Therapeutic Efficacy of an Fc-Enhanced TCR-like Antibody to the Intracellular WT1 Oncoprotein

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Introduction

Therapeutic monoclonal antibodies (mAb) are highly specific and effective drugs, with pharmacokinetics suitable for infrequent dosing. However, all current marketed therapeutic anticancer mAbs target extracellular or cell-surface molecules, whereas many of the most important tumor-associated and oncogenic proteins are nuclear or cytoplasmic (1, 2). Intracellular proteins are processed by the proteasome and presented on the cell surface as small peptides in the pocket of major histocompatibility complex (MHC) class I molecules (HLA in humans), allowing recognition by T-cell receptors (TCR; refs. 3 and 4). Therefore, mAbs that mimic the specificity of TCRs (i.e., recognizing a peptide presented in the context of a specific HLA-type) can bind cell-surface complexes with specificity for an intracellular protein. A “TCR-mimic” (TCRm) antibody was first reported by Andersen et al. (5), and several have since been developed by various groups (6–11).

We recently reported the first fully human TCRm mAb, called ESK1, that specifically targets RMFPNAPYL (RMF), a peptide derived from Wilms’ tumor gene 1 (WT1), presented in the context of HLA-A*02:01 (RMF/A2; ref. 12). WT1 is an important, immunologically validated oncogenic target that has been the focus of many vaccine trials (13). WT1 is a zinc finger transcription factor with limited expression in normal adult tissues, but is overexpressed in the majority of leukemias and a wide range of solid tumors, especially mesothelioma and ovarian cancer (14–16). WT1 was ranked as the top cancer antigenic target for immunotherapy by an NIH-convened panel (17); furthermore, WT1 expression is a biomarker and a prognostic indicator in leukemia (18, 19). ESK1 mAb specifically bound to the Intracellular WT1 Oncoprotein

Abstract

**Purpose**: RMFPNAPYL (RMF), a Wilms’ tumor gene 1 (WT1)–derived CD8 T-cell epitope presented by HLA-A*02:01, is a validated target for T-cell–based immunotherapy. We previously reported ESK1, a high avidity (Kd < 0.2 nmol/L), fully-human monoclonal antibody (mAb) specific for the WT1 RMF peptide/HLA-A*02:01 complex, which selectively bound and killed WT1+ and HLA-A*02:01+ leukemia and solid tumor cell lines.

**Experimental Design**: We engineered a second-generation mAb, ESKM, to have enhanced antibody-dependent cell-mediated cytotoxicity (ADCC) function due to altered Fc glycosylation. ESKM was compared with native ESK1 in binding assays, in vitro ADCC assays, and mesothelioma and leukemia therapeutic models and pharmacokinetic studies in mice. ESKM toxicity was assessed in HLA-A*02:01+ transgenic mice.

**Results**: ESK antibodies mediated ADCC against hematopoietic and solid tumor cells at concentrations below 1 μg/mL, but ESKM was about 5- to 10-fold more potent in vitro against multiple cancer cell lines. ESKM was more potent in vivo against JMN mesothelioma, and effective against SET2 AML and fresh ALL tumor cell lines. At therapeutic doses of ESKM, there was no difference in half-life or biodistribution in HLA-A*02:01+ transgenic mice compared with the parent strain. Importantly, therapeutic doses of ESKM in these mice caused no depletion of total WBCs or hematopoietic stem cells, or pathologic tissue damage.

**Conclusions**: The data provide proof of concept that an Fc-enhanced mAb can improve efficacy against a low-density, tumor-specific, peptide/MHC target, and support further development of this mAb against an important intracellular oncogenic protein. *Clin Cancer Res; 20(15): 4036–46. ©2014 AACR.*
**Translational Relevance**

Wilms’ tumor protein (WT1) is an intracellular, onco-
genic transcription factor that is overexpressed in a wide range of cancers, but has limited expression in normal adult tissues. ESK1, a human IgG1 mAb, mimics T-cell receptor specificity for a WT1-derived peptide (RMF) presented by HLA-A*02:01 and kills cancer cells via antibody-dependent cellular cytotoxicity (ADCC), suggesting that it could treat a wide range of cancers while sparing normal tissue. However, RMF/HLA-A*02:01 epitope levels are far lower than other therapeutic mAb targets, so enhanced ADCC activity should be more valuable clinically. ESKM, a construct with altered Fc glycosylation, demonstrated increased ADCC activity in vitro and greater potency and efficacy in mice. ESKM was not toxic to human HLA-A*02:01 transgenic mice. This study provides proof of concept that an ADCC-enhanced mAb construct can effectively treat cancers while targeting a low-density peptide/HLA epitope (500–6,000 per cell), and supports the clinical utility of ESKM.

leukemias and solid tumor cell lines that are both WT1 + and HLA-A*02:01 + and showed efficacy in mouse models in vivo against several WT1 + HLA-A*02:01 + leukemias (12). Therefore, ESK1 is a useful therapeutic platform for further clinical development, and improvements to the native antibody could help potentiate its effect and improve clinical efficacy.

The mechanisms of action of mAbs can be enhanced through Fc region protein engineering (20), or by modification of Fc-region glycosylation (21, 22). Removal of fucose from the carbohydrate chain increases mAb binding affinity for the activating FcγRIIA receptor and enhances antibody-dependent cellular cytotoxicity (ADCC; refs. 23–26). The addition of bisecting N-acetyll-β-glucosamine (GlcNAc) can also significantly enhance ADCC (26–28).

However, removal or replacement of the terminal galactose residues present on endogenous IgG reduces complement-dependent cytotoxicity (CDC) activity (22, 29).

TCRm antibodies are potentially limited by the extremely low number of epitopes presented on the target cell, which may be as few as several hundred sites (30). Therefore, mechanisms to enhance potency may be essential to their success in humans as therapeutic agents against cancer. The ESK1 mAb works primarily through ADCC and therefore we hypothesized that an Fc-glycosylation altered version of the antibody would improve efficacy in vivo. An Fc-modified antibody was generated by expressing the ESK1 construct in MAGE 1.5 CHO cells (Eureka Therapeutics, Inc.), resulting in a consistent pattern of defucosylation and exposed terminal hexose (mannose and/or glucose), allowing higher affinity for activating human FcγRIIA and murine FcγRIV while decreasing affinity for inhibitory FcγRIIB. The modified antibody, “ESKM,” mediated ADCC at lower doses than native ESK1 and was more potent in human tumor models in vivo. Furthermore, ESKM had similar pharmacokinetics and biodistribution to the native antibody. ESKM showed no observable off-target tissue sink in wild-type mice, and at therapeutic doses there was no difference in half-life or biodistribution in HLA-A2.1 + transgenic mice compared with the parent strain. Importantly, therapeutic doses of ESKM in these mice caused no depletion of total WBCs or hematopoietic stem cells (HSC), or pathologic tissue damage. The retained specificity, enhanced potency, favorable pharmacokinetics and distribution, and lack of toxicity in these models support ESKM as a promising lead clinical drug candidate to treat a wide variety of cancers and leukemias.

**Materials and Methods**

**Oligosaccharide analysis and FcR binding assays**

N-Glycan from ESK1 or ESKM was cleaved from antibody by PNGase F, and measured by HPAEC-PAD using PA200 column. Binding of ESK1/ESKM to mouse FcγRI and mouse FcγRIIB were measured by ELISA. Briefly, 2 μg/mL recombinant mouse FcγRI or FcγRIIB were coated onto ELISA plate. Various concentrations of ESK1 or ESKM antibodies were added to the wells for 1 hour at room temperature, then detected by secondary antibody (HRP-conjugated antihuman IgG Fab’2 fragment). Binding of ESK1/ESKM to human FcγRs was measured by flow cytometry (Guava easyCyte HT; Millipore) against CHO cells expressing human FcγR. Binding of ESK1/ESKM to human FcγRI, FcγRIIA, FcγRIIIA, FcγRIIIB, FcγRIIIB, and human FcRn were measured directly using ESK1 or ESK1 antibody, followed by the second antibody (FITC-conjugated Fab’2 fragment anti-human IgG Fab’2). For human FcγRIIB, dimers were formed first by mixing ESK1 or ESKM to a phycoerythrin-conjugated Fab’2 fragment anti-human IgG Fab’2 at 2:1 ratio at RT for 2 hours. Binding of dimeric complex of ESK1 or ESKM to human FcγRIIB were measured directly by flow cytometry using the immunocomplex.

**Cells and reagents**

Cell lines were from laboratory stocks, and were passaged in RPMI with 10% FBS for less than 1 month before experiments. Cell lines were from the same stocks as published previously (12), and were not tested and authenticated. An ovarian patient sample (WT1 positive by immunohistochemistry, and strongly positive for HLA-A2 and ESK mAb binding by flow cytometry) was obtained under MSKCC IRB approved protocols. For acute lymphoblastic leukemia (ALL) leukemia animal studies, fresh pre-B-cell ALL cells were obtained under MSKCC IRB approved protocols. From the CNS relapse of a female pediatric patient after treatment with a chemotherapy induction regimen and bone-marrow transplant. Leukemia cells were transduced with a lentiviral vector containing a plasmid encoding luciferase/GFP (kindly provided by Vladimir Ponomarev, MSKCC). Luciferase +/GFP + leukemia was then expanded in NSG mice, luciferase signal was confirmed by bioluminescent imaging, and tumor cells were harvested and sorted for CD45. Peptides for T2 pulsing assays were purchased and
synthesized by Genemed Synthesis, Inc. Peptides were >90% pure. GFP-luciferase-expressing SET2 and JMN cells were generated as described previously (12). All cells were HLA typed by the Department of Cellular Immunology at Memorial Sloan-Kettering Cancer Center.

Animals
C57BL/6j and C57BL/-Tg (HLA-A2.1) 1 EngenI (6- to 8-week-old male) and NOD.Cg-PrkdcscidIl2rgtm1Wjl/szJ mice (6- to 8-week-old male), known as NOD SCID (NSG), were purchased from Jackson Laboratory. Functionally equivalent NOD.Cg-PrkdcscidIl2rgtm1JicTac (6- to 8-week-old male), known as NOG, and C.B-Igh-1/JcrTac-Prkdcscid (6- to 8-week-old male), known as CB17 SCID, were purchased from Taconic. All studies were conducted in accordance with IACUC-approved protocols.

Antibody-dependent cellular cytotoxicity
After informed consent on Memorial Sloan-Kettering Cancer Center Institutional Review Board (MSKCC IRB) approved protocols, peripheral blood mononuclear cells (PBMC) from healthy donors were obtained by Ficoll density centrifugation. Target cells used for ADCC were T2 cells pulsed with or without WT1 or RHAMM-3 peptides, and cancer cell lines or primary ovarian cancer sample without peptide pulsing. ESK1, ESKM, or isotype control human IgG1 (Eureka Therapeutics, Inc.) at various concentrations were incubated with target cells and fresh PBMCs at different effector:target (E:T) ratio. Cytotoxicity was measured by standard 4-hour 51Cr-release assay.

Therapy of ESK1 and ESKM in human mesothelioma, acute myeloid leukemia, and ALL xenograft mouse models
Luciferase-expressing JMN cells (3 × 10^5) were injected into the intraperitoneal cavity of CB17 SCID mice. On day 4, tumor engraftment was confirmed by luciferase imaging, signal was quantified with Living Image software (Xenogen), and mice were sorted into groups with similar average signal from the supine position. Mice were injected intraperitoneally with 50 µg ESK1, ESKM, or human isotype IgG1 antibody twice weekly beginning on day 4.

For acute myeloid leukemia (AML) leukemia studies, luciferase-expressing SET2 (AML) cells (3 × 10^5) were injected intravenously via tail vein into NSG mice. Animals were sorted and, where indicated, treated with intraperitoneal injections of 100 µg ESKM twice weekly beginning on day 6. For ALL studies, fresh leukemia cells were obtained as described above (cell lines and reagents) then injected intravenously into NSG mice (55 × 10^6/animal), and engraftment was confirmed by bioluminescent imaging on day 2 after injection. Animals were sorted into 2 groups (n = 5 each) so that average signal in each group was equal. ESKM or isotype control antibody (100 µg/animal) was administered via retro-orbital injection on days 2, 5, 9, 12, 14, and 23, and leukemia growth was followed by bioluminescent imaging. On day 41, animals were sacrificed and bone marrow cells were harvested and pooled. After dissection and homogenization, cells were centrifuged, subjected to Ficoll density centrifugation, and counted after red blood cell lysis (acetic acid). An equal number of cells from each treatment group was resuspended in matrigel (200 µL/injection) and engrafted subcutaneously into the opposite shoulders of NSG mice (n = 4). No further treatment was given, and tumor growth was followed by bioluminescent imaging.

Pharmacokinetic and biodistribution studies
Antibody was labeled with 125I (PerkinElmer) using the chloramine-T method. One hundred micrograms of antibody was reacted with 1mCi 125I and 20 µg chloramine-T, quenched with 200 µg Na metabisulfite, then separated from free 125I using a 10DG column equilibrated with 2% bovine serum albumin in PBS. Specific activities of products were in the range of 4 to 8 mCi/mg. Radiolabeled mAb was injected into mice retro-orbitally, and blood and/or organs were collected at various time points, weighed and measured on a γ counter.

Toxicity studies
For isolated cell binding studies, C57BL/6j or HLA-A2.1^+ transgenic mice were sacrificed, and cells were harvested from spleen, thymus, and bone marrow. After red blood cell lysis, cells (10^6 per tube, in duplicate) were incubated with 125I-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 set of cells was assayed after preincubation in the presence of 50-fold excess unlabeled ESK1 for 20 minutes on ice. Bound radioactivity was measured by a γ counter, specific binding was determined, and the number of bound antibodies per cell was calculated from specific activity.

For toxicity studies, 100 µg of ESKM or isotype control mAb was injected into human HLA-A:02:01 transgenic mice (Jackson Labs) on days 0 and 4, to mimic the maximum dose and therapeutic schedule used in the therapy experiments. Mice were sacrificed on day 5 for collection and analysis of whole blood and bone marrow leukocytes. Whole blood was analyzed with a Hemavet system (Drew Scientific). Bone marrow cells were harvested from both femurs and tibias of mice and subjected to red blood cell lysis, then analyzed by flow cytometry (see Antibodies and flow cytometry analysis).

Antibodies and flow cytometry analysis
For cell surface staining, cells were blocked with human FcR blocking reagent (Miltenyi Biotec), then incubated with appropriate Abs for 30 to 60 minutes on ice. Flow cytometry data were collected on a FACS Calibur or LSRFortessa (Becton Dickinson) and analyzed with FlowJo software (TreeStar). APC-labeled ESK1 and hIgG1 isotype (Eureka Therapeutics, Catalog No. E1901) were generated with Lightning-Link Kit (Innova Biosciences). For human leukemia stem cell studies, cells were stained with the following antibodies, and corresponding fully stained minus one controls: PE-Cy5 anti-human lineage cocktail (BioLegend;
anti-CD3, CD14, CD16, CD19, CD20, CD56), FITC-labeled anti-CD38, PE-labeled anti-CD34, PE-Cy7-labeled BB7.2, and APC-labeled ESK1.

For HSC toxicity studies, mouse bone marrow cells were stained with the following antibodies: (Lineage; CD3, CD4, CD8, Gr1, B220, CD19, TER119, all conjugated with PE-Cy5), Sca-Pacific Blue, CD34-FITC, SLAM-APC, CD48-PE, CD8, Gr1, B220, CD19, TER119, all conjugated with PE-Cy7, CD33 (NKp46)-PE and CD49b/VLA-2a (Pathway; BB7.2, and APC-labeled ESK1).

ESKM antibody has enhanced binding affinity for FcγRIla and reduced affinity for FcγRIib

ESKM mAb was produced in MAGE 1.5 CHO cells, with the homogenous oligosaccharide structure (Supplementary Fig. S1A) and no detectable fucose or galactose. ESKM had 80% higher affinity for activating human FcγRIla (158V variant), 3.5-fold higher affinity for the FcγRIla 158F variant, and 50% reduced affinity for inhibitory FcγRIib. Importantly, ESKM affinity for FcRn was unchanged (Table 1 and Supplementary Fig. S1B). Similarly, ESKM had 51% higher affinity for activating mouse FcγRI, and half the affinity for inactivating mouse FcγRIib (Table 1 and Supplementary Fig. S1C). Changes in FC glycosylation pattern did not affect antigen binding, as avidity of ESKM against WT1 HLA-A*02:01+ JM cells was nearly identical to the native ESK1 (0.2–0.4 nmol/L; Table 1 and Supplementary Fig. S1D).

ADCC mediated by ESKM in vitro

We investigated the relationship of cell surface antigen density with ESKM ADCC efficacy using T2, a TAP-deficient cell line that expresses HLA-A*02:01 but does not present peptides through the ER pathway, and thus can be loaded with exogenous peptides for presentation in a dose-dependent manner. To determine whether ESKM could better mediate ADCC against cells with low antigen density, we fixed the dose of ESK1 and ESKM mAbs and tested them against T2 cells loaded with titrated RMF peptide. Both antibodies were effective against T2 cells pulsed with high peptide concentrations, but ESKM was able to mediate greater ADCC against cells with fewer RMF/A2 complexes (Fig. 1A).

We next determined the in vitro ADCC activity of ESK1 and ESKM against cell lines presenting a range of levels of cell surface RMF/A2 (12). ESKM showed both increased potency and efficacy against 6 leukemia and mesothelioma cell lines in an HLA-restricted manner. ESKM effectively mediated ADCC against BA-25, an ALL cell line expressing approximately 1,000 to 2,000 RMF/A2 targets per cell; both antibodies were similarly effective at ADCC at concentrations above 1 μg/mL, but ESKM was more potent at concentrations down to 100 ng/mL of mAb (Fig 1B). Against AML-14 and SET2 AML cell lines, which both bind ~5,000 mAb per cell, ESKM mediated higher cell lysis than ESK1 at the highest antibody concentrations, and showed cytolytic efficacy down to doses as low as 100 ng/mL (Fig. 1B). As we

| Table 1. Summary of ESK1 and ESKM binding to mouse and human FcγRs, and RMF/A2⁺ target cells |
|-------------------|-------------------|-------------------|-------------------|
| Receptor          | ESK1               | ESKM               | Ratio of affinity constants (ESKM/ESK1) |
| Mouse             |                   |                   |                                |
| FcγRIib           | 32.0 ± 0.454       | 62.3 ± 7.27       | 0.51                          |
| FcγRIV            | 3.34 ± 0.193       | 2.21 ± 0.153      | 1.51                          |
| Human             |                   |                   |                                |
| FcγRI             | 5.81 ± 0.113       | 0.680 ± 0.125     | N.C.                          |
| FcγRIla           | 106 ± 15.5         | 58.3 ± 8.80       | 1.81                          |
| FcγRIib           | 1338 ± 253         | 2644 ± 438        | 0.51                          |
| FcγRIla (158V)    | 92.6 ± 15.0        | 50.4 ± 8.35       | 1.84                          |
| FcγRIla (158F)    | 19.0 ± 2.38        | 5.53 ± 0.741      | 3.45                          |
| FcRn              | 824 ± 102          | 780 ± 97.5        | N.C.                          |
| Cell line         |                   |                   |                                |
| JMN               | 0.2                | 0.4               |                                |

NOTE: Anti-mouse FcR binding was assessed by ELISA, whereas anti-human FcR binding was determined by FCM titration on FcγR-expressing CHO cells. N.C., no change. To determine affinity for the RMF/A2 epitope, 125I-labeled ESK1 and ESKM mAbs were titrated against JMN cells. All curves were fit with a nonlinear single-site total binding saturation curve, and $K_a$ was calculated using Prism software.
have shown previously for ESK1 (12), ESKM did not kill leukemia cells not expressing HLA-A*02:01 (Fig. 1C). Furthermore, ESKM mediated higher specific lysis at nearly all doses tested against 3 HLA-A*02:01+ mesothelioma cell lines: JMN, Meso37, and Meso56 (Fig. 1D). Importantly, ESKM was also more potent against a WT1+, HLA-A*02:01+ primary human ovarian cancer sample (Fig 1D), which was positive for ESK binding by flow cytometry. To assess the human effector cell populations responsible for ADCC in vitro, we separated whole blood from healthy donors into neutrophils and PBMCs, which were further sorted into macrophage/monocyte and NK-cell populations (see Supplementary Methods). Only isolated NK cells were capable of ADCC directed by ESKM in vitro against BV173 target cells; Supplementary Fig. S2). These data showed that ESKM was both more potent—as illustrated by its ability to kill cells with lower mAb concentrations and fewer cell surface targets—and more effective than ESK1, as demonstrated by higher specific lysis attained at the highest concentrations.

**Potency of ESKM against human mesothelioma and leukemia models in mice**

Data from several experiments in vitro and in vivo provided strong evidence that ADCC was the dominant mechanism of therapeutic action of the ESK1 mAb (12). Here, we utilized a SCID mouse model to investigate whether ESKM offered a consistent and significant improvement over native ESK1 in vivo in mice with more available effector cells capable of ADCC than NSG/NOG mice (12). ESKM more effectively engages activating murine FcγRIIa and

![Figure 1.](image)

**Figure 1.** ESKM is more efficacious and potent in ADCC assays with human PBMC effectors at the indicated mAb concentrations and effector/target (E:T) ratios. Cytotoxicity was measured by 4-hour 51Cr release assay. A, T2 cells were pulsed with RMF peptide and incubated with 3 μg/mL mAb. B, HLA-A*02:01+ human leukemia cell lines: BA25 ALL, AML14, and SET2 AML. C, HLA-A*02:01+ HL60 promyelocytic leukemia. D, HLA-A*02:01+ human mesothelioma cell lines: JMN, Meso37, and Meso56; and a HLA-A*02:01+ primary human ovarian cancer. Data presented are averages of triplicate measurements, with spontaneous 51Cr release subtracted, from representative experiments, all with isolated PBMCs from the same donor. All cell lines, with exception of Meso37 and Meso56, were repeated 3 or more times with multiple donors.

**Table 2.** Intraperitoneal effector cell populations in BALB/c, SCID, and NOG mice

<table>
<thead>
<tr>
<th>Parent population</th>
<th>Cell type</th>
<th>Marker phenotype</th>
<th>BALB/c</th>
<th>CB17 SCID</th>
<th>NOG</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b+</td>
<td>Granulocyte</td>
<td>Gr-1+ F4/80+</td>
<td>46.6 ± 28.3</td>
<td>7.45 ± 2.71</td>
<td>0.215 ± 0.137</td>
</tr>
<tr>
<td></td>
<td>Macrophage</td>
<td>Gr-1+ F4/80+</td>
<td>10.5 ± 6.87</td>
<td>19.0 ± 1.29</td>
<td>96.3 ± 1.06</td>
</tr>
<tr>
<td></td>
<td>Monocyte</td>
<td>Gr-1+ F4/80+</td>
<td>39.2 ± 21.1</td>
<td>70.2 ± 2.73</td>
<td>1.25 ± 0.562</td>
</tr>
<tr>
<td>CD11b−</td>
<td>NK cell</td>
<td>NKP46+ CD3ε−</td>
<td>1.68 ± 0.250</td>
<td>36.2 ± 12.0</td>
<td>0.278 ± 0.413</td>
</tr>
</tbody>
</table>

**NOTE:** Intraperitoneal cells were isolated from mice (n = 3 each strain) and analyzed by multicolor flow cytometry. Cell type was determined by the indicated markers, and quantified as percentage of total leukocytes isolated.
FcγRIV, therefore murine cells should serve as potent effectors in vivo. Mice were engrafted with luciferase− JMN mesothelioma cells in the intraperitoneal cavity (simulating this serosal cavity cancer). To determine the relative abundance of murine effector cell populations in the intraperitoneal cavity, we analyzed extracted cells with common murine immunophenotyping markers (31). SCID mice contain intraperitoneal macrophages, neutrophils, and NK cells (Table 2). This flow cytometry analysis also confirmed the presence of murine monocytes, macrophages, and NK cells, but lack of B and T cells in the spleen and peripheral blood, as expected (32). Biweekly 50 μg treatment with ESKM was more effective than ESK1 against intraperitoneal JMN, and significantly improved survival over isotype control antibody (Fig. 2A). Furthermore, ESKM was able to reduce tumor burden during the treatment course, whereas ESK1 merely slowed growth (Supplementary Fig. S3A). In a third experiment at the same dose and schedule, neither antibody construct showed efficacy against intraperitoneal JMN in NOG mice (Supplementary Fig. S3B), which lack NK cells and intraperitoneal neutrophils (Table 2), indicating that these cell populations likely play an important role in efficacy in the intraperitoneal model.

We previously reported that ESKM is effective at a low dose against disseminated bcr/abl-suppressed BV173 ALL in a NSG mouse model, where FcγRIV+ monomyeloid cells are present in the spleen (33). We therefore used NSG mice to evaluate ESKM efficacy against disseminated leukemia models. We first investigated ESKM against SET2, an AML that grew more aggressively than BV173 in the NSG mouse model (Supplementary Fig. S3C). ESKM significantly reduced tumor growth (Fig. 2B). ESKM also significantly

![Figure 2](https://www.aacrjournals.org/ClinCancerRes;20(15)August1,2014/4041)

**Figure 2.** ESKM is superior to ESK1 in vivo, and is effective against multiple tumor models. Tumor burden was determined by luciferase imaging of mice in the supine position (n = 5 per group). Data points are averages of each group, and error bars represent SEM. Arrows indicate treatment with mAb.

A, ESKM significantly reduced mean tumor growth of intraperitoneal JMN mesothelioma as assessed by total luminescence (P < 0.05, multiple T tests on and after day 18), and also significantly improved survival (P = 0.016 vs. isotype, P = 0.095 vs. ESK1), with events representing death or terminal morbidity as assessed on protocol by veterinarians. B, ESKM is effective against SET2 AML (P < 0.05, multiple T tests). C, ESKM is effective against a disseminated fresh, patient-derived pre-B ALL (P < 0.05, P < 0.01, multiple T tests). D, leukemia burden in the bone marrow was quantitated by imaging on day 40, then bone marrow cells were harvested from mice in C and transplanted as subcutaneous tumors into NSG mice. Bone marrow cells from the isotype-treated mice were injected into the right shoulder (viewed from above), whereas an equal number of bone marrow cells from the ESKM-treated mice were injected into the left shoulder. Subcutaneous tumors were then quantitated 28 days after transplantation. In a multicolor flow cytometry analysis of a frozen stock of the same fresh primary ALL sample (before passage in animals), ESK mAb bound the lineagelow/CD34−CD38+ subset.
reduced initial burden and slowed outgrowth of a patient-derived human pre-B-cell ALL (Fig. 2C). Leukemia relapsed after treatment was stopped, allowing us to collect leukemia cells from the bone marrow and transplant to new animals to assess outgrowth from remaining progenitors. Total bone marrow signal in ESKM-treated mice was lower at time of transplant, but equal numbers of ESKM-treated and isotype-treated bone marrow cells were engrafted into recipient animals. Subcutaneous leukemia tumors from isotype-treated leukemia cells grew to 50 times the total signal compared with tumors from ESKM-treated cells (Fig. 2D). In this same patient ALL sample, ESK bound a lineage^low^, CD34^+, CD38^−^ population, characterized as a cancer stem cell population in ALL (34), at nearly the same level as lineage^+^ populations (Fig. 2D and Supplementary Methods). This suggests that ESKM therapy could target this population, but does not imply that ESK mAbs can differentiate between leukemic stem cell and normal HSC populations. Further supporting the hypothesis that ESK mAbs can target leukemic progenitor cells, ESK1 bound CD33^+^/CD34^−^ cells from a separate HLA-A^02:01^+^ AML patient with ~8-fold shift in median fluorescence intensity of ESK1 compared with isotype control antibody (12).

Pharmacokinetics and biodistribution of ESK1 and ESKM

Altering Fc glycosylation could potentially change pharmacokinetic properties of the mAb, thereby affecting its therapeutic utility. Trace ^125^I-labeled antibodies were injected intravenously into mice and blood levels of mAb were measured over 7 days. Both ESK1 and ESKM exhibited biphasic clearance, with initial tissue distribution and an α half-life of 1.1 to 2.4 hours, followed by a slower β half-life of several days (Fig. 3A). ESKM had a shorter β half-life than ESK1 (4.9 days vs. 6.5 days). The biodistribution patterns of the antibodies were determined using the same radiolabeled constructs. Both antibodies displayed similar patterns of organ distribution and clearance (Fig. 3A). Although increased FcγR binding or manose receptor binding could create a sink for ESKM, we found no increase in ESKM distribution to the liver, spleen, thymus, or bone marrow that could account for the shortened serum half-life.

As the ESKM antibody targets a human-specific epitope, the C57BL/6J mouse model cannot recapitulate possible on-target binding to normal tissues that could alter antibody pharmacokinetics and biodistribution. Therefore, we used a transgenic mouse model based on the C57BL/6J background that expresses human HLA-A^02:01^ driven by a lymphoid promoter. We confirmed the presence of surface HLA-A^02:01^ on murine cells by flow cytometry with the BB7.2 antibody. Leukocytes isolated from thymus tissue showed low but detectable binding, whereas cells isolated from the bone marrow and spleen showed appreciable binding, with a 5- to 10-fold shift in median fluorescent intensity over isotype control antibody, comparable to surface HLA-A^02:01^ levels observed on human PBMCs from healthy donors (12). The 9-mer RMF sequence is identical in human and mouse, and therefore this transgenic model could recapitulate antigen presentation of the RMF/A2 epitope in healthy cells. There was no difference between wild-type and HLA-A^02:01^ transgenic mice in blood pharmacokinetics of ESKM, indicating that there was no significant antibody sink. Furthermore, at a therapeutic dose of antibody (100 μg), there was no difference in biodistribution in transgenic compared with wild-type mice (Fig. 3B).

At low doses of trace-labeled ESKM (2 μg), there was a small yet detectable increase in uptake of antibody in the spleen of transgenic mice compared with wild-type mice (Fig. 3C). The additional splenic binding was small, accounting for only 16 ng of antibody, which could be due to RMF presentation in HLA-A^02:01^+ cells, or to an unknown cross-reacting epitope. Splenic uptake was not because of Fc glycosylation pattern alone, as the native ESK1 mAb also showed increased uptake in transgenic spleens at 24 hours (Supplementary Fig. S4A). In addition, increased spleen uptake in transgenic mice seemed to be partly related to strain differences in clearance, as the isotype control antibody also showed 60% increased splenic uptake at 24 hours in transgenic compared with wild-type mice (Fig. 3D and Supplementary Fig. S4A). Furthermore, we observed no binding of ESK1 to cells isolated from transgenic mouse spleen cells by either flow cytometry (12) or specific binding assay with ^125^I-labeled ESK1 (Supplementary Fig. S4B), suggesting that if a cross-reacting epitope was present, it was not expressed in detectable amounts on a specific cell type.

Toxicity of ESKM in a HLA-A^02:01^ transgenic mouse model

WT1 is reported to be expressed in hematopoietic stem cells (HSC; ref. 35), so the C57BL/6J transgenic mouse model with human HLA-A^02:01^ driven by a lymphoid promoter provides an opportunity to assess possible toxicity against progenitor cells in the hematopoietic compartment that, given the high potency of ESKM, might occur even at low epitope density. White blood cell and bone marrow cell counts were assessed 1 day after the final of 2 therapeutic doses of ESKM or isotype control mAb on the same schedule as previously described therapy experiments (12). There were no differences in total white blood cell count, or lymphocyte, neutrophil, monocyte, eosinophil, or basophil cell counts (Fig. 4A). Within the bone marrow compartment, we found equivalent absolute number and frequency of HSC progenitors (LSK; Lineage^−^, c-kit^+, Sca1^+^; ref. Fig. 4B) and HSCs (CD150hi, CD48^−^, LSK; ref. Fig. 4C) in the ESKM treated and isotype control-treated mice.

Finally, we assessed gross and microscopic pathology of lymphoid and major organs of HLA-A^02:01^ transgenic mice treated with ESKM or isotype mAb on the same schedule. No striking differences between ESKM- and isotype-treated groups were observed by a trained hematopathologist (Supplementary Table S1). The bone marrow sections of both groups showed trilineage hematopoiesis with adequate maturation of the myeloid and erythroid lineages. The megakaryocytes were adequate in number...
with normal morphology. Thymus sections showed a well-defined cortex and medulla with few Hassall’s corpuscles, which is normal for rodent histology (36). The kidney sections showed no pathologic findings such as glomerulosclerosis, congestion, or inflammation. All spleen sections showed a normal distribution of red and white pulp. Occasional scattered megakaryocytes were seen in the red pulp consistent with extramedullary hematopoiesis (37).

Discussion

The generation of TCRm antibodies allows use of the mAb to target cell-surface fragments of intracellular proteins, provided that they are processed and presented on MHC class I molecules. ESK1 was the first TCRm antibody reported against a peptide derived from WT1, an important oncogene expressed in a wide variety of cancers, but not normal adult tissues. WT1 seems to be expressed in leukemic stem cells (35), raising the possibility that the mAb could ultimately eliminate clonogenic leukemia cells in patients. Other therapeutic TCRm mouse antibodies, human ScFv, and Fab fragments have been previously described (6–8, 10, 11). However, ESK1 is the first and only fully human therapeutic TCRm mAb reported. Features of the RMF/A2 epitope, especially the low levels of expression on the cell surface, require selection of a highly
both human and murine FcγRIIb should reduce inhibitory receptor activation. As expected, ESKM was both more potent and effective in vitro even at very low epitope density. ESKM was also more effective than ESK1 in vivo, and was able to treat peritoneal mesothelioma in SCID mice, modeling the clinical situation. Furthermore, ESKM significantly slowed leukemia growth of disseminated SET2, a human AML cell line with much more aggressive in vivo leukemia growth kinetics than BV173, and a fresh patient-derived pre-B-cell ALL in xenograft models. In the fresh ALL model, tumor relapsed after mAb therapy was stopped, but leukemia cells extracted from the bone marrow of ESKM-treated mice and transplanted as subcutaneous tumors showed minimal outgrowth. This suggests that ESKM may target a progenitor population of leukemia cells. This supposition is supported by binding of ESK to a lineage^{low-}, CD34^+, CD38^- population by flow cytometry, and consistent with the hypothesis that WT1 expression in HSCs could allow reduction of this population. However, cells collected from the bone marrow after relapse were not phenotyped and sorted, so we cannot determine the exact cell population targeted. ESKM was not curative in the models tested, possibly because of the lack of NK cells in NOG/NSG mice and short treatment courses. In this system, an incomplete effect is not surprising, and may not accurately model the effect in humans.

ESKM therapy was not effective against peritoneal mesothelioma in NSG or NOG mice, which lack NK cells and neutrophils, although naked ESK1 did previously show potent activity against a disseminated leukemia model in these mice. This discrepancy could be due both to the tumor model—leukemia cells could have different sensitivity to effector-mediated cytotoxicity—and to the availability of intraperitoneal effector cells in the NSG/NOG model: our assays indicated that the intraperitoneal cavity of NOG mice contained predominately macrophages, whereas neutrophils were present in the blood and spleen, as previously reported (33). The marked improvement in efficacy with the Fc-enhanced mAb in SCID mice indicated that NK cells and/or monocytes (both with FcγRIV) are important to therapy in the intraperitoneal mesothelioma model, whereas splenicytotic and circulating neutrophils presumably mediate ADCC against disseminated leukemias in NSG/NOG mice.

Altering Fc glycosylation could potentially change pharmacokinetic properties of the mAb through a number of mechanisms, including altered FcRn binding and antibody recycling, modified binding to circulating effector cells, and differential engagement with clearance mechanisms, such as mannose receptors. Similar afucosylated, Fc-modified antibodies with improved ADCC have been investigated in pharmacokinetic studies in vivo (41, 42). ESKM had nearly identical biodistribution to ESK1, but a shortened blood half-life. We saw no change in biodistribution pattern that could account for this altered half-life. IgG half-life is regulated by the neonatal Fc receptor, FcRn (43, 44); however, ESKM had identical affinity for FcRn as ESK1. The altered pharmacokinetics is possibly because of interaction with mannose receptor on macrophages, a known mechanism of glycoprotein clearance (45–47). ESK1 and ESKM
half-lives could be quite different in humans—where higher affinity binding to Fc receptors and clearance through human mannose receptors could have a role—as seen with other high-mannose IgG antibodies in humans (48).

Because ESK mAbs target a human HLA-specific epitope, we utilized the human HLA-A*02:01+ transgenic mouse strain for toxicology studies. WT1 is reportedly expressed in hematopoietic tissues such as bone marrow, thymus, or spleen, where WT1+ HSCs could be expected, and where HLA-A*02:01 expression is highest because the transgene is driven by a lymphoid promoter. This transgenic system is the best available for predicting potential off target toxicity, but is limited to hematopoietic tissues. Despite this limitation, the lack of ESKM toxicity against those tissues shown to express human HLA-A*02:01 suggests that ESKM may be well tolerated in humans.

Selection of ESKM as a lead construct for human clinical development required us to consider its features of a moderately decreased half-life yet increased potency and broader applicability. The potential enhanced efficacy against tumors expressing fewer RMF/A2 sites could expand the number of patients and cancer types eligible for this therapy as well as increase efficacy. In addition, the MAGE 1.5 CHO engineering technology generates mAbs that effectively engage FcγRIIIa (CD16), regardless of amino acid 158 polymorphism. Carriers of CD16-158F are less responsive than CD16-158V/V individuals to human IgG1 therapeutics such as rituximab and trastuzumab (49, 50). WT1 is reportedly expressed in other high-mannose IgG antibodies in humans (48).

Disclosure of Potential Conflicts of Interest

N. Veomett, T. Dao, L. Dubrovsky, and D.A. Scheinberg are inventors of intellectual property related to ESKM that is owned by Sloan Kettering and licensed to Novartis. H. Liu, J. Xiang, and C. Liu have ownership interest (including patents) in Eureka Therapeutics Inc. No potential conflicts of interest were disclosed by the other authors.

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Therapeutic Efficacy of an Fc-Enhanced TCR-like Antibody to the Intracellular WT1 Oncoprotein

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