The Use of Nucleotide Salvage Pathway Enzymes as Suitable Tumor Targets for Antibody-Based and Adoptive Cell Therapies

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Edwin J. Velazquez

A dissertation submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

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ABSTRACT

The Use of Nucleotide Salvage Pathway Enzymes as Suitable Tumor Targets for Antibody-Based and Adoptive Cell Therapies

Edwin J. Velazquez
Department of Microbiology and Molecular Biology, BYU
Doctor of Philosophy

Despite the progress made in cancer research, cancer remains one of the leading causes of death worldwide. Although the development of new cancer treatments has improved cancer patients' survival rate, a significant number of patients experience refractory and recurrence events with serious side effects. It is known that the immune system actively participates in eliminating cancer. However, cancer cells can develop mechanisms to evade the immune system resulting in immunotolerance. Immunotherapy aids the patient's immune system's ability to recognize and eliminate cancer cells. During the last three decades, immunotherapy has gradually emerged as an effective and more specific approach to treat cancer. Particularly monoclonal antibodies and adoptive cell therapies such as chimeric antigen receptor (CAR) T-cells have proven highly effective. Nevertheless, the success of these novel therapies depends on discovering suitable tumor targets. Recently, we reported localization of Thymidine Kinase 1 (TK1) to the plasma membrane of certain cancer cells but have not found such localization on normal cells. Similarly, another nucleotide salvage pathway enzyme Hypoxanthine Guanine Phosphoribosyltransferase (HPRT), has also been reported to be localized to the plasma membrane of certain cancer cells. Thus, TK1 and HPRT membrane-associated forms can be potential tumor targets for cancer immunotherapy.

This dissertation describes the immunotargeting of TK1 for the selective elimination of tumor cells and the surface localization of HPRT on the plasma membrane of cancer cells. Using hybridoma and phage display technologies, we developed monoclonal antibodies (mAb) and isolated human single domain antibodies (sdAb) specific to human TK1. We confirmed that antibodies and sdAbs could target TK1 on the plasma membrane of lung, breast and colon cancer cells, but not on healthy cells. In addition, we demonstrated that cancer cells expressing membrane-associated TK1 (mTK1) co-cultured with human mononuclear cells (MNC) were selectively eliminated through antibody-dependent cell-mediated cytotoxicity (ADCC) when anti-TK1 mAbs were added. Furthermore, we designed novel TK1 specific tumor targeting receptors and expressed them in human T cells and human macrophages. Finally, we proposed using both TK1 and HPRT as biomarkers for the early detection and monitoring of follicular lymphoma (FL), a disease that is usually detected at advanced stages. The knowledge generated from the data presented in this dissertation indicates that TK1 and HPRT may be suitable immunotherapeutic targets for antibody-based and adoptive cell-based therapies against both liquid and solid malignancies. It also proposes the incorporation of TK1 and HPRT as molecular biomarkers for the early detection and monitoring of FL.

Keywords: Thymidine Kinase 1, Hypoxanthine Guanine Phosphoribosyltransferase, tumor biomarker, monoclonal antibody, single domain antibody
I want to thank God for giving me the opportunity of coming to Brigham Young University and this great country. Without his divine intervention, it would have been impossible to complete my Ph.D. and survive all the challenges I went through as a foreigner, student, husband, and father. With no relatives in the US, the need to learn a new language, and scarce financial resources, it was indeed a miracle that my wife and I could complete graduate school simultaneously as we took care of our two children in the middle of a pandemic.

I want to thank brother Terry and Vicky Johnston, who, without expecting anything in return, extended their friendship to us, making possible what for a moment seemed impossible to access for a person like me. They are a true example of Christlike altruism and have inspired us to return the help we have received to others.

I want to thank my mentor Dr. Kim O’Neill for accepting me into his lab and giving me his friendship, support, and encouragement through my Ph.D. I will always be grateful to him for seeing potential in me. I’m incredibly thankful to him for teaching me to be professional and providing the tools and environment that I needed to grow as a scientist. I also appreciate the multiple times he shared his testimony of the restored gospel of Jesus Christ, which lifted me up in moments of spiritual need.

I would also like to thank the other members of my graduate committee: Dr. Scott Weber, Dr. Richard Robison, Dr. Bradford Berges, and Dr. Juan Arroyo, for their constructive suggestions, insights, and academic advice. I thank them for the time they have invested in me as
a Ph.D. student and person. For the correction and direction, I have received from them. Specially I thank them for their example and the positive influence they have been in my life.

I thank the national council of science and technology of Mexico (CONACyT), the Simmons center for cancer research, and the Jau-Fei Chen scholarship program for immunology related research for supporting my scientific career through multiple fellowships and scholarships that allowed me to focus on my cancer research.

I want to thank all of the companions in my lab that work next to my side for the scientific and non-scientific conversations we had. I especially would like to thank Evita G. Weagel for her unconditional friendship and support. Also, to Israel Guerrero, Antonio Solis, Claudia M. Tellez, Alex Benedict, Caleb Cornaby, Taylor D. Brindley, Gaju Shrestha, Eliza E. Bitter, Michelle H. Townsend and all undergraduate students who helped me and assisted me with my research. I enjoyed working and developing friendships with many of them, particularly John Lattin, Daniel Villanueva, Corbin Lee, Brandon Garcia, Liu Lu, Jordan Cress, Tyler Humpherys, Toni Mortimer, Rachel Morris, Kathryn Smith, David Bellini, Zachary Reinstein, Spencer Sabey, Kelsey Bennion and Zachary Ewell.

I want to thank the Department of Microbiology and Molecular Biology, the faculty, and staff for all their support thorough my Ph.D. without their support and encouragement it would not have been possible for me to get to this point in my career.
I want to thank my mother, Rosalina Espinoza, and my father, Jesus Velazquez, for sacrificing so much to help me get this far in my life. Even though our family went through financial hardships, they always did everything in their power to support my education and gave me a home where I could develop my potential.

I want to thank my beautiful wife, Fernanda, for all her unconditional love and support towards me. Her selfless dedication to our family has been truly remarkable. Even though she had enough work doing her master’s program, and being a mother for our two children, she always made herself available and supported all my career decisions.

Finally, I would like to thank my little Mariana and Isaac for all the joy they brought into my life. They were one of my strongest motivations to complete my Ph.D., be a better scientist, father, and human being.
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CHAPTER 1: Introduction to the clinical relevance of the nucleotide salvage pathway enzymes and their potential as tumor targets for cancer immunotherapy.

This chapter introduces TK1 and HPRT and their role in nucleotide salvage pathways and as potential cancer biomarkers. It also describes the initial findings of surface localization of TK1 and HPRT to the cell membrane. Finally, it discusses their potential as cancer immunotherapy targets for antibody-based and adoptive cell therapies. We reported this evidence in three scientific publications. The publications, where I share partial authorship with other members of our research group have been included in this dissertation document as appendixes 1-3.

Wherever appropriate, the work of other authors has been properly cited.
Abstract

Nucleotides are essential biomolecules that constitute the building blocks of DNA. However, nucleotides also participate in different processes in mammalian cells, including energetic metabolism, cell to cell communication, healing, enzymatic regulation, and immune modulation. Nucleotides can be synthesized through the *de novo* pathway and the salvage pathway. It is known that cancer cells rewire the *de novo* and salvage pathways to keep a constant flow of nucleotides to meet their enhanced metabolic demands. This metabolic rewiring in cancer cells results in nucleotide synthesis enzymes' upregulation. Thus, nucleotide synthesis enzymes have become appealing molecular targets for the development of cancer therapies. In recent years, two nucleotide synthesis enzymes from the salvage pathway, thymidine kinase 1 (TK1) and hypoxanthine phosphoribosyltransferase (HPRT), have increased in their clinical relevance. Both TK1 and HPRT enzymes have shown to be upregulated in various liquid and solid malignancies. Moreover, multiple studies have demonstrated the usefulness of TK1 and HPRT as cancer biomarkers for the early detection of disease and monitoring of cancer patients. Recently, we have reported the localization of TK1 and HPRT to the cell membrane of malignant cells, but not on normal cells. This chapter discusses the clinical relevance of TK1 and HRPT as cancer biomarkers and their potential use as therapeutic targets for antibody-based and cell adoptive therapies.
Introduction

The role of nucleotides in cell metabolism

Nucleotides are essential biomolecules that serve as building blocks for DNA and RNA molecules\(^1\). However, in mammalian cells, nucleotides also participate in multiple cellular processes, including cell signaling, energy metabolism, cell and organ healing, and cell to cell communication\(^2,3\). In addition, nucleotides participate as structural components of coenzymes, regulate enzymatic activity, and act as carriers of carbohydrates, amino acids, lipids, sulfate, and methyl groups\(^1,4\). Furthermore, nucleotides are ubiquitous molecules that are present intracellularly, and in the extracellular environment, where their concentrations significantly influence cell homeostasis and immune cell function (Fig. 1-1)\(^5,6\).

Figure 1-1. The role of nucleotides in cell metabolism. Nucleotides are essential molecules composed of a sugar (ribose or deoxyribose) linked to a nitrogen base and phosphate molecules. Nucleotides play an essential role in cell metabolism, cell to cell communication, tumor microenvironment, cell and organ healing, enzymatic activity, protein, and nucleic acid synthesis.
Structurally, nucleotides are composed of one of the five nitrogen bases; adenine, thymine, guanine, cytosine (only in DNA), or uracil (only in RNA) linked to a sugar molecule (deoxyribose or ribose) and one phosphoric acid (Fig. 1-2)\(^7\). When cells need to repair their DNA or proliferate, an increase in the demand for nucleotides occurs due to DNA replication, RNA production, and protein synthesis. Thus, to maintain homeostasis and meet their metabolic demands, cells replenish and keep a balanced supply of nucleotides using energy-demanding, and strongly regulated nucleotide synthesis processes\(^8\).

Figure 1-2. The chemical structure of nucleotides. Nucleotide molecules are composed of a sugar (ribose in the case of RNA and deoxyribose in the case of DNA), a nitrogen base (pyrimidine or purine) linked to the sugar molecule through its 1’ carbon, and one or more phosphate groups attached to the 5’ carbon. There are three known pyrimidines: cytosine, thymine, and uracil, and two purines: Adenine and Guanine.
There are two metabolic pathways through which nucleotides can be synthesized, the *de novo* and the salvage pathway. In the *de novo* pathway, each nucleotide component is synthesized from small intracellular organic molecules such as activated ribose (PRPP), amino acids, ATP, and carbonate\textsuperscript{9,10}. However, the metabolic energy required for this pathway is high since it utilizes energy from both glycolysis and oxidative phosphorylation\textsuperscript{8}. A detailed and elegant description of the *de novo* pathway can be found in a mini-review article written by Robinson et al.\textsuperscript{11}. In the second metabolic pathway, the salvage pathway, the nitrogen bases from existing nucleosides and nucleobases are recycled. Thus, cells can save energy by reutilizing bases derived from catabolic reactions to make new nucleotides\textsuperscript{12}. This recycling is facilitated by enzymes such as adenine phosphoribosyltransferase (APRT), hypoxanthine-guanine phosphoribosyltransferase (HPRT), thymidine phosphorylase (TP), cytidine deaminase (CDA), uridine-cytidine kinase (UCK1), deoxycytidine kinase (DCK), and thymidine kinase 1 (TK1)\textsuperscript{13-19}

Dysregulation of nucleotide synthesis enzymes in cancer

Since cancer cells are generally fast-growing, they have a high demand for DNA, RNA, and protein molecules as well as energy molecules such as, ATP and GTP. As a result, cancer cells often have dysregulation of nucleotide synthesis enzymes because of their high demand for nucleotides\textsuperscript{20}. Alteration in the expression levels of nucleotide synthesis enzymes can lead to perturbations in the nucleotide pools of cells. These perturbations can result in exogenous addition of surplus deoxynucleosides, which causes the misincorporation of bases into DNA\textsuperscript{21}. Thus, dysregulation of nucleotide synthesis enzymes can fuel genome instability and malignant transformation in cells. Multiple studies have shown that multiple enzymes from the *de*
nov 

purine and pyrimidine synthesis pathways are upregulated in cancer cells, resulting in significantly higher quantities of nucleotides being produced to meet the demands of tumor cells. Among the enzymes that are upregulated in the de novo nucleotide synthesis pathway are thymidylate synthase and inosine synthetase, which are upregulated via MYC transcription factor. There are other enzymes such as phosphoribosyl aminoimidazole succinocarboxamide synthase (SAICAR synthetase), glutaminase (GS1) and phosphoribosyl pyrophosphate amidotransferase (PPAT) that have also been shown to be upregulated in the de novo nucleotide synthesis pathway. Scientific evidence indicates that the dysregulation of the de novo pathway is influenced by a combination of oncogenes and tumor suppressors genes, including ras, akt, ampk, and mtor.

The relevance of nucleotide salvage pathway enzymes in cancer

Although most of the research involving nucleotide synthesis enzymes in cancer has been focused on enzymes from the de novo pathway, there is evidence that in many cases cancer cells may have a higher preference to use enzymes from the salvage pathway compared to enzymes from the de novo pathway. Early studies conducted by Weber et al. showed that malignant cells had higher nucleotide salvage pathway enzyme activity than enzymes from the de novo pathways during neoplastic transformation and in hepatomas. Another study done by Qi et al. showed that differential expression of 5'-nucleotidase (5NUC) and the xanthine oxidase/dehydrogenase (XD) enzymes resulted in increased usage of the salvage pathway in colorectal carcinoma. Dysregulation of the salvage pathway enzymes has been observed in lymphocytic and myeloid leukemia cells when compared to normal leukocytes. Other studies have repeatedly shown increased levels of nucleotide salvage pathway enzymes in malignant
cells due to chromosome imbalance and alteration in gene regulatory elements\textsuperscript{30}. These observations suggest that targeting the salvage pathway enzymes may be used as a suitable approach for cancer treatment. Several substrate analogs have been developed to target enzymes involved in the salvage pathway including, 5-fluorouracil (5-FU), trifluridine (FTD), 13-fluorouracil, gemcitabine, and cytarabine\textsuperscript{31,32}. In addition, multiple studies have explored the targeting of salvage pathway enzymes using RNA silencing and CRISPR tools\textsuperscript{33-36}. As a result of these investigations, nucleotide salvage pathway enzymes have increased in clinical relevance\textsuperscript{37,38}.

Two nucleotide salvage pathway enzymes, TK1 and HPRT have been shown to be dysregulated in both hematological and solid malignancies, and their expression levels correlate with cancer progression and patient prognosis\textsuperscript{36,39-41}. Most recently, TK1 and HPRT have been reported to be localized on the plasma membrane of multiple cancer cell lines, including tumor cells from clinical samples\textsuperscript{42-46}. These new findings suggest that TK1 and HPRT may have potential not only as tumor biomarkers, but also as cancer immunotherapy targets. This dissertation describes the use of the nucleotide salvage pathway enzymes TK1 and HPRT as potential tumor targets for cancer immunotherapy. Furthermore, it proposes the use of TK1 and HPRT as biomarkers for the early detection and monitoring of FL.

\textit{Thymidine kinase 1}

TK1 is a cell cycle-regulated salvage pathway enzyme involved in the synthesis and repair of DNA\textsuperscript{47}. It specifically catalyzes the phosphorylation of deoxythymidine to deoxythymidine monophosphate (dTMP), which is then phosphorylated by thymidylate kinase\textsuperscript{48},
producing deoxythymidine diphosphate (dTDP), and then nucleoside diphosphate kinase converts dTDP to deoxythymidine triphosphate (dTTP)\(^4\). The \(tk1\) gene is located on chromosome 17 (17q25), and its expression and activity are tightly regulated throughout the cell cycle, remaining low in \(G_0\) and early \(G_1\) and then significantly increasing during late \(G_1/G_2\) phases with its maximum peak occurring during \(S\) phase, after which it is degraded in the mitotic phase\(^3\)^\(^0\)^\(^5\).

TK1 regulation in healthy cells is a complex process at both genetic and protein levels. However, in cancer cells, this regulation is disrupted, and TK1 is upregulated at all cell cycle stages\(^5\). Several explanations have been proposed for the upregulation of \(tk1\) gene expression involving inversion of sequences in its promoter, point mutations in phosphorylation sites, loss of regulatory elements in its coding sequence, or simply a chromosome imbalance\(^3\)^\(^0\). Because TK1 is upregulated in multiple cancers, and is present in cancer patients' serum, it has acquired relevance as a cancer biomarker\(^5\)^\(^2\)-\(^5\)^\(^4\). Scientific research has shown that the levels and activity of TK1 enzyme in serum (sTK1) significantly increase in cancer patients as an early event in the development of malignancy\(^5\)^\(^5\)-\(^5\)^\(^7\). These levels correlate directly with cancer stage, \(^2\)^\(^6\),\(^5\)^\(^3\) thus allowing the possible monitoring of disease progression. Together, the staining of malignant tissues and the quantification of sTK1, have demonstrated that TK1 is a reliable cancer biomarker. Initially, several studies have shown that TK1 levels in serum could be used for the diagnosis and prognosis of patients with hematological malignancies, including non-Hodgkin's lymphoma, chronic lymphocytic leukemia, and acute lymphoblastic and myeloid leukemia\(^5\)^\(^8\)-\(^6\)^\(^2\). However, it has also been shown that sTK1 and \(tk1\) mRNA levels are useful for diagnosis and monitoring of cancer patients with solid malignancies such as breast cancer, lung cancer, colon
cancer, malignant melanoma, gastric cancer, head and neck squamous carcinoma (HNSCC), liver cancer, and pancreatic cancer. In addition to its capacity to monitor cancer patients, studies involving a significant number of patients have demonstrated that TK1 enzyme levels can be used to detect premalignant tissues, predict response to therapy, predict survival, and anticipate relapse events. Moreover, when TK1 is combined with other biomarkers such as Ki67, prostate specific antigen (PSA), or carcinoembryonic antigen (CEA), cancer diagnosis precision increases.

As TK1 concentration increases, the enzyme tends to make homomeric complexes of two and four identical chains that differ in their enzymatic activity. The tetrameric form is about 100 kDa, while the dimeric form is 50 kDa. The tetrameric form has approximately 30-fold higher activity than the dimeric form (Fig. 1-3). TK1 activation states are dependent on the concentration of ATP and thymidine. At concentrations of 2-3 mM of ATP and higher concentrations of thymidine (15-17 μM), TK1 is present as a dimer, while at lower concentrations of thymidine (0.5 μM), it is a tetramer. ATP presence is also required for the tetrameric form. However, at concentrations above 0.2 mg/ml of the enzyme, TK1 tends to oligomerize in its tetrameric form in an ATP-independent manner. TK1 activation states are also influenced by the cell cycle, the status of the P53 gene, and concentrations of dTTP. When dTTP and ATP are both bound to TK1, its enzymatic activity is inhibited.
In cancer cells, TK1 has higher activity when compared to TK1 in normal cells\textsuperscript{78}. It has been reported that TK1 has isoenzymes that are specific to malignancy, whose activity appears to be higher than TK1 from normal cells\textsuperscript{59}. Some studies have reported TK1 molecular forms of 45 kDa and 200 kDa in leukemic cells\textsuperscript{79}. In addition, analysis of serum from cancer patients has revealed TK1 complexes of higher molecular weight than its tetrameric form (100 kDa), ranging from 200 kDa to 720 kDa\textsuperscript{78,80}. This suggests that TK1 may have a tendency to homooligomerize, complex with other proteins, or present different forms in cancer cells that are not present in healthy cells. Recently, we have reported that monomeric and dimeric forms of TK1 were able to associate with the cell membranes of Burkitt’s lymphoma and acute lymphoblastic leukemia cells\textsuperscript{42,81}. When analyzed in scanning electron microscopy (SEM), the non-Burkitt’s
lymphoma cell line Raji showed surface binding of anti-TK1 antibody significantly higher than that of normal lymphocytes. Further staining of mononuclear cells (MNCs) from acute lymphoblastic leukemia (ALL) patients with anti-TK1 mAbs revealed a positive population for TK1 surface expression varying from 8% to 43.5% (averaging 28.01%). The study also demonstrated that TK1 found on the cell membrane fraction of Raji, Jurkat, and HL60 cell lines had activity (using a traditional TK1 radio assay) while negligible TK1 activity levels were found on the membrane fraction of normal MNCs (see appendix 1)43.

After confirming that TK1 overexpression on the cell membrane of malignant cells had clinical relevance for hematological malignancies, we decided to explore if solid tumors also presented TK1 localization to the plasma membrane. In a further study, we found that in the cancer cell lines NCI-H460 (lung), HT-29 (colon), and MDA-MB-231 (breast), all had localized TK1 in the plasma membrane 82. In addition to multiple cell lines, we also analyzed cells from both patient’s colon tumor tissue and healthy colon tissue. The isolated colon cells were analyzed for surface expression of TK1. Flow cytometry analysis revealed that malignant colon cells from colon cancer patients had significantly higher levels of TK1 on the cell membrane than colon cells from healthy patients ($P=0.0002$, $N=7$) (see appendix 2). These results suggested that TK1 had clinical relevance not only in hematological malignancies but also in solid malignancies. It also showed that the association of TK1 to the cell membrane seems to be an event restricted to malignancy but may not be present in all malignancies. Thus, the scientific evidence suggests that membrane-associated TK1 (mTK1) is a potential target for cancer immunotherapy and could be used in mono or dual targeting. The development of therapeutics such as antibody fragments or chimeric antigen receptor (CAR) specific for TK1 holds potential for the treatment of liquid and solid cancers.
Hypoxanthine guanine phosphoribosyltransferase (HPRT)

Purines participate in multiple cellular processes including, the production of energy, the purinergic signaling, and as cofactors of enzymes that sustain cell growth and proliferation\textsuperscript{83,84}. In the extracellular environment and on the cell membranes, purines also participate in immune responses and host-tumor interactions\textsuperscript{85,86}. An appropriate supply of purines is necessary to maintain various cellular activities, including proliferation and function of mitochondria, and activation or inhibition of apoptosis\textsuperscript{87}. In human cells, purines are supplied through the \textit{de novo} and salvage pathways. These two pathways maintain the cellular pools of adenylate and guanylate via synthesis and degradation of purine nucleotides\textsuperscript{88}. However, because the \textit{de novo} purine synthesis is an energy-demanding metabolic pathway, the majority of the purines are generated by recycling of degraded bases with the help of the salvage pathway enzymes HPRT and adenine phosphoribosyltransferase (APRT)\textsuperscript{37}.

HPRT is a useful enzyme as it can synthesize precursors for adenosine triphosphate (ATP) and guanosine triphosphate (GTP). The mechanism through which HPRT recycles nucleotides involves transferring a phosphoribosyl group from phosphoribosylpyrophosphate (PRPP) to hypoxanthine and guanine bases to form inosine monophosphate (IMP) and guanine monophosphate (GMP), respectively\textsuperscript{89}. The human \textit{hprt1} gene is 47,827 bp and resides in the long arm of the X chromosome, specifically at the Xq26.2-q26.3 locus\textsuperscript{90}. The protein is 217 amino acids long and is composed of ten \(\beta\) strands and six \(\alpha\) helices. The enzyme contains a core region of a twisted parallel \(\beta\) sheet of five \(\beta\) stands surrounded by four \(\alpha\) helices which resemble
a nucleotide-binding pocket (amino acids 137-189). This pocket is where the GMP molecule binds, apparently through a hydrogen bond with amino acid Lys-165 (Fig 1-4).91

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**Figure 1-4.** The crystal structures of HPRT in its tetrameric and monomeric forms. HPRT in its active form is a tetramer composed of four identical chains. The monomeric form is shown on the right, with GMP bound to the enzyme's active site. The active site of HPRT is a characteristic nucleotide-binding pocket similar to other nucleotide synthesis enzymes.

Besides its role as a salvage pathway enzyme, HPRT has received attention due to its participation in other relevant processes, including neuronal development, supporting of hematopoietic stem cell function, and sustained proliferation of tumor cells.92-95 Initially, HPRT was mainly considered to be a reporter gene for mutagenesis and endogenous control for gene expression assays.96 However, recent studies conducted by several research groups including ours, indicate that HPRT has an emerging role as a cancer biomarker and could be a potential therapeutic target for several cancers. Previously, we observed that TK1 upregulation was an early event in malignancy, and that TK1 levels in serum of cancer patients correlated with the stage of disease.39 Therefore, we wondered whether other salvage pathway enzymes were upregulated along with TK1. Initial studies conducted in our lab by Townsend et al.
demonstrated that tissue in a subset of patients with lung, colon, breast, and prostate cancers had HPRT protein levels elevated when compared to normal tissues\textsuperscript{97}. Further Western blot analyses of HPRT protein in cancer cells indicated that lung, breast, colon, prostate cancer and non-Burkitt’s lymphoma cells showed significantly higher HPRT expression levels when compared to those of healthy MNCs. In addition, these analyses revealed a possible relationship with the expression levels of P53, indicating increased levels of both p53 and HPRT in breast, colon, prostate, and non-Burkitt’s lymphoma cancer cells (Fig 1-5).

In addition to protein levels, analysis of \textit{hprt1} mRNA levels in breast, colon, lung, prostate cancers, and normal tissues also showed significant differences between normal and cancerous tissues. These findings have directly led to the conclusion that \textit{hprt1} is not an appropriate housekeeping gene for cancer-related studies, but more importantly, that HPRT protein may be useful as a cancer biomarker\textsuperscript{98}. A further study was conducted at Texas Tech University involving the analysis of \textit{hprt1} mRNA expression levels in all cancers using The Cancer Genome Atlas (TCGA) database\textsuperscript{41}. This study confirmed that when \textit{hprt1} mRNA levels in various cancers were compared to those found in normal tissues from the GTEx database, \textit{hprt1} mRNA levels were upregulated across all cancers compared to healthy tissues (Fig 1-6).
Figure 1-5. Levels of HPRT enzyme and P53 protein in lung, breast, colon, prostate, and non-Burkitt’s lymphoma cancer cells compared to normal MNCs.

Figure 1-6. Expression of hprt1 mRNA levels across all cancers from TCGA database. The mRNA levels of hprt1 are compared with the hprt1 levels in normal samples from the Genotype-Tissue Expression (GTEx) database. All cancers from the TCGA database showed upregulation of HPRT. Modified from Sedano et al.99.

Although the discovery of HPRT upregulation in malignant tissue was insightful, the mechanism through which HPRT is upregulated in cancer and its role in tumorigenesis has not yet been described. However, a recent study conducted by Wang et al. has shown a possible mechanism through which upregulation of HPRT supports proliferation, survival, and epithelial-mesenchymal transition (EMT) in neck and head squamous cell carcinoma (NHSCC). The study
found that HPRT levels correlated with the poor prognosis of cancer patients. Furthermore, experiments involving gain and loss of function demonstrated that depletion of HPRT activity in cancer cells resulted in suppression of cell growth in vivo. In addition, Co-IP experiments revealed that HPRT directly interacted with STAT3, maintaining its phosphorylation in a stable state. It is known that STAT3 has a cancer-promoting effect in most tumors. Stabilization of STAT3 phosphorylation by HPRT promoted IL-6 and EGFR signaling. The study proposes HPRT as a potential therapeutic target.

Considering the new evidence presented for HPRT, we decided to investigate further if HPRT was also localized to the cell membrane of cancer cells. Analysis of A549 and NCI-H460 non-small cell lung cancer (NSCLC) cell lines by confocal microscopy and scanning electron microscopy showed that HPRT was localized to the cell membrane (see appendix 3). Further analysis of clinical samples from ALL samples revealed expression of HPRT (average fluorescent population shift of 25%) on the cell membrane of malignant cells. When MNCs from healthy donors were stained with anti-HPRT mAbs, no membrane associated HPRT (mHPRT) was found on the plasma membrane.

Using dissociated cells from colorectal cancer tissue, Townsend et al. further demonstrated that HPRT surface localization was also present in solid malignancies. Flow cytometry analysis revealed that a significant fraction of malignant colon cells was positive for expression of mHPRT. Unlike cells coming from metastatic colon tissue, cells from healthy colon tissue did not present mHPRT at significant levels. This data suggested that the clinical relevance of mHPRT as a therapeutic target may extend to both liquid and solid tumors.
The evidence reported here reveals an emerging role of HPRT as a tumor biomarker and as a potential target for therapy. Moreover, the localization of HPRT to the cell membrane of tumor cells suggests that HPRT may be used as an immunotherapeutic target. The development of molecules that can specifically target HPRT in cancer cells will enable the evaluation of HPRT as a tumor target for both hematological and solid malignancies.

Cancer immunotherapy

While surgery, chemotherapy, radiotherapy, endocrine therapy, and tyrosine kinase inhibitors have significantly impacted cancer survival rates, a substantial portion of patients remain unresponsive to these therapies or experience recurrence events\textsuperscript{103}. Overall, approximately 40\% of all cancer patients in the US will not survive ten years following diagnosis\textsuperscript{104}. Thus, alternative treatments are necessary, especially for those patients that are unresponsive to standard cancer therapies. It is known that the immune system has the capacity to eliminate cancer cells. Through receptors, ligands, and other secreted molecules, immune cells can distinguish cancer cells from normal cells and selectively remove them in a process called immunosurveillance\textsuperscript{105}. However, as cancer progresses, it evades the immune system, through immunosuppressing signaling and the creation of a hostile tumor microenvironment that converts immune cells into pro-tumor activation states. This process is called immunoescape\textsuperscript{106}. Cancer immunotherapy is a paradigm-shifting approach that restores and boosts the patient’s immune system capacity to eliminate tumor cells\textsuperscript{107}. Cancer immunotherapy treatments include mAbs, cytokines, genetically engineered T-cells and cell therapies, cancer vaccines, bi-specific T-cell engagers, and oncolytic viruses\textsuperscript{108}. During the last two decades, cancer immunotherapy has demonstrated unprecedented success in the clinical setting and gradually reemerged as a
powerful approach for the treatment of cancers\textsuperscript{109}. As of December 2021, there were more than 4,720 drugs in development in the global immuno-oncology (IO) pipeline and approximately 11,643 active clinical trials testing cancer immunotherapy agents\textsuperscript{110}. Among the leading six classes of cancer immunotherapeutics, antibody-based and cell adoptive therapies are the two fastest-growing groups in the IO field.

Antibody-based therapies

The development of hybridoma technology in 1975 by Georges Kohler and Cesar Milstein at the MRC laboratories at Cambridge University was a scientific breakthrough that marked a new era in the production of mAbs and antibody research\textsuperscript{111}. Since then, monoclonal antibodies (mAbs) have been utilized for research, diagnostic and therapeutic purposes. Humanized mAbs have been used for the treatment of several diseases, including cancer\textsuperscript{112}. Today there are more than 2,000 mAbs being tested in the clinical setting\textsuperscript{110}. As of May 2021, the FDA has approved a total of 100 therapeutic mAbs, of which more than 30\% are for the treatment of cancer\textsuperscript{113}. Antibody-based therapies can be used to target proteins that are uniquely found on cancer cells or to block immune checkpoint molecules that tune the immune response in the tumor microenvironment\textsuperscript{114,115}. Thus, mAbs can be used to engage immune cells with tumor cells, boosting the immune response against cancer cells by identifying markers that the immune system can recognize, thereby stimulating an antitumor response\textsuperscript{116}.
Chimeric Antigen Receptors (CAR) T-cell therapies

CAR T-cells are genetically engineered T-cells that express a tumor-targeting receptor\textsuperscript{117}. CAR T-cells are composed of antibody fragments specific for a tumor target, a hinge connected to a transmembrane domain, an activation domain that triggers immune cell activation, and a co-stimulatory domain that supports the activation of the immune cell\textsuperscript{118}. In CAR T-cell therapy, T-cells are taken from the patient; they are genetically engineered to express a specific receptor (CAR) molecule, and re-introduced into the patient\textsuperscript{119}. As of November 2021, there are more than 900 active clinical trials testing CAR T-cells and five CAR T-cell therapies approved by the FDA\textsuperscript{120}. CAR T-cell therapy has proven very successful in hematological malignancies, mainly targeting CD19 (ORR up to \(\sim\)90\%), and most recently CD20 and B-cell maturation antigen (BCMA)\textsuperscript{121,122}. The success of CAR-T cell therapy in hematological malignancies has made scientists try to extrapolate its benefits to the context of solid malignancies. Although CAR T-cell therapy has made significant progress in liquid tumors, there are several challenges related to the nature of solid malignancies that still need to be overcome\textsuperscript{123}. Among these challenges are antigen escape and tumor heterogeneity\textsuperscript{124,125}. Frequently, tumor cells have been shown to lose expression of a particular tumor target, which renders CAR-T cells ineffective. Moreover, solid tumors are in their majority composed of heterogeneous cell populations expressing different markers. Hence, to be effective, future CAR T-cell therapies need to target more than one tumor specific antigen\textsuperscript{126}. Therefore, the expansion of suitable targets that can be used in both hematological and solid malignancies continues to be critical for the success of CAR T-cell therapy\textsuperscript{127}.
**Immunotherapy needs of suitable tumor targets**

Although promising, the efficacy of antibody-based and cell-based therapies is often hampered by tumor antigen escape or off-target effects in part due to the limited number of clinically proven targets. Thus, the discovery of novel tumor targets whose expression is restricted to malignancy is critical to overcome some of the challenges of tumor heterogeneity, antigen escape/loss, and to expand the spectrum of targetable tumors\(^{128}\). Thus, the critical initial steps in developing effective cancer immunotherapies are: 1) The identification of specific tumor antigens and 2) The generation of high affinity antibodies against these antigens. During the last decade, an explosion of new targetable tumor antigens have become available on various human malignancies\(^{127}\). However, a common limitation is that the majority of these tumor targets are also expressed on normal tissues, thus precluding the successful extrapolation of antibody-based and CAR T-cell therapies to a broader spectrum of tumor types and sometimes generating side effects related to cytotoxicity\(^{129}\).

**TK1 and HPRT as potential tumor targets**

We have previously described the surface expression of mTK1 and mHPRT in both hematological and solid malignancies. In tissue staining, Western blot, and mRNA studies, TK1 and HPRT have been shown to be upregulated in malignant tissue but not in normal tissue. Moreover, surface expression of mTK1 and mHPRT seems to be present only on tumor cells and absent or at negligible levels on healthy cells. Therefore, TK1 and HPRT may be suitable immunotherapeutic targets for antibody-based and cell-based therapies. Our hypothesis is that:
The targeting of cancer cells with TK1- and HPRT-specific immunotherapeutics will trigger a significant anti-tumor response by immune cells and initiate the elimination of TK1 and HPRT positive cancer cells without attacking non-cancerous tissues (Fig. 1-7).

In this dissertation we will discuss the development and preclinical evaluation of both antibody-based and cell adoptive therapies targeting membrane associated TK1 and HPRT forms in lung, breast, and colon cancer cells.

**Summary of research chapters**

Chapter 2 describes the immunotargeting of TK1 on the plasma membrane of cancer cells and the development of a panel of novel mAbs against human TK1. We present preclinical data regarding the validation of the TK1 mAbs for both diagnostic and therapeutic purposes. The antibodies produced had high specificity and sensitivity comparable to commercially available anti-TK1 antibodies. In addition, we found novel targetable epitopes in the TK1 molecule.
exposed on the plasma membrane of cancer cells, thus, adding new knowledge regarding the characterization of mTK1 on cancer cells. Moreover, we provide evidence that cancer cells expressing mTK1 can be targeted using mAbs and selectively eliminated by human MNCs through an antibody-dependent cell-mediated cytotoxicity (ADCC) response. To our knowledge, this is the first time that the immunotargeting of TK1 on the plasma membrane of cancer cells has been proven as a feasible approach for the treatment of solid tumors in a preclinical context.

This paper was published in Cancer Cell International (DOI: 10.1186/s12935-020-01198-8).

In Chapter 3, we implemented phage display technology to isolate human single domain antibodies (sdAbs), also known as nanobodies, against TK1. Although the development of mAbs through conventional hybridoma technology has been used successfully for decades, mAbs need further humanization before use in the clinical setting. Using human antibody libraries and phage display technology, we describe the isolation of human anti-TK1 sdAbs from a dAb library. The sdAbs were successfully expressed and validated through enzyme-linked immunoassays (ELISA), Western blot, siRNA knockdown, and flow cytometry. To date, partially humanized or completely human TK1 mAbs and dAbs have not been developed or used for the immunotargeting of cancer cells. We demonstrate that human anti-TK1 dAbs are able to bind to mTK1 on cancer cells. Since the anti-TK1 dAbs were ten times smaller (12kDa) than previously developed anti-TK1 mAbs, they represent an appealing approach for targeting tumor cells. We demonstrated that the anti-TK1 sdAbs could easily be reformatted and fused to custom engineered human IgG Fc regions. Furthermore, we provided evidence that human engineered anti-TK1-dAb-IgG1 antibodies can efficiently target mTK1 without the immunogenicity risks associated with previously developed anti-TK1 mAbs from animal hosts. The data presented
here strengthens the concept of using mTK1 as an immunotherapeutic target and proposes the use of human anti-TK1 nanobodies versus conventional full-length antibodies. The associated article was published in PLOS ONE (https://doi.org/10.1371/journal.pone.0264822).

Chapter 4 is a comprehensive but concise review of follicular lymphoma (FL) biomarkers describing the etiology and pathology of follicular lymphoma through its different stages. We also analyze the efforts made to develop prognostic indexes for the management of FL patients and the existing challenges regarding early detection and management of the disease. We discuss the current progress of follicular lymphoma biomarkers during the age of precision medicine for early detection and prediction to therapy response. In this paper, we point out that the development of novel cancer immunotherapy treatments requires new biomarkers and studies that reflect FL's current course. New biomarkers for FL include circulating tumor DNA, microRNAs, exosomes, cytokines, specific subsets of immune cells, and specific epigenetic-related genes. We discuss the clinical relevance of the nucleotide salvage pathway enzymes as biomarkers for hematological malignancies. We propose the use of TK1 and HPRT to be considered potential biomarkers for the early detection of FL and the monitoring of disease, thus expanding the cancer types where TK1 and HPRT can be utilized for diagnostic purposes. Following peer review by a journal, the reviewers concerns are currently being addressed for resubmission.

Summary of appendices

Appendix 1 is a scientific study published in OncoTargets and Therapy. The study reports the surface localization of TK1 to the cell membrane of Burkkit’s lymphoma and ALL.
cells. The study further reports that surface localization of TK1 seems to be an event restricted to malignancy. The study expands the role of TK1 from a biomarker to a potential immunotherapeutic target for blood malignancies. (https://doi.org/10.2147/OTT.S141239)

Appendix 2 is a scientific paper published in *Cancer Cell International*. The study provides evidence of TK1 expression on the cell membrane of lung, breast, and colorectal malignancies. It reports TK1 membrane expression on HT-29, SW620, MBA-MD-231, MCF7, NCI-H460, and A549 cells. It also demonstrates the expression of TK1 on the cell membrane of malignant colon cells from cancer patients. TK1 expression was not found on cells from healthy patients. The study suggests that TK1 may have potential therapeutic use as a target for solid malignancies. (https://doi.org/10.1186/s12935-018-0633-9).

Appendix 3. Is a study published in *OncoTargets and Therapy*. The study describes the initial experiments conducted to characterize the surface expression of HPRT on the cell membrane of non-small cell lung carcinoma cells. Significant localization of HPRT was found on both cell lines. No HPRT expression was found on cells from healthy donors. This study suggests HPRT may have a dual role as a cancer biomarker and a therapeutic target. (https://doi.org/10.2147/OTT.S128416).

Appendix 4 Contains data from an abstract published in *Cancer Research* entitled “Development of a TK1 specific chimeric antigen receptor T-cell for the treatment of non-small-cell lung cancer”. This poster was presented at the annual meeting of the American Association for Cancer Research in 2017, Washington DC. DOI: 10.1158/1538-7445.AM2017-5619.
Appendix 5 contains data of an abstract published in *Cancer Research* entitled “Macrophage Toll-like receptor-chimeric antigen receptors (MOTO-CARs) as a novel adoptive cell therapy for the treatment of solid malignancies.” This poster was presented at the annual meeting of the American Association for Cancer Research in 2018, Chicago.

DOI: 10.1158/1538-7445.AM2018-2563

Appendix 6 summarizes the number of publications, book chapters, and presentations at both regional and international conferences utilizing data produced by my research. It also contains a list of the awards received and the total amount of funding attracted through grant and fellowship applications during the course of my Ph.D. program.
CHAPTER 2: Novel monoclonal antibodies against thymidine kinase 1 and their potential use for the immunotargeting of lung, breast and colon cancer cells


The following chapter is taken from an article published in Cancer Cell International and included as a book chapter at the Prime Archives in Cancer Research. All content and figures have been formatted for this dissertation. I hereby confirm that the use of this article is compliant with all publishing agreements.
Abstract

Thymidine kinase 1 (TK1) is a pyrimidine salvage pathway enzyme that is up-regulated in malignant tissues and elevated in the serum of cancer patients. While TK1 has been well established as a tumor biomarker, little has been done to explore its potential as a tumor target. Recently, we reported the membrane expression of TK1 on malignant cells, but not on normal cells. This study explores the possible use of monoclonal antibodies for the targeting of membrane associated TK1 in lung, breast, colon and prostate cancer cells. We generated and evaluated a panel of monoclonal antibodies against six different epitopes exposed in the tetrameric form of TK1. Antibodies were developed with hybridoma technology and validated with Western blot, siRNA TK1 knockdown, enzyme-linked immunosorbent assay (ELISA) and flow cytometry. The therapeutic potential of the antibodies was evaluated in vitro in antibody-dependent cell-mediated-cytotoxicity (ADCC) experiments. Binding of the antibodies to TK1 was confirmed by Western blot in purified recombinant protein, cancer serum, and cell lysate. After a TK1 knockdown was performed, a reduction of TK1 expression was observed with five antibodies. Using indirect ELISA, we identified 3B2E11, 9C10, 7H2, 3B4, 8G2 among the most sensitive antibodies (LOD = 10.73–66.9 pg/ml). Surface expression of TK1 on the membrane of various cancer cell lines was analyzed with flow cytometry. Antibodies 8G2, 3B4, 7HD and 5F7G11 detected TK1 on the membrane of various cancer cell lines, including lung, prostate, colon and breast. No significant binding was detected on normal lymphocytes. Increased cytolysis of lung (~ 70%, \( p = 0.0001 \)), breast (~ 70%, \( p = 0.0461 \)) and colon (~ 50% \( p = 0.0216 \)) cancer cells by effector cells was observed when anti-TK1 antibodies were added during ADCC experiments. The antibodies developed showed potential to be used to detect and target TK1 on
the membrane of various tumor cells. The targeting of TK1 in malignant cells using monoclonal antibodies may be a feasible approach for the elimination of high TK1 expressing tumor cells.

Introduction

With the number of clinical and preclinical agents exponentially increasing every year, cancer immunotherapy is currently one of the fastest growing areas in global oncology. From cell adoptive therapies to monoclonal antibodies, the efficacy of most cancer immunotherapies primarily relies on the discovery of suitable tumor targets and the development of highly specific agents against these targets. During the last decade the list of tumor antigens available for immunotherapy have gone from dozens to hundreds, allowing us to treat a broader spectrum of human malignancies. However, a common limitation that many of these tumor targets face is their expression on normal tissues. Thymidine Kinase 1 (TK1) is a cell cycle regulated DNA synthesis enzyme that is up-regulated in malignant tissues during early stages of cancer development. Multiple studies have shown that TK1 levels in serum (sTK1) and tissues correlate with cancer progression, patient outcome and recurrence events. Although TK1 was initially proposed as a cancer biomarker for several blood cancers, it has also been shown to be a reliable biomarker for a wide variety of solid malignancies.

While TK1 levels have been primarily used to monitor the development of malignancy, it has been suggested that overexpression of TK1 or malignant associated forms of the enzyme could be used for the targeting of cancer. Recently, the expression of membrane associated TK1 forms in both cancer cell lines and clinical samples has been reported. In one study it was shown that monomeric and dimeric forms of TK1 can be detected on the cell membrane of...
mononuclear cells (MNC) from patients with acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML)\textsuperscript{81}. While this membrane associated TK1 form was present on malignant cells, it was absent on normal proliferating B cells\textsuperscript{81}. According to another study, membrane expression of TK1 was also found in lung cancer cell lines and cells from breast and colon tumors\textsuperscript{82}. These findings indicate that TK1 may be a potential immunotherapeutic target for antibody-based and adoptive cell therapies.

Despite the large number of studies demonstrating the value of TK1 as a cancer biomarker and its potential as a tumor target, there are a limited number of clinically tested antibodies for the detection and targeting of TK1\textsuperscript{152}. To our knowledge, no TK1 antibody-based therapeutics have yet been developed or tested in a preclinical setting yet\textsuperscript{153}. Moreover, most of the existing antibodies for detection of TK1 primarily target a cell cycle regulatory region of the TK1 molecule at the C-terminus\textsuperscript{154}. It has been reported that TK1 in malignant cells has different isoenzymes and that its activity levels differ significantly from TK1 in normal cells\textsuperscript{59,155}. It is also known that TK1 can form different complexes, with some in serum having abnormal molecular weights of up to 240 kDa and 730 kDa\textsuperscript{156-159}. Therefore, antibodies targeting only one region of the TK1 protein may limit the detection of TK1 to specific forms of the enzyme. Thus, the existing antibodies for TK1, may not be sufficient to detect some of the complex TK1 forms that exist in cancer patients. Broadening the spectrum of targetable TK1 specific epitopes could help us increase our ability to target TK1 in cancer patients.

In this study, we identified six epitopes that were exposed in the tetrameric form of TK1 and generated monoclonal antibodies specific for these regions. Seventeen antibodies were
chosen for characterization and validation through Western blot, siRNA TK1 knockdown, ELISA and flow cytometry. In addition, the potential of the anti TK1 antibodies for the targeting of malignant cells was tested by measuring the antibody-dependent cell-mediated cytotoxicity (ADCC) responses of mono nuclear cells (MNC) against high-level TK1-expressing cancer cells.

Materials and methods

Cell lines and isolation of MNC

The NCI-H460 (ATCCHTB-177™), A549 (ATCC® CCL-185), MDA-MB-231 (ATCC® CRM-HTB-26), PC3 (ATCC® CRL-7934™) and HT-29 (ATCC® HTB-38™) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained according to ATCC recommendations. A549, NCI-H460, PC3 and HT-29 were cultured in RPMI-1640 media (ThermoFisher scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-Glutamine. MDA-MB-231 cells were cultured in DMEM with 2 mM L-glutamine and 10% FBS. Cell lines were grown in an incubator at 37 °C and 5% CO2. All cell lines used were tested for TK1 surface expression with flow cytometry with a commercial antibody Abcam (91651) (Abcam, Cambridge, UK) to confirm the presence of TK1 on the cell membrane. Human MNC were isolated from whole blood from healthy donors with the high affinity CD16 V158 FcγIIIa receptor variant160. The CD16 V158 FcγIIIa variant was confirmed by polymerase chain reaction (PCR) from cDNA of the donor’s MNC and DNA sequencing. MNC were isolated with lymphocyte separation media (Corning, NY) following manufacturer instructions, red blood cells were depleted with red blood cell lysis buffer (Biolegend, San Diego, CA) and the MNC were resuspended in RPMI media with 2 mM L-glutamine and 10% heat inactivated human AB serum (Millipore SIGMA, St Louis, MO, USA).
Blood withdrawal was done under the Brigham Young University’s institutional review board approval No. 1734.

Production of recombinant TK1 in *E. coli* and *Saccharomyces cerevisiae*

The production of human TK1 was carried out in an *E. coli* expression system by Genscript’s recombinant protein service. In-house production of human TK1 was done using the pESC-URA (Genscript, Piscataway, NJ) yeast expression system and a Saccharomyces cerevisiae yeast strain with a REG-1 mutation. Briefly, the coding sequence of the human TK1 (Genbank NM_003258.5) was synthesized and placed into the pESC-URA vector flanked by the Sal I restriction sites. A tag of 6 histidines was included at the C-terminus of the TK1 sequence to facilitate His-tag purification. The TK1-pESC-URA vector was introduced in electrocompetent yeast using the lithium acetate procedure and an Eporator system (Eppendorf, Hamburg, Germany). After electroporating, the cells were plated in synthetic complete (SC) drop-out Ura-plates (Takara Bio USA Inc, Mountain View, CA) and grown for 36 h at 30 °C. Yeast culture was scaled up to 500 ml and induced for protein expression with galactose. After 36 hr, yeast cells were harvested and lysed in lysis buffer with the Halt™ protease inhibitor cocktail (ThermoFisher Scientific, Waltham, MA) in a French press. Recombinant TK1 was purified from cleared lysate using Ni-NTA-agarose bead columns (Qiagen, Hilden, Germany) and validated with commercially outsourced TK1 produced in *E. coli* by Genscript and the commercial anti TK1 antibody ab91651 in Western blot.
Epitope selection

Epitopes on the active form of TK1 that could be accessible to were determined analyzing the 1XBT crystal structure of the tetrameric form of TK1 using the PyMOL software. The epitope sequences were then analyzed using the protein BLAST tool from NCBI with the non-redundant protein sequences and the Homo sapiens (taxid9606) data bases to see the epitopes’ similarity with other human proteins. The sequences of the mouse, rabbit, dog and human TK1 isoform 1 (Genebank, NM_003258.5) were aligned and analyzed using the Geneious software to identify regions across the human TK1 sequence that significantly differ between species.

Production of hybridomas and selection of antibody clones

Antibodies were generated in mice and rats that were immunized with 6 different TK1 peptide sequences that were selected as described in the previous section. The peptide sequences for TK1 and the hybridoma cell lines were produced using the monoclonal antibody generation service MonoExpress™ Premium (Genscript, Piscataway, NJ). Briefly, the production of hybridomas consisted of four phases as follows. Phase one was the preparation of the immunogen. In this case the synthesis of six TK1 peptides using the PepPower™ peptide synthesis service (Genscript, Piscataway, NJ). Phase two involved the immunization of 3–5 Balb/c mice or rats with the MonoExpress™ immunization protocol. After the immunization regimen was completed, splenocytes were isolated and fused to myeloma cells using polyethylene glycol (PEG) and electrofusion. The cells were then cultured in hypoxanthine-aminopterin-thymidine medium (HAT) to select only the myeloma-lymphocyte hybrids. During phase three, individual hybridoma cells were isolated through limiting dilutions and their
supernatants were tested for binding to 1 μg/ml of each TK1 peptide used for immunization by indirect enzyme-linked immunosorbent assay (ELISA). The ten hybridomas with the best screening results were then selected for isotyping. The supernatants were then evaluated for binding to TK1 in Western blotting. After antibody binding to TK1 was confirmed through indirect ELISA and Western blotting, phase four involved the subcloning of each hybridoma by the limiting dilution technique. Each hybridoma was then expanded and frozen. From 44 hybridomas, a total of 17 clones were selected based on Western blot and Indirect ELISA results. The monoclonal antibodies were then purified from hybridoma supernatant with protein G and protein L columns (ThermoFisher scientific, Waltham, MA) and eluted in phosphate-buffered saline (PBS) buffer.

Western blot

Western blot was performed to validate the binding of the custom antibodies to TK1 using recombinant human TK1 produced with a yeast expression system to mimic the TK1 folding in human cells. The antibodies were also tested for their capacity to bind to TK1 in serum samples from lung cancer patients. Briefly, 0.5 μg of TK1 or 15 μg of serum samples were mixed with 6× Laemmli buffer (Millipore SIGMA, St Louis, MO). The protein samples were then heated at 100 °C for 5 min and loaded into a 12% SDS-PAGE gel. The proteins from the gel were then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5% milk in PBS tween-20 (PBS-T) buffer for 1 hr at room temperature, the blocking solution was poured out and primary antibody solution (1–2 μg/ml) was added. Membranes were incubated at 4 °C overnight. After overnight incubation, membranes were washed 3 times with PBS-T buffer. The bound proteins were detected using a 1:20,000 solution
of horseradish peroxidase-conjugated (HRP) anti-mouse or anti-rat antibody (Advansta Corporation, San Jose, CA). The proteins were then detected through the peroxidase reaction using enhanced chemiluminescence (ECL) (Advansta Corporation, San Jose, CA). Films were exposed for different amounts of time depending on the antibody being tested, with times ranging from 30 s to 5 min. The films were scanned, and the images were analyzed using the software ImageJ from NIH164.

siRNA TK1 knockdown

For our TK1 siRNA experiments we used the validated TK1 siRNA s14160 Silencer® Select (ThermoFisher Scientific, Waltham, MA). The Silencer™ GAPDH (Cat. No. 4390849) siRNA was used as positive control and the Silencer™ siRNA control No. 1 (Cat. No.4390843) was used as negative control. A total of $2.5 \times 10^5$ MDA-MB-231 cells were seeded in a 6 well plate and incubated overnight at 37 °C and 5% CO2. Cells were then transfected using 7.5 μl of lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) and 30 pmol of each corresponding siRNA following the manufacturer's protocol. After 24 hr., transfection was repeated. Cells were harvested after 24 hr. after second transfection for subsequent experiments and cell lysate preparations. Cell lysates were prepared using NP-40 cell lysis buffer with Halt™ protease inhibitor cocktail (100×) (ThermoFisher Scientific, Waltham, MA) and 1 mM of phenylmethylsulfonyl fluoride (Millipore SIGMA, Burlington, MA). Lysates were cleared by centrifugation and flash frozen with liquid nitrogen and stored at −80 °C. The cell lysates were then analyzed with Western blot comparing cell lysates treated with TK1 siRNA with the GAPDH siRNA and siRNA negative controls for six custom TK1 antibodies and the commercial TK1 antibody Ab76495 (Abcam, Cambridge, UK).
The sensitivity of each antibody to detect TK1 protein was estimated by indirect ELISA. In order to determine the limit of detection (LOD), antibodies were tested with individual dose–response curves and a four-point parameter logistic curve (4PL). Costar 96-well plates (Corning, NY, USA) were coated overnight with 100 μl of 1:1 serial dilutions of TK1 in PBS buffer at 4 °C. Dilutions ranged from 5000 to 26 ng/ml or from 6000 to 5 pg/ml, depending on the antibodies’ sensitivity. After overnight incubation, the plates were washed 3 times with PBS-T and blocked for 1 h with PBS 1% BSA at room temperature on a shaker with gentle agitation. The plates were then washed 3 times with PBS-T, and 100 μl of a 1 μg/ml solution of primary antibody were added into each well. The plates were incubated for 1 hr. at room temp on a shaker with gentle agitation. After incubating, the plates were washed 3 times with PBS-T, and 100 μl of a 1:40,000 dilution of secondary HRP-conjugated antibody (Advansta Corporation, San Jose, CA) solution were added into each well. Plates were incubated for 1 h at room temperature on a shaker. The plates were washed 3 times with PBS-T, and 100 μl of 1-step ultra TMB substrate (ThermoFisher scientific, Waltham, MA) were added to each well. The plates were then incubated for 10 min at room temperature, protected from light. The reaction was stopped by adding 100 μl of 0.5 M sulfuric acid solution. The absorbance was measured using a Synergy HT Microplate Reader (Bio-Tek Winooski, VT) at 450 nm and 650 nm. Samples were run in triplicate and confirmed with at least two independent experiments.

From the antibodies that showed high sensitivity in indirect ELISA, multiple antibody pairs were tested in sandwich ELISA format. One hundred μl of a 0.5 μg/ml solution of the capture antibodies were allowed to adsorb overnight at 4 °C. After overnight incubation, the
plates were washed 3 times with PBS-T buffer and blocked with PBS 1% BSA as previously described. TK1 protein or cancer serum was serially diluted in PBS buffer and then allowed to bind to the primary antibody for 1 hr at room temperature on a shaker. The wells were then washed 3 times with PBS-T buffer and 100 μl of biotinylated detection antibody solution (0.5 μg/ml) was added and allowed to bind for 1 hr at room temperature. After incubation, the plates were washed with PBS-T buffer and 100 μl of Pierce high sensitivity streptavidin-HRP solution (ThermoFisher scientific, Waltham, MA) was added to each well. The plates were incubated for 30 min. and washed with PBS-T buffer 3 times. After washing, 100 μl of 1-step ultra-TMB substrate solution (ThermoFisher scientific, Waltham, MA) was added to the wells and the plates were incubated with the substrate for 10 min. The reaction was quenched by adding 100 μl of 1 M sulfuric acid. Absorbance was measured using a Synergy HT Microplate Reader (Bio-Tek Winooski, VT) at 450 nm and 570 nm.

For each antibody, the limit of quantification (LOQ) and the LOD were determined by ELISA using the following formulas: LOQ = 10 (s/f’) and LOD = 3.3(s/f’)\(^{165,166}\) where s is the standard deviation from the residuals and f’ is the slope of a calibration curve. A 30% maximum value for relative standard deviation for the concentration estimates was set for the LOD and a 10% relative standard deviation for the LOQ, according to Hayashi et al.

Flow cytometry

Surface expression of TK1 was analyzed through flow cytometry. The antibodies’ binding capacity to detect membrane associated forms of TK1 was compared to the binding capacity of the commercial TK1 antibodies Ab91651 and Ab76495 (Abcam, Cambridge, UK).
For the experiment, four different cancer cell lines expressing different levels of TK1 were used. The cell lines used were the non-small cell lung carcinoma cell line NCI-H460, the breast cancer cell line MDA-MB-231, the prostate cancer cell line PC3 and the colorectal cancer cell line HT-29. Since membrane expression of TK1 appears to be restricted to malignancy, MNC from healthy donors were used as a negative control. Each one of the cancer cell lines was stained with each one of the anti-TK1 antibodies in at least 3 independent flow cytometry experiments.

For the analysis, cancer cells and normal MNC were collected as follows. Cancer cells were grown as monolayer cultures using DMEM or RPMI1640 with 10% FBS and 10 mM L-glutamine. The cells were grown until they reached 70–80% confluency and detached using accutase (ThermoFisher scientific, Waltham, MA). The cells were then collected and washed 3 times with cell staining buffer (PBS buffer, pH7.4, BSA 1% and sodium azide 0.1%). MNCs were isolated from whole blood as previously described and washed 3 times with cell staining buffer. All cells were resuspended at a concentration of $5 \times 10^5$ cells/ml, placed in individual microcentrifuge tubes and incubated with 5 μl of Human TruStain Fc block (BioLegend, San Diego, CA) for 10 min at room temperature. In the case of MNC, the cells were incubated with 10% heat inactivated human serum 30 min on ice. Unstained, isotype, and positive controls (CD44, CD45, GAPDH) were included in all experiments. After incubation with Fc block, the cells were stained with 1 or 2 μg of the custom and commercial TK1 antibodies and incubated for 30 min on ice. The cells were then washed 3 times and resuspended in 100 μl of cell staining buffer. Secondary antibodies conjugated to Alexa Fluor 647 (Abcam, Cambridge, UK) were added and the cells were incubated on ice for another 30 min, protected from light. After incubating with the secondary antibodies, the cells were washed 3 times, resuspended in 100 μl
of cell staining buffer and analyzed. Dead cell discrimination was performed by adding 15 μl of a 10 μg/ml PI solution (Millipore SIGMA, St Louis, MO) 1 min before analysis. A Cytoflex flow cytometer machine (Beckman Coulter, Brea, CA) was used to collect the data. The data analysis was performed using FlowJo software (FlowJo, Inc., Ashland, OR).

Detection of membrane associated TK1 and isolation of plasma membrane protein

To further confirm the levels of membrane associated TK1 from cancer and normal cells, we isolated plasma membrane protein fractions from cancer and normal cells. For this experiment, the non-small cell lung cancer cell line NCI-H460 was chosen for presenting high levels of membrane associated TK1. MNCs from a healthy individual were used as normal cells. To isolate the plasma membrane protein, we used the extraction kit (Abcam, Cambridge, UK) and followed the manufacturer’s instructions. Briefly 2 × 10^8 cells were harvested and gently lysed with a Dounce homogenizer in 2 ml of lysis buffer with protease inhibitors. After lysis, the cells were centrifuged at 700g for 10 min at 4 °C and the supernatants were transferred to a new tube. The cleared supernatants were then centrifuged at 10,000g for 30 min at 4 °C. The supernatant containing the cytosolic fraction was completely aspirated and the cell pellet containing the membrane protein fraction was resuspended and mixed with upper and lower phase solutions as instructed from the manufacturer. The total plasma membrane protein was then quantified using the BCA assay kit (ThermoFisher scientific, Waltham, MA). A Total of 30 μg of protein for each sample was loaded into an SDS-PAGE gel. Separated proteins were transferred to a nitrocellulose membrane and Western blots were performed as previously described. To detect TK1, we used a validated TK1 antibody. As negative control, we used an
anti-GAPDH antibody (Cell Signaling, Danvers, MA) and as positive control we used an anti-
sodium/potassium ATPase antibody MA3-928 (ThermoFisher scientific, Waltham, MA).

Antibody-dependent cell-mediated cytotoxicity experiments

The capacity of the custom antibodies to elicit ADCC responses was tested in vitro. For
these experiments the A549 cell line which expresses high levels of TK1 on the cell membrane
was chosen as a target cell. To measure cell death overtime, we used the real time cell analysis
platform ExCELLigence (ACEA biosciences, San Diego, CA). The ExCELLigence platform
works on the principle of impedance. Tissue culture plates with nanogold electrodes were used to
measure impedance caused by target cells adhering to the plates. The impedance reflects cell
growth and cell viability. The cell growth is then expressed as a normalized cell index (NCI) that
is measured over time. The higher the cell index (CI), the more cell growth. A decrease in the
cell index reflects cell death or cytotoxicity caused by a treatment, in this case, an ADCC
response[167]. For the ADCC experiments, a max of $8 \times 10^3$ target cells were seeded in each well
in a total volume of 100 μl of RPMI media. Experiments were run for 48–72 hr. CI were
normalized to 1 at the time when effector cells and antibodies were added. After sufficient
growth (CI 0.5–1.0), 50 μl of freshly isolated MNC cells were added at different effector-target
ratios (E:T). E:T ratios of 5:1, 2.5:1, 1.25:1 and 0.625:1 were tested. After finding the optimum
E:T ratio, dose response curves were generated by testing different concentrations of the TK1
antibodies to find the minimum amount of antibody that could produce a significant increase in
specific cell death. All experiments included cells treated with the IgG2b negative control
antibody (BIO-RAD, Hercules, CA). Experiments also included controls of target cells without
antibody and a full cell lysis control. The effect of the TK1 antibodies in target and effector cells
alone were also tested and included as additional controls. The percentage of specific cytolysis was determined using the immunotherapy module from the RTCA pro software (ACEA biosciences, San Diego, CA). The signal from effector and target cells with no antibody and from effector alone was subtracted. According to the user’s manual, the software subtracts the values from the reference wells using the following formula:

\[
\% \text{ Cytolysis}_{st} = \frac{1 - \text{Norm}(\text{CI}_{st} - \text{AvgEff}_\text{Sub}_{t})}{\text{AvgNorm}(\text{CI}_{Rt} - \text{AvgEffR}_\text{Sub}_{t})} \times 100
\]

where \( \text{Norm}(\text{CI}_{st} - \text{AvgEff}_\text{Sub}_{t}) \) is the Normalized value of the sample Cell Index minus the Average Cell Index for the matching selected Effector alone control at time \( t \) and \( \text{AvgNorm}(\text{CI}_{Rt} - \text{AvgEffR}_\text{Sub}_{t}) \) is the average normalized cell index value of the reference wells minus the Average Cell Index of the matching effectors used in the reference at time \( t \). If target alone is used as reference, there is no subtraction of \( \text{AvgEffR}_\text{Sub}_{t} \).

To add a qualitative element to our ADCC experiments and visually monitor the ADCC responses of MNCs against different target cells, we also used the ImageXpress® Pico system (Molecular devices, San Jose, CA). For this assay, we chose two cell lines positive for TK1 on the cell membrane, the MDA-MB-231 and the HT-29 cell lines. To allow the ImageXpress® Pico system to count individual cells, the cells were engineered with the IncuCyte® Nuclight Green Lentivirus reagent (Sartorius, Gottinga, Germany) to express nuclear restricted green fluorescent protein (Nuc-GFP). Cells were seeded at the same density described above and grown overnight under controlled environmental conditions (37 °C, 5% CO2).
Effector cells and anti-TK1 antibodies were then added to the target cells and the target cells were counted each hr. for 48–72 hr. Normalized GFP cell count was compared between treated and untreated cells. Antibody isotype controls were included as negative controls.

Statistical analysis

All the statistical analyses were performed using GraphPad Prism software (GraphPad, San Diego, CA). For the analysis of the dose–response curves from the indirect ELISAs, the data were log-transformed and analyzed with a 4-parameter non-linear regression analysis with a 95% confidence interval (CI). The slope, the R square, and standard deviation of the residuals were calculated. A test for goodness of fit, replicates test for lack of fit and test for homoscedasticity were performed. For ADCC experiments involving dose–response curves, two-way repeated measures ANOVA analyses were performed to compare different treatments during a course-time. Analysis of multiple comparisons was performed comparing the mean of each treatment with every other treatment mean. Correction of multiple comparisons was done with the Tukey test. For cytolysis experiments, isotype control vs treatment group were compared using multiple two tailed T-tests. Non-consistent standard deviation was assumed.

Results

Production and validation of recombinant human TK1

About 2 mg/ml of soluble protein was obtained from a 500 ml liquid culture. In-house produced TK1 in yeast was then validated with Western blot using commercially outsourced TK1 (< 80% pure) produced by Genscript in an E. coli expression system as positive control. A
band of 25 kDa was be observed in both TK1 sources, commercially outsourced (bacteria) and in-house produced (yeast) (Additional file 2-1) TK1 was detected with the anti-TK1 antibody ab91651, thus, validating our TK1 production process. In addition, a 50 kDa dimer band was observed in the TK1 produced in our yeast expression system, showing the ability of our in-house TK1 to properly fold and make TK1 complexes.

Selection of targetable regions in the active form of TK1

To identify regions specific for human TK1, alignments of the human TK1 sequence with those of mice, dog and rabbit showed conserved and variable regions among species. However, the epitopes of our interest were localized in the most variable regions of the TK1 human sequence when compared with the other species (Fig. 2-1A). The similarity of the human TK1 sequence to other species is shown in Table 2-1. Six different epitopes across the TK1 molecule were chosen for antibody generation (Table 2-2). The epitopes covered accessible regions in the tetrameric form of the human TK1 isoform 1, from the N-terminus to the C-terminus (Fig. 2-1B). BLAST results showed 100% coverage and identity for most of the epitopes’ hits specific for TK1 protein with E values ranging from $10^{-5}$ to $10^{-15}$. We set an E value of $1e^{-6}$ as a tolerance value for all the hits the epitopes had as recommended by the NCBI BLAST QuickStart tutorial\textsuperscript{169}. We did not see any non-TK1 related hits equal or below the cut-off value from the six epitope sequences. Thus, the epitope sequences seemed to be unique to the human TK1, and no significant similarities with other human proteins were found.
Figure 2-1. Alignment of the amino acid sequences of thymidine kinase 1 from 4 different species, *Canis lupus familiaris*, *Oryctolagus cuniculus*, *Mus musculus* and *Homo sapiens*. A, The conserved regions are highlighted and the differences between species are shown in the identity top bar. TK1 epitopes are annotated in yellow. B, The tetrameric form of TK1 was 3D modeled using the PyMOL software and the 1XBT crystal structure from the NCBI protein data bank. The peptide sequences utilized in the generation of monoclonal antibodies were highlighted in red. The epitopes selected for antibody generation were all peptide sequences that were exposed and hypothetically accessible to antibody binding. Epitope 6 is localized at the end of the C-terminus of the TK1 molecule but not visible in the available crystal structures of TK1.

Table 2-1. Percentage of similarity between the TK1 sequences of different animal species.

<table>
<thead>
<tr>
<th></th>
<th>O. cuniculus</th>
<th>C. lupus</th>
<th>M. musculus</th>
<th>H. sapiens</th>
</tr>
</thead>
<tbody>
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<td>O. cuniculus</td>
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<td>86.27</td>
<td>91.03</td>
<td></td>
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<tr>
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<td>88.84</td>
<td></td>
</tr>
<tr>
<td>M. musculus</td>
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<td>83.47</td>
<td>86.32</td>
<td></td>
</tr>
<tr>
<td>H. sapiens</td>
<td>91.03</td>
<td>88.84</td>
<td>86.32</td>
<td></td>
</tr>
</tbody>
</table>
Table 2-2. The amino acid sequences and regions of the selected TK1 epitopes.

<table>
<thead>
<tr>
<th>Epitope</th>
<th>Sequence</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CYAKDTRYSSSFCTHDRNTME</td>
<td>55-74</td>
</tr>
<tr>
<td>2</td>
<td>MEALPACLRLRDVAQEALGVC</td>
<td>73-91</td>
</tr>
<tr>
<td>3</td>
<td>CPDIVEFCEAMANAGK</td>
<td>103-117</td>
</tr>
<tr>
<td>4</td>
<td>CAYTKRLGTEKEVEV</td>
<td>161-174</td>
</tr>
<tr>
<td>5</td>
<td>CASGQPAGPNKENCVPVGKP</td>
<td>193-212</td>
</tr>
<tr>
<td>6</td>
<td>KPGEAVAARKLFAPQQILQC</td>
<td>211-230</td>
</tr>
</tbody>
</table>

Validation of TK1 antibodies through western blotting

An initial screening using supernatants from the hybridomas clones was conducted to identify antibody candidates. After this initial screening, a panel of 17 hybridoma clones was selected for antibody production. To determine if these antibodies had the capacity to bind human TK1 protein, we first performed western blotting with purified recombinant TK1 from our yeast expression system and compared each of the antibodies with the commercial TK1 antibody Abcam (91651). The results showed that all antibodies except 10H2, 6A11, and 8F12 showed binding to the recombinant purified TK1 protein (Fig. 2-2A). It is known that TK1 exists in multiple forms such as the monomeric, dimeric, and tetrameric form. We found that antibodies 4G10, 10E8, 6E10, 7D1, 8G2, 2E8 and 9A9 were able to bind multiple forms of TK1, including the dimer and tetramer. In addition, the majority of the antibodies demonstrated ability to detect TK1 in the serum of cancer patients (a representative sample using serum from a stage IV lung cancer patient is shown in Fig. 2-2B). Although the antibodies mainly detected the dimeric form of TK1 in serum, we observed that antibodies 4G10, 3G7, and 3B4 were able to detect the TK1
monomeric form while the antibodies 5F7G11 and 2E8 seemed to detect a broader range of TK1 complexes from 25 kDa to 100 kDa or larger forms.

Figure 2-2. Western blot analysis of the generated antibodies for all 6 epitopes. The antibodies’ capacity to detect human TK1 was tested. A, An amount of 0.5 μg of recombinant human TK1 was loaded in an SDS-PAGE and transferred to nitrocellulose membranes. B, An amount of 20 μg of serum from a stage IV lung cancer patient was loaded in each lane. The membranes were probed with the custom TK1 antibodies and compared with a commercial TK1 antibody. For serum, a transferring antibody was included as a serum loading control and to test serum integrity.

Antibody validation with a siRNA TK1 knockdown

After having confirmed the binding of the custom antibodies to recombinant TK1 and TK1 in human serum samples, we decided to validate the specificity of six antibodies by silencing the expression of TK1 using TK1 siRNA. In order to do that, we first tested if we were able to successfully knockdown TK1 in the breast cancer cell line MDA-MB-231. We observed a significant reduction in the expression of TK1 in the cells treated with the TK1 siRNA, while expression of TK1 using GAPDH and the non-specific siRNAs was significantly higher. Similarly, the cells treated with GAPDH siRNA showed a decrease of GAPDH expression
according to Western blot analysis. GAPDH expression was not affected by TK1 siRNA or the negative siRNA control (Fig.2-3A).

**Figure 2-3.** Validation of the custom anti-TK1 antibodies with a TK1 siRNA knockdown experiment in MDA-MB-231 cells. A, GAPDH positive, SiRNA negative controls and TK1 siRNA s14160 were tested with commercial anti GAPDH and anti TK1 antibodies. B, Validation of 6 custom TK1 antibodies with Western blot analyzing cell lysates from siRNA negative control and TK1 siRNA treated MDA-MB-231 cells. C, Normalized TK1 expression in reference to GAPDH and comparison of expression levels between cells treated with TK1 siRNA and untreated.

Six different antibodies against the six different TK1 epitopes were tested including antibodies: 10E8 (Epitope 1), 10H2 (Epitope 2), 3B4 (Epitope 3), 5F7G11 (Epitope 4), 2E8 (Epitope5) and 3B2E11 (Epitope 6). Each antibody was tested using cell lysate from cells treated with a negative siRNA control compared with a cell lysate from cells that were treated with TK1 siRNA. A significant reduction in the detection of TK1 was noticed with antibodies 10E8, 8G2, 3B4, 2E8 and 3B2E11. This difference was particularly pronounced with antibody 3B2E11, which is the most sensitive antibody clone. No difference in the TK1 expression between cell lysates was observed with antibody 5F7G11 (Fig. 2-3B, C). We observed that the main forms of
TK1 present in cell lysate were the dimeric and the tetrameric forms. In the case of antibodies 10E8, 8G2 and 3B4, we could also observe TK1 forms of high molecular weight around or above 200 kDa. Such bands were not detectable in cell lysates of siRNA TK1 treated cells. While antibodies 10E8, 8G2 and 3B4 were able to detect multiple forms of TK1 in cell lysates, antibodies 2E8 and 3B2E11 detected only tetrameric and monomeric forms, respectively.

Novel anti-TK1 antibodies detect TK1 surface expression in lung, breast, colon and prostate cancer cells but not on normal MNCs.

During flow cytometry experiments, we found that several of the anti-TK1 antibodies were able to bind to membrane-associated TK1 across four different cancer cell lines (Additional files 2-2 to 2-6). In the case of NCI-H460 cells, which had the highest TK1 levels, at least 1 antibody from each of the six TK1 epitopes show binding to membrane associated TK1. Although in this cancer line, TK1 was targetable with all six epitopes, only epitope three seemed to be targetable on the cell membrane of all the cancer cell lines. Moreover, the expression levels of membrane associated TK1 (mTK1) changed depending on the cancer cell type. The maximum number of cells positive for membrane associated TK1 was 95.6% for NCI-H460, 72.2% for PC3, 62.4% for HT-29 and 49.1% for MDA-MB-231 cells. Two of the custom antibodies, 3B4 and 5F7G11 showed consistent binding to MDA-MB-231 (breast) and PC3 (prostate) cancer cell lines, although antibody 5F7G11 showed low affinity for TK1 in indirect ELISA (Fig. 2-5). This antibody also showed low specificity in antibody validation with an siRNA TK1 knockdown. Thus, we excluded this antibody as a candidate for the immunotargeting of TK1. In the case of the HT-29 (colon) cells, the antibodies with the highest binding for TK1 on the cell membrane were 10E8, 7H2 and 3B4. In general, these four antibodies, 10E8, 3B4, 8G2 and 7H2 showed
similar or even higher binding levels compared to antibody ab91651 and were selected as candidates to be used in ADCC experiments (Fig. 2-7). None of the custom antibodies mentioned above showed significant binding to normal MNC. In the case of antibody 3B2E11 that showed binding to H460 and PC3 cells, we decided not to use it as candidate for ADCC experiments, due to its rat isotype. In multiple times, we observed that our Rat isotype control bound to PC3 cells at similar levels as the antibody 5F7G11 which was generated in rat. Although this happened only in PC3 cells, this antibody was excluded from our subsequent antibody-dependent cell-mediated cytotoxicity (ADCC) experiments.
Figure 2-7. Detection of TK1 surface expression through flow cytometry. Four different cancer cell lines expressing membrane associated TK1 were stained with the custom TK1 antibodies. The binding capacities of the custom antibodies were compared to a commercial TK1 antibody. A, The average percentage value of positive cells to TK1 from three independent flow cytometry runs across 4 cancer cell types, with lung cancer cells expressing the maximum percentage (95.6%) followed by prostate (72.2%), colon (62.4%) and breast (49.1%). B, Clones 3B4, 8G2, 10E8 and 7H2 showed consistent binding for detection of TK1 surface expression in lung, prostate, breast and colon cancer cell lines. In the case of H460 cells, which had the highest expression levels of TK1 on the cell membrane, antibodies from epitope 1 and 6 showed binding consistently. Although 5F7G11 did not show high specificity to TK1 when tested with a TK1 knockdown, the antibody showed consistent binding to the surface of all cancer cell lines analyzed. In addition, antibody 8F12 showed consistent binding too but did not produce any signal in indirect ELISA.
Antibody validation through ELISA, targeting of different TK1 regions and sensitivity of the antibodies

To find the most sensitive antibodies for the detection of TK1, each of the antibodies was first individually tested by indirect ELISA using a dose–response curve (Fig. 2-4). The curves were then analyzed with a 4-parameter non-linear regression and the limit of detection (LOD) of each antibody was determined. From the 17 antibodies 13 fit the shape of a sigmoidal curve and passed the goodness of fit test. The R squared values ranged from 0.9696 to 0.9989. For the clone 9A9, we could see the bottom and the steepest part of the curve, but not the top plateau region. This was due to the available range of the TK1 dilutions. However, a slope could be calculated from the linear portion of the curve. Antibody clones 6A11 and ID3G4 did not develop a detectable signal in ELISA. Thus, these clones were excluded from further testing. For 15 of the 17 TK1 antibodies, the inter-assay CV% was between 1.52 and 17.32, while the intra-assay CV% was between 0.45 and 11.40.

Figure 2-4. Calibration curves of indirect ELISAs. Each antibody’s binding capacity to TK1 was tested in indirect ELISA. A 4-point non-linear regression analysis was performed for each dose–response curve. Antibody curves have been grouped according to their epitope specificity.
The limit of detection was calculated for each of the antibodies as previously described in the methods section. The interpolated values, standard deviations and percentage of the inter and intra coefficient of variance for each antibody are shown in Table 2-3. From the indirect ELISA, we found that the most sensitive antibodies were produced by the clones 3B2E11, 9C10, 7H2, 2E8, and 1B12, with clone 3B2E11 producing the most sensitive antibody, having a LOD between 8.87 and 12.58 pg/ml (Table 2-3 and Fig 2-5). These antibodies were able to detect concentrations of TK1 in the picomolar range, thus making them candidates for clinical and therapeutic applications.

Table 2-3. Sensitivity of the TK1 antibodies. The LOD values are expressed as mean values from two independent immunoassays. Precision and uncertainty are reflected in the coefficient of variance between assays. The most sensitive antibodies are marked with stars.

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Isotype</th>
<th>Host</th>
<th>Mean LOD</th>
<th>SD±</th>
<th>Inter CV</th>
<th>Intra CV</th>
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<td>Ms</td>
<td>44.67</td>
<td>4.33</td>
<td>9.69</td>
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<td>Ms</td>
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<td>0.55</td>
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In order to test the potential of the custom antibodies as diagnostic tools, we tested different antibody pairs in sandwich ELISA. The detection antibodies were biotinylated to facilitate the union of streptavidin-HRP protein. The antibody pairs that showed consistent results were 7H2 as capture (C) and 3B2E11 as detection (D), 10E8 (C) and 3B2E11 (D) and 2E8 (C) and 3B2E11 (D) (Fig. 2-6A). The sensitivity of the antibody pairs ranged from 0.12 to 0.04 ng/ml. The signal generated at the LOD of each antibody was at least three standard deviations far from the blanks (Fig. 2-6B).
Figure 2-6. Evaluation of three antibody pairs for TK1 detection in sandwich ELISA. A, Dose response curves and 4-point non-linear regression analysis of three antibody pairs. B, The signal obtained from TK1 detection in serial dilutions with three antibody pairs is proportional to the concentration of antigen. C, Optimization of capture and detection antibodies concentration ratios. D, Spiking recovery assay. Assessment of the interference of human serum with the antibodies’ capacity to detect a known concentration of TK1. Recovery was around 100% with 10% serum dilution. E, Specificity of the immunoassay to TK1. Using cell lysate from a high expressing TK1 cancer cell line and lysate from cells treated with siRNA TK1, the specificity of the immunoassay was evaluated. Signal from the siRNA TK1 knockdown cell lysate was significantly lower than the untreated cell lysate.

To evaluate the effect that human serum could have on the antibodies’ detection capacity, we performed a spiking recovery assay to determine the ability of these three antibody pairs to detect TK1 in human serum. A 10% serum dilution was spiked with a known amount of TK1 (10 ng/ml). We then determined how much of this added TK1 could be detected, expressed as a recovery percentage. For the pairs 10E8 (C) and 3B2E11 (D), 7H2 (C) and 3B2E11 (D) and 2E8 (C) and 3B2E11 (D), the recovery percentages were 96%, 113% and 91%, respectively (Fig 2-6D). To further test the specificity of the antibody pairs, we choose the antibody pair 2E8 (C) and 3B2E11 (D) and compared the signal generated by this pair from a cell lysate positive for
TK1 and a cell lysate from cells treated with siRNA TK1. Both cell lysates were diluted to the same protein concentration. As an additional control, we included spiked serum with a known concentration of TK1. We observed that there was a significant difference in the signal (about sixfold) obtained between normal cell lysate and cell lysate from cells treated with TK1 (Fig. 2-6E).

TK1 can be detected in plasma membrane protein extracts from cancer cells, but not in extracts from normal cells.

We found that TK1 was present in the plasma membrane protein fraction of H460 cells, but we could not detect TK1 in the plasma membrane fraction of normal MNC cells. We did not see signal coming from the GAPDH negative control, thus indicating low contamination levels of cytosolic proteins in the plasma membrane protein extract. The plasma membrane protein fractions were positive for Na/K ATPase, which is present on the cell membrane (Fig. 2-8).

Anti-TK1 monoclonal antibodies are able to elicit ADCC responses
Since membrane expression of TK1 was detected with several of the anti-TK1 antibodies, we decided to test their potential to target TK1 and elicit an in vitro ADCC response against cancer cells. We selected the antibodies 10E8, 8G2 and 3B4 for their specificity and capacity to detect membrane expression of TK1. For these experiments, two real-time cell analysis systems were used. For our initial ADCC experiments involving A549 cells, we used the ExCELLigence platform. For our subsequent ADCC experiments we used the ImageXpress®Pico system, which allowed us to incorporate cell imaging along with our cell measurements. The A549 (lung), the MDA-MB-231 (breast) and the HT-29 (colon) cell lines used in these experiments were previously screened to confirm the expression of membrane associated TK1 (Fig. 2-9).

Figure 2-9. Expression of TK1 on A549 cells. A, Surface expression of TK1 on A549 cells detected by flow cytometry with TK1 antibody ab91651. B, Optimization of effector and target cell ratios for ADCC assays using the ExCELLigence platform. Four different E:T ratios were tested. E:T ratios close to 1:1 showed reduced non-specific cell killing by MNCs. Eight thousand target cells (A549) were used in all wells.

In order to find the right effector cell/target cell ratio, we first optimized the amount of effector (E) and target (T) cells without an antibody present. In the case of the ExCELLigence system, we found that E:T ratios of 1.25:1 and 0.625:1 produced less background of non-specific cell killing (Fig. 2-9B). After finding the optimum E:T ratios, we tested different concentrations
of the custom TK1 antibodies 3B4 and 8G2. The purpose of this was to find the lowest concentration of antibody at which a significant ADCC response was produced, in comparison to control groups. In the case of the antibody 3B4 (isotype IgG2b), we found that concentrations of 5.0 and 7.5 μg/ml caused significant increases in cell killing of A549 cells by MNC, compared to IgG2b isotype control ($p = 0.0001$) for both concentrations. In the case of the 8G2 (isotype IgG2b) antibody, a concentration of 2.5 μg/ml was enough to elicit a response significantly different from the IgG2b isotype controls ($p = 0.0003$). However, higher concentrations of 8G2 yielded significantly stronger ADCC responses (Fig. 2-10B). After adding the TK1 antibodies, the maximum amount of specific cell killing induced by antibodies 3B4 and 8G2 was observed at 36 h and 48 h (Fig. 2-10E, F). The normalized cell indexes of the target cells and effector cells were not affected by the antibodies themselves. Once we found the minimum concentration of antibody that was necessary to elicit a significant ADCC response, we proceeded to measure the percentage of specific cytolysis. In the case of antibody 3B4, the maximum percent of cytolysis was observed after 36 h of treatment, while for 8G2 antibody, this occurred at 48 hr. An increase in specific cytolysis around 70% was observed with the antibody 8G2 ($p = 0.001$) while this value was 60% with the antibody 3B4 ($p = 0.0001$) (Fig. 2-10C, D).
Figure 2-10. ADCC assay using TK1-specific monoclonal antibodies 3B4 and 8G2. MNC and A549 cells were co-cultured in the presence or absence of the TK1 monoclonal antibodies 3B4 and 8G2. A, On the left, a 3B4 antibody concentration dose–response curve can be observed. The ADCC response started after 24 hr. of treatment. C and E, A significant cytolysis percentage is observed at 36 hr. with a 7.5 μg/ml 3B4 antibody \( (p = 0.0001) \). Graph B shows a dose-response curve for antibody 8G2. D and F, A significant increase in the cytolysis percentage compared to the isotype controls \( (p = 0.0003) \) was observed at 48 hr. with a 2.5 μg/ml antibody concentration.

For our subsequent ADCC experiments, we evaluated the ADCC response generated with the antibodies 3B4 and 10E8 against breast and colon tumor cells using the ImageXpress® Pico system. The cell lines MDA-MB-231 and HT-29 were previously engineered to express nuc-GFP, which allowed monitoring of cell death and proliferation. We found that a 5:1 E:T ratio worked better for our GFP-based assays. A concentration of 5 μg/ml was enough to elicit an ADCC response with antibody 10E8, while 7.5 μg/ml was utilized for antibody 3B4. A cytolysis around 70% of the MDA-MB-231 cells was observed when antibody 3B4 was added (Fig. 2-
The ADCC response was significant when compared to the isotype control ($p = 0.0172$). In the case of HT-29 cells, a cytolysis about 50% was observed using antibody 10E8 ($p = 0.0216$), which was significantly different from the isotype control (Fig. 2-11B). Cell imaging revealed an increased migration and clustering of MNCs around target cells when TK1 antibodies were added. Cell killing was visually confirmed by the presence of apoptotic cells and a significant reduction in the average fluorescence intensity of the target cells.

Figure 2-11. ADCC experiments on the MDA-MB231 breast cancer and the HT-29 colon cancer cell lines using the ImageXpress® Pico real time cell imaging system. A, The ADCC response against MDA-MB231 cells by MNCs using antibody 3B4. A significant decrease in the number of target cells was detected at 72 hr. compared to the isotype control ($p = 0.0172$). B, The ADCC response against HT-29 cells by MNCs using antibody 10E8. A decrease in the number of target cells of about 50% was detected at 48 hr. ($p = 0.0216$).
Discussion

Scientific evidence points to TK1 is a suitable tumor biomarker for the early detection of malignancy, tumor progression, prediction of recurrence and patient outcome\textsuperscript{54,170,171}. However, new evidence indicates an emerging role of TK1 as a possible tumor target. Previous findings confirming the presence of cell membrane associated TK1 forms in tumor cells from patients and recent studies reporting the targeting of TK1 expression inside cells, have strengthened the clinical relevance of TK1 as a tumor target\textsuperscript{81,82}. In this study, we have explored the targeting of TK1 with monoclonal antibodies. To our knowledge, this is the first time that the targeting of TK1 with monoclonal antibodies in lung, breast and colon cancer cells has been reported. In addition, we have described the generation and characterization of a new panel of high-affinity TK1 monoclonal antibodies, targeting six different epitopes, for the detection and immunotargeting of cancer.

Our results found that TK1 could be potentially targeted with antibodies at 6 different regions. In particular, antibodies against TK1 epitope two, five and six showed the highest affinities (below 50 pg/ml) (Fig.2-5). Since these are regions that appear to be on the exterior of the tetrameric form of TK1, antibodies may have easier access to these particular epitopes. In the past, TK1 has primarily been targeted towards the C-terminus, and although there are some commercial antibodies that target regions at the N-terminus, there is no available data indicating that these antibodies can reliably detect TK1 in serum or membrane-associated TK1 forms. To our knowledge, there is only one clinically tested TK1 immunoassay currently available and it targets a region called XPA-210 towards the C-terminus\textsuperscript{172}. Thus, the combination of antibodies
targeting multiple regions of the TK1 molecule could help us increase our ability to target TK1 in cancer cells.

The potential of these antibodies to detect and target cancer cells was initially tested with flow cytometry (Fig. 2-7). We found that the antibodies developed were able to detect membrane TK1 expression in lung, breast, prostate and colon cancer cells. This may indicate the possibility of targeting a broad spectrum of tumors. In addition, we found specific epitopes that seem to be exposed in the membrane associated form of TK1. According to our flow cytometry data, the regions of the membrane associated TK1 molecule that seem to be exposed were mainly epitope two, three and four (Table 2-2). Epitope two, is an exposed ribbon in the tetrameric form of TK1 located towards the N-terminus while, epitope three is a ribbon towards the center of the molecule. Epitope four contains a lasso and the active site of the molecule, suggesting that membrane associated TK1 may keep kinase activity. However, depending on which cancer cell line was analyzed, we observed that other epitopes were exposed as well. This was the particular case for H460 and HT-29 cells, where anti-TK1 antibodies for epitopes one and six showed binding to the cell membrane, while these epitopes were not detectable in MB-MDA-231 cells and PC3 cells. It is important to emphasize that NCI-H460 cells were the cells with the highest levels of TK1 on the cell membrane. Thus, it is possible that the higher expression levels of surface TK1 make these epitopes more accessible for antibodies. The absence of significant binding of the anti-TK1 antibodies to normal cells confirms our previous reports that TK1 membrane expression may be restricted to malignancy. This was further confirmed by Western blot analysis of membrane associated TK1 protein levels in NCI-H460 cells plasma membrane protein extracts and plasma membrane protein extracts from normal MNCs (Fig. 2-8). This, makes TK1 an immunotarget with low off-target effects that could be used for antibody-based or
cell adoptive therapies such as chimeric antigen receptor (CAR) T cell therapy\textsuperscript{173}. Furthermore, if membrane expression of TK1 is restricted to malignant cells, then the detection of membrane associated TK1 forms may be a hallmark linked to the development of particular cancers.

Although the role of TK1 as a tumor biomarker has extensively been studied, little has been done to explore the potential of TK1 as a tumor target. Previous studies have suggested that TK1 in malignant cells may be different than TK1 in normal cells. In fact, the targeting of malignant forms of TK1 was suggested almost three decades ago by other research groups\textsuperscript{150,151}. Due to their high specificity, monoclonal antibodies are ideal candidates for the specific targeting of TK1 in cancer cells. In addition, we did not find any available studies about targeting TK1 with monoclonal antibodies particularly targeting its membrane associated forms in malignant cells. Thus, our ADCC experiments were designed to explore this possibility.

During this study three of our custom TK1 antibodies, antibodies 10E8 (targets epitope one), 8G2 (targets epitope 2) and 3B4 (targets epitope three) were selected for their specificity and capacity to detect membrane associated TK1 on malignant cells. In this case, the anti-TK1 antibodies’ ability to elicit an ADCC response was measured. It is important to mention that even though the commercial anti-TK1 antibody ab91651 could detect surface expression of TK1, its particular rabbit isotype made it unsuitable for our ADCC experiments. We found a significant increase in the cytolysis of lung, breast and colon cancer cells by MNC when the anti-TK1 antibodies were added in comparison to controls (Figs. 2-10 and 2-11), thus confirming previous reports of the membrane expression of TK1 and opening the door for additional studies exploring the targeting of TK1 in cancer cells.
The mechanisms through which TK1 is expressed on the surface of cancer cells remains unknown. However, there are some indications of its associations with the cell membrane. In the past, other pyrimidine salvage pathway enzymes were found to be associated with the membrane of cancer cells\textsuperscript{46}. Other studies using yeast two-hybrid experiments have found TK1 interacting with other membrane proteins such as SEZL6 that are upregulated in lung cancer cells\textsuperscript{174,175}. Therefore, the characterization of novel TK1 protein–protein interactions on the membrane of cancer cells can possibly lead to the development of more specific targeted therapies. The dual targeting of tumor antigens is a feasible strategy that has been tested before in cancer immunotherapy\textsuperscript{176}.

The experiments presented here provide early evidence that the targeting of membrane-associated TK1 with monoclonal antibodies may be a feasible approach for the treatment of cancer. In addition, we have developed a novel panel of TK1 antibodies that may be useful for TK1-based diagnostics. Future directions of this research will focus on the targeting of several other cancer cell lines, \textit{in vivo} ADCC experiments and the development of both antibody-based and cell adoptive therapies targeting TK1.

\textit{Conclusions}

The antibodies presented here had the ability to detect and quantify TK1 in the picomolar range and potentially could allow us to measure TK1 levels in cancer patients. In addition, the antibodies were able to detect multiple forms of TK1, including membrane-associated TK1 in lung, breast, colon and prostate cancer cells. Thus, TK1 may be a tumor target that can be used
for therapy in multiple solid malignancies. The increased cytolysis of A549, MDA-MB-231 and HT-29 cells by MNCs in the presence of anti-TK1 monoclonal antibodies during the in vitro ADCC experiments suggests that TK1 monoclonal antibodies have potential not only as diagnostic tools, but also as immunotherapeutic agents for the treatment of cancer. Therefore, the immunotargeting of TK1 may be a feasible approach for the elimination of cancer cells. The exploration of TK1 as a tumor target may lead to the development of other TK1-based immunotherapeutics.

Acknowledgements

We acknowledge the valuable contributions of Kiara V. Whitley during our first attempts to develop TK1 antibodies. We thank Corbin Lee, Rachel A. Skabelund, Kelsey Bingham and Kai Li Ong for their help in the production of recombinant TK1 and assistance with other technical aspects of this research. We also thank to the BYU’s Simmons Center for Cancer Research who kindly provided a cancer research fellowship that gave support in the initial stages of this project. Funding was also provided by the department of microbiology and molecular biology at Brigham Young University.
Supplementary figure 2-1. Production and validation of human recombinant TK1 in a yeast-based expression system. The sequence of human TK1, transcript variant 1, (Accession No. NM_003258.5) was codon optimized to be expressed in a *Saccharomyces cerevisiae* yeast strain with a REG-1 mutation. A 6xHis tag was added at the C-terminus to facilitate His-tag purification. The optimized TK1 sequence was synthesized and cloned into the pESC-URA vector (Genscript, Piscataway, NJ) and was purified by affinity chromatography using NI-NTA-agarose beads columns (Qiagen, Hilden, Germany). In-house produced TK1 was validated along with commercially outsourced recombinant TK1 in Western blot using the anti-TK1 antibody ab91651 (Abcam, Cambridge, UK).

Supplementary figure 2-2. Flow cytometry analysis of NCI-H460 cells with the custom TK1 antibodies. See Table 2-3 to find the name of each clone associated with its respective ID.
Supplementary figure 2-3. Flow cytometry analysis of MDA-231 cells with the custom TK1 antibodies. See Table 2-3 to find the name of each clone associated with its respective ID.

Supplementary figure 2-4. Flow cytometry analysis of PC3 cells with the custom TK1 antibodies. See Table 2-3 to find the name of each clone associated with its respective ID.
Supplementary figure 2-5. Flow cytometry analysis of HT-29 cells with the custom TK1 antibodies. See Table 2-3 to find the name of each clone associated with its respective ID.

Supplementary figure 2-6. Flow cytometry analysis of normal MNC cells with the custom TK1 antibodies. See Table 2-3 to find the name of each clone associated with its respective ID.
CHAPTER 3: Selection of Human Single Domain Antibodies (sdAb) Against Thymidine Kinase 1 and Their Incorporation Into sdAb-Fc Antibody Constructs For Potential Use In Cancer Therapy.

https://doi.org/10.1371/journal.pone.0264822.

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Abstract

Thymidine Kinase 1 (TK1) is primarily known as a cancer biomarker with good prognostic capabilities for both hematological and solid malignancies. However, recent studies targeting TK1 at protein and mRNA levels have shown that TK1 may be useful as a therapeutic target. In order to examine the use of TK1 as a therapeutic target, it is necessary to develop therapeutics specific for it. Single domain antibodies (sdAbs), represent an exciting approach for the development of immunotherapeutics due to their cost-effective production and higher tumor penetration than conventional antibodies. In this study, we isolated sdAb fragments specific to human TK1 from a human sdAb library. A total of 400 sdAbs were screened through 5 rounds of selection by monoclonal phage ELISA. The most sensitive sdAb fragments were selected as candidates for preclinical testing. The sdAb fragments showed specificity for human TK1 in phage ELISA, Western blot analysis and had an estimated limit of detection of 3.9 ng/ml for the antibody fragments 4-H-TK1_A1 and 4-H-TK1_D1. The antibody fragments were successfully expressed and used for detection of membrane associated TK1 (mTK1) through flow cytometry on cancer cells [lung (~95%), colon (~87%), breast (~53%)] and healthy human mononuclear cells (MNC). The most sensitive antibody fragments, 4-H-TK1_A1 and 4-H-TK1_D1 were fused to an engineered IgG1 Fc fragment. When added to cancer cells expressing mTK1 co-cultured with human MNCs, the anti-TK1-sdAb-IgG1_A1 and D1 were able to elicit a significant antibody-dependent cell-mediated cytotoxicity (ADCC) response against lung cancer cells compared to isotype controls ($P<0.0267$ and $P<0.0265$, respectively). To our knowledge this is the first time that the isolation and evaluation of human anti-TK1 single domain antibodies using phage display technology has been reported. The antibody fragments isolated here may represent a valuable resource for the detection and the targeting of TK1 on tumor cells.
Introduction

Efficient DNA repair and synthesis requires a balanced supply of nucleotides and coordination of the metabolic pathways utilized for their production \(^\text{177}\). In order to sustain proliferation, malignant cells have significant alterations in the activity levels of several of their nucleotide synthesis enzymes \(^\text{20,178,179}\). These alterations, particularly in the pyrimidine salvage pathway, can lead to an imbalance in the cell’s nucleotide pools which could lead to error prone DNA replication and genome instability, a hallmark of cancer \(^\text{134,180,181}\). Thymidine Kinase 1 (TK1) is a pyrimidine salvage pathway enzyme that catalyzes the phosphorylation of thymidine to thymidine monophosphate \(^\text{182}\). In healthy cells, TK1 is only elevated during the S phase, but low or absent during other cell cycle stages \(^\text{183,184}\). However, in malignant cells TK1 expression levels are upregulated and the enzyme seems to lose its normal cell cycle regulation control elements \(^\text{51}\). Increased levels of TK1 in both tumors and serum are associated with disease stage and, as cancer progresses, serum TK1 levels increase with disease stage \(^\text{146,185,186}\). During the last 3 decades scientific evidence has shown that TK1 levels in the serum of cancer patients can be used as a biomarker for early cancer detection \(^\text{137,187}\). As TK1 levels in serum are correlated with tumor progression, patient response and cancer recurrence, TK1 has also been proposed as a suitable tumor biomarker for the continued monitoring of patients \(^\text{139,140,142,145,188}\).

While the usefulness of TK1 as a tumor biomarker has been the main focus of many studies, in recent years the interest in using TK1 as a therapeutic target for multiple cancers has gradually increased \(^\text{153}\). In a study conducted by Malvi et al., the silencing of TK1 in lung adenocarcinoma (LUAD) cell lines inhibited the cell growth, migration and invasion capacities of LUAD cells both in vitro and in vivo \(^\text{189}\). Similarly, another study showed that targeting of
TK1 through genetic knockdown significantly reduced cell proliferation of pancreatic ductal \(^{151}\). In addition, it has been reported, that some forms of TK1 seem to be able to associate to the cell membrane of several cancer cell types, including leukemia, breast, lung and colon tumor cells possibly through protein-protein interaction or transitory membrane localization through exosomes \(^{81,82,190}\). Moreover, early experimental data has shown that membrane associated TK1 (mTK1) in lung, colon and breast cancer cells could be targeted using monoclonal antibodies. However, the study was limited to some extent because the antibodies used were produced in mice and humanized antibodies are still required to better evaluate potential therapeutic use in humans \(^{191}\). This evidence together suggests that the targeting of TK1 both inside the cell and its mTK1 form could be possible approaches for the development of novel cancer therapies. Therefore, the generation of therapeutics specific for TK1 could enable us to explore the potential of TK1 as a tumor target.

Monoclonal antibodies (mAbs) are suitable candidates for the development of cancer therapies due to their high specificity and affinity for their molecule targets \(^{192}\). The majority of current therapeutic antibodies have been produced with hybridoma technology or in transgenic mice, these approaches require the use of special animals, time consuming protocols and humanization or reformatting through complicated techniques such as CDR engraftment before any therapeutic use is possible \(^{193,194}\). Recently, phage display technology has been incorporated in the production pipeline of therapeutic antibodies by many pharmaceutical companies \(^{195,196}\). This technology offers the possibility to explore vast human antibody libraries in a relatively short period of time compared to hybridoma technology and does not require animals and can isolate antibodies against weakly immunogenic antigens \(^{197}\). In addition, the antibody fragments
can be isolated in convenient formats that facilitate further modifications for therapeutic applications\textsuperscript{198,199}. The use of phage display technology to obtain single domain antibody fragments (sdAbs), also called nanobodies, against TK1 is a convenient and appealing approach to isolate and develop biopharmaceuticals specific for the targeting of TK1. In this study we isolated human antibody fragments against the tumor proliferation biomarker TK1 from a sdAb library. The antibody fragments were evaluated for their capacity to bind and detect TK1 in monoclonal phage ELISAs, Western blot and flow cytometry. The antibody fragments were then incorporated into engineered IgG1 constructs and tested for their capacity to target TK1 on malignant cells and elicit an ADCC response from human MNCs against cancer cells. We hypothesize that engineered single domain antibodies (sdAb) specific for TK1 can efficiently target tumor cells expressing high levels of TK1. Thus, the use of human sdAb molecules targeting TK1 may enable us to better explore the potential of TK1 as a tumor target in proliferating malignant cells.

\textit{Materials and methods}

Isolation of anti-TK1 sdAb fragments through phage display

A phage display library of human single domain antibodies developed by Dr. Daniel Christ at the MRC laboratory of molecular biology was used to select high affinity anti-TK1 sdAbs (Source Bioscience, Cambridge, U.K) as previously described\textsuperscript{200}. The full repertoire of sdAbs was contained within a single human VH framework (V3-23/D47) fused to the gene III protein of the M13 filamentous phage. The sdAb library was constructed into the pR2 (MYC VSV-G tag) plasmid and had a diversity of 3x10\textsuperscript{9} fragments. Each sdAb fragment contained three diversified complementary regions (CDR1, CDR2 and CDR3). Ubiquitin and Galactosidase sub
libraries were also included as positive controls and run before using the dAb library to test the viability of the components of the library and verify that the selection process and testing of the sdAbs would be performed correctly.

To initially amplify the full repertoire of sdAbs, one aliquot of the sdAb library, which was contained in *E. coli* TG1 bacteria, was grown in 500 ml of 2xTY media supplemented with 4% glucose and 100 μg/ml of ampicillin until the culture reached an OD$_{600}$ of 0.5. An amount of 2x10$^{12}$ KM13 helper phages were added to 500 ml of each TG1 bacteria culture, and the culture was incubated for 1 hour at 37 ºC without agitation. After infection, the media was replaced with 2xTY media containing 0.1 % glucose, 100 μg/ml of ampicillin and 75 μg/ml of kanamycin and the library was grown for 24 hours at 25 ºC on a shaker at 250 rpm. The phages displaying the sdAb fragments were then purified using polyethylene glycol 6000 (PEG) solution (Millipore SIGMA, St Louis, MO, USA). The purified phages were then quantified by infecting TG1 bacteria with serial dilutions of the phage and plating the infected TG1 on TYE amp plates. Full-length human recombinant TK1 (>80% pure) produced in *E coli* (Genscript, Piscataway, NJ) was diluted in phosphate buffered saline (PBS) buffer to a 50,000 ng/ml concentration. TK1 was then immobilized on Maxisorp plates (ThermoFisher Scientific, Waltham, MA) by adding 100 ul of above TK1 solution and incubating them at 4 ºC overnight. The plates were then blocked with 5% milk PBS buffer (MPBS) for 45 minutes at room temperature on a shaker followed by 15 minutes at 37 º C. The plates were then washed 3 times with PBS. Approximately 5x10$^{10}$ phages displaying the full repertoire of sdAbs, were applied to each well that had been coated with TK1. To increase the surface area available for capture of the whole repertoire of the sdAb library, each phage dilution was added to four wells. Phages were allowed to bind for 1 hour at room
temperature with moderate shaking. After incubation, the wells were washed 15 times with PBS-T buffer (0.1% Tween) and 2 times with PBS buffer. The phage-sdAbs that remained attached were then eluted by adding 100 μl of a 0.1 mg/ml trypsin-TBSC buffer solution (Millipore SIGMA, St Louis, MO, USA) and incubation for 1 hour at room temperature with moderate shaking. The eluted phages were then recovered and used to infect a 30 ml TG1 bacteria culture 0.5 OD₆₀₀. The infected culture was then incubated for 1 hour at 37 °C without shaking. After infection, the TG1 bacteria were harvested and resuspended in 1 ml of 2xTY media. The cells were then plated on TYE, 4% glucose plates with ampicillin (100 μg/ml). The next day, colonies were scraped off and grown in 500 ml of 2xTY until the cultures reached at 0.5 OD₆₀₀. The cultures were then infected with 1x10¹² KM13 phages and grown at 25 °C for 24 hours on a shaker at 250 rpm. After incubating for 24 hours, the phage-antibody sub library was purified using PEG 6000 and the phage titer was determined for both the eluted phages and the purified phages. This process of selection was repeated 5 times. To eliminate antibodies that could possibly bind to the 6xHis-tag in TK1, the last two rounds of selection were done using TK1 produced in HEK 293expi cells without a 6xHis-tag (Origene, Rockville, Maryland, USA).

Polyclonal and monoclonal phage ELISAs

To monitor the increase in the overall number of TK1 binders between rounds of selection the isolated phages from each round of selection were screened using a polyclonal phage ELISA. For the polyclonal phage ELISA 96-well Costar plates (Corning, NY, USA) were coated with 100 μl of serial dilutions of TK1 (50,000 ng/ml-400 ng/ml) in duplicates. Uncoated wells (without antigen) were included as blanks in each ELISA. After the wells were coated, the plates were incubated overnight at 4°C with gentle agitation. After incubating, the plates were
washed 3 times with PBS buffer and blocked with 240 μl of MPBS for 45 minutes at room temperature followed by 15 minutes at 37°C. Wells were washed 3 times with PBS and purified phages diluted in MPBS (1:1 ratio) were added into each well. The plates were incubated for 1 hour at room temperature with gentle agitation. After incubating the plates were washed with PBS-T five times and 100 μl of Horse Radish Peroxidase (HRP) conjugated anti-M13 antibody solution (1:2,000 dilution in MPBS) was added into each well. Following addition of the HRP conjugated antibody, 100 μl of Tetramethyl Benzidine (TMB) substrate (ThermoFisher scientific, Waltham, MA, USA) was added into each well and the color was allowed to develop for 30 minutes. The reaction was stopped with 50 μl of 1M sulfuric acid, and the absorbance values were measured using a Synergy HT Microplate Reader (Bio-Tek Winooski, VT) at 450 nm and 650 nm.

Eighty individual clones were tested using monoclonal phage ELISA following every round of selection (biopans). After each biopan and before scraping off the colonies, eighty clones were picked and grown overnight at 37°C in 200 μl of 2xTY media supplemented with 4% glucose and ampicillin (100 μl/ml). Culture dilutions (1:40) from the overnight cultures were made by diluting 5 μl of the overnight culture in 200 μl of 2xTY 4% glucose and ampicillin and grown at 37°C for three hours. After reaching a 0.5 OD₆₀₀, 50 μl of 2xTY containing 4 x 1₀⁶ KM13 helper phages were added into each well to produce phage-sdAb fragments. The infected cultures were incubated at 37°C for one hour without shaking and the media was changed with 200 μl of 2xTY with 0.1% glucose, ampicillin (100 μg/ml), and kanamycin (75 μg/ml). Cultures were grown at 25°C for 24 hours at 250 rpm. After 24 hours the plates were centrifuged at 3200 x g for 10 minutes and the supernatants were recovered from each well. Each supernatant was
then mixed with MPBS in 1:1 ratio. To test each clone for its capacity to bind to TK1, 96 well plates were coated with 100 μl of a 10,000 ng/ml solution of TK1 per well as previously described. The same protocol as described for the polyclonal phage ELISA was used for the monoclonal phage ELISA.

Detection of phage-sdAbs by dot blot

Dot blot was used to confirm the expression of sdAb fragments in TG1 supernatants. After recovering supernatants containing phage-sdAb fragments, 2-3 μl of each supernatant containing the phage-sdAbs was immobilized on a nitrocellulose membrane (BIO-RAD, Hercules, CA). The membrane was then blocked with 5% MPBS for 1 hour at room temperature on a shaker. The membrane was then incubated with a 1:20,000 anti-VSV-G-HRP antibody solution at 4 ºC overnight with moderate shaking. The next day, the membrane was washed 3 times with 240 μl of PBS-T for 5 minutes. After the membranes were washed, 2 ml of enhanced chemiluminescence substrate (Advantsta Corporation, San Jose, CA) was added so that the membrane was completely covered. The membranes were incubated for 2 minutes, and the excess of reagent was poured off. Membranes were covered in plastic wrap and light sensitive films were placed on the membranes for different exposure times and measured using an imaging developer.

Sensitivity of TK1 specific antibody fragments

The sensitivities of the TK1 sdAbs were determined using dose-response curves and monoclonal phage ELISA. Briefly, Costar 96-well plates (Corning) were coated with serial dilutions of TK1 in duplicate overnight at 4ºC with gentle agitation. The dilutions ranged from
23,600 ng/ml-23 ng/ml for E-TK1 and 500 ng/ml-3.9 ng/ml for H-TK1. The next day, plates were blocked with 240 μl of MPBS/well for 45 minutes at room temperature and 15 minutes at 37°C. After blocking, the wells were washed three times with PBS and 100 μl of a 1:1 dilution of phage supernatant in MPBS was added into each well. Subsequent steps in the ELISA were carried out as previously described. The curves were then analyzed using a four-point parameter logistic curve and the limit of detection of each sdAb fragment was determined. A signal that was at least three standard deviations away from the signal of the blank was used as the limit of detection, as previously described 201. The sensitivities of the clones were then compared.

Validation with a TK1 siRNA and non-specific binding controls

In order to confirm specific binding to TK1, individual clones were screened against lysate from a siRNA TK1 knockdown cell line and compared to wild type cell lysate. The TK1 knock down cell lysate was prepared as previously described 191. TK1 forms produced in bacterial, yeast, and mammalian expression systems were used as positive controls and uncoated wells were used as negative controls. The monoclonal phage ELISA was performed as described above.

Sequencing Analysis of sdAb fragments

Plasmids were isolated from the clones that showed the highest affinity for TK1 using the PureYield Plasmid Miniprep system (Promega). Samples were prepared for sequencing using the primer (5’ CCCTCATAGTTAGCGTAACGA 3’) and the universal M13 reverse primer (5’ CAGGAAACAGCTATGAC 3’). Sequencing data was analyzed using Genious prime software 163.
Anti-TK1-sdAbs protein modeling and docking analysis of sdAb-TK1 complexes

The structures of the anti-TK1-sdAb fragments were analyzed using the GalaxyWEB TBM web server. The most stable structures of each anti-TK1 sdAb fragment were analyzed using the Visual Molecular Dynamics (VMD) software developed by the computer science and biophysics department of the University of Illinois. The CDR regions were mapped by analyzing the deduced amino acid sequences of each anti-TK1-sdAb fragment in the IgG Blast tool from NCBI. The sequences of the anti-TK1-sdAb fragments were aligned using Genious prime software. In silico analysis of the interaction between the anti-TK1-sdAb fragments and the crystal structure of TK1 was performed using the high ambiguity driven protein-protein docking (HADDOCK) web server. Visualization of the anti-TK1-sdAb-TK1 complexes was also performed with VDM software.

PCR amplification and cloning into pET-scFv-T

Sequences of the sdAb fragments were amplified from the phagemid plasmids corresponding to the isolated positive clones in phage ELISA. The sequences were amplified using primers containing the NcoI restriction site (5’ GAACATATGATGAAAAAATTATTA 3’) and the NotI restriction site (5’ GAAGGATCCTGCGGCCCCCTTTC 3’). PCR products were run in a 1% agarose gel and desired sequences were extracted using the Zymoclean DNA Gel Recovery Kit (Zymo research, Irvine, CA, USA). Following gel recovery, sdAb sequences were digested using NcoI and NotI restriction enzymes (New England Biolabs, Ipswich, MA, USA). Digested sequences were then ligated into the pET-scFv-T backbone (Addgene, Watertown, MA). Ligation was carried out using the Quick Ligation kit (New England Biolabs, MA). The ligated plasmids were then cloned into Dhα5 competent cells. Transformed colonies
were grown for 16-20 hours and plasmids were isolated and analyzed by restriction enzyme analysis to verify the presence of the insert. Positive clones were then sequenced.

Expression of antibody fragments in Rosetta 2(DE3) pLysS E. coli cells and His-tag purification

The pET-TK1-sdAb-6xHis constructs were cloned into Rosetta blue (DE3) pLysS E. coli cells (Millipore SIGMA, St Louis, MO). Individual colonies were selected and grown in a culture overnight at 37°C. The overnight culture was then scaled up and grown until the OD$_{600}$ reached 0.6. Expression of the sdAb fragments was induced by addition of 0.4 mM Isopropyl β-d-1-thiogalactopyranoside (IPTG). After IPTG induction, the cultures were grown for 24 hours at 28°C. The Rosetta blue (DE3) pLysS E. coli cells were pelleted, and the supernatant was saved for later purification. The cells were subjected to osmotic shock by resuspending cells in TES buffer (20 mM Tris-HCl pH 7.6, 5 mM EDTA, and 20% sucrose). After one hour incubation on ice, the sample was centrifuged at 14,000 x g for 20 minutes. The pellets were resuspended in ddH$_2$O and incubated on ice for 30 minutes. Centrifugation was repeated and the supernatant was preserved. The cell pellet was lysed using 10x bug buster reagent (Millipore SIGMA, St Louis, MO) diluted in 20 mM Tris–HCl (pH 7.8) buffer containing 15 mM NaCl, 5 mM MgCl$_2$, DNase (25 U/ml) and protease inhibitors. Cells were lysed for 20 minutes on shaker with moderate agitation. The lysed cells were spun down at 16,000 x g for 20 minutes and the supernatant was recovered. Supernatants were mixed with equilibrated Ni-NTA agarose beads (Qiagen, Hilden, Germany) for 3 hours at 4 °C. After incubating the NI-NTA beads were washed twice with cell lysis buffer and placed into 5 ml polypropylene columns. The Ni-NTA beads were then washed with 50 ml of wash buffer and then the His-tagged proteins were eluted with elution buffer in 0.3
ml fractions. The fractions were analyzed by SDS-PAGE and Western blot to detect the purified anti-TK1 sdAb fragments and estimate their purity.

sdAb ELISA

TK1 sdAbs expressed in a pET system and purified were tested again in ELISA to confirm that their binding properties to TK1 were kept when expressed without being fused to the PIII coat protein of the phages. The ELISA was performed as previously described for phage ELISA, except the blocking solution was replaced with 5% BSA. Because these antibodies were expressed with a VSV-G-tag this time the detection antibody was an anti-His-HRP (Biolegend, San Diego, CA) or anti-VSV-G-HRP (Bethyl, Montgomery, TX) rather than an anti-M13-HRP antibody.

Western blot with purified anti TK1-sdAb fragments

The purified fragments were tested for their capacity to bind to purified TK1 and TK1 in cell lysate using Western blotting. Recombinant human TK1 produced in bacteria and Expi293F cells were used together with cell lysate from A549 lung cancer cells, including a siRNA TK1 knockdown cell lysate. Briefly, 0.5 μg of TK1 or 20 μg of cell lysate were mixed with 6x Laemmli buffer (Millipore SIGMA, St Louis, MO). The protein samples were then heated at 100 ºC for 5 minutes and loaded into a 12 % SDS-PAGE electrophoresis. The proteins from the gel were then transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). After blocking with 5 % milk in MPBS buffer for 1 hour at room temperature the blocking solution was poured off and anti-TK1 sdAb fragment solution (1 μg/ml-2 μg/ml) was added. Membranes were incubated at 4 ºC overnight. After overnight incubation membranes were washed 3 times
with PBS-T buffer. The bound proteins were detected using a 1:20,000 solution of an anti-VSV-G-HRP and anti-His-HRP antibody (Bethyl, Montgomery, TX, USA). The proteins were then detected through the peroxidase reaction using enhanced chemiluminescence (ECL) (Advansta Corporation, San Jose, CA). Films were exposed for differing amounts of time depending on the antibody being tested, times ranged from 30 seconds to 5 minutes. The films were scanned, and the images were analyzed using the software ImageJ from NIH.

Cell lines and Isolation of human MNCs

The NCI-H460 (ATCCHTB-177™), A549 (ATCC® CCL-185), HCC1806 (ATCC® CRL-2335™) and HT-29 (ATCC® HTB-38™) cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained according to ATCC recommendations. A549, NCI-H460, HCC1806 and HT-29 were cultured in RPMI-1640 media (ThermoFisher scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS) and 2mM L-Glutamine. All cell lines were grown in an incubator at 37 °C and 5% CO₂. All cell lines used were tested for TK1 surface expression with flow cytometry with the commercial antibody ab91651 (Abcam, Cambridge, UK) to confirm the presence of TK1 on the cell membrane. MNCs were isolated using lymphocyte separation media (Corning, NY) following the instructions from the product’s manual, red blood cells were depleted with red blood cell lysis buffer (Biolegend, San Diego, CA) and the MNCs were resuspended in LGM-3 (Lonza, Basel, Switzerland). Isolation of human MNCs and serum from blood was done under approval of the Brigham Young University Institutional Review Board number 1734.

Flow cytometry
The purified phage-sdAb fragments, anti-TK1 sdAb fragments, and anti-TK1-sdAb-IgG1 fusions were all tested for their capacity to detect mTK1 on the cancer cell lines NCI-H460, HCC1806, HT-29, and normal MNCs. For this analysis 1x10^6 cells per sample were analyzed. Cells were washed twice with PBS and resuspended in 200 μl of cell staining buffer. After resuspension, the cells were stained using PEG purified phage-TK1-sdAb fragments (100 μl), purified sdAb fragments (5-10 μg), or purified sdAb-IgG1 antibodies for 40 minutes. The cells were then washed 3 times with 200 μl of cell staining buffer and stained for 30 minutes with anti-his-APC or anti-VSV-G-FITC antibodies or, in the case of purified phage-sdAb fragments, anti-M13-FITC secondary antibody. To detect binding of sdAb-IgG1 antibody fusions anti-Human IgG-FITC antibody (Abcam, Cambridge, UK) was used. After incubation with secondary antibody, the cells were washed 3 times with 200 μl of cell staining buffer. Before analysis samples were stained with 10 μg/ml PI solution and the samples were analyzed in a Cytoflex flow cytometer machine (Beckman Coulter, Brea, CA). The FCS files were analyzed using the FlowJo software (FlowJo, Inc., Ashland, OR).

Incorporation of TK1-sdAb fragments into pFUSE-IgG1 constructs and antibody expression in CHO cells

The DNA sequences of the best two anti-TK1 sdAbs were cloned between the NcoI and EcoRI restriction sites of the pFUSE-hIgG1e5-Fc2 vector (InvivoGen, San Diego, CA). This vector is designed for the production of human recombinant antibodies in mammalian cells and contains a human IgG1 heavy chain mutated at the S239D/A330L/I332E sites which confers an increased binding to FcγIIIa receptors in macrophages (MO) and natural killer cells (NK). Thus, the recombinant antibodies fused to this engineered IgG1, can elicit an enhanced antibody-
dependent cell-mediated cytotoxicity (ADCC). The primers used for the amplification of the sdAb fragments for this construct were primer Fw- 5’
GAAGAATTCCATGGCAGGAGGTGCAG 3’ and primer Rv- 5’ GGCCCATGGCGCTCGAGACGGTGAC 3’. The two TK1-scFv-hIgG1 DNA constructs were introduced into CHO.K1 cells (ATCC, Manassas, VA, USA) by lipofection using the lipofectamine LTX reagent (ThermoFisher scientific, Waltham, MA). After 48 hours the media was changed and Zeocin selection antibiotic (InvivoGen, San Diego, CA, USA) was added at a concentration of 100 μg/ml. After 10 days in selection the cells were expanded. The media was then replaced with ProCHO™ AT (Lonza, Basilea, Switzerland) and the cells were incubated for 48-96 hours or until cell viability was about 50%. The media was collected and cleared from cells by centrifuging at 200 x g. The anti-TK1-scFv-hIgG1 antibodies where then purified from cleared media using protein A purification columns (ThermoFisher scientific, Waltham, MA). Characterization of the purified recombinant antibodies was carried out with Western blot and flow cytometry as described above.

In vitro testing of anti-TK1-sdAb-IgG1 Antibodies through ADCC

The capacity of the TK1-sdAb-IgG1 antibodies to target mTK1 on cancer cells and elicit an ADCC response was evaluated in vitro. For this experiment NCI-H460 cells, which expressed high levels of mTK1, were engineered to express cytosolic GFP. The cells were then co-cultured with human MNCs and anti TK1-sdAb-IgG1 antibodies were added in various concentrations. Cell death was then measured using a real time cell imaging system. The experiments were conducted as follows. One day before treating the cells with antibodies and controls 5000 NCI-H460 GFP+ cells were seeded per well in a 96-well tissue culture plate (MIDSCI, St. Louis,
MO), placed inside an ImageXpress® Pico system. The GFP+ cells were counted every hour for the next 8-12 hours. After initial growth human MNCs were added at two different ratios, 5:1 and 10:1. The cells were co-cultured using LGM-3 media to sustain MNCs and antibodies were added at various concentrations (20, 10, 5, 2.5 μg per well). An optimized 5:1 effector:target ratio and a concentration of 10 μg of antibody/ml were used for experiments. The cells were monitored for 72-96 hours under environmentally controlled conditions. The number of cells from each treatment including controls were analyzed and compared over time. Each treatment in the assay was run in duplicate wells and the experiment was repeated twice.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism software (GraphPad, San Diego, CA). ELISA data from dilution curves was log-transformed and analyzed with a 4-parameter non-linear regression analysis with a 95% confidence interval (CI). To compare the different treatments of the ADCC experiments, the data was normalized in reference to the moment MNCs and antibodies were added and analyzed using a two-way ANOVA with repeated measure analysis. Analysis of multiple comparisons was performed comparing the mean of each treatment with every other treatment mean in each time point and over the total course of time. One-way ANOVA was performed to compare normalized GFP+ cell counts at specific time points.
Results

Antigen validation

All of the antigens used for the selection of the sdAbs were validated before the biopanning process. Recombinant human TK1 was produced in *E. coli* (E-TK1) and was used during early rounds of selection at high concentrations while TK1 produced in human Expi293F cells (H-TK1) was used for the last two rounds of selection at lower concentrations. This is because H-TK1 produced in human cells is properly folded. Both antigens were validated using Western blot with the KO validated anti-TK1 antibody ab91651 (Abcam, Cambridge, UK). Both E-TK1 and H-TK1 purified fractions were positive for TK1 and showed bands respective to the monomer and dimer of TK1. The purity of the antigens was shown to be higher than 80% based on the SDS page and Coomassie blue staining (Fig 3-1A, right).

Figure 3-1. Antigen validation and quantification of eluted phages thorough rounds of selection. A, Antigen validation, the purity and integrity of human recombinant TK1s produced in *E. coli* cells and Expi293F cells were assessed in SDS-PAGE and validated using Western blot with the anti-TK1 antibody ab91651. B, Enrichment of TK1 binders through 5 rounds of selection. The number of binders was estimated based on titrations of eluted phages used to infect TG1 bacteria. C, A representative image of a viral titration to determine the number of eluted phages. TG1 bacteria were infected with serial dilutions of the eluted phages after each biopan. The infected TG1 bacteria were then plated on TYE amp plates.
Isolation and validation of anti-TK1 sdAb fragments

The isolation of anti-TK1-sdAbs was monitored after each biopan by determining the phage titer from the eluted phages. As expected, the number of eluted phages per ml increased exponentially through the 5 rounds of selection (Table 3-1, Fig 3-1B). This trend could be observed by infecting TG1 bacteria with the eluted phages, plating the bacteria in selective agar plates and then determining the number of CFU/ml (Fig 1C). After 3 consecutive rounds of selection with recombinant human TK1 produced in E. coli the enrichment factor of TK1 binders went from 1 to 48.9. (Table 3-1). Two more rounds of selection were performed using recombinant H-TK1 to eliminate non-specific sdAb fragments that could possibly bind to the His-tag present in E-TK1 and also to obtain fragments that could bind to properly folded human TK1. We observed that the enrichment factor increased 3-fold after 1 round of selection with H-TK1 and then doubled in the subsequent round of selection using H-TK1.

Table 3-1. Enrichment of anti-TK1-sdAb phages through 5 rounds of selection.

<table>
<thead>
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<th>Round of selection</th>
<th>Input phages</th>
<th>Eluted phages</th>
<th>Ratio (eluted/input)</th>
<th>Enrichment factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.00E+12</td>
<td>4.12E+04</td>
<td>8.24E-09</td>
<td>1.00</td>
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<td>1.60E+06</td>
<td>3.20E-07</td>
<td>38.83</td>
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<tr>
<td>3</td>
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<td>2.01E+06</td>
<td>4.03E-07</td>
<td>48.91</td>
</tr>
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<tr>
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<td>1.13E+07</td>
<td>2.27E-06</td>
<td>275.49</td>
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</tbody>
</table>

An exponential increase in the number of positive clones after each biopan was observed. About 50 % of the clones produced a positive signal by the 4th biopan and about 90% of the clones were positive in the 5th biopan (Fig. 3-2A). A similar trend was observed in polyclonal phage ELISA (Fig. 3-2B).
Figure 3-2. Selection and expression of anti-TK1-sdAb phages. A, Analysis of 80 clones using monoclonal phage ELISA was performed after each round of selection. B, Polyclonal phage ELISA using the total purified phages per round of selection. In both A and B the overall signal and number of positive clones increased after each round of selection. C, Detection of anti-TK1-sdAb phages using dot blot with an anti-VSV-G-HRP antibody. Also, verification of packaging of the library from PEG purified phages after initial amplification of the sdAb library. D, Representative image of monoclonal phage ELISAs during the rounds of selection.

In addition to monoclonal and polyclonal phage ELISAs, we screened purified phages and individual supernatants containing phages with Western blot and dot blot. Since each antibody fragment displayed in the M13 phages had Myc and VSV-G tags, we detected the production of phage-antibodies using anti-VSV-G-HRP conjugated antibody. Western blot analysis of the purified phages revealed the presence of bands corresponding to phage-sdAb fusions. Dot blot analysis of individual clones showed the successful production of individual phage-sdAbs (Fig 3-2C). After the initial screens using monoclonal phage ELISA, 26 clones were chosen for their ability to produce high signals. These included clones from the 2^{nd}, 3^{rd} and 4^{th} rounds of selection. Clones from the 5^{th} biopan were excluded due to a decrease in the
diversity of the sdAb sequences. The clones were re-tested to confirm their capacity to bind TK1 and reproduce a positive signal. From the 26 selected clones, 14 clones were able to reproduce a positive signal after the initial screen (Fig 3-3).

Figure 3-3. Confirmation of positive clones. To verify the positive clones found during the initial screening, their capacity to reproduce a positive signal was tested. The strongest positive clones were selected through the different rounds of selection. Bacteria corresponding to each of these clones were streaked for a second time on TYE amp plates. New cultures were grown from a single colony and infected with KM13 helper phage to induce the production of their respective anti-TK1 phage-sdAbs. A, Positive anti-TK1 phage-sdAbs tested in monoclonal phage ELISA. The capacity to bind TK1 and the stability of each clone was confirmed. B, Representative image showing the color development generated by the positive clones and plate layout indicating the position of each clone that was tested.

After confirming their binding to TK1, the positive clones were evaluated using dose calibration curves to determine if they bound to TK1 quantitatively and to determine their sensitivity. The 14 clones showed all sigmoidal curves according to our non-linear 4-point logistic analysis. The goodness of fit test showed R squares ranging between 0.9964-0.9772. The curves behaved according to receptor-ligand interactions models, showing that the binding of the anti-TK1-sdAbs were proportional to the concentration of TK1 in each well (Fig 3-4A). This could also be visually appreciated in the colorimetric reactions in the dilution curves for each clone (Fig 3-4B). There was no significant background signal produced by the clones in the blanks.
Figure 3-4. Testing of the anti-TK1 sdAbs with dose response curves. A, Dose response curves corresponding to anti-TK1 sdAb fragments obtained through various rounds of selection. The data was Log transformed and the curves were analyzed using a 4-parameter non-linear regression. B, A representative image of the colorimetric reaction showing that the signal of each anti-TK1 sdAb fragment is proportional to the concentration of TK1 protein. Negligible or no significant signals were produced in the blanks.

The most sensitive clones were 4-H-TK1_A1 and 4-H-TK1_D1. Using the phage supernatant from these clones we were able to detect TK1 protein levels as low as 23 ng/ml in monoclonal phage ELISA (Fig 3-5A). Further sequencing of the clones revealed that the sequences of clones 4-H-TK1_A1 and 4-H-TK1_D1 were different.
Figure 3-5. Sensitivity and specificity of the anti-TK1-sdAb fragments. A, The 14 anti-TK1 sdAb fragments were tested against a minimal fixed concentration of 23 ng/ml of human TK1 produced in human cells (H-TK1). The sdAbs 4-H-TK1_A1 and 4-H-TK1_D1 produced the highest signals. B, Dilution curves with H-TK1 and the best clones. Concentrations ranged from 500 ng/ml to 3.9 ng/ml, the fragments kept their binding properties after being expressed as sdAb fragments. C, siRNA TK1 knock down validation. TK1 was knocked down in A549 cells. The knockdown was validated using the commercial anti-TK1 antibody ab91651. D, Validation of the best 2 anti-TK1-sdAbs. The binding capacity of the sdAb fragments was tested against 3 different sources of human recombinant TK1, cell lysate from the cancer cell line A549 and cell lysate from A549 TK1 knockdown. It can be seen that both fragments bind to all recombinant TK1 proteins. A significant difference can be seen in the signal coming from the normal cell lysate in comparison with the TK1 knockdown cell lysate.

Anti-TK1-sdAbs phages 4-H-TK1_A1 and 4-H-TK1_D1 demonstrated the capacity to bind H-TK1 in phage ELISA. The signal was proportional to H-TK1 concentration (Fig 3-5B). Validation of the clones 4-H-TK1_A1 and 4-H-TK1_D1 using a siRNA TK1 knockdown and different sources of recombinant human TK1 revealed that the clones were able to bind to E-TK1 (produced in E. coli), H-TK1 (produced in Expi293F cells) and TK1 produced in a yeast system. Moreover, the signal with cell lysate from A549 cells was significantly stronger (~10-fold) to the signal coming from the cell lysate of A549 TK1 knockdown for both 4-H-TK1_A1 (P<0.0296) and 4-H-TK1_D1 (P<0.0129). A TK1 knock down was produced as previously described for this experiment (Fig 3-5C and D) 191.
Flow cytometry

Membrane expression of TK1 on NCI-H460 cells was detected by staining the cells with PEG purified phages from each respective clone. We observed that after subtracting the background binding from the anti-M13-APC antibody there was an increase in binding to the cells stained with the anti-TK1-sdAbs of 20% and 25% for H-4-TK1_A1 and H-4-TK1_D1 sdAbs respectively (Fig 3-10A). Therefore, the anti TK1 sdAbs showed a capacity to detect membrane expression of TK1 on NCI-H460 cells. NCI-H460 cells were simultaneously screened with the commercial anti-TK1 antibody ab91651.
A Detection of mTK1 using purified anti-TK1 dAb phages

B Detection of mTK1 using recombinant anti TK1-dAbs on NCI-H460 (Non-small cell lung carcinoma cells)
Detection of mTK1 using recombinant anti TK1-dAbs on HT-29 (colon adenocarcinoma cells)

Detection of mTK1 using recombinant anti TK1-dAbs on HCC1806 (Triple negative breast cancer cells)
Figure 3-10. Detection of mTK1 on cancer cells and healthy MNCs using anti-TK1 sdAb fragments. A, NCI-H460 cells were stained with the purified phage-anti-TK1 sdAb fragments, a shift in the population could be observed using both phage-sdAb fragments. B-E, Expressed and purified anti-TK1-sdAb_A1 and D His-tagged and VSV-G tagged were used to stain NCI-H460 (lung), HT-29 (colon), HCC1806 (Triple negative breast cancer) and healthy lymphocytes. The TK1 levels detected using the anti-TK1 sdAb fragments were comparable to the levels detected by commercial anti-TK1 antibody. The highest levels of mTK1 were detected on NCI-H460, followed by HT-29 and then HCC1806. No significant binding was detected on normal MNCs with the anti-TK1 sdAb fragments nor with the commercial anti-TK1 antibody. F, The expression levels of mTK1 on cancer cells and the binding of anti-TK1 sdAb fragments. Only A1-His sdAb showed consistent binding similar to commercial antibody ab91651.
After confirming the capacity of the TK1-sdAb-phages A1 and D1 to detect mTK1 on cancer cells the fragments were expressed as sdAbs and used to stain different cancer cell lines to confirm their ability to detect mTK1. The sdAbs were expressed as two different versions; His-tagged and VSV-G tagged. Among the 4 sdAbs, TK1-D1-His showed the most consistent binding to mTK1 on NCI-H460 (~95%), HT-29 (~87%) and HCC1806 (~53%) (Figs 3-10B-10F). These expression levels were comparable to those seen with the commercial TK1 antibody ab91651; 97%, 72% and 55% for NCI-H460, HT-29 and HCC1806 respectively. For NCIH460, which had the highest percentage of cells positive for mTK1, the A1 His-tagged sdAb fragment and both the A1 and D1 VSV-G tagged sdAbs showed binding (Fig 3-10B). D1-VSV-G fragment showed no binding to HT-29 cells or HCC1806 while A1-VSV-G and A1-His showed variable levels of binding to both cell lines. However, not consistently as the D1-His fragment. This may be due to differences in their tags, expression levels in each cell line and possible binding to different epitopes. No significant binding of the anti-TK1 sdAb fragments and the commercial anti-TK1 antibody was found on normal lymphocytes after subtracting non-specific binding of secondary antibody anti-Human IgG FITC (Fig 3-10E). It can be appreciated that the cancer cell lines expressed variable levels of mTK1 NCI-H460 being the one with the highest percentage of positive cells followed by HT-29 and HCC1806 (Fig 3-10F).

Amplification of sdAB fragments, sequencing and cloning into pET-scFv-T vector

The dAb fragments were amplified by PCR and ligated into the pEt-scFv-T vector successfully as shown in figure 3-6. The anti-TK1-sdAb vectors were sequenced, and the amino acid sequences were deduced from their nucleotide sequences (Table 3-2).
Figure 3-6. Amplification and ligation of anti-TK1-dAb fragments into the pET-scFv-T expression vector. A, a representative image of a PCR showing amplification of anti-TK1 dAb fragments. B, Restriction enzyme analysis with NcoI and NotI enzymes of pET-anti-TK1-dAb constructs. C, Map of a pET-anti-TK1-dAb construct. Fragments are ligated into the pET-scFv plasmid using NcoI and NotI restriction sites. D) Alignment of the 4-H-TK1_A1 and 4-H-TK1_D1 sdAb sequences shows that the differences of the sdAbs are in their CDRs.

Further analysis of the nucleotide sequences of the Anti-TK1 dAb fragments with the IgG blast tool from NCBI revealed the specific sites for their respective CDRs. The annotated sequences are found in Table 3-2. In addition, the alignment of the sequences using Genious software confirmed that the annotated CDRs were the regions with most differences among the sequences while the heavy chain framework regions remained conserved (See Fig. 3-6D). This is consistent with the original description of the library which was built in a human VH framework and introduced diversity in the CDRs.
Table 3-2. The deduced amino acid sequences of the best two anti-TK1 sdAb fragments isolated through phage display.

<table>
<thead>
<tr>
<th>Anti-TK1 dAb</th>
<th>FR1</th>
<th>CDR1</th>
<th>FR2</th>
<th>CDR2</th>
</tr>
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<tr>
<td>4-H-TK1_A1</td>
<td>MAQVQLLESGGGLVQPGGSLRLSCAA</td>
<td>SGDRTFTDEN</td>
<td>MSWVRQAPGKGEWVSA</td>
<td>IDNADGST</td>
</tr>
<tr>
<td>4-HTK1_D1</td>
<td>MAQVQLLESGGGLVEPGGSLRLSCAA</td>
<td>GDSFTTKN</td>
<td>MAWVRQAPGKGEWVSA</td>
<td>ISKRSGST</td>
</tr>
</tbody>
</table>

Analysis of protein structure of Anti-TK1-sdAb fragments and modeling of sdAb-TK1 complexes

After submitting the corresponding amino acid sequences from the anti-TK1 sdAb fragments A1, and D1 into the GalaxyTBM server, 5 model structures were generated for each fragment. The most stable structure from each anti-TK1-sdAb was then visualized using the VMD software. The anti-TK1-sdAb fragments presented a typical structure of a sandwich of two antiparallel β sheets according to previously reported single domain structures. Their CDRs were contained in the loops connecting their antiparallel β sheets with CDR3 displaying a longer loop as expected and in accordance with the sdAb library design (Figs 3-7A and 3-7B).

Furthermore, analysis of the interaction between the anti-TK1_4-H-A1 sdAb and the 1XBT crystal structure of TK1 using the high ambiguity driven protein-protein docking (HADDOCK) web server revealed the interaction of the anti-TK1 sdAb 4-H-TK1_A1 through its CDRs with TK1. The CDRs seemed to interact with the α1-ribbon towards the N-terminus and two regions close to the β-ribbons at the c-terminus of the TK1 molecule (Fig 3-7C and 3-7D).
Figure 3-7. The 3D structure of the two best anti-TK1-sdAb fragments based on their deduced amino acid sequences. The anti-TK1-sdAb fragments were modeled using the GalaxyWeb TBM server. The most stable structures were then visualized using the VMD 1.9.3 software. CDRs were mapped by analyzing the anti-TK1 sdAb amino acid sequences with the IgBlast tool from NCBI. A, H-4-TK1_A1 sdAb. B, H-4-TK1_D1 sdAb. C, High ambiguity driven protein-protein docking analysis using the HADDOCK 2.4 web server. The most stable structures of the H-4-TK1-A1 sdAb fragment and human TK1 protein monomer were analyzed to predict their protein-protein interactions. The analysis shows that in the most stable TK1-TK1sdAb complex, the TK1-sdAb would bind to TK1 through its CDRs. The CDRs would interact with the α1-ribbon towards the N-terminus and two regions close to the β-ribbons towards the c-terminus of the TK1 molecule. D, Docking between the anti-TK1 sdAb H-4-TK1_A1 and the monomer of human TK1 from the 1XBT crystal structure.

Expression and characterization of purified sdAb fragments

The anti-TK1 sdAbs H-4-TK1_A1 and H-4-TK1_D1 were successfully expressed using the pET-scFv-T system. The fragments were detected with anti-His-HRP and anti-VSV-G-HRP antibodies in Western blot as could be observed (Fig. 3-8A). Moreover, coomassie blue staining showed successful purification of a 12-15 kDa band which matched the size of the band shown in Western blot. Purification of sdAb fragments with Ni-NTA bind-His resin yields were
between 1 up to 4 mg/ml of purified fragment (Fig. 3-8B). Alternatively, the fragments that are VSV-G-tagged were purified using protein A columns (Fig. 3-8C). Although, the yields in protein A purification were lower, about 0.4 mg/ml (Fig. 3-8D).

Figure 3-8. Expression and purification of anti-TK1 sdAbs. A, Detection of anti-TK1 sdAb fragments with anti-His-HRP antibody in Western blot and their respective SDS-PAGE analysis. B, Quantification of the His-tag purified sdAb fragments with BCA assay. The protein yields ranged between 1-4 mg/ml of purified sdAb. C, Western blot and SDS-PAGE analysis of protein A purified anti-TK1 sdAb H-4-TK1_A1. The antibody fragments can alternatively be purified with protein A purification. D, Quantification of protein A purified anti-TK1 sdAb H-4-TK1_A1.

Western blot and sdAb ELISA

After being expressed as sdAb fragments without a PIII gene fusion, the purified anti-TK1-sdAbs retained binding properties to TK1 similar to those observed when expressed as PIII fusions displayed on filamentous KM13 phage. Thus, indicating that their binding is due to the sdAb sequence and not through non-specific interaction of the coat proteins of the KM13 phage. This was tested using sdAb ELISA. As shown, the anti-TK1 sdAbs bound proportionally to the
concentration of TK1 in the wells. Concentrations ranged between 500 ng/ml-3.9 ng/ml of H-TK1. The anti-TK1-sdAbs produced signals significantly higher than the blanks at concentrations of 3.9 ng/ml for H-4-TK1-A1 and for H-4-TK1-D1, with both signals reaching plateau phase after 125 ng/ml (Fig 3-9A).

Figure 3-9. sdAb ELISA and Western blot analyses. A, Purified recombinant anti-TK1 sdAbs kept their binding properties to H-TK1 after being expressed in *E. coli* and His-tag purified as observed using sdAb ELISA. B, The anti-TK1 sdAbs showed binding to recombinant human TK1 produced in both *E. coli* (E-TK1) and human cells (H-TK1). The fragments were validated by comparing their binding to TK1 in cell lysates of A549 cells (100 μg) and A549 TK1 knockdown (100 μg). C, Detection of TK1 in cell lysates of 4 different cancer cell lines and normal MNCs (20 μg each). The fragments were able to detect the tetrameric form of TK1, controls included commercial anti-TK1 abcam91651 and GAPDH.

The anti-TK1-sdAbs were then tested in Western blot. As can be observed in Fig. 3-9B, the anti-TK1-ssdAbs showed binding to recombinant human TK1 produced in *E. coli*, and Expi293F cells. Moreover, the Anti-TK1 sdAb fragments showed a significantly higher signal in A549 cell lysate compared to A549 TK1 siRNA knockdown, particularly fragment 4-H-TK1_A1 showed specificity for TK1 (Fig. 3-9B).
Once we confirmed the binding of the fragments to purified recombinant TK1 and validated their specificity using a TK1 siRNA knockdown we proceeded to test their capacity to detect TK1 in cell lysates of different cancer cell lines, including, NCI-H460, HT-29, MDA-MB-231, PC3 and human MNC. It could be observed that the anti-TK1 sdAb OriTK1_A1-VH was able to detect bands corresponding 100 kDa in several cell lysates except in MDA-MB-231 cells where it seems to detect a 50 kDa band instead. Also, the signals for cell lysates from HT-29 and H460 were weaker than the signals coming from other cell lysates. In the case of the anti-TK1 sdAb OriTK1_D1-VH we obtained stronger signals in all cell lysates compared to the signal of OriTK1_A1-VH. OriTK1_D1-VH also detected a 50 kDa band in MDA-MB-231 cells in addition to the 100 kDa band (Fig 3-9C). These may correspond to the active dimer and tetramer forms of TK1.

Cloning of the Anti-TK1-sdAb fragments into the pFUSE-IgG1e5 vector and expression of recombinant antibody in CHO.K1 cells.

Before cloning the anti-TK1-sdAb sequences into an expression vector, site directed mutagenesis (SDM) was performed to change the amber stop codons present in the sdAb sequences to glutamic-acid. The anti-TK1-sdAb fragments were then successfully ligated into the pFUSE-IgGe5-IL2 expression vector at the NcoI and EcoRI restriction sites. Restriction analysis of these constructs showed the successful insertion of the sdAb genes into the pFUSE-IgGe5-IL2 vector (Fig 3-11A). After 72 and 96 hours of transfecting CHO.K1 cells with the pFUSE-4-H-TK1_A1 and D1, and a control without sdAb construct, the collected supernatants were purified using a protein A column. The recombinant antibodies were successfully purified from supernatant with a yield of 0.4-0.26 mg/ml. Western blot analysis and SDS page showed
for both sdAb-Fc antibodies a ~38-40 kDa band which is the expected size for the monomer of the anti-TK1-sdAb-IgG1 fusion. In the case of the control vector containing an IgG1 sequence without being fused to a sdAb sequence an IgG1 fragment of smaller size (~26 kDa) was produced. The recombinant antibodies were all positive in Western blot to anti-human IgG antibody confirming the presence of the engineered IgG1 heavy chain fused to the sdAbs (Fig 3-11B). Furthermore, the anti-TK1-sdAbs once fused to IgG1 Fc were able to detect recombinant purified human TK1 (H-TK1). The signal produced in Western blot was proportional to the concentration of antigen and was higher than the signal produced with anti-TK1 sdAbs without FC fusion (Fig 3-11C). In addition, the recombinant antibodies showed capacity to detect mTK1 on cancer cells in flow cytometry (Fig 3-11D). A shift in the population of NCI-H460 cells could be seen when they were stained with the TK1-A1-IgG1 and D1 antibodies while no shift was detected using IgG1 fragments without fusion to TK1-sdAbs.

Figure 3-11. Expression and testing of the Anti-TK1 sDab-IgG1 antibodies. A, Construction of an anti-TK1 sdAb fragment. Restriction analysis of pFUSE-anti-TK1-sdAb plasmids and their respective maps. B, Expression and detection of anti-TK1-sdAb-IgG1 antibodies. As can be observed sdAb-IgG1 fusions produced higher molecular weight bands compared to IgG1-no sdAb constructs. No human IgG was detected in non-transfected CHO.K1 cells. C, After fusing to an Fc the anti-TK1-sdAb antibodies were able to detect mTK1 on NCI-H460 cells. IgG1-no sdAb control did not bind to NCI-H460 cells.

Anti-TK1-sdAb antibodies elicited in vitro ADCC responses of human MNC against cancer cells expressing mTK1
To test the potential use of the anti-TK1-sdAb antibodies for the immunotargeting of cancer cells that express mTK1, we co-cultured tumor cells expressing high levels of TK1 with human MNCs, added anti-TK1-sdAb-Fc antibodies and monitored the ADCC response over time. The NCI-H460 cell line was chosen as this cell was previously shown to express the highest levels of mTK1 on the cell surface in flow cytometry (Fig 13-1F). An initial test using different concentrations of anti-TK1-sdAbs showed that the cell killing was proportional to the concentration of antibody used (Fig 3-12A) and it was found that a concentration of 10 μg/ml of the engineered anti-TK1 antibodies was necessary to cause a significant ADCC response. Cells treated with the anti-TK1-sdAb-IgG1_A1 and D1 antibodies at 10 μg/ml and co-cultured with human MNCs had a significant ADCC response against the cancer cells when compared to isotype (P<0.0395) and no antibody controls (P<0.0038) after 88 hours (Fig 3-12B). Although the 2-way ANOVA analysis did not show the difference to be significant in the case of the anti-TK1-sdAb-IgG1_D1 antibody, a reduction of more than 50% of target cells was observed compared to isotype controls. Imaging of the cells revealed that after 96 hours there was a difference in the cell health and number of MNCs clustering targets cells. It could be seen that cells treated with anti-TK1-sdAb-IgG1 experienced a more severe ADCC response than those treated with the isotype control (Fig 3-12B). Statistical analyses at individual time points indicates that the percentage of cell killing is significantly higher at 96 hours when TK1 is targeted using the anti-TK1-sdAb-IgG1_A1 (P<0.0267) and D1 (P<0.0265) compared to controls and at 72 hours (P<0.0207 and P<0.0246 respectively) (Fig 3-12C).
Figure 3-12. Anti TK1-sdAb-Fc antibodies elicit ADCC responses against NCI-H460 cells expressing mTK1. A, Optimization of several antibody concentrations. The decrease in GFP+ NCI-H460 cells co-cultured with MNC over time is proportional to the concentration of anti-TK1-sdAb-IgG1/ml used. B, A significant ADCC response is elicited by MNCs against NCI-H460 cells when anti-TK1-sdAb-IgG1 antibodies are added compared to controls. C, After 96 hours the cell killing can visually be appreciated to be more severe in the cells treated with the anti-TK1-sdAb-IgG1 antibodies. D, Percentage of cell killing calculated every 24 hours. The percentage of cell killing was significantly higher after 72 and 96 hr. when anti-TK1-sdAbs were added in comparison to IgG1 isotype control and effector only-no sdAb control.
Discussion

From its early beginnings, studies involving TK1 have been focused mainly on its use as a tumor biomarker. However, new evidence has shown that TK1 may have an emerging role as a target for cancer therapy. Recent studies on the suppression of TK1 in cancer cells have shown that the silencing of TK1 decreases the capacity of lung adenocarcinoma, pancreatic and thyroid carcinoma cells to proliferate, migrate or make mesenchymal transitions. Additionally, some TK1 forms seem to be able to associate to the cell membrane of cancer cells, an event that is apparently restricted to malignancy. Thus, it is clear that the development of therapeutics that can specifically target TK1 are necessary to explore the potential of TK1 as a target. Monoclonal antibodies are the fastest growing biopharmaceuticals in immuno-oncology. Previously it has been shown that anti-TK1 monoclonal antibodies could be used for the immunotargeting of TK1 on several cancer types. Although some monoclonal antibodies against TK1 have been developed there are currently no humanized versions of TK1 antibodies suitable for therapy. Moreover, the recent increase in the use of phage display antibody libraries has proven the advantages of using sdAb fragments in the development of therapeutic antibodies for cancer treatment. Single domain antibodies are characterized for their smaller size while keeping all the binding properties of full-length antibodies. In this study anti-TK1 sdAbs were isolated from a sdAb library through phage display. This is the first time that the isolation and characterization of 100% human sdAb fragments specific for TK1 has been reported. This study also provides evidence that single domain antibodies or nanobodies can be used to target mTK1 on cancer cells, an antibody approach that has not been previously used for the targeting of TK1. Furthermore, this study shows evidence that TK1 sdAbs can be
incorporated in engineered IgG1 antibody constructs to generate molecules for potential immuno-oncology applications.

It is important to mention that the process through which we selected the antibodies using TK1 produced in bacteria and TK1 produced in human cells was chosen to ensure that we obtained a sufficient number of sdAbs and that the anti-TK1 sdAbs were able to bind to properly folded human TK1. ELISA data in this study has shown that the anti-TK1 sdAbs fit the receptor-ligand model and that the binding of the anti-TK1-sdAb fragments was dependent on the amount of available antigen. This study also showed that the anti-TK1-sdAbs were able to be expressed without the PIII fusion while keeping their binding properties previously shown in phage monoclonal ELISA. Validation with an siRNA TK1 knockdown indicated that the antibody fragments developed were specific for human TK1. Furthermore, the flow cytometry data showed that the nanobodies can potentially be used to target cancer cells expressing TK1, particularly mTK1. This early evidence indicates that anti-TK1-sdAbs could be used for the development of experimental TK1-based therapeutics such as anti-TK1-sdAb fragments that could be conjugated with toxins or gold nanoparticles for anticancer photothermal therapy. Moreover, these anti-TK1-sdAb fragments could also be used for the development of immuno-oncology therapeutics such as engineered antibodies or chimeric antigen receptors.

As this study has shown, anti-TK1-sdAb-IgG1 antibodies are capable of targeting mTK1 on cancer cells and elicit an ADCC response by human MNCs against TK1 high-level expressing cancer cells building on previous findings. Unlike previous anti-TK1 antibodies generated through conventional hybridoma technology, these engineered antibodies are completely human,
are significantly smaller than full length antibodies and have an engineered IgG1 to enhance the
ADCC response. Thus, they can have better tumor penetration than conventional antibodies, and
can be used to better engage the immune system with tumor cells. Although it remains unclear
why TK1 is localized to the cell surface of multiple cancer cell lines, the flow cytometry data and
ADCC results described here suggest that it could be feasible to harness the immune system
against tumor cells expressing mTK1. It is not the first time that a protein thought to be limited
to the interior of the cell has been reported to be on the cell surface. Examples can be found in
the HSP70 family of proteins. Although HSP70 proteins were thought to be limited to function
only inside cells, it is well documented that they are secreted and localized on the cell membrane
of cancer cells. Recent studies have shown that TK1 is present in exosomes. As with
HSP70 proteins a possible explanation could be that it is transitorily localized on the cell
membrane as exosomes exit the cell fusing to the cell membrane, and through non-conventional
protein-protein interactions. Moreover, it is also important to point out that other nucleotide
salvage pathway enzymes have been reported to be localized on the cell membrane e.g.,
hypoxanthine-phosphoribosyl transferase (HPRT). Targeting of this enzyme with monoclonal
antibodies has also been recently reported.

Conclusion

This study reports the isolation and evaluation of human single domain antibodies against
TK1 and their binding to the tumor proliferation biomarker TK1 on lung, colon and breast cancer
cells. The antibody fragments have potential as diagnostic and therapeutic agents, although
additional in vivo studies are required to confirm their efficacy. The antibody fragments can be
successfully incorporated into IgG1 Fc constructs for the production of completely human
engineered antibodies able to elicit significant ADCC responses from human MNCs against cancer cells expressing mTK1. The antibody fragments could potentially be used in other therapies such as chimeric antigen receptors (CAR) for T cells or other recombinant antibody constructs. The use of TK1 as a therapeutic target will enable the testing of experimental TK1-based therapies.

Acknowledgments

We acknowledge the valuable contributions of Zachary D. Ewell, Kelsey B. Bennion, and Naomi Rapier-Sharman, for their technical support during the early stages of this research.
Abstract

Follicular lymphoma (FL) is the most common indolent Non-Hodgkin’s lymphoma diagnosed in both the US and Western Europe and comprises about 70% of all cases. Although the combination of different chemotherapy regimens with rituximab have had a significant impact on progression free survival rates, FL remains a treatable but incurable disease where patients frequently experience relapse and refractory events. Survival of patients decreases as they age, and disease evolves towards aggressive diffuse large B cell lymphoma. Extensive efforts have been made to timely diagnose and stratify the risk of FLs using biopsy, CT and PET scans, ultrasound, MIR and traditional histology. In addition, the use of several prognostic indexes such as FLIPI, FLIPI-2, and tumor burden measurements has helped physicians and scientists in the management of FL patients. Because of its indolent nature and the lack of clinically proven biomarkers, more than 50% of FL cases are detected in advanced stages. Moreover, a subset of FL patients (~20%) experience poor survival rates (2 years). With the recent development of new tyrosine inhibitors, epigenetic drugs, immunomodulators, immuno checkpoint antibodies and Chimeric Antigen Receptor (CAR) T cell therapies, there is an increased necessity to predict FL patients' response. Gene expression profiles, mutational landscape of relevant genes, serum cytokines, immune cell subsets, exosomes, microRNAs, circulating tumor DNA and nucleotide salvage pathway enzymes are among potential predictive FL biomarkers. In this review, we examine their predictive capabilities for early detection, risk stratification, selection of therapeutic regimen, prediction of patient response and monitoring of FL disease.
Introduction

In the US and western Europe, Follicular lymphoma (FL) encompasses 20-40% of all non-Hodgkin’s lymphomas (NHL) and 70% of all indolent lymphomas worldwide\textsuperscript{217,218}. FL originates in mature B cells at the bone marrow with the initial t(14;18)(q32;q21) translocation resulting in placing the $BCL2$ gene under the control of the immunoglobulin heavy-chain enhancer during defective VDJ recombination\textsuperscript{219}. It is believed that B cells with this genetic lesion (70-95% of all FL cases) then migrate from the bone marrow to lymph nodes where they undergo malignant transformation during aberrant somatic hypermutation (aSHM) and class switch recombination (CR) at germinal centers (GC) in the follicles\textsuperscript{220}. Eventually, enough genetic lesions accumulate and FL evolves to an aggressive form of lymphoma called diffuse large B cell lymphoma (DLBCL) or undergoes histological transformation (HT), causing bone marrow failure, susceptibility to infections, secondary cancers and death\textsuperscript{221,222}.

Although FL has a high 5-year survival rate, because of its indolent nature 68 % of the cases are diagnosed in stages II, III and IV when the cancer has spread to multiple regions\textsuperscript{223}. The majority of FL cases are diagnosed after age 50 after which the survival rate decreases. When transformation of FL to diffuse large B cell lymphoma (DLBCL) takes place, most treatments of FL do not generate patient response. Moreover, about 20 % of FL patients have a poor prognosis and an overall survival rate (OS) of 2 years or less\textsuperscript{224}. Even though FL can become a manageable chronic condition, patients frequently experience relapse and refractory events which many times have a detrimental effect on the patients’ life quality\textsuperscript{225}.  

Traditional diagnosis of FL lymphoma involves biopsy from surgery or bone marrow aspirate, staining of cell surface markers and BCL2 cytosolic expression followed by imaging using ultrasound, magnetic resonance imaging (MRI), computed tomography (CT) and positron emission tomography (PET). Although prognostic indexes and histologic analysis along with imaging have been helpful in the management of FL patients, currently there is not a consensus on how to treat FL patients. Also, prognostic indexes have shown a limited capacity to predict patient response to therapy, detect FL at early stages or identify FL patients that are prone to relapse or DLBCL transformation. In addition, as new treatments for FL are available such as Bruton’s tyrosine inhibitors, epigenetic drugs, phosphoinositide 3-kinase (PI3K) and BCL2 inhibitors, immunomodulators, immune checkpoint antibodies and cell adoptive therapies more predictive biomarkers for FL are needed. Among new potential predictive FL biomarkers are, gene expression profiles, mutational landscape of relevant genes, serum cytokines, immune cell subsets, exosomes, microRNAs, circulating tumor DNA and nucleotide salvage pathway enzymes. In this review we discuss the current status of predictive biomarkers for the detection and monitoring of FL.
Figure 4-1. Biomarkers through the course of follicular lymphoma (FL). Follicular lymphoma genetic lesions originate in B cells at the bone marrow. Defective V(D)J recombination causes t(14;18)(q32;21) translocation placing BCL2 gene under the IgGH gene enhancer rescuing B cells from apoptosis. B cells carrying the t(14;18)(q32;21) translocation enter the lymph nodes either into the borders of the T- cells zone or into the follicles. In the follicles t(14;18)+ B cells further differentiate by interacting with follicular helper T cells (TFH) and follicular dendritic cells (FDC). The t(14;18)+ B cells then undergo intensive proliferation forming a germinal center. The same B cells go multiple rounds of somatic hyper mutation (SMH) and class switch recombination (CSR). This causes the accumulation of genetic lesion that eventually leads to evolution of the disease to histological transformation or diffuse large B cell lymphoma (DLBCL). Besides t(14;18)(q32;21) translocation other early genetic lesions are found in chromatin modifying genes. Other subsequent mutations are found in genes P16/INK4, MEF2, BCL6, MYC, KLF4, MYD88, TP53, some of them have been associated to later stages of FL and transformation to DLBCL. Infiltration of high number of cytotoxic T cells are related to good prognosis. High numbers of CD163+ and CD68+ macrophages are related to both poor and good prognosis. Expression of SIRP-α in high levels in CD14 monocytes and macrophages is correlated to unfavorable outcome while low expression of SRIP-α is related favorable outcome. The presence of FOXP3+ Treg cells has been reported with good prognosis. CD8+ T cells expressing high levels of PD-L1 have been correlated with significant lower response to immunocheckpoint therapy. IL1R and IL2R, IL12, IL15 and other cytokines have shown predictive value in FL. Mutations specific to FL can be detected by analyzing ctDNA. Exosomes and miRNA can also be found in serum and utilized as prognostic biomarkers. Pyrimidine salvage pathway enzymes such as TK1 and HPRT whose serum and expression levels are upregulated at early stages of FL and correlate with cancer stage and aggressiveness.
The FL international prognostic index (FLIPI) was created using data from 4,167 patients in an international cooperation of 5 research groups between both the US and Europe. FLIPI uses 5 parameters: Age >60 years, hemoglobin <12g/dl, serum LDH normal, Ann Arbor stage III/IV and number of involved nodal areas >4. Three risk groups defined as low (presence of 0-1 adverse factors), intermediate (2 factors), and poor risk (≥3 factors). The FLIPI has been validated in clinical trials with chemotherapy and chemoimmunotherapy using both OS and progress-free survival (PFS). After the inclusion of rituximab for the treatment of FL, the same group that created FLIPI proposed a new index that could better reflect the course of FL disease in the era of immunochemotherapy. FLIPI-2 considers five parameters: 1) β2-microglobulin (β2M) higher than the upper limit of normal; 2) longest diameter of the largest involved lymph node longer than 6 cm; 3) bone marrow involvement; 4) hemoglobin level <120 g/l and 5) age >60 years. FLIPI-2 has been validated clinically showing correlation with FLIPI with some studies suggesting that FLIPI-2 is somewhat superior to FLIPI. Although FLIPI and FLIPI-2 have been used with some success to classify patients into risk groups there has been difficulty in using them to choose treatment or predict patient response to therapy. Moreover, these prognostic indexes (PI) lack molecular elements and are not able to accurately predict relapse events or DLBCL transformation. F-fluorodeoxyglucose (FDG)-PET has been used to detect active disease and determine total metabolic tumor volume (TMTV). TMTV in combination with FLIPI-2 has shown to help in identifying patients with severe prognosis. Another PI, the PRIMA-PI is derived from FLIPI-2 and uses only β2M and bone marrow involvement. The PI has 3 risk categories, high with a β2M 3mg/L with bone high with β2M level >3mg/ml and bone marrow involvement; intermediate with β2M ≤3mg/L with bone marrow involvement; and low
with β2M ≤ 3mg/L without bone marrow involvement. Although simpler and promising, its
prognostic capacity seems limited and further validation is required before its clinical
implementation\textsuperscript{234}.

\textit{CD163+ and CD68+ macrophages, CD4+ and CD8+ T cells, and other immune cell subsets}

Since FL originates in multiple lymphoid organs (bone marrow, lymph nodes, spleen, and
MALT), FL B cells are often surrounded by other non-malignant immune cells including
different subsets of T cells, B cells, macrophages, and follicular dendritic cells (FDCs). The
amounts of infiltrating immune cells and their activation states/phenotypes have a profound
effect in the course of FL. The amount of CD68+ and CD163+ tumor associated macrophages
(TAMs) have shown to be useful to predict OS and PFS. Multiple studies have shown that FL
patients with a higher number of CD68+ and CD163+ TAMs receiving chemotherapy had poor
prognosis whereas patients receiving immunochemotherapy had longer survival rates\textsuperscript{235,236}. Thus, the prognostic value of CD68+ and CD163+ TAMs may be dependent on therapeutic
regimens. Another biomarker useful to identify monocyte and macrophage subsets with
prognostic value in FL is SIRPα. Using CD14 and the expression levels of SIRPα three subsets
of macrophages can be identified in FL (CD14+SIRPα\textsuperscript{hi}, CD14−SIRPα\textsuperscript{low}, and CD14−SIRPα\textsuperscript{neg}).
Increased numbers of CD14−SIRPα low cells seem to correlate with better OS\textsuperscript{237}. Infiltration of
cytotoxic T cells positive for granzyme B are an indication of an outgoing immune response at
the tumor site.. CD4+ and CD8+ cells positive for PD-L1 expression were found in higher
amounts in FL patients that responded well to immune checkpoint therapy\textsuperscript{238}. Another T cell
subset that has prognostic value are FOXP3+ regulatory T cells, whose presence is positively correlated with OS\textsuperscript{239}.

\textit{m7-FLIPI, mutational status of relevant genes, and gene expression profiling}

Using deep DNA sequencing Pastorea et al. analyzed the mutation status of the coding sequence of 74 recurrently mutated genes in lymphoma from two independent patient cohorts. Clinical factors were used to establish a clinicogenetic risk model based on failure-free surviving that included the mutation status of seven genes (\textit{EZH2}, \textit{ARID1A}, \textit{MEF2B}, \textit{EP3000}, \textit{FOXO-1 CREBBP}, and \textit{CARD11}), FLIPI and Eastern Cooperative Oncology Group (ECOG) performance status\textsuperscript{19}. Even though m7-FLIPI is the first known clinicogenetic model, there is some controversy if it accurately can be used for risk stratification or implemented in the clinical setting on a routine basis. It seems that m7-FLIPI is dependent on therapeutic regimen and may need additional evaluation and adjustments\textsuperscript{240}.

Besides the genes considered in m7-FLIPI, there have been other genes whose expression signatures and mutational status have shown clinical relevance as biomarkers for FL. Rearrangements and mutations in genes regulating proliferation such as the \textit{MYC} have been reported in indolent lymphomas and associated with poor prognosis in patients with advanced stages of FL\textsuperscript{241}. Mutations in genes involved in cell cycle regulation and programmed cell death have been also reported in FL including \textit{CDNK2A}, \textit{CDKN2B}, \textit{TP53} and \textit{BCL2}. Among early lesions of FL that are reported are deletions of 1p36, gains of 7p and 12q chromosomes in related onco and tumor suppressors genes including \textit{TNFRSF14}, \textit{EZH2} and \textit{MLL2} genes\textsuperscript{242}. Other genetic lesions can occur later in the course of FL during aSMH in the proto-oncogenes \textit{BCL6}, \textit{RHOH/TFF}, \textit{PIM1}, \textit{PAX5}, \textit{IRF4}, \textit{ST6GAL1}, \textit{BCL7A}, \textit{CIITA}, \textit{LRMP} and \textit{SOCS1}. Some of these
mutations are frequently associated with malignant transformation of FL to DLBCL\textsuperscript{220}. Other relevant mutations with prognostic relevance in FL are mutations in *MYD88, CXCR4, BCL6, STAT6, TNFAIP3, IRF8* and *ETSI* genes\textsuperscript{243}.

The use of gene expression profiles for the management of FL patients has been assessed using microarray and RNAseq data from patients of the Primary Rituximab and Maintenance (PRIMA) trial. In a study conducted by Bolen et al. the impact of mutational load and of a 6-gene (*GZMA, GZMB, PRF1, IFNg, EOMES*, and *CD8A*) effector T cell (T\textsubscript{eff}) signature was utilized to classify patients into 2 groups, “inflamed” (T\textsubscript{eff}-high) and an “uninflamed” (T\textsubscript{eff}-low), finding that patients in the inflamed group had longer PFS\textsuperscript{244}. Huet et al. using data from the same PRIMA trial identified 23 genes whose expression levels were associated with PFS and reflected B cell biology and tumor microenvironment. The data obtained from the patients was used to build a model that was able to identify the population of patients that were at high risk\textsuperscript{245}.

**Immunohistological and cell surface markers**

Immunohistochemical staining and analysis of FL cells by flow cytometry is, for almost all of the cases, positive for cell surface staining of CD19, CD20, CD22, CD79a, CD10 and monoclonal immunoglobulin. Additionally, about 85%-90% of FLs overexpress cytoplasmic BCL2 although it can vary depending on the grade of the disease. FL cells usually are negative for CD5, CD11c CD23 and CD43. Loss of CD10 expression is usually an indication of transformation of FL to DLBCL\textsuperscript{226,246}. With the FDA approval of immunocheckpoint therapies, the importance of PD-1 and PD-L1 expression on both immune cells and tumor cells has acquired increased relevance in diagnosis and prognosis of B cell lymphomas. However, among
B cell lymphomas FL expresses the lower levels of plasma PD-L1 and the expression of PD-L1 in tissue and peripheral blood may have independent prognostic value\textsuperscript{247}.

*Epigenetic changes*

Mutation on chromatin modifying enzymes and dysregulation in methylation patterns of tumor suppressor, cell cycle regulators and DNA repair genes have been reported as epigenetic hallmarks of FL\textsuperscript{248}. Misregulation of histone lysine methylation has been reported to be involved in cancer and developmental defects. Particularly, methylations in H3K4, H3K36 and H3K79 are related to active transcription, while methylations in H3K9, H3K27 and H4K20 are associated with silenced chromatin states\textsuperscript{249}. EZH2 is a H3K27 methyltransferase that when mutated is related to favorable outcome and low risk. It is also a potential biomarker for DLBCL and therapeutic target\textsuperscript{250}. MLL2/KMT2D is another methyltransferase and potential biomarker that is mutated in about 90% of FL cases. Deregulation of KMT2D has been reported to produce reduced trimethylation of H3K4 and has therapeutic potential as a target. Mutations in KMT2D along with CREBBP are suggested to be early oncogenic events\textsuperscript{251}. Hypermethylation of MGMT promoter has been associated with good prognosis\textsuperscript{252}. Among tumor suppressor genes with epigenetic modification is KLF4, which is hypermethylated in B cells in FL. Epigenetic silencing of KLF4 may be beneficial for patients and has potential prognostic value\textsuperscript{253}. P16/INK4 is a CDK4 inhibitor, methylation in its promoter leads to inactivation of the gene, which according to some studies may be an indication of poor prognosis in FL patients\textsuperscript{254}. MEF2 encodes transcription factors that control cell differentiation and recruit histone-modifying enzymes, mutations in MEF2 are associated with malignant transformation of germinal center (GC) B cells and dysregulation of BCL6 a sign of DLBCL transformation\textsuperscript{255}. Non-silent-mutations in other
relevant epigenetic modifiers are found in *ARID1A*, *EP300*, *KTM2C* and *BCL7*, some of them part of the m7-FLIPI\(^{256}\).

*IL2-R*

Interleukin-2 Receptor (IL-2) has been another promising potential biomarker that has been linked to FL in multiple studies. In one study soluble IL-2 (sIL-2) was significantly correlated with the clinical stages of FL, as well as the quantity of nodal regions\(^{257}\). Another study showed that the levels of IL-2R regressed over time as patients received rituximab treatment, and that increased levels of IL-2R was an indicator of disease progression. Among a comparison of other cytokines, this study also reported that IL-2R levels were the only ones to achieve statistical significance in a cohort of patients (84% elevated, \(P=0.04\))\(^{258}\). An additional study showed the usefulness of sIL-2R as a prognostic index for FL in the gastrointestinal tract\(^{259}\). Together these studies showed the usefulness of IL-2R as a biomarker before treatment, but it wasn’t until 2017 that a study demonstrated its usefulness post-treatment as well. This study involved rituximab in combination with other drugs to provide chemotherapy (R-CHOP), and high post-treatment sIL-2R was shown to be correlated with inferior Progression-free survival (PFS), demonstrating IL-2R’s usefulness in post-treatment assessment as well\(^{260}\). In addition, high serum sIL-2R has been shown not only to be correlated with PFS, but with Overall Survival (OS) and Disease Specific Survival (DSS) as well\(^{261}\).

*Other Serum cytokines*

Serum cytokines levels can be useful to predict how patients may respond to certain treatments. When compared to healthy controls, FL patients have higher levels of IL-1Ra, IL-6,
IL-7, IL-10, IL-13, TNF-α, VEGF, and PDGF. Further studies at the University of Iowa and Mayo Clinic have linked the expression of these cytokines to poor disease outcomes. Upregulation of IL-1Ra indicated negative patient outcome in newly diagnosed patients. IL-12 and CXCL9 were also associated with decreased survival. In addition, increased levels of CXCL9 and CXCL10 correlated with a lower lymphocyte to monocyte ratio, another indicator of poor prognosis. Multivariate analysis of TGF-β and VEGF is another predictive method, with high TGF-β and low VEGF resulting in improved outcomes. Patients with higher concentrations of serum IL-6 and TNF-α displayed higher tumor burden and inferior survival.

Studies have also investigated the impact of cytokines on different treatment options. The University of Iowa/Mayo Clinic study reported poor outcomes of alkylator and anthracycline-based chemotherapies with high levels of IL-1Ra and IL-12. Another article demonstrated that increased IL-12 negatively impacted responses to rituximab antibody therapy. In a CD19 CAR T cell clinical trial, it was found that IL-15 was positively associated with the efficacy of the treatment. Overall, serum cytokines are promising biomarkers because of their potential to represent the tumor microenvironment and predict disease outcomes.

Circulating tumor DNA

Circulating cell free DNA (cfDNA) are DNA fragments coming from cells that are released to the blood plasma. The cfDNA coming from tumor cells is called tumor circulating DNA (ctDNA) and can be released to the blood plasma even from deep tissues. This ctDNA can carry all the specific genetic aberrations characteristic of the cancer type, making it a highly specific biomarker. Through quantitative PCR (qPCR), digital droplet PCR (ddPCR) and next generation sequencing (NGS) specific mutations or chromosomal rearrangements unique to
specific for FL can be detected such as the t(14;18) translocation. In a recent study conducted at Stanford University ctDNA was used to differentiate patients with indolent FL from those undergoing transformation to DLBCL. Using NGS the authors found ctDNA carrying FL and DLBCL specific genetic aberrations in 100% of the patients and were able to differentiate indolent FL cases from those transitioning to DLBCL with a 99.8% specificity. The use of ctDNA has demonstrated capacity to predict clinical outcomes and represents a non-invasive method to diagnose and manage FL patients (Table 4-1).
Table 4-1. Predictive biomarkers for follicular lymphoma and their detection methods.

<table>
<thead>
<tr>
<th>Type</th>
<th>Biomarker</th>
<th>Detection method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translocations and gene rearrangements</td>
<td>t(14;18)(q32;q21), MYC, BCL2</td>
<td>PCR, FISH</td>
</tr>
<tr>
<td>Histological biomarkers</td>
<td>CD19, CD20, CD22, CD79a, CD10, BCL2, CD5, CD11c, CD23, CD43 and CD10</td>
<td>IHC, flow cytometry</td>
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<tr>
<td>Mutational status of FL related genes</td>
<td>CDKN2A, CDKN2B, TP53, BCL2, TNFRSF14, BCL6, RHOH/TFF, PIM1, PAX5, IRF4, ST6GAL1, BCL7A, CIITA, LRMP, SOCS1, MYD88, CXCR4, STAT6, TNFAIP3, IRF8 and ETS1</td>
<td>NGS</td>
</tr>
<tr>
<td>Epigenetic changes</td>
<td>EZH2, MLL2/KMT2D, CREBBP, MGMT, KLF4, P16/INK4, MEF2, BCL6, ARID1A, ARID1A, KTM2C, BCL7</td>
<td>NGS, methyl-seq</td>
</tr>
<tr>
<td>Gene expression signatures</td>
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<td>Microarray, NGS</td>
</tr>
<tr>
<td>Serum cytokines</td>
<td>IL1R, IL2R, IL-6, IL-7, IL-10, IL-13, TNF-α, VEGF, PDGF3, CXCL9, CXCL10, TGF-β, IL-12, IL-15</td>
<td></td>
</tr>
<tr>
<td>-----------------</td>
<td>-----------------------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Non-malignant immune subsets</td>
<td>MO&amp;MO[CD14^+\text{SIRP}^\text{α}\text{hi}/\text{CD14}^-\text{SIRP}^\text{α}\text{low}, \text{MO CD163/CD68, CD4+ and CD8+ T cells}</td>
<td></td>
</tr>
<tr>
<td>ctDNA</td>
<td>t(14;18), IGH-BCL2, MYD88 L265P, CARD11, PIM1</td>
<td></td>
</tr>
<tr>
<td>MicroRNAs</td>
<td>44 signature, miRNA-17-92, miR-9, miR-155, miR-217, miR-222, miR-223, and miR-494</td>
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<tr>
<td>Exosomes</td>
<td>tetraspanins, CD63, CD81 and CD9, CD20, MHC II, AKT mRNA, BCL6 mRNA, MYC mRNA, PTEN mRNA, BCL-XL mRNA</td>
<td></td>
</tr>
<tr>
<td>Pyrimidine salvage pathway</td>
<td>TK1, HPRT</td>
<td></td>
</tr>
</tbody>
</table>

Immunohistochemistry (IHC), Flow cytometry, Immunoassay, cytokine microbeads array and flow cytometry, Flow cytometry, histology, NGS, PCR, droplet PCR, NGS, microRNA profiling (NGS, microRNA microarray), Exosome isolation, immunoassay, microRNA profiling (NGS, microRNA microarray), Immunoassay, IHC, Flow cytometry
MicroRNAs

MicroRNAs are small non-coding RNAs that regulate gene expression silencing messenger RNA (mRNA) by binding to their 3’ untranslated region\textsuperscript{270}. MicroRNAs (miRNA) participate in tumorigenesis and have a characteristic signature in FL. Wang et al. researched the role of miRNAs in FL and identified 44 miRNAs that were divided in three signatures that can be used to identify patients that have an improved response to chemotherapy. The study further identified microRNAs with tumor suppressor properties, cell cycle regulation and cytokine signaling\textsuperscript{271}. Among the most important miRNAs that can have potential as biomarkers are the miR-17-92 cluster which includes six different miRNAs. This set of miRNAs are found to be overexpressed in FL and promote proliferation, inhibit apoptosis, induce angiogenesis, activate transcription signaling and cooperates with \textit{MYC}, possibly targets \textit{PTEN}, \textit{p21}, \textit{Bim} and \textit{E2F}\textsuperscript{272}. Other miRNAs with diagnostic potential in FL are miR-9, miR-155, miR-217, miR-222, miR-223. And miR-494\textsuperscript{273}.

Exosomes

Exosomes are extracellular vesicles (40-120 nm) from endocytic origin that facilitate the communication between cells at close and far proximity. Because they can be found in different body fluids, there has been an increasing interest in using them as a source of biomarkers\textsuperscript{274}. Among some of the common proteins found in exosomes are endosome-associated proteins, tetraspanins, CD63, CD81 and CD9. However, exosomes can also transport microRNAs, DNA, and other proteins\textsuperscript{275}. They also can deliver antigens to tumors and help promote anti-tumoral responses, transport pathogenic proteins or transport molecules that help tumors to thrive.
Quantification of lymphoma cell-derived exosomes (LCEV) and analysis of their cargo may be used as predictive tools. Characterization of exosomes from FL B cells showed that LCEVs expressed high levels of MHC I, low levels of MHC II and high levels of CD86. Researchers from the Spanish lymphoma oncology group analyzed the content of mRNA in exosomes derived from FL cells in 166 patients and found that the presence of AKT mRNA predicted poor response to rituximab. They also found that presence of BCL6 and C-MYC mRNA was indicative of lower PFS and that low PTEN and BCL-XL mRNA may be related with relapse events. Aung et al found that the release of exosomes carrying CD20 were used by B-Cell lymphoma cells as a shield against anti-CD20 monoclonal antibody therapy and that blocking ABCA3 reduced the production of exosomes and increase the susceptibility of lymphoma B cells to immunotherapy.

Nucleotide salvage pathway enzymes

A significant amount of scientific evidence has shown that Thymidine Kinase 1 (TK1) is a sensitive and reliable tumor biomarker for a broad range of malignancies including B cell lymphomas. Prochazka et al demonstrated that high TK1 serum levels in FL patients predicted lower complete response (CR), OS and PFS. Moreover, TK1 levels were able to predict relapse events and when combined with β2M and FLIPI help to refine risk assessment. Silencing of TK1 has shown to decrease tumor cell proliferation, cell migration and mesenchymal transformation of both pancreatic and lung cancer cells. Recent findings have shown that TK1 mRNA is present in exosomes and some forms of the enzyme seem to be associated with the cell membrane of GC B cell lymphoma cells such as Burkitt’s lymphoma. Furthermore immunotargeting of TK1 with monoclonal antibodies lead to increased killing of cancer cells by
human MNC cells. Another nucleotide salvage pathway enzyme with an emerging role as a tumor biomarker is hypoxanthine phosphoribosyltransferase (HPRT). Although it was initially considered as a housekeeping gene, recent evidence shows that HPRT is upregulated multiple cancers and may be a suitable biomarker for B cell lymphomas. In addition, HPRT surface expression has also been reported in lymphoma B cells and may have a dual role as both a tumor biomarker and a therapeutic target.

Conclusion

Over the last two decades the prognostic models FLIPI, FLIPI-2, tumor burden measurements and traditional histologic analysis have been the main tools to diagnose and manage FL patients. Although successful in some extent, these diagnostic tools have not been sufficient to reach a consensus about therapy regimens, nor increase our ability to anticipate relapse and refractory events, and transformation to DLBCL. With the discovery of new drugs and development of novel immunotherapies the prognostic tools of the past may not reflect the current course of FL disease. A more detailed genetic profiling, characterization of mutational landscape and epigenetic modifications of FL in combination with a better understanding of the tumor microenvironment has the potential of producing better prognostic models to diagnose, manage and treat FL patients in the era of precision medicine.
APPENDIX 1: Biomarker analysis and clinical relevance of TK1 on the cell membrane of Burkitt's lymphoma and acute lymphoblastic leukemia.


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Biomarker analysis and clinical relevance of TK1 on the cell membrane of Burkitt's lymphoma and acute lymphoblastic leukemia

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Abstract: TK1 is an enzyme involved in DNA synthesis and repair. TK1 is usually found elevated in cancer patients' serum, which makes it a useful tumor proliferation biomarker that strongly correlates with cancer stage, metastatic capabilities, and recurrence risk. In this study, we show that TK1 is upregulated and localizes on the plasma membrane of Burkitt's lymphoma, acute promyelocytic leukemia, T cell leukemia, and acute lymphoblastic leukemia (ALL). Using flow cytometry, we confirmed that TK1 localizes on the surface of Raji, HL60, and Jurkat cells lines and on ALL clinical samples. Using fluorescence microscopy, we found a strong association of TK1 with the plasma membrane in Raji, HL60, and Jurkat cell lines. These findings were also confirmed by scanning electron microscopy. Our study also shows that this phenomenon does not occur on normal resting or proliferating lymphocytes. In addition, we show that membrane TK1 is found in all oligomeric forms ranging from monomer to tetramer and exhibits enzymatic activity. These findings suggest TK1 as a possible target for immunotherapy with the potential to be utilized in the treatment of hematological cancers.

Keywords: Burkitt's lymphoma, acute lymphoblastic leukemia, ALL, thymidine kinase 1, surface antigen

Introduction

TK1 is a nucleotide salvage pathway enzyme primarily responsible for phosphorylating deoxythymidine to deoxythymidine monophosphate.1 Under normal conditions, TK1 is tightly regulated by the cell cycle. Usually, TK1 levels are low in G1 phase, peak in S phase, and low during the late G2/M phase.2 The low levels of TK1 during the late G2/M phase occur because of degradation, which is believed to be regulated by polyubiquitination that targets degradation via the anaphase-promoting complex/cyclosome (APC/C) pathway.3 The rapid increase in TK1 levels during S phase is believed to be partly mediated by a TK1 regulatory switch, which happens when TK1 switches from a dimer (inactive enzyme) to a tetramer (active enzyme).3-5

In cancer events and upon loss of p53 regulation, TK1's cell cycle regulation is lost and TK1 levels are upregulated.6 TK1 has been found upregulated in tissue and serum in both solid tumors and hematological malignancies, which is why TK1 has been extensively studied as a cancer proliferation biomarker.7,8 The diagnostic and prognostic potential of TK1 has been demonstrated using the traditional TK activity radioassay for hematological malignancies and solid tumors.9 Moreover, TK1 levels in serum have been shown to have diagnostic potential in other cancers such as bladder, cervical carcinoma, gastric, non-small cell lung, renal, and colorectal cancers.9-10 Early
Events in carcinogenesis show an upregulation of TK1 in the serum as well, so TK1 has also been studied as a prognostic marker in many cancer types. In summary, high TK1 levels correlate with tumor aggressiveness and can be indicative of early events in carcinogenesis.

Burkitt’s lymphoma (BL) and acute lymphoblastic leukemia (ALL) are some of the most highly proliferative hematological malignancies and primarily affect children, with a small incidence in adults too. BL is an aggressive non-Hodgkin lymphoma that affects B cells. BL is the most common type of pediatric cancer in malaria-endemic regions, such as equatorial Africa, Brazil, and Papua New Guinea, affecting over 40 million children every year. While the currently available multi-agent chemotherapy treatment (cyclophosphamide, vincristine, prednisolone, and doxorubicin) has a 5-year event-free survival rate of over 99% in high-income countries, the survival rate is much lower in low-income countries (50% for 1-year event-free survival rate). ALL is the most common hematological malignancy diagnosed in children in the USA with over 6,000 cases every year. The highest peak of incidence occurs between the ages of 3–9 years, and it is the number one cause of death from cancer in people under 20 years old. One of the major complications with ALL in children is the infiltration of leukemic cells in the central nervous system (CNS), which usually occurs in relapse. In the past few decades, there have been major improvements in ALL treatments including an intensive 8-week chemotherapy regimen that has increased ALL 5-year remission rates to up to 85% and overall survival rates from 10% to 90%. These new treatments include a combination chemotherapy regimen during the 6–8 months after remission designed to prevent relapse and CNS leukemic infiltration. Unfortunately, 15%–20% of children with ALL will relapse and their survival rates drop to 20%–30%. More investigation is needed to understand the unique characteristics of BL and ALL and to find new targets that could help increase the survival.

Due to the proliferative nature of BL and ALL, we hypothesized that TK1 was highly expressed in BL and ALL cells. While investigating this hypothesis, we found TK1 to be overexpressed on the membrane of BL, acute promyelocytic leukemia, acute T cell leukemia, and ALL clinical samples. Of note, TK1 is not readily detectable on the membrane of normal resting or proliferating lymphocytes, and thus TK1 membrane localization appears to be an event exclusive in malignant cells. Moreover, membrane TK1 is found in all oligomeric forms and exhibits enzymatic activity. These findings suggest that TK1 is a tumor-specific antigen on the cell surface and therefore a potential immunotherapy target for BL and ALL.

Materials and methods
Tissue collection, cell lines, and ALL samples
Raji (BL), HL60 (acute promyelocytic leukemia), and Jurkat (acute T cell leukemia) cell lines were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were authenticated by short tandem repeat (STR) analysis at the University of Arizona Genetics Core Facility prior to our study. All cells were grown in Roswell Park Memorial Institute (RPMI) 1640 supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine and incubated at 37°C with 5% CO2. ALL samples were obtained from patients at diagnosis or relapse after informed consent on a biobank protocol at the Huntsman Cancer Institute (Salt Lake City, UT, USA) and frozen with dimethyl sulfoxide (DMSO) and albumin in aliquots. ALL samples were thawed at 37°C and washed with Dulbecco’s phosphate-buffered saline (DPBS) immediately before use.

Mononuclear cell separation
Whole blood was collected from healthy young volunteers aged 18–30 years under institutional review board (IRB) approval from the Office of Research and Creative Activities at Brigham Young University (BYU X090281). All healthy blood volunteers provided written informed consent. Blood was diluted 1:1 with DPBS layered on top of lymphocyte separation medium (LSM) (Celite, Corning Incorporated, Corning, NY, USA) and centrifuged at 400g for 30 minutes without brake or acceleration. The mononuclear cell layer was aspirated and rinsed with DPBS. The cells were treated with red blood cell lysis buffer and resuspended in RPMI 1640 supplemented with 10% FBS and 2% human serum from the original blood donor. After a further incubation for 24 hours at 37°C with 5% CO2, the lymphocytes were aspirated and washed with DPBS and prepared for flow cytometry and scanning electron microscopy (SEM).

B cell magnetic sorting
Lymphocytes (B and T cells) were obtained by mononuclear cell separation from whole blood with LSM. Cells were then washed with magnetic-activated cell sorting (MACS) buffer and stained with anti-CD19 antibody conjugated to biotin. We incubated these cells with streptavidin-gold magnetic MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany), and CD19+ cells were sorted via magnetic selection. Cells were
resuspended in MACS buffer and then washed and resuspended in DPBS for downstream application.

**B cell proliferation**

B cells obtained through magnetic selection were seeded at 2 × 10⁵ cells/ml in a six-well plate in RPMI 1640 supplemented with 10% FBS. We used the CellXVivo Human B Cell Expansion Kit (R&D Systems, Inc., Minneapolis, MN, USA) to induce proliferation. We counted the B cells and incubated them following the kit’s instructions for 5 days. After 5 days, we observed the cells under a microscope to assess proliferation and counted them again to quantify division. Cells were washed and resuspended in DPBS and used in flow cytometry.

**Antibodies**

We used three custom mouse monoclonal antibodies developed in our laboratory against TK1 (CB1, A72, and A74) and a commercially available rabbit monoclonal antibody against TK1 (ab91651; Abcam, Cambridge, UK). CB1 binds to a region in the C-terminal domain of TK1, specifically to the active domain. A72 and A74 are against an immunodominant region beside the TK1 C-terminal domain. The three custom antibodies were conjugated to fluorescein isothiocyanate (FITC) using a conjugation kit (EasyLink, ab102884; Abcam) and stored in the dark at 4°C. The custom antibodies were used for most assays, and the commercially available antibody (ab91651) was used for immunohistochemistry. The ALL samples were stained with CD34-APC-Cy7, HLA-DR-AlexaFluor488, and commercial (ab91651) APC for staining of ALL samples. CD34 and HLA-DR antibodies were purchased from Biologend (San Diego, CA, USA).

**Flow cytometry**

Raji, Jurkat, and HL60 cell lines, normal lymphocytes, and B cells were washed 3x in DPBS. All cells were resuspended in DPBS at 5 × 10⁵ cells/ml and were placed in individual microcentrifuge tubes and incubated with Fc block (Human TrueStain FCx; Biologend) for 10 minutes at room temperature. Cells were then stained with CB1, A72, and A74 conjugated to FITC. Negative controls included unstained cells and cells stained with an isotype antibody. ALL samples were resuspended in Cell Staining Buffer (Biologend), incubated with Fc block for 10 minutes at room temperature and then stained with isotype control, CD34 (APC-Cy7), HLA-DR (AlexaFluor488) to confirm ALL phenotype and with commercial (APC) to check for TK1 expression. For lymphocytes treated with TK1, we incubated 5 × 10⁵ cells/ well in a six-well plate and added DPBS or concentrations of yeast recombinant TK1 at 0.25 μM, 0.5 μM, or 0.75 μM. Cells were incubated at 37°C and 5% CO₂ for 24 hours; then, the cells were washed 3x in DPBS and incubated in Fc block for 10 minutes, after which cells were stained with anti-TK1 antibody ab91651 and then with an anti-rabbit secondary FITC antibody. Negative controls included unstained sample, anti-NFκB (rabbit), and anti-rabbit secondary FITC antibody. We also performed dead cell discrimination using a propidium iodide (PI) solution (2 mg/mL) immediately before analysis. We collected 10,000 events per sample in a flow cytometer (Attune; Thermo Fisher Scientific, Waltham, MA, USA), and the data were analyzed using the FlowJo software (FlowJo, Inc., Ashland, OR, USA).

**Fluorescent microscopy**

Raji, HL60, and Jurkat cell lines and normal lymphocytes were stained with FITC-conjugated antibodies, namely isotype control, anti-NFκB antibody, or anti-TK1 antibody (CB1) for 30 minutes on ice and in the dark. Cells were washed with cold DPBS and then resuspended at 5 × 10⁵ cells/mL. Approximately 20 μL of cell solution was placed on a glass slide, after which a drop of mounting medium containing 4’,6-diamidino-2-phenylindole (DAPI) was added to the sample (Vectashield Antifade Mounting Medium with DAPI; Vectorsheld, Burlingame, CA, USA), and a coverslip was placed on top. Cells were visualized in a light microscope (Zeiss Imager A1 Fluorescence Microscope; Carl Zeiss Meditec AG, Jena, Germany) using different channels to detect fluorescence. Blue represents DAPI fluorescence and green represents FITC fluorescence.

**SEM**

Raji cells and normal lymphocytes were washed 3x in DPBS for 5 minutes to remove medium and cellular debris. The cells were then resuspended in flow cytometry staining buffer (eBioscience; Thermo Fisher Scientific) for 15 minutes and then blocked with 1% bovine serum albumin (BSA) in DPBS (BSA/DPBS) for 5 minutes. We then added anti-TK1 antibody (A72) conjugated to biotin at a concentration of 0.005 μg/mL and incubated the cells for 15 minutes on ice. After incubation with primary antibody, we washed the cells with BSA/DPBS twice. We then stained the cells with anti-biotin conjugated to 2 nm gold nanoparticles (Nanoprobes, Yaphank, NY, USA) at a concentration of 1:500 in BSA/DPBS and incubated for 30 minutes on ice. We then washed the cells with BSA/DPBS for 5 minutes and twice with DPBS to remove any block. After staining, we fixed the cells in
0.2% glutaraldehyde for 15 minutes followed by a wash in 0.02 M glycine for 10 minutes to quench the fixative. We then rinsed the cells 3x in DPBS and used a cytometric centrifuge (Cytopsin; Thermo Fisher Scientific) to spread the cells onto cover slips. Cells were then rinsed 3x with ddH₂O and gold particles, if present, were enhanced for 3 minutes using the GoldEnhance™ EM Plus kit (Nanoprobes). The cells were then rinsed 3x with ddH₂O and dehydrated via sequential washes of 70%, 80%, 90%, and 100% EtOH for 5 minutes each. After the cover slips were dry, we imaged them using an XL-30 ESEM (Philips, Amsterdam, the Netherlands). Pictures provided were taken using a gaseous secondary electron (GSE) detector. Raw images are next to enhanced images showing red dots as gold. Energy-dispersive analysis X-ray (EDAX) was performed on all samples to quantify the levels of gold present. EDAX will provide a k-ratio, a Z-value, an A-value, and an F-value. The k-ratio represents the element’s peak height compared to a sample of the pure element collected under the same conditions. The Z-value represents a correction in the atomic number taking backscattered election yield of the pure element and the sample. The A-value represents a compensation for X-rays generated in the sample that cannot emit energy. The F-value represents a correction for the generation of X-rays. We used these EDAX output values to normalize our samples’ gold weight percentages using the following equation:

\[
\text{Normalized weight percentage} = \frac{Z \times A \times F}{k - \text{ratio} \times 100}
\]

**Plasma membrane and cytoplasmic protein isolation and Western blotting**

To separate the plasma membrane and cytoplasmic proteins, we used the Pierce™ Cell Surface Protein Isolation Kit (Thermo Fisher Scientific; catalog number, 89881). For this procedure, we used 4x10⁷ Raji cells or normal lymphocytes per test. Normal lymphocytes were isolated as described in the “Monoclonal cell separation” section. To label the cells with biotin, cells were incubated with 40 mL of biotin solution in a 75 flask. Biotin solution was made by dissolving the contents of one vial of Sulfo-NHS-SS-Biotin (provided in the kit) in 48 mL of ice-cold PBS. The flask was incubated on a rocking platform with gentle agitation for 30 minutes at 4°C. After incubation, 500 μL of quenching solution was added to the flask to quench the reaction. Then, cells were transferred to a conical tube and pelleted at 500xg for 3 minutes, after which the supernatant was discarded. Cells were then washed with 5 mL of tris-buffered saline by pipetting up and down twice with a serological pipette and pelleted at 500xg for 3 minutes. A cocktail of protease inhibitors was added to 500 μL of lysis buffer (provided in the kit) and added to the cell pellet. The cells in lysis buffer were transferred to a microcentrifuge tube and resuspended in the fluid by pipetting up and down. The cells were then disrupted using a cell disrupter (Sonicator 3000; Misonix, Inc., Farmingdale, NY, USA) at low power (1.5 amplitude) on ice using five 1-second bursts. Cells were incubated for 30 minutes on ice, vortexed every 5 minutes for 5 seconds, and sonicated for 1 second at low power (1.5 amplitude) every 10 minutes. The cell lysate was centrifuged at 10,000xg at 4°C for 30 minutes at 4°C, and the clarified supernatant was transferred to a new microcentrifuge tube. To isolate the biotin-labeled proteins, the clarified supernatant was added to a column that contained 500 μL of NeutrAvidin Agarose that had been previously washed with 500 μL of wash buffer and centrifuged for 1 minute at 1,000xg. The column was capped and incubated for 60 minutes at room temperature with end-over-end mixing using a rotator. Then, the column was centrifuged for 1 minute at 1,000xg, and the flow-through was kept for cytoplasmic protein analysis. The column was washed 3x with 500 μL of wash buffer containing a cocktail of protein inhibitors to remove any other cytoplasmic proteins. To elute the membrane proteins, a 50 mM dithiothreitol (DTT) solution was made by adding 2.7 μL of 1 M DTT to 450 μL sodium dodecyl sulfide polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. Approximately 450 μL of DTT solution was added to the column and incubated for 60 minutes at room temperature with an end-over-end mixing on a rotator. The column was then centrifuged for 2 minutes at 1000xg, and the flow-through was collected and stored at −20°C. For Western blot analysis, samples were thawed on ice after which they were boiled for 5 minutes and then run on a 12% acrylamide SDS gel at 90 V for 2-3 hours. The proteins on the gel were transferred onto a nitrocellulose membrane at 90 V for 50 minutes. The membrane was blocked with 5% nonfat milk in DPBS for 1 hour at 4°C on a rotating platform and then incubated in anti-TK1 commercial antibody (ab91651) in a 1:10,000 dilution in milk overnight at 4°C on a rotating platform. Membrane was washed 3x in DPBS for 3 minutes each in a rotating platform and then incubated with an IRDye 800 donkey anti-rabbit secondary antibody for 1 hour at 4°C on a rotating platform (LI-COR, Lincoln, NE, USA). Finally, the membrane was washed 3x with DPBS and imaged in an Odyssey CLx Imaging System (LI-COR).
Membrane separation and TK1 activity radioisotope assay

Plasma membranes were separated using a sucrose gradient. First, Raji, Jurkat, and HL60 cell lines were grown to an exponential growth phase. A total of 17×10^7 cells were pelleted at 2100×g for 20 minutes, and the pellets were resuspended in 6 mL of a buffer consisting of 20% (v/v) sucrose–Tris buffer (pH 6.8). The cell suspension was freeze-thawed in liquid nitrogen and then at 37°C 3× and centrifuged at 600×g for 10 minutes at 4°C to collect cell membranes. The supernatant was discarded, and the pellet, containing the membranes, was washed with Tris buffer (pH 6.8) and then resuspended in 9 mL of 20% (w/v) sucrose–Tris buffer. A two-step sucrose gradient was prepared in a Beckman open-top tube (Beckman Coulter, Brea, CA, USA; catalog number, 22337922) by adding 13 mL of 70% (w/v) sucrose–Tris buffer, followed by 12 mL of 50% (w/v) sucrose–Tris buffer, followed by 9 mL of 20% (w/v) sucrose–Tris buffer containing the membranes. The gradients were centrifuged at 25,000 rpm for 14 hours in a Beckman L8-60M ultracentrifuge (Beckman Coulter). The plasma membranes are found at the 50%–70% (w/v) sucrose–Tris buffer interface. The membranes were collected by the bottom drop method, which consists of using a hot needle to create a hole at the bottom of the tube and collecting each layer separately drop-by-drop. The 50%–70% (w/v) sucrose–Tris buffer interface was diluted to below 20% (w/v) sucrose–Tris buffer using Tris buffer (pH 6.8). The diluted interface was centrifuged at 47,000 rpm for 1 hour, and the pellet was resuspended in 1 mL Tris buffer (pH 6.8). This solution containing the membranes was used in the TK radioisotope assay. The TK radioisotope assay was previously described by O’Neill et al.2 Briefly, reactions were set up with 100 μL of the solution containing the membranes and 100 μL of a solution containing 2 μM [γ-32P]-thymidine, 0.02 M Tris, 20 μM MgCl2, 0.2 M KCl, 0.1 M NiCl2, 5 mM β-mercaptoethanol or DTT, and 4 mM adenosine triphosphate (ATP) or 4 mM cytidine triphosphate (CTP) for 1 hour at 37°C. After incubation, four 25 μL of each sample were applied to 2.5 cm DE-81 paper disks (Sigma-Aldrich Co., St. Louis, MO, USA; catalog number, Whatman Article No. 28419431), and the disks were washed 3× in 20 mL ammonium formate (Sigma-Aldrich Co.; catalog number, 156264), followed by a wash in 95% ethanol and a wash in distilled water. The disks were placed in scintillation vials with 3 mL of CytoScint (MP Biomedicals, Santa Ana, CA, USA; catalog number, 0188245301), and the radioactivity was measured using a wet scintillation counter (Beckman Coulter; model number LS6500).

Statistical analysis

Flow cytometry and TK1 activity data were analyzed using an unpaired t-test comparing the isotype control to each of the samples. Error bars represent the standard error of the mean. EDAX data were analyzed using a Kruskal–Wallis one-way analysis of variance (ANOVA) test. Two-tailed P-values <0.05 were determined to be significant.

Results

Flow cytometry shows a significant fluorescent shift in Raji, HL60, and Jurkat cells bound to anti-TK1 antibodies suggesting that TK1 is localized on the plasma membrane of these cells

Using standard surface staining and flow cytometry, we discovered that Raji cells have a significant fluorescent shift compared to controls when bound to TK1 antibodies, suggesting that TK1 is found on the outer cell membrane of Raji cells (Figure 1A–C). We used an anti-NFκB antibody as a control to confirm the integrity of the cell membrane since TK1 is also a cytoplasmic protein. The cells were NFκB negative, which suggests that our anti-TK1 antibodies were indeed bound to the surface of the cell and not internalized (Figure 1A). We also used CD19 as positive surface staining.
Figure 1: Flow cytometry analysis of Raji cells.

Notes: (A) Controls used for flow cytometry analysis of Raji cells. Cells are NK cell and CD19. (B) Histograms and density plots showing a fluorescence intensity shift in Raji cells stained with anti-CD19 antibodies. A72, A74, and CB1. Cells show a significant fluorescent shift when bound to anti-CD19 antibodies. Isotype control and NFk-B fluorescent shifts are nonsignificant, suggesting low nonspecific binding and integrity of the cell membrane. (C) Quantification of the percentage of cells showing a positive fluorescent shift. Raji cells show a significant fluorescent shift when bound to anti-CD19 and anti-TH1 antibodies when compared to controls. *P < 0.05, **P < 0.01, ***P < 0.001.

Abbreviation: FITC, fluorescein isothiocyanate.
control. This finding that TK1 is on the surface of Raji cells was confirmed using three different monoclonal antibodies against different TK1 epitopes (Figure 1B). Quantification and statistical analysis of the fluorescent shifts show a significant difference in the percentage of Raji cells positive for TK1 compared to isotype control (Figure 1C).

To confirm these findings, we also stained other hematological cancer cell lines such as HL60 (acute promyelocytic leukemia) and Jurkat (acute T cell leukemia) with CB1 conjugated to FITC and a commercially available anti-TK1 antibody conjugated to FITC. We used CD45 and Na^+K^+ ATPase as positive controls, as both cells express them highly on their membrane. Both HL60 and Jurkat cells showed a significant fluorescent shift when bound to TK1 antibody (Figure 2).

**Fluorescent microscopy suggests that TK1 is associated with the plasma membrane**

To confirm that TK1 was associated with the membrane, we produced images using a fluorescent microscope to visualize this membrane localization phenomenon. We stained Raji, Jurkat, and HL60 cell lines and normal lymphocytes with CB1 antibody conjugated to FITC (Figure 3). Our isotype control shows minimal fluorescent background, while the samples treated with antibodies against Na^+K^+ ATPase and TK1 showed strong FITC signals on the cell membranes, suggesting a strong association of TK1 with the cell membranes. Normal lymphocytes show minimal FITC signal associated with TK1, indicating minimal presence of TK1 on their surface.

**TK1 is absent on the plasma membrane of normal lymphocytes**

To test whether TK1 membrane localization was exclusive to malignant cells, we stained normal resting lymphocytes from whole blood with our antibodies. We isolated mononuclear cells from whole blood using LSR. We analyzed these cells immediately using flow cytometry. These normal lymphocytes were CD45^+ and TK1^−. We also tested whether the localization of TK1 on the surface was due to proliferation. We magnetically sorted CD19^+ cells from mononuclear lymphocytes isolated from whole blood by using an anti-CD19 antibody conjugated to biotin which we bound to streptavidin magnetic beads. We stimulated these cells to proliferate using the CellXivo Human B Cell Expansion Kit (R&D Systems, Inc.). We then stained the cells with the same antibodies used with the resting lymphocytes using CD45 as positive control and analyzed the cells through flow cytometry. Flow cytometry analysis confirmed that normal lymphocytes do not significantly upregulate TK1 on their surface even when undergoing cell division (Figure 4A). These results indicate that the localization of TK1 on the outside layer of the plasma membrane may be an event exclusive to malignant cells.

In addition, to test whether extracellular TK1 interacts with membranes, we incubated normal lymphocytes with 0.25 μM, 0.50 μM, 0.75 μM, and 1 μM of purified TK1 for 24 hours and then probed the cells with anti-TK1 antibody. Flow cytometry analysis showed that there is no significant expression of TK1 on the surface of these lymphocytes when exposed to supraphysiological levels of TK1 (Figure 4B). These results suggest that there is no interaction of membrane with extracellular TK1.

**SEM shows further confirmation of TK1 localization on the surface of cancer cells**

To further confirm the location of TK1 on the surface of cancer cells, we developed an SEM protocol and used gold nanoparticles to visualize TK1. For this procedure, we used our A72 antibody. TK1 is shown to be expressed on the surface of Raji cells (Figure 5A and B) and is absent in normal resting lymphocytes (Figure 6A and B). EDAX helped us quantify the amounts of gold present on the cell surface. The Raji cells probed with A72 antibody show significantly higher amounts of gold bound to their membrane than Raji cells probed isotype control. Raji cells probed with A72 antibody show similar amounts of gold...
to Raji cells probed with Na⁺K⁺ antibody (Figure 5C). In normal lymphocytes, the amounts of gold in Raji cells probed with A72 are similar to those probed with isotype control, suggesting that TK1 is absent on the surface of normal lymphocytes (Figure 6C).

ALL clinical samples also express TK1 on their plasma membrane.

To ensure TK1's relevance in clinical samples, we tested ALL samples (n=9) obtained from the Huntsman Cancer Institute. These samples were CD34⁺ and HLA-DR⁺ as...
Figure 5 SEM of Raji cells.
Notes: (A) Cells were stained with anti-β1 integrin gold, anti-NF-κB-biotin + anti-β1 integrin gold, and anti-TK1 (A72)-biotin + anti-β1 integrin gold visualized through a GIE detector. White spots represent gold bound to the membrane of the cells. (B) Images were filtered so the white spots (gold) could be shown in red for better visualization. (C) EDAX quantification of gold-wt percentages in Raji cells stained with anti-β1 integrin gold, anti-NF-κB-biotin + anti-β1 integrin gold, A72-biotin + anti-β1 integrin gold, and a positive control NaK. A72-biotin + anti-β1 integrin gold. The amount of gold particles found on the surface of Raji cells when stained with anti-TK1 A72 antibody is significantly higher than background. *P<0.05; **P<0.01
Abbreviations: EDAX, energy-dispersive analysis X-ray; GIE, gaseous secondary electron; SEM, scanning electron microscopy.

Figure 6 SEM of normal lymphocytes.
Notes: (A) Cells were stained with anti-β1 integrin gold, anti-NF-κB-biotin + anti-β1 integrin gold, and anti-TK1 (A72)-biotin + anti-β1 integrin gold visualized through a GIE detector. White spots represent gold bound to the membrane of the cells. (B) Images were filtered so the white spots (gold) could be represented in red for better visualization. No gold can be visualized in these samples. (C) EDAX quantification of gold-wt percentages in normal lymphocytes stained with anti-β1 integrin gold, anti-NF-κB-biotin + anti-β1 integrin gold, A72-biotin + anti-β1 integrin gold, and a positive control NaK. A72-biotin + anti-β1 integrin gold. The amount of gold particles found on the surface of normal lymphocytes when stained with anti-TK1 A72 antibody is the same as the background indicating the absence of TK1 in these cells. ***P<0.001.
Abbreviations: EDAX, energy-dispersive analysis X-ray; GIE, gaseous secondary electron; SEM, scanning electron microscopy.
expected (Figure 7A). We performed alive/dead cell discrimination to prevent any false positives in these samples. All ALL samples tested were positive for TK1. Cell viability impacted the analysis as we got lower event counts when we discriminated dead cells. However, even in samples with low viable cell count, TK1 was expressed on the cell membrane. Percentage of cells positive for TK1 varied from 8% to 43.5%, averaging 28.01% (Figure 7B). We believe that we see this big range due to disease progression and grade.

TK1 on the membrane is found in different oligomeric

To further characterize TK1 on the membrane, plasma membrane and cytoplasmic proteins were isolated from Raji cells and normal lymphocytes. Both the membrane and the cytosolic proteins were run on a 12% acrylamide SDS gel and then transferred to a nitrocellulose membrane and probed with anti-TK1 antibody at 191651. Results show that the Raji cell membrane protein fraction contains mostly monomers.

Figure 7 Flow cytometry analysis of ALL clinical samples.

Notes: (A) Density plots of flow cytometry controls used in the analysis of ALL samples. Cells are CD45 and HLA-DR - Controls indicate an insignificant fluorescent shift in cells stained with isotype IgG control and anti-NF-kB antibody. Cells appear to shift in fluorescence when bound to anti-TK1 antibody. (B) Quantification of cells shifting toward a greater fluorescence in ALL samples. ALL samples have a significant percentage of cells with a greater fluorescent shift compared to controls. **P<0.01.

Abbreviations: ALL, acute lymphoblastic leukemia; PI, propidium iodide.
and dimers of TK1 (Figure 8A). On the other hand, the normal lymphocyte membrane protein fraction contains minimal traces of TK1 (Figure 8B).

**TK1 on the membrane shows activity**

Membrane fractions of Raji, Jurkat, and HL60 cell lines and normal lymphocytes were used in a radioassay to measure TK1 activity. TK1 activity was measured by subtracting the enzymatic activity with CTP as the phosphate donor from the total enzymatic activity with ATP as the phosphate donor. The reasoning behind this is that TK2, an isozyme form of TK1 found in mitochondria, favors CTP as the phosphate donor. By subtracting the enzymatic activity with CTP as the phosphate donor, we ensure that the remaining enzymatic activity is purely due to TK1 activity, as described by Lee and Cheng. Our data show that the membrane fractions of Raji, Jurkat, and HL60 cell lines show TK1 enzymatic activity (Figure 9). Moreover, these membrane fractions show higher TK1 activity levels than those in normal lymphocytes. This could be due to minimal presence of TK1 on the surface of normal lymphocytes.

**Discussion**

BL and ALL are complex diseases that affect millions of children worldwide. They are also two of the fastest proliferating tumors in humans. Rapid cellular proliferation and resisting apoptosis are two of the hallmarks of cancer that we can clearly see in hematological malignancies, especially in BL and ALL. Early events in the deregulation of the cell cycle can lead to rapid proliferation and possible cancer. Current treatments for BL and ALL include combination chemotherapy drugs that cause debilitating side effects in children; therefore, there is an immediate need to identify new targets to treat BL and ALL through immunotherapy. This study provides a potential novel target against these malignancies.

Our study shows that upregulation of TK1 can also be found on the membrane of hematological malignancies. This upregulation can be due to increased levels of TK1 in the cell during cellular division, an event that happens very frequently in most cancer, especially in BL and ALL.

In this study, we show the novel finding that multiple hematological cancer cell lines and ALL clinical samples express...
high levels of TK1 on their surface. Interestingly, this is not seen in resting or proliferating normal lymphocytes. TK1’s expression appears to be similar to that of CD229 in multiple myelomas, where CD229 is upregulated only in malignant cells and remains low/absent in normal lymphocytes. In addition, our data suggest that membrane TK1 is found in mononuclear and dimer form and has enzymatic activity suggesting that TK1 has a function on the membrane. The actual function of membrane TK1 and the mechanism/pathway by which TK1 reaches the membrane remain unknown. However, our data suggest that the origins of membrane TK1 are endogenous and do not come from serum TK1. We believe that during rapid proliferation events in malignancy, as TK1 protein levels increase, TK1 interacts with one or several membrane proteins in the endoplasmic reticulum (ER). For example, SEL16 and ODC1 are membrane proteins known to interact with TK1. Both of these proteins also upregulate during malignancy events and are found in greater quantities on the cellular membrane. Perhaps their interaction results in TK1 being transported to the membrane, remaining there, or perhaps being released into the bloodstream to be later found in the serum.

The findings presented in this study are important as they represent the discovery of a novel antigen on the surface of some cancer cells, which if used in conjunction with current immunotherapeutic treatments, such as chimeric antigen receptor (CAR) T cells, has the potential to improve treatments for different hematological malignancies.

Conclusion
TK1 is localized on the surface of Raji (BL), HL60 (acute promyelocytic leukemia), and Jurkat (acute T cell leukemia) cell lines and ALL clinical samples, and not on the surface of normal resting or proliferating lymphocytes, and may be used as a target to treat hematological malignancies.

Acknowledgments
This study was supported by funding from the Brigham Young University Department of Microbiology and Molecular Biology, the Brigham Young University College of Life Sciences, and the Simmons Center for Cancer Research. We thank Dr Juan Arroyo for his technical assistance in microscopy imaging/processing and Jaden Evans for his assistance with the radioactive assays.

Disclosure
The authors report no conflicts of interest in this work.

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APPENDIX 2: Membrane expression of thymidine kinase 1 and potential clinical relevance in lung, breast, and colorectal malignancies.


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Membrane expression of thymidine kinase 1 and potential clinical relevance in lung, breast, and colorectal malignancies


Abstract

Background: Lung, breast, and colorectal malignancies are the leading cause of cancer-related deaths in the world causing over 2.8 million cancer-related deaths yearly. Despite efforts to improve prevention methods, early detection, and treatments, survival rates for advanced stage lung, breast, and colon cancer remain low, indicating a critical need to identify cancer-specific biomarkers for early detection and treatment. Thymidine kinase 1 (TK1) is a nucleotide salvage pathway enzyme involved in cellular proliferation and considered an important tumor proliferation biomarker in the serum. In this study, we further characterized TK1's potential as a tumor biomarker and immunotherapeutic target and clinical relevance.

Methods: We assessed TK1 surface localization by flow cytometry and confocal microscopy in lung (NCI-H460, A549), breast (MDA-MB-231, MCF-7), and colorectal (HT-29, SW620) cancer cell lines. We also isolated cell surface proteins from HT-29 cells and performed a western blot confirming the presence of TK1 on cell membrane protein fractions. To evaluate TK1's clinical relevance, we compared TK1 expression levels in normal and malignant tissue through flow cytometry and immunohistochermistry. We also analyzed RNA-Seq data from The Cancer Genome Atlas (TCGA) to assess differential expression of the TK1 gene in lung, breast, and colorectal cancer patients.

Results: We found significant expression of TK1 on the surface of NCI-H460, A549, MDA-MB-231, MCF-7, and HT-29 cell lines and a strong association between TK1's localization with the membrane through confocal microscopy and Western blot. We found negligible TK1 surface expression in normal healthy tissue and significantly higher TK1 expression in malignant tissues. Patient data from TCGA revealed that the TK1 gene expression is upregulated in cancer patients compared to normal healthy patients.

Conclusions: Our results show that TK1 localizes on the surface of lung, breast, and colorectal cell lines and is upregulated in malignant tissues and patients compared to healthy tissues and patients. We conclude that TK1 is a potential clinical biomarker for the treatment of lung, breast, and colorectal cancer.

Keywords: TK1, Surface expression, Membrane TK1, Thymidine kinase 1, Lung cancer, Breast cancer, Colon cancer
Background
Lung, breast, and colorectal malignancies are the leading causes of cancer-related deaths in the world. These three cancers account for over 4.86 million cases diagnosed and over 2.8 million cancer-related deaths worldwide every year [1]. Thus, lung, breast, and colorectal cancers are a major health concern as over 11.7 million people are currently diagnosed and living with these diseases worldwide and represent a substantial economic burden in countries of all incomes [2].

Despite efforts to improve methods of prevention, early detection, and treatment, survival rates for advanced stage lung, breast, and colon cancer remain low at 4%, 26%, and 58% respectively [3]. Therefore, there is an urgent need to identify cancer-specific biomarkers for early detection and treatment of the leading cause of cancer-related deaths such as lung, breast, and colorectal cancers [4, 5].

Thymidine kinase 1 (TK1) is a nucleotide salvage pathway enzyme involved in cellular proliferation and considered an important tumor proliferation biomarker [6–9]. In serum, TK1 has been shown to be elevated in early events of malignancy, and thus, TK1 can serve as an early detection biomarker [9–11]. Moreover, serum TK1 has been found to be elevated in several hematological and solid tumors including breast, lung, colorectal cancer, among others, and high serum TK1 levels usually correlate with cancer grade and stage, increased T-values, and increased tumor size [7–9, 12–14]. Serum TK1 can be also used as a prognostic tool to monitor responses to chemotherapy or surgery [14, 15].

To further characterize TK1’s potential as a tumor biomarker, we evaluate TK1 as a potential immunotherapeutic target. In this study, we evaluate the expression levels of membrane TK1 on lung, breast, and colorectal cell lines using flow cytometry. We also show evidence that TK1 is localized on the surface of lung, breast, and colorectal cell lines. In addition, we evaluate TK1 expression levels in normal and malignant tissue to determine TK1’s clinical relevance. These results suggest TK1 as a potential immunotherapeutic target.

Materials and methods
Cell lines and cell culture conditions
Lung cancer cell lines NCI-H460 (ATCC® HTB-177™) and A549 (ATCC® CCL-185™), breast cancer cell lines MCF7 (ATCC® HTB-22™) and MDA-MB-231 (ATCC® HTB-132™), and colon carcinoma cell lines SW620 (ATCC® CCL-221™) and HT-29 (ATCC® HTB-38™) were purchased from ATCC (Rockville, MD). NCI-H460 and HT-29 cell lines were grown in RPMI 1640 medium (Corning Life Sciences, VWR International, Radnor, PA) supplemented with 2 mM L-glutamine and 10% fetal bovine serum. MDA-MB-231, MCF7, and SW620 cell lines were grown in DMEM medium (Gibco, Thermo Fisher, Waltham, MA) supplemented with 4 mM L-glutamine and 10% fetal bovine serum. A549 cells were grown in DMEM/F-12 medium (Gibco, Thermo Fisher, Waltham, MA) supplemented with 4 mM L-glutamine and 10% fetal bovine serum. L-glutamine and fetal bovine serum were purchased from Thermo Fisher (Waltham, MA). The media was renewed every 2–3 days. For subculturing, cells were detached using Accutase (Stem Cell Technology, Vancouver, Canada) and seeded in 1:3 or 1:6 ratios. All cells were cultured at 37 °C with 5% CO₂. All cell lines were authenticated by short tandem repeat (STR) analysis at the University of Arizona Genetics Core Facility during our study.

Antibodies
We used three custom mouse monoclonal antibodies developed in our lab against TK1 (CB1, A72, and A74) and a commercially available rabbit monoclonal antibody against TK1 (ab91651) (Abcam, Cambridge, United Kingdom). CB1 binds to the C-terminal domain of TK1, specifically to the active domain. A72 and A74 are against an immunodominant region not on the TK1 C-terminal domain. These antibodies have been previously tested to work in ELISA, immunohistochemistry, and Western blots to confirm their specificity [6, 16, 17]. The three custom antibodies were conjugated to FITC using a conjugation kit (EasyLink, Abcam, ab102884) and stored in the dark at 4 °C. The commercially available antibody (ab91651) was conjugated to FITC or APC using a conjugation kit (EasyLink, Abcam, ab102884) and stored in the dark at 4 °C. We used FITC-conjugated CB1, A72, A74, and APC-conjugated ab91651 for flow cytometry, FITC-conjugated A72 for confocal microscopy, and unconjugated ab91651 was used for Western blotting and immunohistochemistry.

Flow cytometry
Cells were rinsed with Dulbecco's phosphate-buffered saline (DPBS) and treated with Accutase (Stem Cell Technology, Vancouver, Canada) at 37 °C for 5–10 min to allow for detachment and then rinsed with their respective complete medium. Cells were pelleted and resuspended at 1 × 10⁶ cells/mL in Cell Staining Buffer (BioLegend, San Diego, CA) and 200 µL of cells were placed in individual microcentrifuge tubes and stained with 1 µg of FITC-conjugated CB1, A72, A74, or APC-conjugated ab91651 for 30 min on ice in the dark. Negative controls used were unstained cells, cells stained with isotype mouse and rabbit antibodies, and NFκB to confirm the integrity of the cell membrane. Cells were then washed with Cell Staining Buffer and resuspended
in 500 µL of FACS buffer. FACS buffer was made with phosphate-buffered saline (PBS), 2% calf serum (Thermo Fisher, Waltham, MA), 1 mM EDTA (Thermo Fisher, Waltham, MA, CAS 6851-92-6), and 0.1% sodium azide (Sigma Aldrich, St. Louis, MO, CAS 26282-22-8). We collected 1×10^6 events per sample in a flow cytometer (Attune, Life Technologies, Carlsbad, CA) and data was analyzed using the FlowJo software (FlowJo, Ashland, OR).

Confocal microscopy
Cells were grown on glass coverslips for 48 h. Coverslips containing cells were washed in DPBS and then incubated with either isotype control, NKβ1, or A22-FTTC for 30 min at 4 °C on a rocking platform. Coverslips were then washed with 3 times with DPBS for 10 min on a rocking platform. We then dipped the coverslips in 1× CellMask™ Deep Red Plasma membrane stain (Thermo Fisher Scientific, Waltham, MA) for 10 min at 37 °C. The coverslips were rinsed in DPBS and imaged immediately using an Olympus Fluoview FV1000 confocal laser scanning microscope (Olympus, Tokyo, Japan). Images were obtained using the Laser Sharp Computer Software (Bio Rad Laboratories, Hercules, CA) and later processed in Photoshop (Adobe Systems, San Jose, CA).

Cell surface protein isolation and Western blotting
To isolate the cell surface proteins, we used the Pierce™ Cell Surface Protein Isolation Kit (Thermo Fisher Scientific, Waltham, MA, Cat # 88981). Briefly, the HT-29 cell line was grown to 90% confluency in T75 flasks and the media was removed. The cells were washed with ice-cold PBS (provided in the kit) and then the PBS was removed within 5 s. To label the cells with biotin, cells were incubated with a biotin solution made by dissolving the contents of one vial of Sulfo-NHS-SS-Biotin (provided in the kit) in 48 mL of ice-cold PBS. 10 mL of the biotin solution was added to each flask of cells and the flasks were incubated on a rocking platform with gentle agitation for 30 min at 4 °C. After incubation, 500 µL of quenching solution (provided in the kit) were added to each flask to quench the reaction. Then, cells were gently scraped and transferred to a conical tube and pelleted at 500×g for 3 min, after which the supernatant was discarded. Cells were then washed with 5 mL of Tris-buffered saline (TBS, provided in the kit) by pipetting up and down twice with a serological pipette and pelleted at 500×g for 5 min. A cocktail of protease inhibitors (Halt™ Protease & Phosphatase Inhibitor Cocktail, Thermo Fisher, Waltham, MA, product # 78440) was added to 500 µL of lysis buffer (provided in the kit) and added to the cell pellet. The cells in lysis buffer were transferred to a microcentrifuge tube and resuspended in the fluid by pipetting up and down. The cells were then disrupted using a cell disruptor (Sonicator 3000, Misonix, Inc., Farmingdale, NY) at low power (1.5) on ice using five 1-s bursts. Cells were incubated for 30 min on ice, vortexed every 5 min for 5 s, and sonicated for 1 s at low power (1.5) every 10 min. The cell lysate was centrifuged at 10,000×g for 2 min at 4 °C and the clarified supernatant transferred to a new microcentrifuge tube. To isolate the biotin-labeled proteins, the clarified supernatant was added to a column that contained 500 µL of NeutrAvidin agarose that had been previously washed with 500 µL of wash buffer and centrifuged for 1 min at 1000×g. The column was capped and incubated for 60 min at room temperature with end-over-end mixing using a rotator. Then, the column was centrifuged for 1 min at 1000×g and the flow-through was discarded. The column was washed 3 times with 500 µL of wash buffer containing a cocktail of protein inhibitors to remove any other cytoplasmic proteins. To elute the membrane proteins, a 50 mM dithiothreitol (DTT) solution was made by adding 23.7 µL of 1 M DTT (provided in the kit) to 450 µL SDS-PAGE sample buffer. 450 µL of DTT solution were added to the column and incubated for 60 min at room temperature with an end-over-end mixing on a rotator. The column was then centrifuged for 2 min at 1000×g and the flow-through collected and stored at −20 °C. For Western blot analysis, samples were thawed on ice after which they were boiled for 5 min and then run on a 12% acrylamide SDS gel at 90 V for 2–3 h. The proteins on the gel were transferred onto a nitrocellulose membrane at 90 V for 50 min in a cold room. The nitrocellulose membrane was blocked with 5% non-fat milk in DPBS for 1 h at 4 °C in a rotating platform and then incubated in anti-TK1 commercial antibody (ab91651) in a 1:1000 dilution in milk overnight at 4 °C in a rotating platform. The nitrocellulose membrane was washed 3 times in DPBS for 3 min each in a rotating platform and then incubated with an IRDye 800 donkey anti-rabbit secondary antibody for 1 h at 4 °C in a rotating platform (LI-COR, Lincoln, NE). Finally, the membrane was washed 3 times with DPBS and imaged in an Odyssey CLX Imaging System (LI-COR, Lincoln, NE).

Tissue dissociation and analysis
Healthy and malignant colon tissues were obtained from Utah Valley Regional Medical Center in Provo, UT under informed consent and following a protocol established by Utah Valley Regional Medical Center. Tissues were minced into 3–4 mm pieces with a sterile scalpel. Minced tissue was washed with 1× Hank's Balanced Salt Solution (HBSS) (Thermo Fisher, Waltham, MA) containing 5% FBS. Collagenase type II or type IV (both from Thermo Fisher, Waltham, MA, product # 17101015 and # 17104019, respectively) was added to the minced tissue.
and incubated at 37°C for 4–8 h to allow for cell dissociation. To obtain a cell suspension and separate dispersed cells from larger tissue pieces, cells were filtered through a 100 μm nylon mesh cell strainer (BD Biosciences, San Jose, CA). To prepare cells for flow cytometry analysis, cells were washed twice with HBSS and then resuspended in Cell Staining Buffer. Cells were treated with Fc block (Human TruStain FcX™, BioLegend, San Diego, CA), anti-human CD45 antibody (clone 2D1, eBioscience, San Diego, CA) and PI to gate out resident lymphocytes and dead cells. We collected 2 × 10⁶ events per sample in a flow cytometer (Attune, Life Technologies, Carlsbad, CA) and data was analyzed using the FlowJo software (FlowJo, Ashland, OR).

Analysis of RNA expression data
First, we evaluated differences in expression levels of the TK1 gene in 2645 tumor samples and 264 normal samples from The Cancer Genome Atlas [18]. We used RNA-sequencing data that had been summarized at the gene level to transcripts-per-million units and processed using the featureCounts algorithm [19, 20]. We also log-transformed the data values. These data included expression values from tumor-adjacent and blood samples; these samples were often, but not necessarily, matched to the tumor samples. Second, we evaluated breast-cancer samples from The Cancer Genome Atlas for which hormone-receptor status had been determined via immunohistochemistry; we limited this analysis to tumors that either were (1) positive for HER2 expression or (2) negative for HER2, ER, and PR expression. In calculating differences in expression between these groups, we used a two-sided, Mann–Whitney U Test. We also evaluated the level of correlation between TK1 and six stemness and EMT genes (CD44, SNAI1, SNAI2, TWIST1, ZEB1, TGFβ1) using Spearman's method. We wrote scripts in the Python programming language (https://python.org, v3.6.1) to parse and prepare the data. We used the R (v3.2.2) statistical software and the ggplot2 software package (v2.2.1) to generate graphs illustrating these expression levels [21, 22].

Immunohistochemistry
Lung, breast, and colorectal tissue microarrays were purchased from US Biomax (Rockville, MD, catalog # LC2004, #BR721, and #C001002b). Each slide contains at least 40 tissue cores with tissues varying in grade and stage and, and normal healthy tissues. The slides were incubated in Histo-Clear (National Diagnostic, Atlanta, GA) for 10 min for three changes to remove paraffin. The slides were then incubated in 100% ETOH for two changes for 5 min each, then in 90% ETOH for two changes for 1 min each, then in 70% ETOH for one change for 1 min. The slides were then washed in ddH₂O for 5 min. To perform antigen retrieval, the slides were incubated in DIVA Decloaker (Biocare Medical, Pacheco, CA) at 80–95°C for 30 min and let them cool for 10 min. Then washed 2 × in ddH₂O for 5 min. To perform

Fig. 1 Membrane TK1 expression in of colon, breast, and lung-cancer cell lines. Flow cytometry analysis of cell lines treated with anti-TK1 antibodies: a Quantification of TK1 expression on the cell membrane of HT-29 and SW620 cell lines stained with FITC or APC-conjugated anti-TK1 antibodies. b Quantification of TK1 expression on the cell membrane of MCF7 and MDA-MB-231 cell lines stained with FITC or APC-conjugated anti-TK1 antibodies. c Quantification of TK1 expression on the cell membrane of NCI-H460 and A549 cell lines stained with FITC or APC-conjugated anti-TK1 antibodies. Statistical analysis was performed by comparing the mouse isotype control fluorescent levels to those of AK2, AK4, C81, or ab91651: *P < 0.05, **P < 0.005, ***P < 0.0001; ns = P > 0.05.
peroxidase quenching, the slides were washed in Tris-buffered saline (TBS) containing 3% hydrogen peroxide for 20 min and then washed 2× with TBS-T for 5 min. The slides were blocked for 15 min using a Background Sniper Block (Biocare Medical) to reduce background and then washed with TBS twice for 3 min. We incubated the slides with a 1:200 dilution of anti-TK1 (Abcam, ab91651) anti-GAPDH (positive control, mouse monoclonal, Cell Signaling Technologies), and universal negative control serum (negative control, Biocare Medical) and put them in a humidity chamber to prevent drying. After primary antibody staining, the slides were washed 3× for 5 min with TBS and incubated with MACH 4 Universal Horseradish Peroxidase (HRP) Polymer (universal for rabbit/mouse secondary, Biocare Medical) for 30 min in a humidity chamber and then washed again 2× for 3 min with TBS. We developed the slides with ImmunFACT DAB Peroxidase (HRP) substrate (Vector Laboratories, Burlingame, CA) and then washed them 2× for 5 min with TBS. We then stained the slides with Hematoxylin (Biocare Medical) and rinsed them for 5 min in running water. We mounted the slides using cover slips and Cyto Seal (Thermo Scientific) and imaged them using a light microscope. Images were analyzed using ImageJ open source software, using the “IHC (more brown)” plug-in to obtain quantification using a gray scale [23]. The lower the gray value, the darker the tissue is stained.

**Statistical analysis**

For our flow cytometry data, we used a multiple-comparisons one-way ANOVA test using the Sidak’s correction for multiple comparisons to compare the expression of TK1 on the surface of lung, breast, and colon cell lines vs. isotype control. All samples were compared against isotype mouse IgG except for Commercial, which was compared against isotype rabbit IgG. We set a significant P-value at ≤ 0.05. Error bars represent the standard error of the mean.
For our IHC analysis, we used Tukey's multiple comparison test to compare the means of each group to one another. One side P-value was set to ≤0.05. Error bars represent the standard deviation of the samples. We used Prism 7 (GraphPad, La Jolla, CA) to perform our statistical analysis and produce graphs for most analysis. We used the R (v3.2.2) statistical software and the ggplot2 software package (v2.2.1) to generate graphs for TK1 gene expression bioinformatics analysis.

Results

TK1 expression is elevated on the surface of NCI-H460, A549, MCF7, MDA-MB-231, SW620, and HT-29 cells

Using flow cytometry, we observed an overall increase in fluorescence intensity in NCI-H460, A549, MCF7, MDA-MB-231, SW620, and HT-29 cells (Fig. 1, Additional file 1). The data represents eight independent staining procedures. Cells stained with A72 showed a significant increase in fluorescence in all cell lines but SW620 and A529 (*P < 0.05; **P < 0.005; ***P < 0.001). Cells stained with C81 showed significant increase in fluorescence in all cells but MDA-MB-231 and A549 (Fig. 1a–c; *P < 0.05; **P < 0.005; ***P < 0.001; ns = P > 0.05). Cells stained with ab91651 all showed a decrease in fluorescence intensity across all cell lines except SW620 cells (**P < 0.005; ***P < 0.001; ns = P > 0.05). Overall, cells bound to C81 had the lowest increase in fluorescent intensity. We used an anti-NEK8 antibody as a non-specific control as well as an intracellular control. It allowed us to test whether the cells were intact and the fluorescent intensity change was due to punctured/dead cells or intact cells.

Data revealed that the lung cell lines NCI-H460 and A549 had the highest TK1 surface expression out of all the other cell lines, followed by breast cell lines MDA-MB-231 and MCF7, and colorectal cell line HT-29 (Fig. 1b, c). The SW620 cell line showed very little expression of TK1 on its surface, only and average of 10% of fluorescence total increase in cells bound to A72, A74, and C81, and only 2.7% in cells bound to commercial antibody ab91651 (Fig. 1a). These results suggest that five of the six cell lines tested expressed TK1 on their cell surface.

TK1 is strongly associated with the membrane of NCI-H460, MDA-MB-231, and HT-29 cells

To visualize TK1's localization and to ensure antibody binding was to the membrane only, we performed confocal microscopy in HT-29, MDA-MB-231, and NCI-H460 cells since these cell lines expressed high levels of TK1 on their membrane in flow cytometry. We stained the cells with isotype control, anti-NEK8, and anti-TK1 (A74) antibodies conjugated to FITC. We used intact cells to ensure FITC signals were not coming from intracellular TK1. We obtained single channel images, rhodamine for membrane, FITC for isotype IgG, A74 and NEK8 antibodies, and overlaid them to observe associations between the signals. We observe minimal FITC signals for cells stained with isotype IgG and NEK8. However, we observe a much stronger signal from cells treated with anti-TK1 antibody (A74) (Fig. 2). These images show a clear colocalization of TK1 antibody signal and membrane dye, confirming the presence of TK1 on the surface of HT-29, MDA-MB-231, and NCI-H460 cells.
Additionally, plasma membrane proteins were isolated from the HT-29 cell line. The membrane proteins and cell extract were run on a 12% acrylamide SDS gel and then transferred to a nitrocellulose membrane and probed with anti-TK1 antibody (ab91651). We observe that TK1 is found in the membrane protein fraction of these cells further confirming the localization of TK1 on the membrane (Fig. 3). Moreover, the plasma membrane and cytosolic protein fractions show oligomeric forms of TK1 (dimer and tetramer).

Membrane TK1 expression is significantly lower in normal colon than malignant colon clinical samples

To maintain a healthy gastrointestinal health, the lining of the gastrointestinal tract needs to undergo constant cell proliferation [24]. Since TK1’s levels are proliferation-dependent, we wanted to test whether healthy normal colon cells expressed TK1 on their membrane to ascertain the clinical relevance of TK1 as biomarker target in colorectal cancer patients. We stained dissociated healthy and malignant colon tissue with anti-human CD45 antibody and PI to gate out resident lymphocytes and dead cells, and with anti-TK1 ab91651 and CD44 (positive control, adhesion protein) antibodies to test for surface expression of these two proteins. Flow cytometry revealed that the healthy normal colon tissue we tested (n = 7) showed negligible expression of TK1 on the membrane when compared to isotype control (rabbit) (P = 0.8004). Malignant colon tissue (n = 7), however, showed significantly higher expression of TK1 on the membrane compared to normal colon tissue (P = 0.0002) (Fig. 4). CD44 expression levels were not significantly different between healthy normal and malignant colon tissues (P = 0.6634). These
results are crucial in establishing clinical relevance of TK1's localization on the membrane as a unique event in cancer and not a proliferation-dependent event.

TK1 levels are significantly higher in malignant vs. normal healthy tissue

We stained lung, breast, and colon tissue arrays containing healthy normal, healthy normal adjacent, cancer adjacent, and malignant tissue with anti-TK1 antibodies to establish overall expression of TK1. We imaged all tissues with a light microscope at 20×. We conducted the analysis using a gray scale. The lower the gray value, the darker the staining. There was a significant increase in TK1 expression in malignant tissues compared to normal healthy tissues, where TK1 expression was negligible (Figs. 5, 6 and 7). However, there was also a portion of malignant tissues that stained negative for TK1 (Table 1). Lung tissue array shows ~50% of the tissues stain positive for TK1 in both adenocarcinoma and squamous cell carcinoma (Fig. 5a, Table 1). We also observe that normal cancer adjacent tissue is negative for TK1 (Fig. 5a). We can observe a clear differential expression between malignant and normal
**Fig. 7** Immunohistochemistry analysis of TK1 expression in colon cancer tissue. Colon cancer tissue arrays were stained with anti-TK1 antibody (ab91651, GAPDH, or isotype antibody). GAPDH was used as a positive control to account for housekeeping gene expression. The isotype antibody was used to account for background noise and non-specific binding. Tissues were imaged in a light microscope at 20×. Analysis was conducted using a gray scale. The lower the gray value, the darker the staining. a: Quantitative analysis of colorectal cancer IHC staining. The top graph shows that there is a statistically significant expression of TK1 in ~74% of the colon adenocarcinoma tissues. The bottom graph shows the TK1 expression near to GAPDH and isotype controls. Background levels show no statistical difference between malignant and normal healthy tissues. Malignant and normal healthy tissue showed no statistical difference in GAPDH expression, whereas TK1 expression did show a statistically significant difference between TK1+ and TK1− tissues. b: Image showing colorectal adenocarcinoma positive for TK1. The yellow circle encloses an atypical glandular structure positive for TK1. Overall, the tissues shown in (b, c) show what we observed in the tissue. Average gray values represented in (a) that some colorectal adenocarcinoma tissues showed strong TK1 staining and some showed negative TK1 staining. **p < 0.001, n = 9**

**Table 1** TK1 staining in normal and malignant tissue

<table>
<thead>
<tr>
<th>Tissue type</th>
<th>Negative</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung adenocarcinoma</td>
<td>20</td>
<td>23</td>
</tr>
<tr>
<td>Lung squamous cell carcinoma</td>
<td>35</td>
<td>94</td>
</tr>
<tr>
<td>Normal lung</td>
<td>40</td>
<td>0</td>
</tr>
<tr>
<td>Cancer adjacent normal lung</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Breast ductal carcinoma</td>
<td>6</td>
<td>19</td>
</tr>
<tr>
<td>Normal breast</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Colon adenocarcinoma</td>
<td>9</td>
<td>26</td>
</tr>
<tr>
<td>Normal colon</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

healthy tissue in both lung adenocarcinoma and squamous cell carcinoma (Fig. 5b, c). In breast tissue arrays, infiltrating ductal carcinoma positive for TK1 has a significantly lower average gray value than normal breast tissue stained for TK1 (Fig. 6a). TK1 staining localizes to the gland structures (Fig. 6a) in TK1+ tissue. Gland structures or stroma did not stain with TK1 antibody in TK1− tissue (Fig. 6b). These binary results are also observed in colorectal tissue arrays (Fig. 7). However, metastatic adenocarcinoma stained negative for TK1 (n = 9) (Fig. 7a). The darkest TK1 staining in colorectal adenocarcinoma can be found in groups of atypical
Fig. 8 Lung, breast, and colorectal cancer patients show upregulated TK1 gene expression compared to normal patients: a TK1 gene expression in lung adenocarcinoma vs. normal lung patients; b TK1 gene expression in lung squamous cell carcinoma vs. normal lung patients; c TK1 gene expression in breast invasive carcinoma vs. normal breast patients; d TK1 gene expression in colon adenocarcinoma vs. normal colon patients.

Fig. 9 Boxplot of RNA-sequencing data from The Cancer Genome Atlas showing TK1 gene expression in HER2+ breast tumor and triple negative breast cancer (TNBC) tumors. The boxplot shows there is a significant upregulation in TK1 expression in TNBC tumors compared to HER2+ tumors.

glandular structures as seen in Fig. 7b. TK1 – colorectal adenocarcinoma tissue shows no staining in the glandular structures (Fig. 7c).

TK1 gene expression levels are upregulated in malignant lung adenocarcinoma, lung squamous carcinoma, breast invasive carcinoma, and colorectal adenocarcinoma.

We also evaluated TK1 expression using RNA-Sequencing data from The Cancer Genome Atlas (TCGA). Our analysis reveals that TK1 levels are upregulated in both lung adenocarcinoma and lung squamous cell carcinoma, breast invasive carcinoma, and colorectal adenocarcinoma patients (Fig. 8). Lung adenocarcinoma and lung squamous carcinoma seem to be the malignancies with the most differential expression of TK1 between normal and malignant patients, followed by breast invasive
carcinoma, where we could also observe clear differential expression (Fig. 8a–c). There was some overlap in TK1 expression in colorectal adenocarcinoma patients vs. normal patients, probably due to the highly proliferative nature of the colon, as TK1 levels rise during proliferation. However, TK1 levels in colorectal adenocarcinoma patients are still more upregulated than in healthy normal patients (Fig. 8d).

**Triple negative breast cancer patients show higher levels of TK1 than HER2+ cancer patients**

We evaluated TK1 gene expression using RNA-sequencing data from TCGA in breast cancer patient available data for which hormone receptor status was available. Data was analyzed by tumors that were either HER2+ status or HER2- and ER-, and PR-status (triple negative breast cancer, TNBC). We found that TNBC tumor samples expressed higher levels of TK1 than HER2+ tumor samples (Fig. 9). These data seem consistent with membrane expression of TK1 in MDA-MB-231 cells (TNBC), which express higher levels of TK1 on their surface than MCF7 cells (HER2-+) when stained with a911651 antibody (Fig. 1c).

**TK1 expression levels in triple negative breast cancer show positive correlation with stem cell and EMT markers**

RNA expression analysis was performed to evaluate the level of correlation between TK1 and six stemness and EMT genes (CD44, SNAI1, SNAI2, TWIST1, ZEB1, TGFBI). We found that in TNBC TK1 expression levels positively correlated with CD44 (rho = 0.24) and SNAI1 (0.15), and negatively correlated with SNAI2 (rho = -0.16), TGFBI (rho = -0.13), TWIST1 (rho = -0.02), and ZEB1 (rho = -0.25). On the other hand, we found that in HER2+ tumors, TK1 expression levels negatively correlated with all the markers (Fig. 10a–f). These results are interesting, as it reveals that TK1 levels may correlate with stemness and invasion potential in cancer cells.

**Discussion**

The salvage pathway enzyme TK1 plays a crucial role in pyrimidine deoxyribonucleotide synthesis during the cell cycle. Because of this critical association to proliferation and the cell cycle, TK1 has been established as a proliferation biomarker in many cancers, including lung, breast, and colorectal. Serum TK1 is used in many applications for the early detection and diagnosis of cancer, as it is found upregulated in cancer patients. In this study, we present data supporting the expression and localization of TK1 on the cellular membrane of lung, breast, and colon cancer, suggesting TK1 as a surface marker for these malignancies and a different function for TK1 never reported before. The expression of TK1 on the surface of these solid malignancies seems to mirror that of other surface or stem cell markers such as NCAM in lung cancer, CD133 in lung and colon cancer, and CD298 in breast cancer [25–28]. Our results also correlated with some of our previous findings, which show that TK1 localizes on the surface of hematological malignancies such as Burkitt’s lymphoma, acute lymphoblastic leukemia, promyelocytic leukemia, and T-cell lymphoma cells [29]. Interestingly, membrane TK1 seems to be found in monomeric and dimer form similar to membrane TK1 found in hematological malignancies, which suggest kinase enzymatic activity [29]. The actual function of membrane-expressed TK1 is still unknown, however.

Moreover, we show that in colon patient tissue, a highly proliferative tissue, TK1 is expressed on the membrane of only malignant cells and not healthy normal cells. This may mean that TK1’s localization to the cell membrane is an event unique to malignancy. We have seen similar results in the expression of TK1 in hematological malignancies vs. normal proliferating lymphocytes, where TK1 only localized to the membrane of the cancer cells [29].

We also report clinical data from The Cancer Genome Atlas (TCGA), where we explore TK1 gene expression levels, showing that TK1 levels are upregulated in lung, breast, and colorectal cancer patients compared to their healthy normal patients. The bioinformatics analysis reveals that the TK1 expression in some normal healthy tissues overlap with that of some malignant tissues. This is an important observation for clinical relevance, as the gene expression results correlate with our IHC results also shown in this study. IHC reveals that normal healthy tissue is negative for TK1, but some malignant tissue stains positive for TK1 and some malignant stains weakly positive or negative for TK1 in all lung, breast, and color tissue arrays. These results suggest that not all malignancies will have TK1 as a biomarker.

Clinically speaking, a good surface biomarker will be one that overexpresses on the membrane, shows stable expression levels in tumors, and is low or absent

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**Figure 10:** Scatterplots of RNA-sequencing data from The Cancer Genome Atlas comparing TK1 expression to six stemness and EMT markers in HER2+ breast tumors and triple negative (TNBC) tumors. a) Cell stemness marker CD44 is positively correlated to TK1 in TNBC tumors and negatively correlated to TK1 in HER2+ breast tumors. b) EMT marker SNAI1 is positively correlated to TK1 in TNBC tumors and negatively correlated to TK1 in HER2+ breast tumors. c) EMT marker SNAI2 is negatively correlated to TK1 in TNBC tumors and in HER2+ breast tumors. d) EMT marker TWIST1 is negatively correlated to TK1 in TNBC tumors and in HER2+ breast tumors. e) EMT marker ZEB1 is negatively correlated to TK1 in TNBC tumors and in HER2+ breast tumors. f) Stemness and EMT marker TGFBI negatively correlated to TK1 in TNBC tumors and in HER2+ breast tumors.
in normal cells/tissues [30]. For example, the Erb-B2 Receptor Tyrosine Kinase 2 (HER2) is overexpressed in subsets of breast, ovarian, gastric, colorectal, pancreatic and endometrial cancers [31]. In breast and colon cancer, HER2+ tumors are treated by targeting HER2 on the membrane [32, 35]. Current treatments include Herceptin (trastuzumab), Perjeta (pertuzumab), Tykerb (lapatinib), and Kadcyla (T-DM1 or ado-trastuzumab emtansine). Comparing HER2 and TK1, both proteins are heterogeneously expressed in cancer tissues [34, 35]. Moreover, surface expression levels of HER2+: cancer cell lines are comparable to surface expression of TK1. In fact, A549 and NCI-H460 cells lines show higher expression of TK1 on the surface to breast and colon cancer cell lines reported in the literature [36–38]. We also report that TK1 gene expression levels are significantly higher in TNBC vs. HER2+: breast tumors, suggesting TK1 as an alternative biomarker and potential target for TNBC. We also show that in TNBC tumors, TK1 levels positively correlate with two stemness and EMT markers, whereas in HER2+: tumors, TK1 levels show the opposite correlation. This suggest that increased levels of TK1 may aid or have a function in invasion and migration potential in cancer cells.

Further research is needed to help elucidate the mechanism by which TK1 reaches the cell membrane and to understand the function of TK1 on the membrane.

Conclusions
This study shows that TK1 localizes on the cell membrane of NCI-H460, A549, MCF7, MDA-MB-231, SW620, and HT-29 cells lines and on the membrane of colorectal cancer cells and not on the membrane of healthy colorectal cells from patients. This indicates that TK1’s localization on the cell surface may be an event unique to malignancy and independent of proliferation. We also show that TK1 is upregulated in a significant population of cancer patient tissues and that TK1 gene expression is also upregulated in cancer patients compared to normal healthy patients. Given these results, TK1 could potentially be used as an immunotherapeutic target, either using antibodies against TK1, a drug-antibody conjugate, or a chimeric antigen receptor (CAR) T cell targeting TK1.

Additional file

Additional file 1. Flow cytometry histograms of cell lines treated with anti-TK1 antibodies. Cells treated with anti-TK1 antibodies (black line) showed a shift in fluorescence compared to isotype controls (gray area).

Abbreviations

Authors’ contributions
EGW and KLD contributed to conception and design. EGW, RB, RJN, NSW contributed to development of methodology and data acquisition. EGW, AMF’ and MW contributed to the acquisition and analysis of IHC, data, IHC, IS, ITC, and SHP performed bioinformatics analysis. EGW, NSW, RAR, and KLD contributed to the analysis and interpretation of data. EGW, ISW, PMW, and KLD contributed to the writing, reviewing and revision of the manuscript and the supervision and direction of the study. All authors read and approved the final manuscript.

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Acknowledgements
We would like to thank Simmons Center for Cancer Research for financial support, Dr. Joan Arroyo for his assistance in tissue staining and confocal microscopy, Dr. Himeida Chanez (Universidad Nacional Mayor de San Marcos, Lima, Peru), for her pathology expertise and help in IHC analysis, and a team of surgeons at Utah Valley Regional Medical Center for providing normal and malignant colon tissue.

Competing interests
Kim O’Neil has an advisory role and owns stock in Thunder Biotech and holds a patent with Brigham Young University.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication
Not applicable.

Ethics approval and consent to participate
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Healthy and malignant colon tissues were obtained from Utah Valley Regional Medical Center in Provo, UT under informed consent and following a protocol established by Utah Valley Regional Medical Center.

Funding
This study was funded by the BYU Simmons Center for Cancer Research.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Received: 4 October 2017 Accepted: 1 September 2018 Published online: 10 September 2018

References


APPENDIX 3: Non-small-cell lung cancer cell lines A549 and NCI-H460 express hypoxanthine guanine phosphoribosyltransferase on the plasma membrane.


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Non-small-cell lung cancer cell lines A549 and NCI-H460 express hypoxanthine guanine phosphoribosyltransferase on the plasma membrane

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Abstract: In both males and females, lung cancer is one of the most lethal cancers worldwide and accounts for >30% of cancer-related deaths. Despite advances in biomarker analysis and tumor characterization, there remains a need to find suitable biomarker antigen targets for treatment in late-stage lung cancer. Previous research on the salvage pathway enzyme TK1 shows a unique relationship with cancer patients as serum levels are raised according to cancer grade. To expand this analysis, the other salvage pathway enzymes were evaluated for possible upregulation within lung cancer. Adenine phosphoribosyltransferase, deoxycytidine kinase, and hypoxanthine guanine phosphoribosyltransferase (HPRT) were assessed for their presentation on two non-small-cell lung cancer cell lines NCI-H460 and A549. In the present study, we show that deoxycytidine kinase and adenine phosphoribosyltransferase have no significant relationship with the membrane of NCI-H460 cells. However, we found significant localization of HPRT to the membrane of NCI-H460 and A549 cells. When treated with anti-HPRT antibodies, the average fluorescence of the cell population increased by 24.5% and 12.9% in NCI-H460 and A549 cells, respectively, in comparison with controls. To ensure that expression was not attributed to cytoplasmic HPRT, confocal microscopy was performed to visualize HPRT binding on the plasma membrane. After staining NCI-H460 cells treated with both fluorescent antibodies and a membrane-specific dye, we observed direct overlap between HPRT and the membrane of the cancer cells. Additionally, gold-conjugated antibodies were used to label and quantify the amount of HPRT on the cell surface using scanning electron microscopy and energy-dispersive analysis X-ray. Further confirming HPRT presence, the gold weight percentage of the sample increased significantly when NCI-H460 cells were exposed to HPRT antibody (P=0.012) in comparison with isotype controls. Our results show that HPRT is localized on the surface of these non-small-cell lung cancer cell lines.

Keywords: non-small-cell lung cancer, NCI-H460, A549, surface antigen, hypoxanthine guanine phosphoribosyltransferase

Introduction

Lung cancer is one of the leading causes of cancer-related deaths in both males and females worldwide. In 2015, 221,200 individuals in the US were diagnosed with lung cancer, while another 158,040 individuals were killed by the disease. Approximately 85% of lung cancer cases are diagnosed as non-small-cell lung cancer, which encompasses squamous cell carcinoma, adenocarcinoma, and large cell carcinoma. Despite advances in combinatorial therapy using both chemotherapy and radiotherapy, patient...
outcome has not improved at a satisfactory rate. Currently, the 1-year survival rate for lung cancer patients is 44%, and the 5-year survival is only 17%. Low survival is largely attributed to late-stage diagnoses. Approximately 57% of patients are diagnosed at a late stage, leading to reduced treatment options and increased mortality. When diagnosed at a late stage, the survival rates are reduced to 26% and 6% for 1-year and 5-year survival, respectively.

Because early detection of lung cancer is integral to patient survival and outcome, substantial efforts have been made to develop noninvasive tests that identify non-small-cell lung cancers, allowing physicians to diagnose the disease at an earlier stage. Although profiling cancer tissues to find circulating biomarkers can aid in identifying tumor-derived proteins, these methods are extremely invasive. As a result, researchers have developed techniques to identify cancer biomarkers in the sputum of patients. These tests utilize DNA-based assays to detect methylated gene promoter regions that are commonly found in tumors and lead to the loss of tumor suppressor function. RARβ is a chief candidate for this type of analysis because it is involved in cellular signaling during embryonic morphogenesis, cell growth, and differentiation. Studies show that 95% of the cancer tissue has upregulated methylation of the RARβ promoter compared to controls, demonstrating its use as an effective biomarker for lung cancer detection. The p16 tumor suppressor gene has also been used in early detection through evaluation of hypermethylation at its locus. This methylation change is often detected in precursor lesions of tumors and serves as an early event in cancer development and progression. In addition, recent advancements have allowed physicians to detect cancer using breath samples from patients by analyzing volatile organic compounds. By evaluating panels of patients, cancer profiles are established that can later be used as reference to aid physicians in early lung cancer detection. While these methods are promising for the early recognition of lung cancer, they are not suitable for the treatment of patients.

Once lung cancer is detected and diagnosed, a majority of patients are treated with surgery, chemotherapy, radiation therapy, and targeted therapy. For patients suffering from non-small-cell lung cancer, the most common treatment is chemotherapy combined with targeted drugs. Although many patients go into remission after initial treatment, a large percentage eventually relapse, and chemotherapy regimens offer little advantage over other treatments for advanced non-small-cell lung cancer. New therapies utilize cancer antigens to target tumors, which enables physicians to personalize treatments. Treatment efficacy is enhanced with tumor biopsies, which classify the individual mutations in a tumor to help determine the best course of treatment. Because of these biopsies, multiple genes have been assessed and shown as biomarkers for lung cancer due to their upregulation in comparison with normal tissue. CTNNB1, CYP24A1, S100P, and ALDH1A1 all have 5- to 10-fold increases in the level of expression in both adenocarcinoma and squamous cell carcinoma samples in comparison with normal tissue. This information leads to personalized treatment and aids physicians in determining effective drug regimens. For example, ~10% of patients with non-small-cell lung cancer have a mutation in the epidermal growth factor receptor (EGFR) that renders them sensitive to tyrosine kinase inhibitor drugs. Although personalizing treatment based on tumor characteristics can be effective and lead to increased survival rates for small subsets of patients, the current targeted treatments lack specificity and can often lead to unwanted off-target effects.

The purpose of this study was to find a lung cancer biomarker on the surface of non-small-cell lung cancer cells. Due to the proliferative capacity of cancer cells and the need for nuclear mitotic division to support rapid division, the salvage pathway enzymes deoxyuridine monophosphate kinase (DCK), deoxycytidine monophosphatase (HPR), and hypoxanthine phosphoribosyltransferase (HPR) were evaluated for potential expression on non-small-cell lung cancer cell lines. DCK functions by transferring a phosphate group to 2-deoxyribose in the production of DNA bases. APRT catalyzes the transfer of a phosphoribosyl group from 2-phosphoribosylpyrophosphate (PRPP) to adenine, forming adenine monophosphate in the production of adenine bases. HPR is a crucial enzyme for the large-scale production of guanine and inosine bases. HPR functions by transferring phosphoribose from PRPP to hypoxanthine or guanine bases to form inosine monophosphate (IMP) and Guanine monophosphate (GMP), respectively. We designed this study to evaluate the potential of these salvage pathway enzymes as possible biomarker targets for the treatment of non-small-cell lung cancer.

We utilized a variety of methods, including flow cytometry, confocal microscopy, and scanning electron microscopy, to determine whether DCK, APRT, or HPR had any significant relationship with the surface of H460 and A549 cells. In addition, we also evaluated HPR expression within patient tissue to determine whether there was a unique elevation in patients with lung carcinoma. Although we found no significant relationship between DCK and APRT with H460 non-small-cell lung cancer cells, HPR had a significant
colocalization with the membrane of both A549 and H460 cancer cells.

**Materials and methods**

**Chemicals**

Mouse-antihuman HPRT monoclonal antibody clone 1F8D11 (Thermo Fischer Scientific, Waltham, MA, USA) was aliquoted and stored at −20°C. DCK antibody clone 2243C2 (Santa Cruz Biotechnology Inc., Dallas, TX, USA) and APRT antibody lot 10196 (Abnova, Taipei City, Taiwan) were stored at −20°C. Mouse-FITC and rabbit-FITC antibody (Sigma Aldrich, St Louis, MO, USA) were stored at 4°C and were used in minimal light conditions. Bovine serum albumin (BSA, Sigma Aldrich) and sodium thiosulfate (Macron Fine Chemicals, Center Valley, PA, USA) were dissolved in phosphate-buffered saline (PBS) at a 1% concentration and stored at 4°C. A 50% glutaraldehyde stock solution (Electron Microscopy Sciences, Hatfield, PA, USA) was stored at −20°C, and workable solutions were diluted to 0.25% in PBS and stored at 4°C. Glycine (Thermo Fischer Scientific) was diluted to 0.2 mM in PBS and stored at 4°C. NF-κB polyclonal antibody (Bioss Antibodies, Woodburn, MA, USA) was stored at −20°C. CD44 monoclonal antibody (One World Lab, San Diego, CA, USA) was stored at −20°C.

**Cell culture conditions**

The human non-small-cell lung cancer cell lines H460 and A549 were obtained from the American Type Culture Collection (Rockville, MD, USA). H460 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine (all from Hyclone, Logan, UT, USA). A549 cells were grown in DMEM/F12 medium supplemented with 10% fetal bovine serum and 4 mM L-glutamine (all from Hyclone). The cell media were replaced every 48 hours, and cells were trypsinized and reduced once 90% confluence was obtained. Cells were treated with Accutase (Stemcell Technologies, Vancouver, Canada) when utilized for flow cytometry, and when plated for all other applications. All cells were grown at 37°C and 5% CO2. Cell lines were authenticated in May 2016 by the University of Arizona Genetics Core.

**Flow cytometry**

The expressions of HPRT, DCK, and APRT in cultured cells were evaluated by measuring the levels of fluorescence in cells treated with each salvage pathway enzyme antibody. All samples were analyzed on a Blue/Red Attune (Applied Biosystems, Foster City, CA, USA), which recorded 25,000–50,000 events per sample. Briefly, 250,000 cells were incubated with 200 µL of PBS containing 1 µg of antibody to DCK, APRT, and HPRT for 15 minutes on ice. Cells were then labeled with FITC-conjugated secondary (mouse or rabbit) antibody for 15 minutes on ice. Isotypic IgG and unainted cells served as negative controls. The forward/side-scatter plots were used to gate out cell doublets and dead cells. Resulting data were analyzed and plotted using FlowJo Software (FlowJo Enterprise, Ashland, OR, USA). CD44 was utilized as a positive control (Figures S1 and S2), and NF-κB was utilized as a negative control.

**Confocal microscopy**

Fluorescently stained cells were examined under an epifluorescence microscope (Olympus, Tokyo, Japan) equipped with a laser confocal system (Bio-Rad Laboratories, Hercules, CA, USA) using a 15 mW Krypton/argon laser. Image processing was carried out with Laser Sharp Computer Software (Bio-Rad Laboratories). After incubation, cells were plated at a concentration of 4·10⁶ cells/mL on glass coverslips. Following 1 day of growth, cells were incubated in 500 µL of PBS containing 2.5 µg of anti-HPR antibody for 15 minutes on a shaker at 4°C. Cells were then labeled with 2.5 µg of FITC-conjugated secondary antibody for 15 minutes on a shaker at 4°C. Then, cells were incubated at 37°C for 10 minutes with a 1:10,000 dilution of a Cell Mask Deep Red plasma membrane dye (Fisher Scientific, Waltham, MA, USA).

**Scanning electron microscopy**

After incubation, cells were plated at a concentration of 400,000 cells/mL on glass coverslips. After 1 day of growth, cells were placed in 6-well plates and washed three times with PBS followed by 1% BSA in PBS wash, 1% sodium thiostolate in PBS wash, and 1% sodium azide wash for 5 minutes each at 4°C. Cells were then incubated with 5 µg of primary antibody conjugated to Biotin for 15 minutes on a shaker at 4°C. After primary incubation, cells were washed with 1% BSA followed by two washes with PBS. Then, cells were washed with 1% PBS–BSA and 1% sodium thiostolate for 5 minutes on a shaker at 4°C. Cells were then incubated with 2.5 µg of a streptavidin–gold conjugate (Nanoprobes, Yaphank, NY, USA) for 15 minutes on a shaker at 4°C. This was followed by a 1% BSA wash and three PBS washes. Cells were then fixed via incubation in a 0.25% glutaraldehyde solution diluted in PBS for 5 minutes. The reaction was extinguished by adding a 0.2 mM glycine diluted in PBS solution and incubating for 10 minutes until...
the solution turned to a slight yellow color. Cells were then washed three times with dH2O. Solutions A and B from the Nanoprobe gold enhancement kit were incubated together for 5 minutes. Solutions C and D were then added, vortexed, and 40 μL of the gold enhancement solution was added to each sample and incubated for 5 minutes. Each sample treated with gold enhancement is coated in a solution of 2 nm gold particles, but only gold already present via secondary antibody binding will be enhanced to form a definitive particle. Each sample was subsequently put through a series of dehydrations with 70%, 80%, 90%, and 100% Ethanol before analysis. Gold-labeled samples were examined under a Phillips XL-30 ESEM using a 15 kV electron stream under low vacuum conditions at 0.8 Torr. A gaseous secondary electron (GSE) detector was utilized to image the cell morphology and topography. A backscattered electron (BSE) detector was utilized to visualize gold particles on the cell surface. Once images for the cells were obtained, the elemental composition was evaluated using energy-dispersive analysis X-ray (EDAX). Because of gold enhancement, the elemental gold percentage of the background levels of gold was ~8%.

Immunohistochemistry
Lung carcinoma tissue arrays were obtained from Cyvhist (Frederick, MD, USA). These tissues contain various stages of cancer along with corresponding benign and normal tissues from 35 different patients. HIPRT levels were assessed utilizing standard immunohistochemistry staining. Tissues were rehydrated in a series of ethanol washes before treatment with a DIVA decloaker solution to retrieve antigen. Tissues were then incubated with a background washer solution to reduce nonspecific antibody binding. Following blocking, a primary antibody is added to the tissue at a concentration of 1:100 to 1:200 and incubated overnight at 4°C. Following primary staining, tissues were washed and then treated with secondary antibody conjugated to an horseradish peroxidase polymer and incubated for an hour. Following washing, a DAB (3,3' diaminobenzidine) peroxidase solution is incubated with the tissues. Areas of antibody binding will convert the colorless substrate to a brown product, effectively highlighting the target protein. Tissues were treated with hematoxylin to stain the nucleus of the cells. Along with HIPRT treatment, a universal negative antibody was used as a negative control.

Tissues were quantified utilizing ImageJ software. All images were evaluated using the IHC toolbox ImageJ plugin. The DAB option is chosen, and the tissue image is then removed of all other staining except for DAB. Following this analysis, the image is then converted to a gray scale and a threshold is applied in order to eliminate areas of white inherent in the tissue. Once the threshold is applied, the average gray value of the tissue is collected. The same threshold is applied to all tissue samples in order to ensure consistency.

Statistical analysis
Analysis of variance (ANOVA) statistical analysis with the multiple comparison method was used to determine the differential surface expression of various treatments for flow cytometry data on both A549 and H460 cells. In addition, two-way ANOVA tests were performed to compare the mean values of HIPRT expression between A549 and H460 cells. EDAX data were analyzed using ANOVA with the multiple comparison method in addition to unpaired t-tests to determine significance between samples. All statistical analyses were performed using GraphPad Prism 7 software. Differences were considered significant when the P-value was <0.05.

Results
DCX and APRT are not found on the surface of non-small-cell lung cancer H460 cells
Flow cytometry utilizing FITC fluorescent antibodies was used to quantify the DCX and APRT surface antigens. Figure 1A and B shows the relative binding of DCX and APRT protein on the surface of H460 cells, while Figure 1C shows the binding of HIPRT. In the presence of anti-DCX and anti-APRT antibody, there was no significant increase in the fluorescent intensity of treated samples and no resulting shift in the cell population. Further statistical analysis revealed that DCX and APRT were not significantly different than the secondary IgG antibody controls. These data show no relevant binding of specific antibodies to the cell surface and suggest that the therapeutic potential of DCX and APRT is minimal for non-small-cell lung cancers.

Flow cytometry shows significant HIPRT expression on the surface of A549 and H460 cells
When treated with anti-HIPRT fluorescent antibodies, both A549 and H460 cancer cells had an increase in the fluorescent population (Figures 1C and 2). A 28% shift in the population is observed in H460 cells (Figure 1C), while a
12% shift is observed in A549 cells (Figure 3). Statistical analysis comparing anti-HPRT-treated cells with isotype IgG controls showed a statistically significant difference in H460 and A549 cells (Figures 1C and 2C). Thus, these data show a significant association between HPRT and the surface of non-small-cell lung cancer cells. This analysis also revealed a significantly higher HPRT surface expression in H460 cells when compared to A549 (Figure 3).
Confocal microscopy confirms that HPRT is bound to the surface of the cell

In order to confirm that HPRT was not bound to cytoplasmic protein, the surface expression of HPRT was further evaluated with confocal microscopy (Figure 4). Images obtained from cells treated with membrane dye and FITC antibody stain were overlapped to show colocalization of treated antigen on the plasma membrane of the cancer cell. When cells are treated with anti-HPRT antibody, a yellow pigment appears in the merged image, which indicates a direct relationship between the plasma membrane dye and the FITC dye. No other treatment experienced this same overlapped pigmentation, which confirms the relationship between HPRT and the plasma membrane of H460 cells.
Figure 4 Plasma membrane colocalization with I-PRMT in H460 cells

Notes: H460 cells were dyed with both a FITC dye and a rhodamine read membrane dye to label antibody treatments and the plasma membranes, respectively. Utilizing unstained cells, I IgG-immune cells, and NF-kB-immune cells as controls, plasma membrane associations were evaluated to determine whether any of the treatments significantly bound to the membrane of H460 cells. (A) Each sample was analyzed and imaged by a 488 nm laser to illuminate FITC-positive cells. These images show the binding of the respective antigen treatment. (B) Samples were also imaged at a 594 nm laser to show rhodamine-positive cells. This dye binds to the plasma membranes of all cells. (C) The two images obtained from columns A and B were merged to show associations between treated antibodies and the plasma membrane of cells. These results show a clear overlap between cells treated with anti-HPRT antibody and those treated with the membrane dye. This demonstrates a clear association between I-PRMT and the plasma membrane of H460 cells.

Abbreviation: I-PRMT, hypoxanthine guanine phosphoribosyltransferase.

HPRT antigen is scattered randomly across the surface of H460 cells

The location of the HPRT protein on the surface of H460 cells was also analyzed with scanning electron microscopy (Figure 5). The gold elemental peak along with the elemental composition of each sample reveals the changes in the surface gold percentages when cells are exposed to primary antibodies. Images obtained from this analysis show HPRT on the cell surface, but there is no apparent clustering of the antigen as gold particles are scattered across the cell randomly. EDAX analysis showed that cells treated with anti-HPRT antibody had an increase in the average gold
Figure 5: Scanning electron microscopy images and resulting EDAX in H460 cells.

Notes: Cells were labeled with gold toward their respective antibody treatment. (A) Images were obtained using a BSE. This detector is specialized to image heavy metals within samples and highlights enhanced gold within the sample. Any distinguishable large particles of gold represent a bound antibody enhanced with gold. (B) Images were also obtained with a GSE, which showed cell morphology to ensure correct cell structure and integrity. (C) EDAX analysis of each sample showed the gold elemental peaks for all the elements present within the sample. Silicon is the highest represented element because cells were mounted on silicon cover slips for analysis. The gold elemental peaks are indicated with a gold error. Images obtained from this analysis show the exact location of the HPRT bound to the surface of the cell and show no clear pattern indicating a random distribution of the antigen across the surface of the cell.

Abbreviations: BSE, back scatter electron; EDAX, energy-dispersive analysis X-ray; GSE, gaseous side electron; HPRT, hypoxanthine guanine phosphoribosyltransferase.

weight percentage of 10.39% in comparison with only 8.75% for IgG controls. With a P-value of 0.012 (Figure 6), these data indicate a statistically significant presence of HPRT on the surface of H460 cells while also demonstrating that the antigen shows no patterns of expression.

**HPRT expression in H460 cells is higher than expression within A549 cells**

While HPRT is present on both H460 and A549 cells, there is a statistically significant difference between the amount of the protein expressed between the two cell lines (Figure 3).
H460 cells have ~50% more protein on the surface when compared to A549 cells. This altered expression may directly correspond to tumor proliferation as H460 cells grow at a much faster rate, approximately double that of A549 cells. These results suggest that HPRT surface expression may be more prevalent in rapidly proliferating cells as the need for protein is increased.

**Discussion**

HPRT is a salvage pathway enzyme involved in the production of both guanine and inosine bases. The enzyme functions by transferring phosphoribose from PRPP to hypoxanthine or
granine bases to form IMP and GMP, respectively.28 Because of the proliferative capabilities of cancer cells and the large demand for nucleotide production, an upregulated expression at the HPRT locus is hypothesized to be present in these environments.29

We have found that there is significant HPRT colocalization with the plasma membrane in H460 and A549 cancer cells. This same expression is not observed for the salvage pathway enzymes DCK and APRT, indicating that HPRT may possess a role in cancer that is not shared by other salvage pathway enzymes and could be a useful biomarker target for non-small-cell lung cancer.

The reason for the surface expression of HPRT is currently unknown, and the purpose of its external presentation in lung cancer cells can only be speculated.30 We hypothesize that this unique surface expression may point to a secondary function of HPRT that goes beyond its primary role as a purine synthesis enzyme. HPRT is already known to have a secondary regulatory role in neural development and purine synthesis as patients who have a deficiency of the enzyme develop a disease known as Lesch–Nyhan syndrome. This disease is characterized by severe neurological illness, hyperuricemia, and purine overproduction. Purine overproduction is directly related to the loss of HPRT function and demonstrates the enzyme’s necessary responsibility in cells to regulate and control certain pathways. The regulatory role of HPRT may be important for its unique role within cancer. Loss of strict HPRT regulation may enhance cellular proliferation and may contribute to tumor development as cells no longer have regulation of processes normally controlled or limited by HPRT. Further defining these secondary functions may provide additional information about the unique cellular interactions present in the tumor microenvironment.27,28

Although there is significant HPRT expression on A549 and H460 cells, the relative protein level is not equal between the cell lines. The differential expression of HPRT between these two cancer cell lines may be attributed to the growing capacity of the cells, as H460 cells grow at a rate that is nearly double the rate of A549 cells. In addition, H460 cells are known to be highly aggressive due to their increased vascularity and ability to metastasize.29,31 It is likely that the surface expression of HPRT may correspond with proliferation and tumor aggressiveness. This is further explored as stage III tissue stained with HPRT appears to be more prevalent in patient tissue.

We have shown this phenomenon in vitro, but further research into the in vivo expression is required to confirm whether HPRT could be utilized as a biomarker within patients, although we report a significant increase in HPRT within some patients. If found expressed in vivo, HPRT could be utilized in therapies to effectively treat non-small-cell lung cancer.

**Conclusion**

HPRT is expressed on the surface of NCI-H460 and A549 non-small-cell lung cancer cells and may be used as a biomarker target.

**Acknowledgments**

We thank the Simmons Center for Cancer Research for funding this work. We also thank Connor Peck for his editorial comments on the manuscript.
Disclosure

The authors report no conflicts of interest in this work.

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Supplementary materials

Figure S1. Salvage pathway expression on the surface of H460 cells.
Notes: CD44 is displayed in these diagrams in order to show a protein that is expressed on >99% of the cell population. (A) APRT surface expression is evaluated against a CD44 (blue) positive control and an NF-kB (aqu) negative control. (B) DCK (purple) surface expression in comparison with a CD44 (blue) positive control. (C) Anti-HPRT treated cells (pink) shift in the population in relation to CD44 (blue).
Abbreviations: APRT, adenosine phosphoribosyltransferase; DCK, deoxycytidine kinase; HPRT, hypoxanthine guanine phosphoribosyltransferase.

Figure S2. HPRT surface expression on A549 non-small-cell lung cancer cells.
Notes: When cells were exposed to anti-CD44 antibody (blue), >98% of the population was positive for surface expression. HPRT binding in A549 cells is compared to CD44 surface expression along with all controls.
Abbreviations: HPRT, hypoxanthine guanine phosphoribosyltransferase.

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APPENDIX 4: Development of a TK1 specific chimeric antigen receptor T-cell for the treatment of non-small-cell lung cancer


The following appendix was a published abstract in Cancer Research. This data was also presented at the American Association for Cancer Research Annual Meeting in 2017. I hereby confirm that the use of this abstract is compliant with all publishing agreements.
Abstract

Our current research explores the development and tumoricidal activity of chimeric antigen receptor T-cells targeting a new immunotherapeutic target, thymidine kinase 1 (TK1), against non-small lung cancer (NSCLC), both in vitro and in vivo. There has been recent success utilizing CAR T-cell therapy in clinical trials, but it has been mainly focused on the treatment of hematological malignancies targeting CD19. Lung cancer is the most common cause of cancer mortality globally and is responsible for more than one million of deaths every year. Moreover, NSCLC comprises approximately 85% of all lung cancers. Clinical trials against NSCLC using engineered T-cells targeting NY-ESO-1, VEGFR2, MAGE-A3, and mesothelin are currently ongoing. In spite of efforts to find new molecular targets, CAR T-cell therapy still faces several challenges in the treatment of solid malignancies due to the lack of specific molecular targets. We have previously reported the up-regulation of TK1 in multiple malignant tissues including lung cancer tissues and the presence of TK1 on the cell surface of different NSCLC cell lines, such as H460 and A549. Flow cytometry, scanning electron microscopy and confocal microscopy showed evidence of TK1 on the surface of these cancer cells lines. We have built third generation TK1-CARs with lentiviral and retroviral vectors. The constructions include a single chain variable fragment for TK1, a CD28 and 4-1BB moieties connected with a CD3ζ signaling domain. Confirmation of the CAR expression on transduced human T cells was performed by flow cytometry and confocal microscopy, and approximately 60% of the transduced T-cells expressed TK1-CARs. Upon co-culturing TK1-CAR T-cells there was a significant increase in T-cell activity and cancer cell lysis elevated as high as 48% in comparison to negative controls. Cytokine profiles revealed a significant increase of the levels of IL-2 and IFN-γ after 24 hr. of co-culturing, indicating T-cell activation. TK1-CAR T-cells, untransduced
T-cells, and transduced T-cells with empty vectors were co-cultured with H460 cells and time-lapse videos were recorded, every 5 minutes between 12 and 24 hr. post transduction. Clustering of TK1-CAR T-cells around lung cancer cells and induction of cell death after T-cell synapsis with target cells was observed. Preliminary in vitro data has shown that TK1-CAR T-cells induce specific cell lysis in NSCLC cells. In vivo experiments using xenografts models in SNG mice will be performed. Statistical differences between survival curves of mice treated with TK1 CAR T-cells, untransduced T-cells and transduced T-cells with empty vectors are expected.
APPENDIX 5: Macrophage Toll-like receptor-chimeric antigen receptors (MOTO-CARs) as a novel adoptive cell therapy for the treatment of solid malignancies


The following appendix is a published abstract in Cancer Research. This data was also presented at the American Association for Cancer Research Annual Meeting in 2018. I hereby confirm that the use of this abstract is compliant with all publishing agreements.
Abstract

Recent clinical trials using chimeric antigen receptors (CAR) T cells have demonstrated tremendous success in eradicating hematologic malignancies. Notwithstanding the excitement generated by CAR T cell therapy, its clinical efficacy has not been effectively translated to the context of solid tumors; the physical barriers of solid malignancies and the immunosuppressive conditions at the tumor site hinder the efficacy of CAR T cells. Macrophages have the ability to infiltrate almost every tissue and frequently are recruited into tumors. Therefore, macrophages are an attractive vehicle for CAR therapy and could help solve current challenges that CAR T cells face in the treatment of solid tumors. MOTO-CAR cells are monocyte-derived human macrophages that are genetically modified by a lentiviral or adenoviral approach to express a synthetic tumor-targeting receptor and to secrete cytokines, ligands or chemokine receptors. MOTO-CAR receptors are composed of a single-chain variable fragment (ScFv) that binds to a specific tumor target, a hinge to link it to a transmembrane domain, and an engineered Toll/Interleukin-1 receptor (TIR) signaling domain. When the ScFv binds to the tumor cell via its tumor target, an activation signal is transmitted. Myd88 dependent and independent signaling cascades are elicited, activating the macrophage and polarizing it towards a proinflammatory phenotype to eliminate cancer cells in a selective way. We previously reported the expression of Thymidine Kinase 1 (TK1) on the cell membrane of the non-small cell lung carcinoma NCI-H460 and A549 cell lines. The in vitro function of TK1 MOTO-CAR cells was evaluated against these cancer cell lines, using GFP-based phagocytosis and killing assays. Additionally, cell migration and interaction was recorded using time-lapse video with a confocal microscope. Upon co-culturing, with its target TK1 specific MOTO-CARs showed a nearly 4-fold increase in killing activity when compared with the controls (p<0.01). MOTO-CAR cells were produced
through a lentiviral approach with around 30% of the cells expressing MOTO-CARs and with an
adenoviral approach using the Ad5f35 vector with an efficiency of 70-80% of cells being
transduced. Furthermore, after transduction MOTO-CAR cells showed a consistent M1
phenotype expressing high levels of CD14, CD80, CD206 and low levels of CD163. Time-lapse
videos showed migration and clustering of MOTO-CAR cells around H460 GFP + cells.
Moreover, cell death was observed upon contact of MOTO-CAR cells with target cells as well as
phagocytic activity. In vivo testing using an orthotopic NOD scid gamma mice model is in
progress. Our preclinical data show evidence that human macrophages are a suitable vehicle for
CAR therapy and have the potential to successfully extrapolate the clinical efficacy of CAR
therapy to the context of solid tumors.
First author publications


Publications as coauthor


Book chapters


**PRESENTATIONS**

Oral presentations

Oral talk given at the Biomedical West Regional Conference on January 19, 2017 entitled, “Chimeric Antigen Receptor (CARs) for Thymidine Kinase 1 (TK1): A novel immunotherapy approach to fight cancer.”
Poster presentations


Biosciences, 7th Annual Cancer and Immunotherapy symposium, Georgia, Atlanta, USA. Winner of the poster session.


expression of HPRT on the surface of colorectal cancer cell lines HT20 and SW620. American Association for Cancer Research annual meeting 2017, Washington D.C., USA.


**AWARDS AND SCHOLARSHIPS**

- 2021 Distinguished Graduate Student Award

*Department of Microbiology and Molecular Biology. April 2021*

- Summer fellowship

Simmons Center for Cancer Research. Apr 2020-Aug 2020

*Winner of the poster presentation session*

Intermountain Biological Engineering Conference 2019. March 2019

*Award for outstanding student presentation*
Microbiology and molecular biology graduate retreat. Aug 2020

*Winner of the poster presentation session*

ACEA Biosciences, a part of Agilent’s, 7th Annual Cancer and Immunotherapy symposium. Apr 2019

*Jau-Fei Chen scholarship for immunology related research*

Microbiology and Molecular Biology Department. Sep 2018-Apr 2019

*Year-round fellowship*

Simmons Center for Cancer Research. Sep 2017-Aug 2018

*Summer fellowship*

Simmons Center for Cancer Research. Apr 2017-Aug 2017

*Winner of the poster presentation session*

American Society of Microbiologists (ASM). March 2017

*Scholarship for the support of doctoral degree studies abroad*

National Council of Science and Technology of Mexico. Sep 2015-Aug 2020

TOTAL FUNDING ATTRACTED

~ $82,500.00
MEMBERSHIPS

American Association for Cancer Research (AACR) Dec 2016-Present

Associate member

CONFERENCES assisted

- American Association for Cancer Research Annual Meeting, Virtual meeting, 2021
- American Association for Cancer Research Annual Meeting, Virtual meeting, 2020
- American Association for Cancer Research Annual Meeting, Atlanta, Georgia, 2019
- ACEA Biosciences, 7th Annual Cancer and Immunotherapy symposium, Atlanta, Georgia, 2019
- Intermountain Biological Engineering Conference Salt Lake City, 2019
- American Association for Cancer Research Annual Meeting Chicago, Illinois, 2018
- American Society of Microbiology intermountain branch regional meeting, Provo, Utah 2018
- American Association for Cancer Research Annual Meeting, Washington D.C., 2017
- American Society of Microbiology intermountain branch regional meeting 2017
- 56th Midwinter Conference of Immunologist, Monterrey, California, 2017
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