Supplementary Materials

Materials and Methods

Cell lines

The cell lines maintained at Sloan Kettering included JMN, VAMT, H2452, H2373, MSTO, Meso 34, Meso 37, Meso 47, and Meso 56. Other lines maintained at Sloan Kettering were the human AML lines HL60, SET2, AML14, B ALL lines Daudi and BA25, CC228 colon, SKOV3 ovarian, RMS-13 sarcoma, OVCAR3 ovarian, and T cell line T2. All cells were HLA typed by the Department of Cellular Immunology at Memorial Sloan-Kettering Cancer Center. Leukemia cell lines LAMA81, BV173, and 697 (all $WT1^+/A0201^+$) were kindly provided by H. J. Stauss (University College London). SKLY16 (B cell lymphoma; $WT1^-/ A0201^+$); K562 and BA25 (both $A0201^+$), and HL60 (A0201-), all $WT1^+$ leukemias, and a TAP-deficient T2 cell line were obtained from the American Type Culture Collection. The cell lines were cultured in RPMI 1640 supplemented with 5% FCS, penicillin, streptomycin, 2 mM glutamine, and 2-mercaptoethanol (β2M) at 37°C/5% CO_2.

ScFv clones specific for WT1 peptide/HLA-A0201 complexes

A human ScFv antibody phage display library (7×10^{10} clones) was used for the selection of mAb clones. Selection and characterization of the ScFv, as well as engineering full-length mAb using the selected ScFv fragments are described in Supplementary methods. In order to reduce the conformational change of MHC1 introduced by immobilizing onto plastic surfaces, a solution panning method was used in place of conventional plate panning. In brief, biotinylated negative antigens (A0201/RHAMM-R3) were first mixed with the human scFv phage library, then the antigen-scFv phage antibody complexes were pulled down by streptavidin-conjugated Dynabeads M-280 through a magnetic rack and discarded. A repeat of this process on A0201/RMF monomers was then conducted. The clones bound to the positive antigen were then eluted and were used to infect *E. coli* XL1-Blue. The scFv phage clones expressed in the bacteria were purified (*42*, *43*). The panning was performed for 3-4 cycles to enrich scFv phage clones binding to HLA-A0201/WT1 complex specifically. Positive clones were determined by standard ELISA method against biotinylated single chain HLA-A0201/WT1 peptide complexes.

The positive clones were further tested for their binding to HLA-A2/peptide complexes on live cell surfaces by flow cytometry, using a TAP-deficient, $HLA\text{-}A0201^+$ human cell line, T2. T2 cells were pulsed with positive and multiple control peptides (table S1) peptides (50 μg/ml) in the serum-free RPMI1640 medium, in the presence of 20 μg/ml β2M overnight. The cells were washed, and the staining was performed in following steps. The cells were first stained with purified scFv phage clones, followed by staining with a mouse anti-M13 mAb, and finally by goat F(ab)'2 anti-mouse Ig conjugated to FITC. Each step of the staining was done for 30-60 minutes on ice, and the cells were washed twice between each step of the staining.

Engineering full-length mAb using the selected ScFv fragments

Full-length human IgG1 of the selected phage clones were produced in HEK293 and Chinese hamster ovary (CHO) cell lines, as described (*44*). ESK1 was derived from one of these clones. In brief, antibody variable regions were subcloned into mammalian expression vectors, with matching Lambda or Kappa light chain constant sequences and IgG1 subclass Fc. Molecular weight of the purified full-length IgG antibodies were measured under both reducing and non-reducing conditions by electrophoresis.

Radioimmunoassays and kinetics

ESK1 was labeled with ^{125}I (PerkinElmer) using the chloramine-T method (*45-46*). Antibody (100 µg) was reacted with 1 mCi ^{125}I and 20 µg chloramine-T, quenched with 200 µg Na metabisulfite, then separated from free ^{125}I using a 10DG column (company) equilibrated with 2% bovine serum albumin in PBS. Specific activities of products were in the range of 4-8 mCi/mg.

Hematopoietic cell lines, adherent cell lines (harvested with non-enzymatic cell dissociation solution (Sigma), PBMCs from normal donors and AML patients were obtained as described above. Cells were washed once with PBS and re-suspended in 2% human serum in PBS at 10^7 cells/ml on ice. Cells (10^6 per tube, in duplicate) were incubated with ^{125}I-labeled ESK1 (1 µg/ml) for 45 minutes on ice, then washed extensively with 1% bovine serum albumin in PBS on ice. To determine specific binding, a duplicate set of cells was assayed after pre-incubation in the presence of 50-fold excess unlabeled ESK1 for 20 minutes on ice. Bound radioactivity was measured by a gamma counter, specific binding was determined, and the number of bound antibodies per cell was calculated from specific activity. Avidity was then determined.

Complement-dependent cytotoxicity (CDC)

BV173 or 697 cells (5×10^4) in RPMI 1640 media were incubated 90-120 min with 10 µg/ml ESK1 mAb or isotype control mAb or positive control mAb W632 (anti-human HLA) and baby rabbit complement (titrated to be non-cytotoxic alone at 1:6 or 1:9 dilution) and assayed by trypan blue exclusion by microscopy. Alternatively, ESK1 was incubated with cells and NSG mouse serum overnight. These assays were confirmed as well with human serum complement (1:5 dilution) using an LDH release assay (Promega) or trypan blue. Complement sources from mouse, human and rabbit were used for other CMC experiments.

Cell viability and antibody crosslinking assays

BV173 or JMN cells were plated in black 96-well flat-bottom plates (10^4 cells/well) and incubated with either ESK1 or isotype control antibody (30 ng – 30 µg/ml) for 24, 48, or 72 hours. For crosslinking assays, BV173 cells were first incubated with ESK1 or isotype control at various concentrations, washed and re-suspended in complete RPMI media, then added to plates containing goat-anti-human antibody (Rockland Immunochemicals) either soluble or absorbed. In other experiments, ESK1 was adsorbed first to the plate to allow cross-linking as well. Primary mAb was used at concentrations of 0.1 to 30 µg/ml and secondary antibody was used at ratios of 0.1:1 up to 10:1. In other assays, mAb was repeatedly added every day. Cell viability was assessed by ATP content using an ATP-Lite assay (Perkin Elmer).

Antibody-dependent cellular phagocytosis (ADCP)

Four methods were used based on previous studies (*2*, *47*, *48*).

- (Method 1) NSG mouse peritoneal macrophages were obtained and incubated with CFSE-labeled target cells with or without ESK1 or isotype control mAb for 2 or 4 hours before examining by microscopy for phagocytosis.
- (Method 2) Carboxyfluorescein diacetate succinimidyl ester (CFSC)–labeled target cells were mixed with mouse macrophages for 2 or 4 hours or overnight and were stained with anti-mouse CD11b mAb or F4/80 conjugated with (Phycoerythrin (PE) or allophycocyanin (APC), then examined by flow cytometry or fluorescence microscopy.
- (Method 3) CFSC-labeled BV173 cells were injected into mice with mAbs for 2 hours or overnight and then spleens and peritoneal macrophages were harvested and stained with mAb to mouse macrophage markers, such as CD11b/PE, and phagocytosis was examined by flow cytometry or fluorescence microscopy.
- (Method 4) CFSC-labeled BV173 cells were injected into mice with the mAb and at 4, 24 and 72 hours, spleens and livers of mice were stained with anti-mouse CD11b mAb, and examined by

immunohistochemistry for the presence of phagocytosis.

Figures

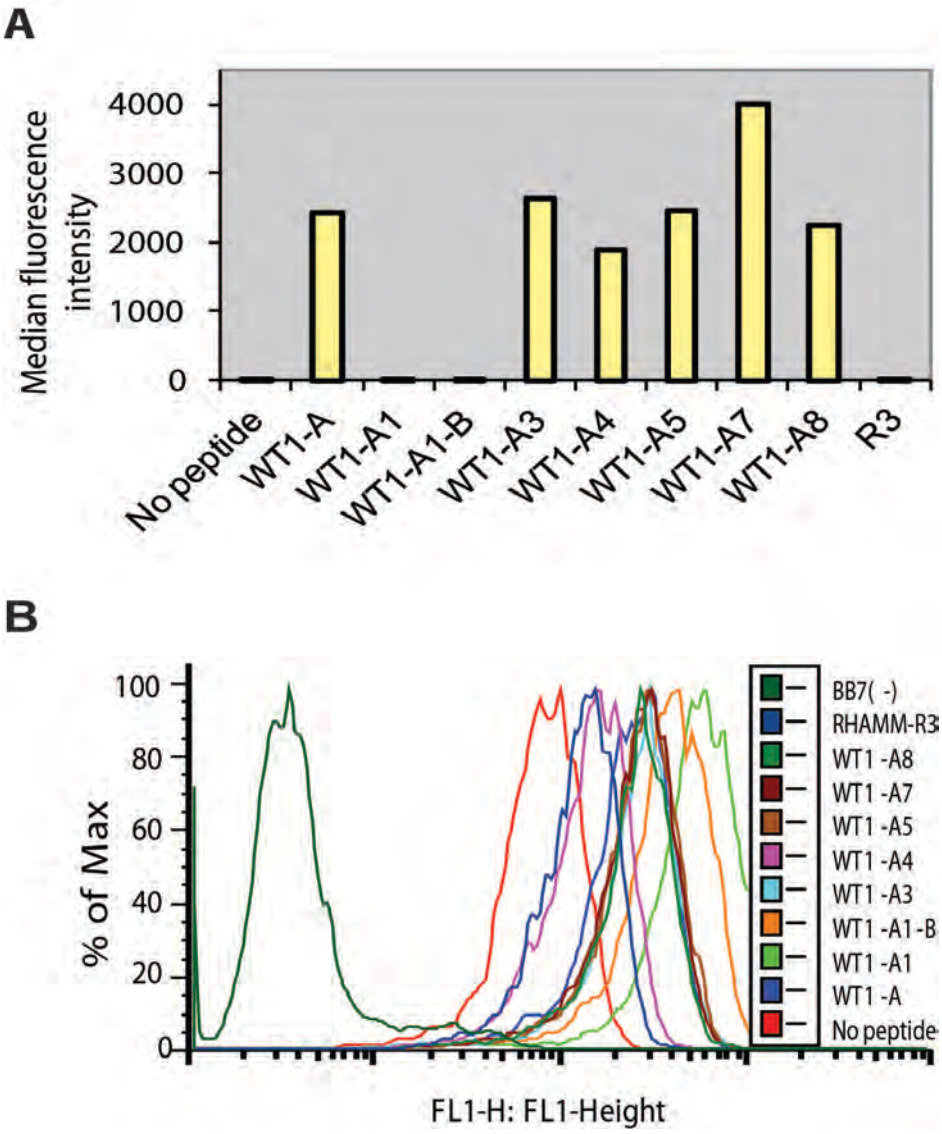


Figure S1. Epitope specificity. The RMF peptide sequence was substituted with alanine at positions 1, 3, 4, 5, 7, or 8, or with tyrosine (WT1-A1B) at position 1 (sequences in table S1.). (**A**) T2 cells were pulsed with indicated peptides at 50 µg/ml and the binding of ESK1 was measured by flow cytometry. (**B**) In order to show that the analog peptides still bound to HLA-A0201, cells were simultaneously stained with anti-HLA-A2 mAb, clone BB7.2 to measure the relative binding of the peptides to HLA-A2 molecule.

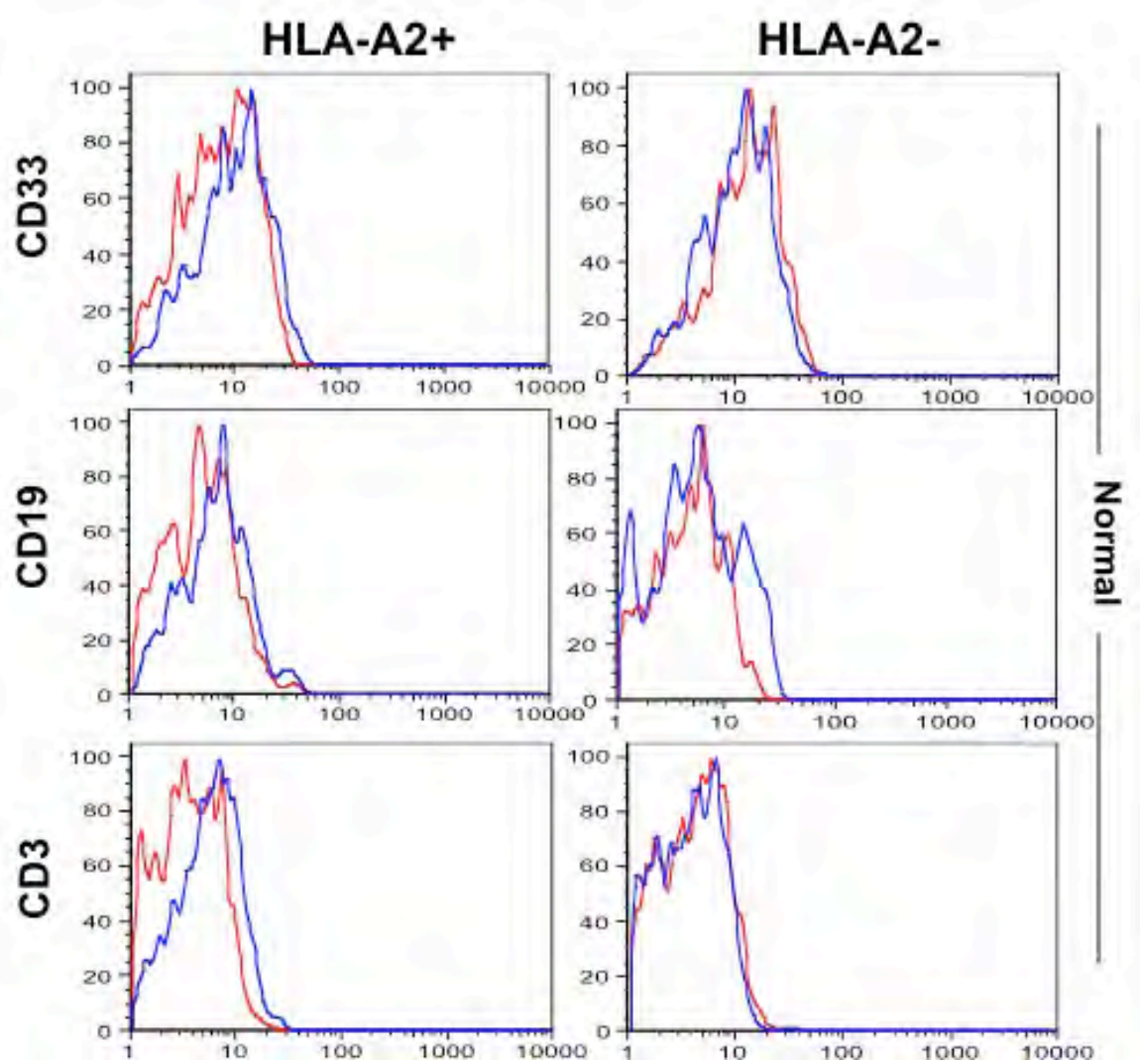


Figure S2. Binding of ESK1 to PBMCs from healthy donors. PBMCs from HLA-A0201^{+} or HLA-A0201^{-} healthy donors were directly stained with ESK1-APC or isotype human IgG1-APC or mAbs to human CD3, CD19, and CD33. Cells were analyzed by flow cytometry. The data show the histogram overlay of the ESK1 (red line) vs hIgG1 (blue line) staining on gated CD33^{+}, CD19^{+}, or CD3^{+} populations. One representative example is shown for 16 different donors tested (n = 9 A02^{+} and 7 A02^{-}).

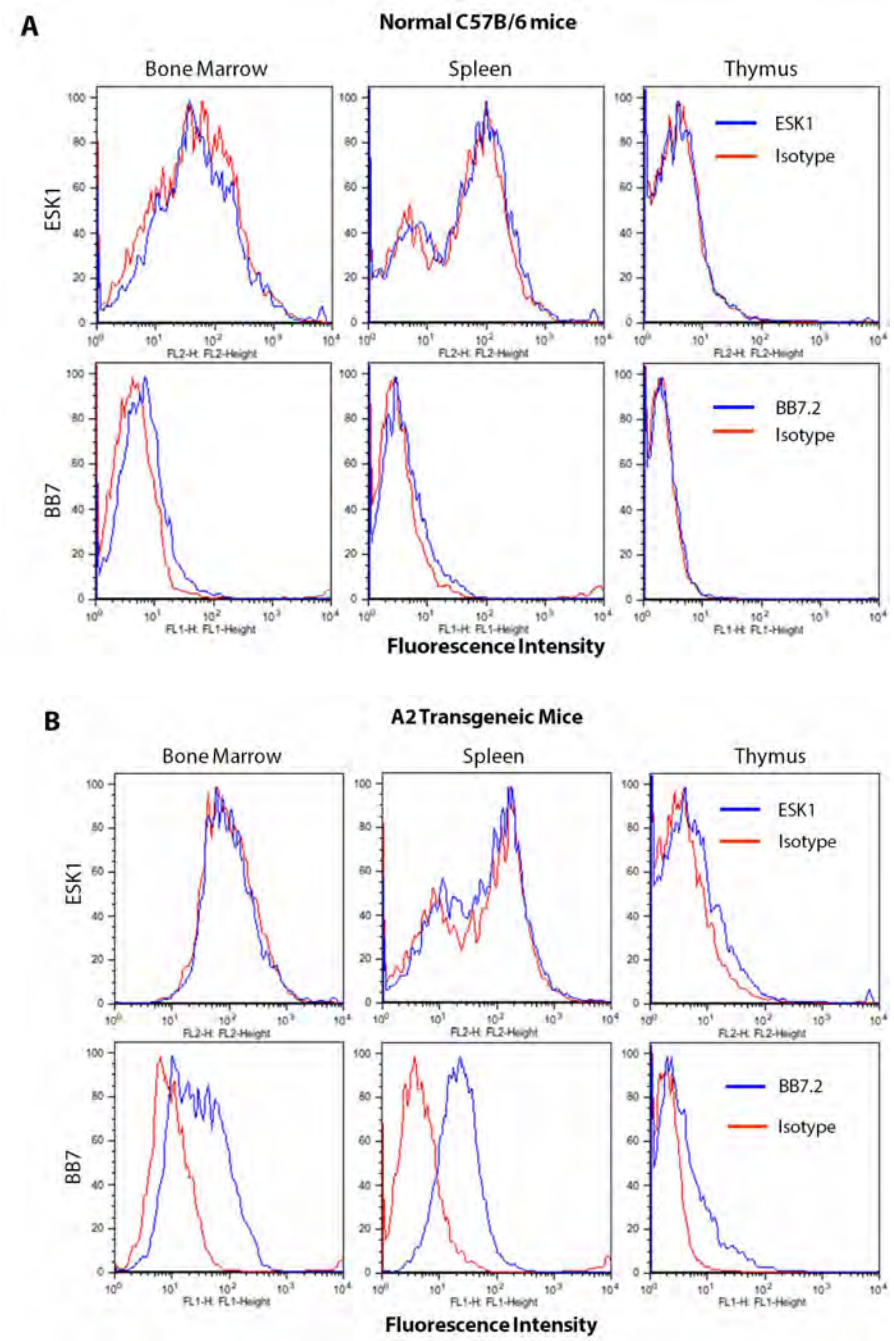


Figure S3. Binding of ESK1 to cells from HLA-A0201 transgenic mice. Single cell suspensions were studied from the spleen, bone marrow, and thymus were isolated from C57BL/6 (**A**) and C57BL/6-HLA-A0201 transgenic mice (**B**). The *WT1* gene with the same RMF sequence as in humans is present in this strain of mice. The cells were stained with BB7.2/FITC, isotype mIgG2b, or mAb ESK1, or its isotype control human IgG1 (all 10 µg/ml), and were analyzed by flow cytometry. The data are representative of four wild-type BL/6 mice and six HLA-A0201 transgenic BL/6 mice.

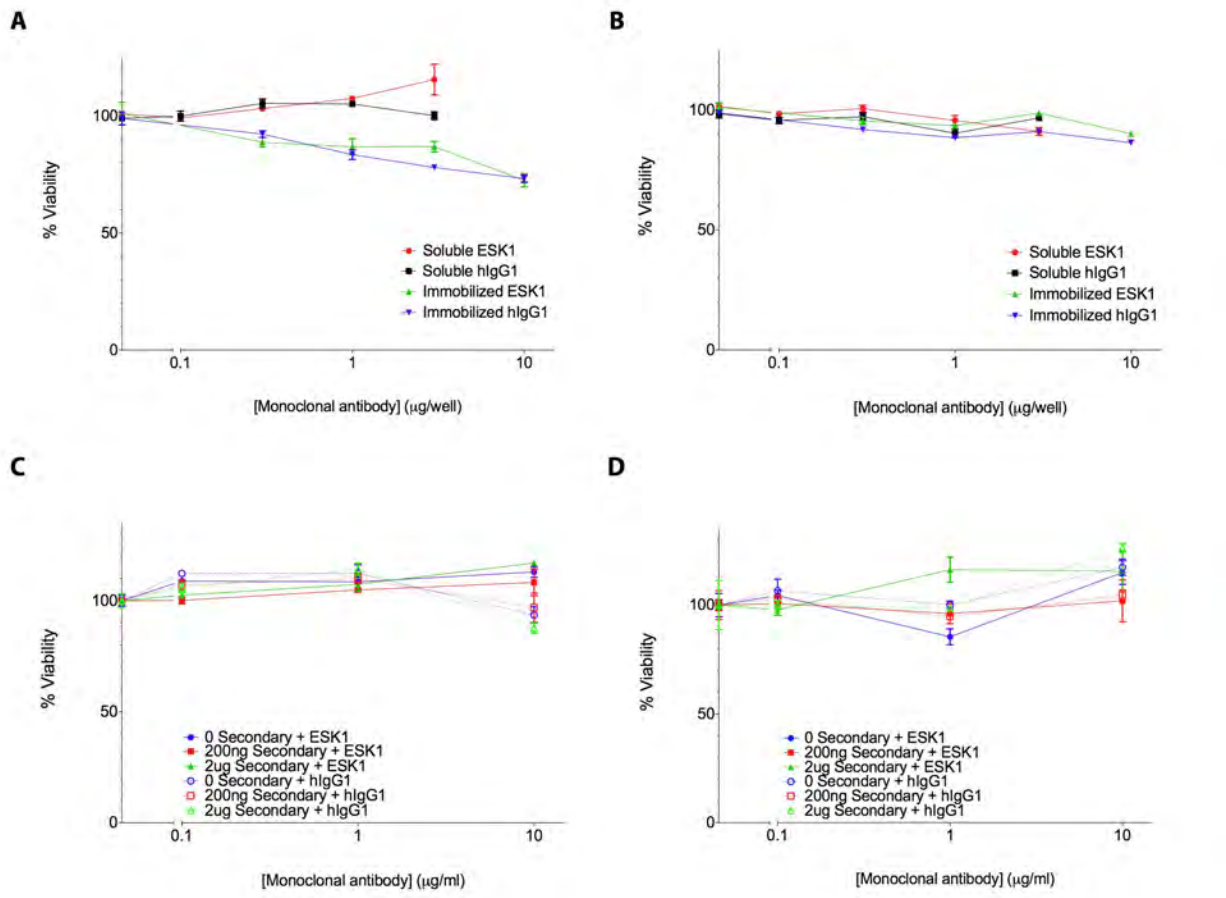
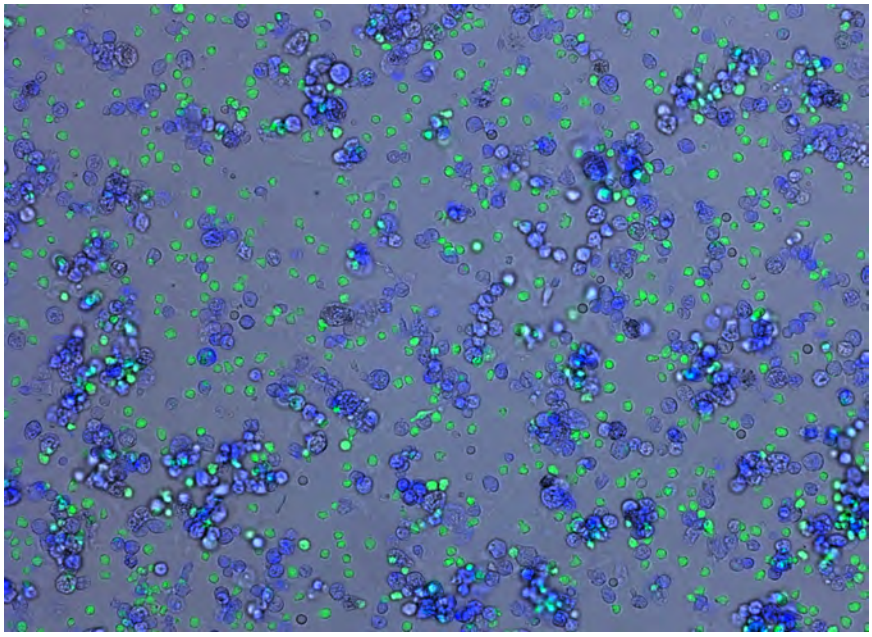
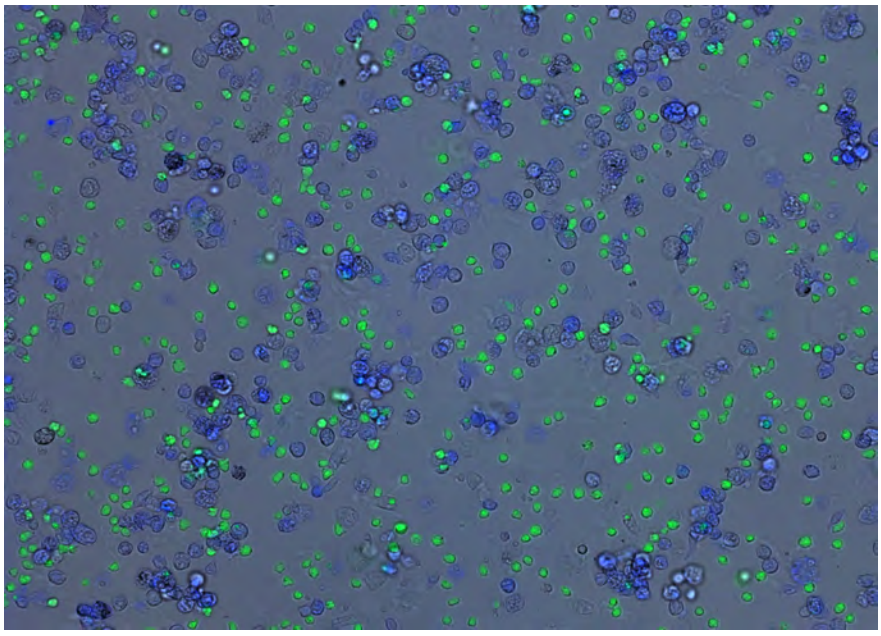


Figure S4. Effects of ESK1 or control IgG, alone or cross-linked, on viability of BV173 cells. Cells were incubated with ESK1 at concentrations indicated with or without goat anti-human IgG as indicated for 24 or 48 hours, as indicated. (See Supplementary Methods.) Data are normalized to no treatment control group, are mean +/- SEM of triplicate data, and are reprentative of 4 or more similar experiments. (A) Soluble or immobilized mAb alone at 24 hours. (B) Soluble or immobilized mAb alone at 48 hours. (C) mAb Cross-linked by immobilized secondary antibody at 24 hours. (D) mAb Cross-linked by immobilized secondary antibody at 48 hours.



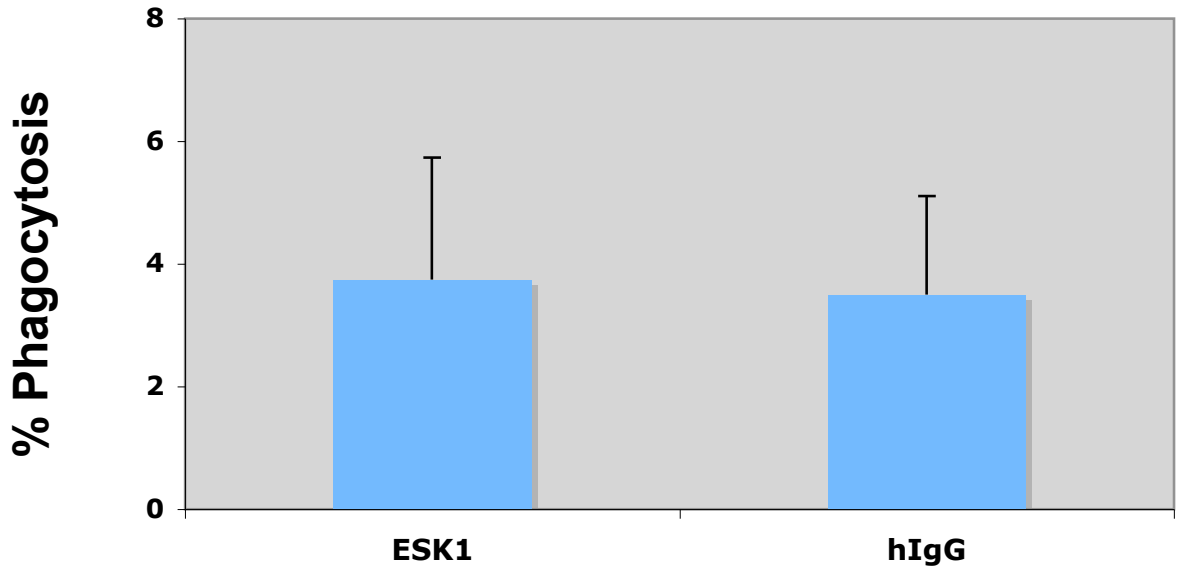
A.

ESK1 group



Isotype control IgG group

Figure S5. ADCP after treatment with ESK1 or control HuIgG. (A) Representative photomicrograph of one experiment of 4 attempts. (See supplemental methods above.) BV173 cells were CFSE labeled (green) and incubated with mouse peritoneal macrophages, stimulated with thioglycolate, that were stained with anti-mouse CD11b /APC (blue), for 2 hrs at a ratio of mac: BV173 2:1, in the presence of the indicated mAb. Phagocytosis was determined by microscopy.



B. BV173 cells were labeled with CFSE (1 uM), washed 3 times and incubated with peritoneal macrophages in the presence of ESK1 or isotype control human IgG1 at 10 ug/ml for 2.5 hours. Phagocytosis was determined by immunofluorescence microscopy by counting the adherent macrophage that had ingested green BV173 cells. More than 70 cells were counted in each field and the data show the average of 4 fields +/- SD. Representative of 4 experiments. These experiments were done at various concentrations of antibody in the presence of naïve macrophages drawn from mice or with macrophages from mice that had been injected first with the monoclonal antibody and target cells to mimic the *in vivo* situation. In no similar case could we observe, by microscopic or cytometric immunofluorescence methods, *in vitro* or from tissues, opsonization or phagocytosis mediated by ESK1.

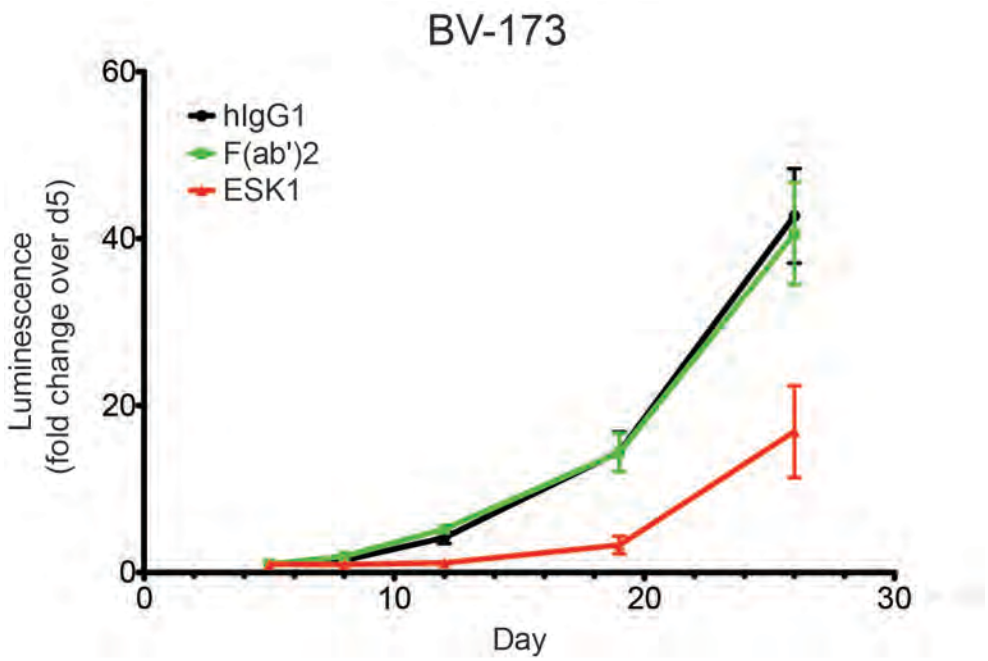


Figure S6. ESK1 efficacy is Fc-dependent. Luciferase-expressing BV173 cells (3×10^6) were injected via tail vein. Xenografts were treated with 100 µg antibody (ESK1 or hIgG1 isotype control) or 73 µg ESK1–F(ab')2 administered intravenously on days 6 and 10. Luminescent signal of each mouse (n =5) in four positions (front, back, left, right) was summed, and fold-change in signal over initial imaging at day 5 was calculated.

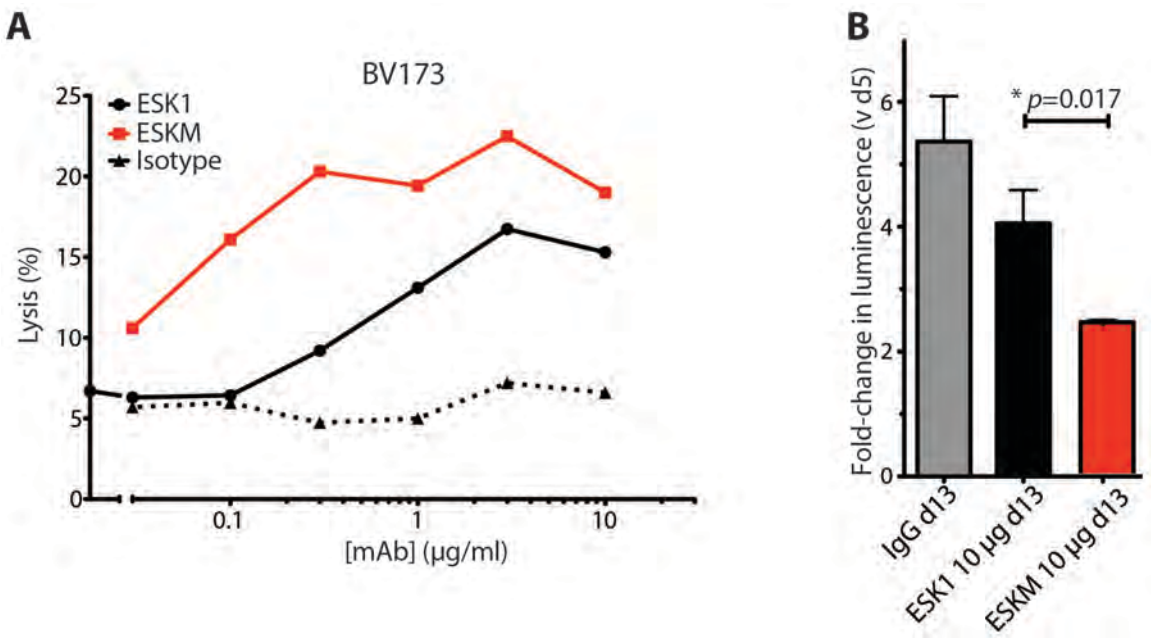


Figure S7. The Fc glycosylation–modified ESK1 antibody with enhanced ADCC activity (ESKM) has increased efficacy *in vitro* and *in vivo*. (A) *In vitro* ADCC mediated by ESK1 or ESKM on BV173 cell lines was measured by 4 hr-^{51}Cr-release assay in triplicate at a E:T ratio of 50:1. Data are representative of 3 experiments. (**B**) Therapy in BV173 xenografted mice. Luciferase-expressing BV173 cells (2×10^6) were injected via tail vein, and 10 µg antibody (ESK1, Fc-enhanced ESKM, or hIgG1 isotype control) was administered intravenously on days 6 and 10. Luminescent signals of each mouse in four positions (front, back, left, right) were summed, and the fold-change in signal over that from initial imaging at day 5 was calculated. *P* value calculated using a *t* test. Data are mean fold-change ± SEM ($n = 5$).

Supplemental Figure S8. Toxicity to A0201 transgenic mice after ESK1 treatment. A microscopic exam of hematoxylin and eosin stained fixed tissues was performed. Representative photomicrographs are shown. A full pathological description is found in Table S3. Power 20X. The left side panels are control treated mice. (A) Liver from IgG treated animal. (B) Spleen from IgG treated animal. (C) Kidney from IgG treated animal. (D) Bone marrow from IgG treated animal. The right side panels are ESK1 treated mice. (E) Liver from ESK1 treated animal. (F) Spleen from ESK1 treated animal. (G) Kidney from ESK1 treated animal. (H) Bone marrow from ESK1 treated animal.

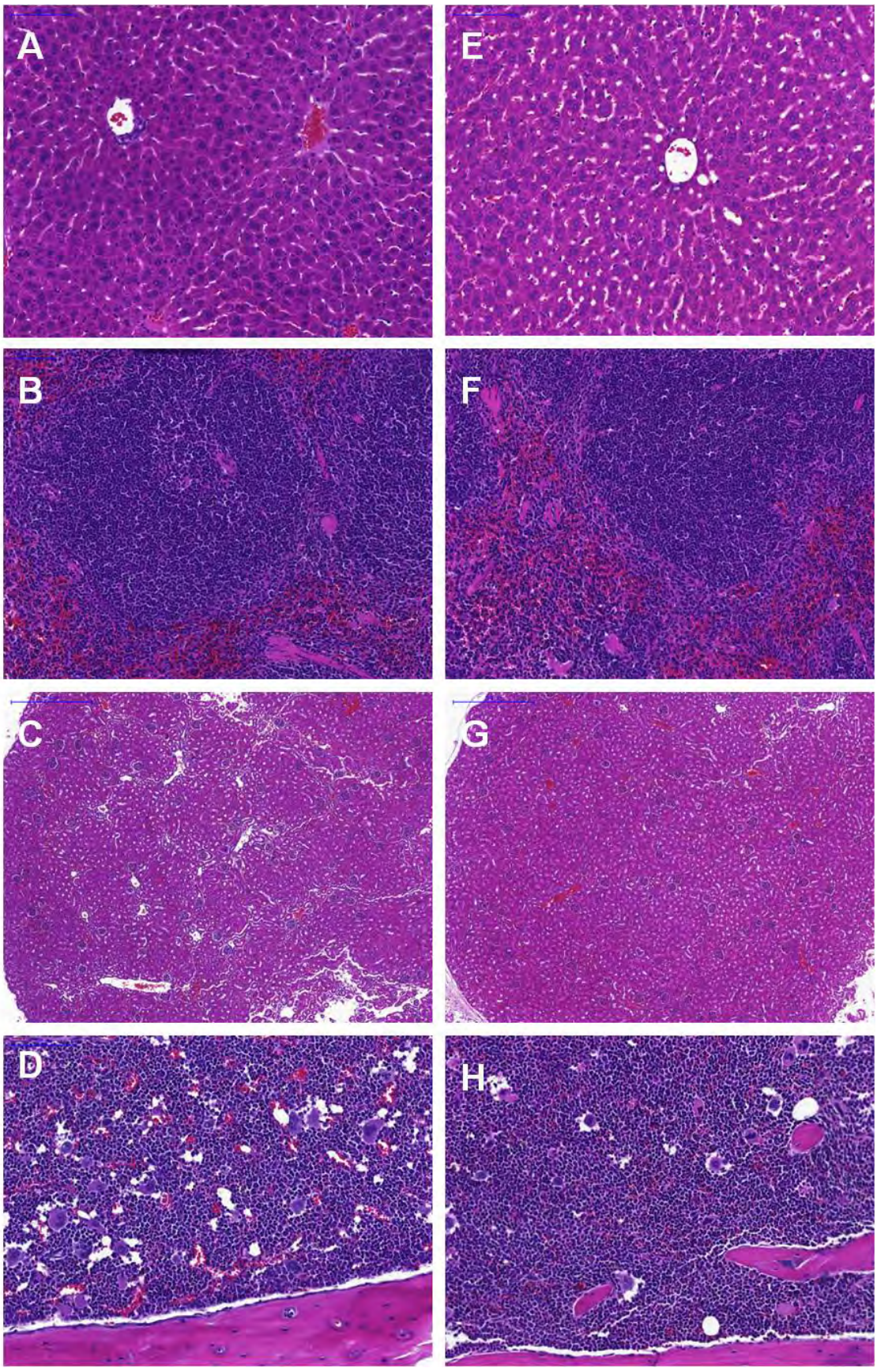

A
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Table S1. Peptide sequences used in these studies. WT1-A and WT1-A1 peptides were named according to our previous studies (*28*). Alanine-mutated peptides were named based on the positions where the substitution was made. RHAMM-R3 (*49*) and EW (*50*) were described previously.

Peptide name	Peptide sequence
WT1-A	RMFPNAPYL
WT1-A1	YMFPNAPYL
WT1-A1-B	AMFPNAPYL
WT1-A3	RMAPNAPYL
WT1-A4	RMFANAPYL
WT1-A5	RMFPAAPYL
WT1-A7	RMFPNAAYL
WT1-A8	RMFPNAPAL
RHAMM-R3	ILSLELMKL
EW	QLQNPSYDK

Supplemental Table S2. Percentage of cells killed by ESK1 with baby rabbit complement. BV173 (ESK1 positive) or 697 (ESK1 low control) leukemia cells were treated with ESK1 and baby rabbit complement (diluted 1:6), under the conditions and concentrations as described. (See supplemental methods above.) Data are mean of a triplicate experiment. Data are representative of 4 experiments.

Cell line	Cells Alone	Cells + Complemen alone	ESK1 + Complemen	HuIgG1 + complement	W632 + complement
BV173	7	11	8	8	91
697	3	10	7	5	Not done

Supplemental Table S3. Toxicity to A0201 transgenic mice after ESK1 treatment. A microscopic exam of hematoxylin and eosin stained fixed tissues was performed and recorded as follows. Representative photomicrographs are shown in Fig S8.

Organ	Day 6 pathology	Day 14 pathology
Bone marrow	Trilineage hematopoiesis with adequate maturation of the myeloid and erythroid lineages. The megakaryocytes are adequate in number with normal morphology	Trilineage hematopoiesis with adequate maturation of the myeloid and erythroid lineages. The Megakaryocytes are adequate in number with normal morphology
Liver	Normal lobular architecture without congestion or inflammation.	Normal lobular architecture without congestion or inflammation
Spleen	Normal distribution of red and white pulp. The red pulp has high numbers of megakaryocytes consistent with extramedullary hematopoiesis.	Normal distribution of red and white pulp. The red pulp has high numbers of megakaryocytes consistent with extramedullary hemaopoiesis
Thymus	Well-defined cortex and medulla with few Hassall's corpuscles, which is normal for rodent histology	Well-defined cortex and medulla with few Hassall's corpuscles, which is normal for rodent histology
Kidney	No glomerulosclerosis, congestion, or inflammation.	No glomerulosclerosis, congestion, or inflammation.
Heart	Normal myocytes without inflammation or fibrosis.	Not done
Small Intestine	Normal villi and crypts.	Not done.
Lung	Normal.	Not done.

Supplemental Table S4. Whole animal and spleen weights after treatment with ESK1 or control HuIgG. 2 animals were sacrificed at day 6 and 3 on day 14. Data are a mean +/- SD of the percentage of mouse weight relative to time 0 (*n =5)* of whole mouse weights from 3 animals (day 14) or 5 animals (day 6) and the mean weights of the spleens (*n* = 2 on day 6 and *n*=3 on day 14)

Treatment group and tissue	Day 6	Day 14
ESK1 treated whole mouse	106+/- 3 %	128 +/- 8 %
HuIgG treated whole mouse	108 +/- 2%	100 +/- 3%
ESK1 treated spleen	86mg	66mg
HuIgG treated spleen	58mg	70mg