) served for detection (Chemidoc XRS Imager, BioRad).

Animal Studies

Female SCID (CB17/Icr-Prkdc^{scid}/Crl) or SCID/beige (CB17.B6-Prkdc^{scid}Lyst^{bg}/Crl) mice (Charles River, Sulzfeld, Germany) were housed under SPF conditions and provided with food and water *ad libitum*. All animal experiments were performed according to the FELASA guidelines and with institutional approval (reference number V312-72241.121-4(72-8/08)). SCID mice were irradiated (2 gray) the day before cell inoculation. In general, 25 x 10^6 INA-6.Tu1 plasma cells in approx. 500 µl sterile PBS were injected intraperitoneally (i.p.). Treatment started one day after cell inoculation. All antibodies were adjusted to a concentration of 1 mg/ml and administered by i.p. or, in the case of F(ab')$_2$ fragments, alternating i.p./intravenous (i.v.) injections. Antibodies were administered 7 times during 14 days, a total of 0.85 mg antibody per mouse with a single dose equaling approx. 6 mg/kg body weight. The fusion protein sgp130Fc was injected i.p. once a week for 4 weeks, 150 µg protein per dose (approx. 7.5 mg/kg). Mice were monitored on a routine basis for signs of tumor development, starting at the site of injection. The survival time was defined as the time between cell inoculation and day of sacrifice when tumor burden caused paraplegia, cachexia, or any other signs of suffering. Animals without any signs of tumors were sacrificed at the end of the experiment, on day 136 latest. Some tumors were explanted and cells retrieved for further studies.

Statistical analysis

For in vitro experiments, an unpaired Student's *t*-test was used to determine the statistical significance of differences between groups. A two-tailed p-value below 0.05 was considered significant. Survival of the mice was analysed according to the Kaplan–Meier method and a log-rank (Mantel-Cox) test was applied to determine differences between groups using the

GraphPad Prism 6.0 software. Survival benefit was determined as significant if log-rank was $p < 0.05$.

1. Baum W, Steininger H, Bair HJ, et al. Therapy with CD7 monoclonal antibody TH-69 is highly effective for xenografted human T-cell ALL. Br J Haematol. 1996;95(2):327-338.

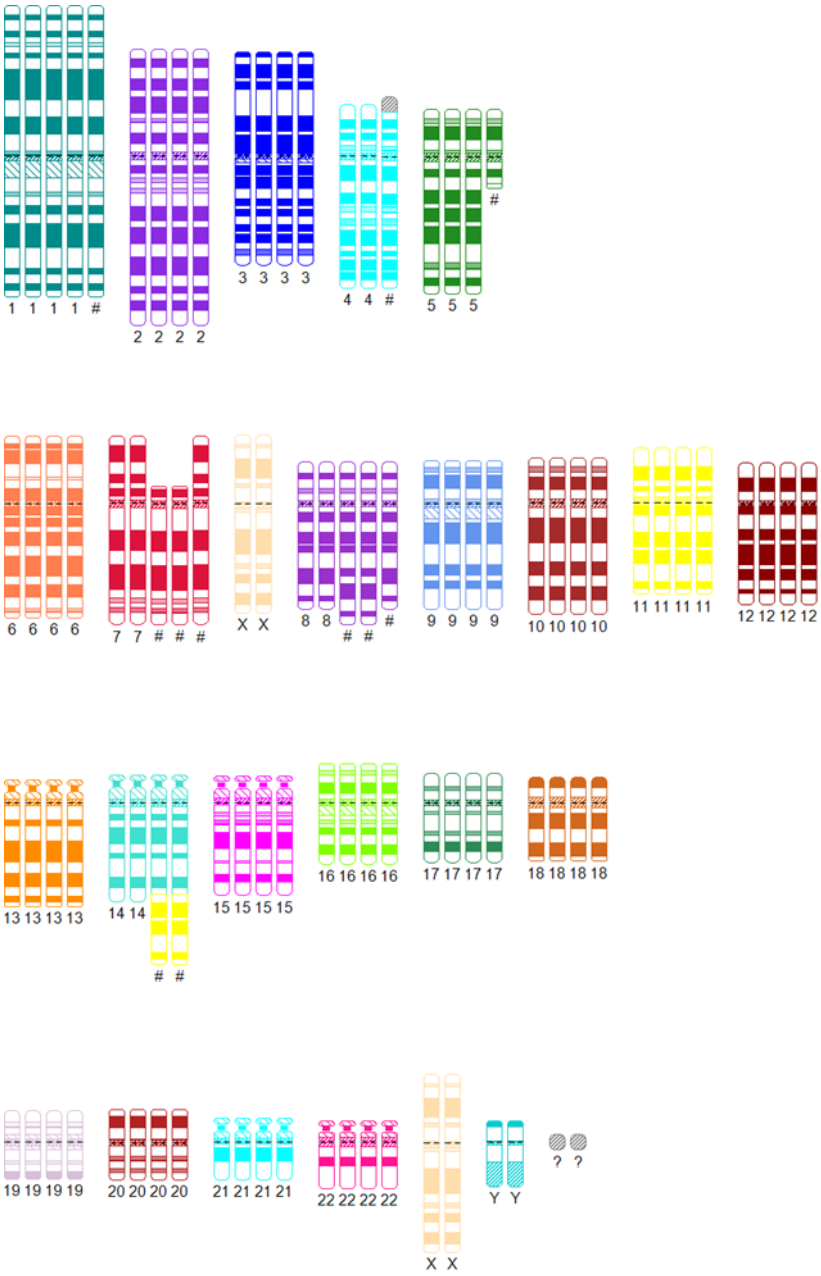
2. Hiller B, Bradtke J, Balz H, Rieder H. "CyDAS Online Analysis Site", 2004. http://www.cydas.org/OnlineAnalysis/

3. Aukema SM, Theil L, Rohde M, et al. Sequential karyotyping in Burkitt lymphoma reveals a linear clonal evolution with increase in karyotype complexity and a high frequency of recurrent secondary aberrations. Br J Haematol. 2015;170(6):814-825.

SUPPLEMENTARY FIGURES

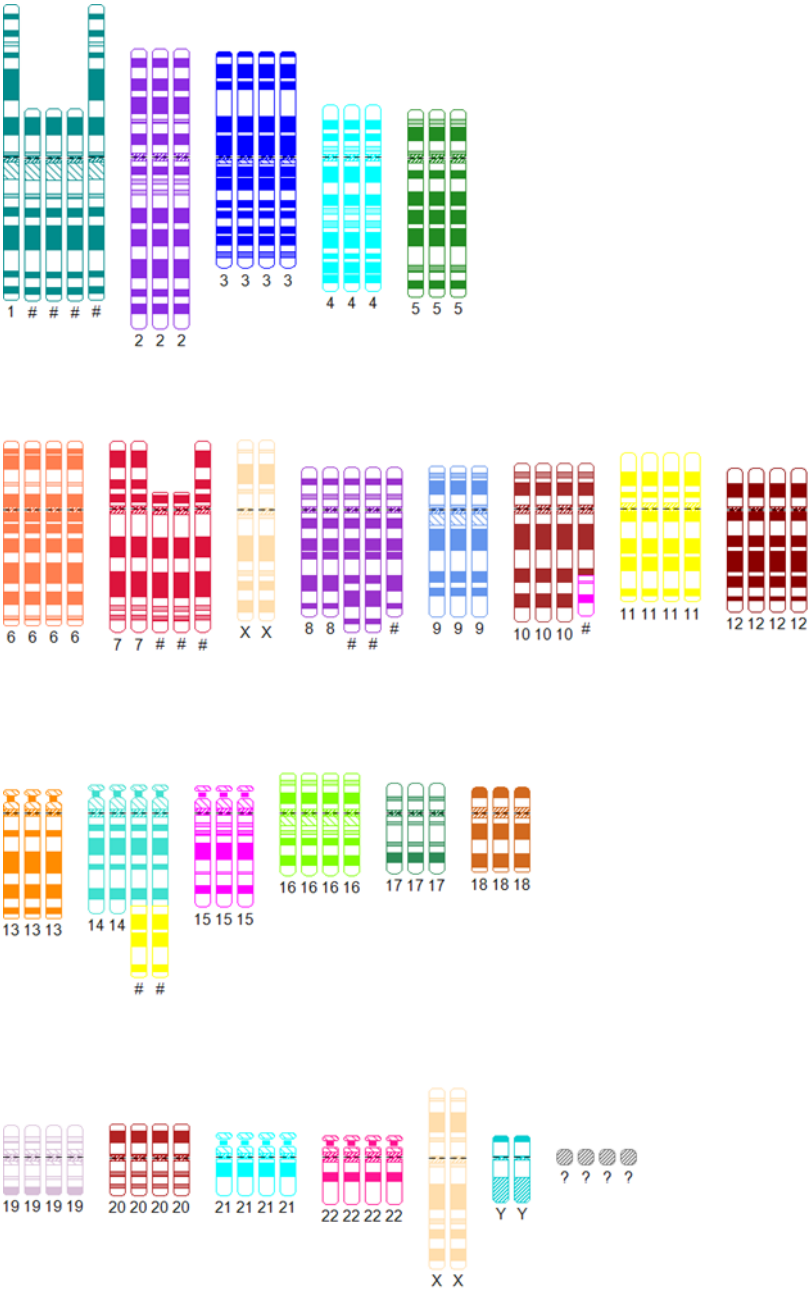
Figure S1. Cytogenetic analysis of INA-6 and subline INA-6.Tu1

INA-6



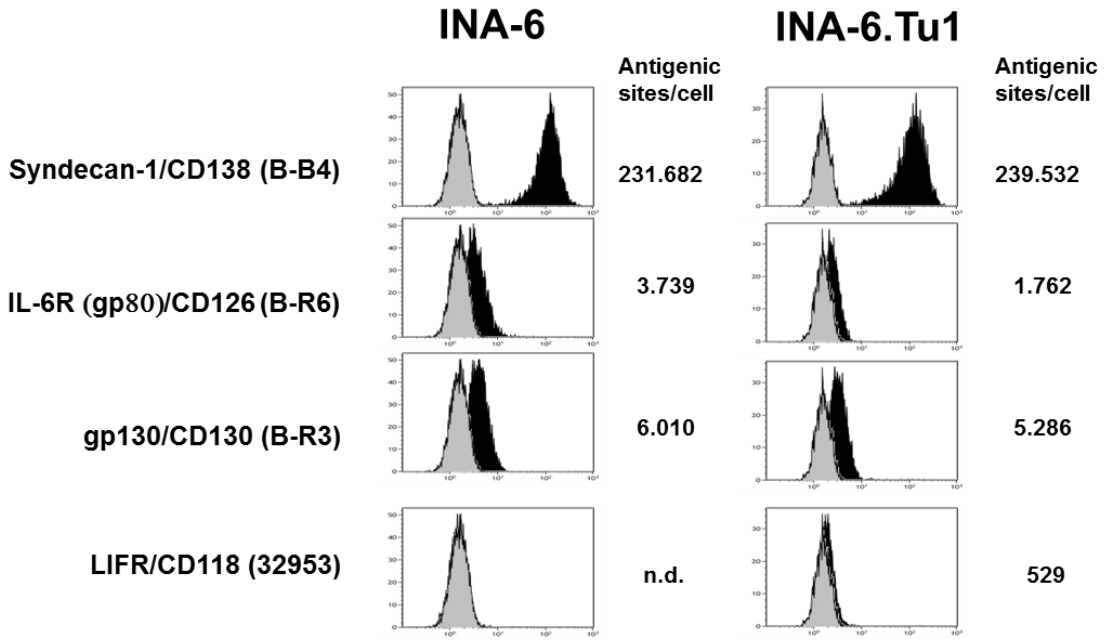
71~92,XXYY,+1,−4,add(4)(p16),del(5)(q13q35),+7,del(7)(p13)x2,+8, dup(8)(q22q23)x2, der(14)t(11;14)(q13;q32)x2,+mar1,+mar2[cp8]. ish **add(4)(p16)** (**MMSET/FGFR3++**). nuc ish 1p36 (**EGFL3/TP73 x 4~5**), 1q (**IRTA1/2 prox x 4~5, IRTA1/2 dist x 4~5, ANGPTL1/ABL2 x 4~5**), 4p16 (**MMSET/FGFR3 x 4**), 8q24 (**MYC prox x 4~5, MYC dist x 4~5**), 11q13 (**CCND1 x 6**), 13q14 (**RB1 x 4**), 14q32 (**IGH x 4**), 17 (**P53 x 4, MPO x 4**), 21q (**LSI 21 x 4**)(**CCND1 con IGH x 2**)

INA-6.Tu1



69~89,XXYY,+1,del(1)(p22)x3,−2,−4,−5,+7,del(7)(p13)x2,+8,dup(8)(q22q23)x2,−9, der(10)t(10;15)(q23;q22),−13,der(14)t(11;14)(q13;q32)x2,−15,−17,−18,+mar1,+mar3,+mar4, +mar5[cp6]. ish 4p16 (**MMSET/FGFR3 x 3**). nuc ish 1p36 (**EGFL3/TP73 x 3~4**), 1q (**IRTA1/2 prox x 4~5, IRTA1/2 dist x 4~5, ANGPTL1/ABL2 x 4~5**), 8q24 (**MYC prox x 4~5, MYC dist x 4~5**), 11q13 (**CCND1 x 6**), 13q14 (**RB1 x 4**), 14q32 (**IGH x 3~4**), 17 (**P53 x 3~4, MPO x 3~4**), 21q (**LSI 21 x 4**)(**CCND1 con IGH x 2**)

Figure S2. Cytokine receptor expression.



Cell surface cytokine receptor expression was analysed by flow cytometry and quantitated using the QIFIKIT (Dako, Glostrup, Denmark). Cells were stained with specific receptor antibodies (black histograms) and an irrelevant antibody (clone TH69) as control (grey histograms). For comparison, cells were stained with an antibody against CD138/syndecan-1. Names of the antibody clones are given in brackets. The specific antibody-binding capacity units of individual antibodies correspond to the mean number of accessible antigenic sites per cell. n.d., not detectable.