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B7-H4 as a Target for Breast Cancer Immunotherapy

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**14. ABSTRACT**

B7-H4 is a recently discovered B7-family molecule that has been shown to inhibit T cell proliferation and secretion of IL-2. Therefore, it has been classified as an immunosuppressive protein. Protein expression has been limited to subsets of activated T cells and is inducible in dendritic cells and macrophages. In contrast, protein expression is abundant on tissues from several malignancies, most notably breast adenocarcinoma. We proposed to generate antagonistic humanized anti-B7-H4 antibodies for the reversal of immune escape generated in breast cancer. Here we report the generation of 84 mouse monoclonal antibodies for the detection of B7-H4 by ELISA, 25 for the detection of cell surface B7-H4 by flow cytometry, and 5 suitable for immunohistochemistry of paraffin embedded tissue. Five antibodies have been subcloned and purified. While the antibodies have no direct cytotoxic effect, 2 of the clones block B7-H4 binding to T-cells. Candidate antibodies will be subsequently humanized using genetic engineering techniques. Here, we also report several novel findings not yet reported in published literature and not anticipated in our grant proposal.

**15. SUBJECT TERMS**
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## Introduction

In order for tumors to survive, the cancer must avoid elimination by the host's immune system either by avoiding detection or by inducing suppressive mechanisms. Inhibition of the immune response in the tumor microenvironment can be accomplished by the secretion of immunosuppressive cytokines, like TGF-β, the recruitment of suppressor cells such as T regulatory cells and myeloid derived suppressor cells (MDSC), or the expression of immunomodulatory molecules at the cell surface like CTLA-4, PD-1, and B7-H4. B7-H4, the most recently discovered of the B7-like molecules, is not as well characterized as other cell surface suppressor molecules. Its mRNA expression is ubiquitous but its protein expression has been limited to subsets of antigen presenting cells [1]. Many human cancers also express B7-H4 such as renal cell carcinoma [2], melanoma [3], prostate cancer [4], ovarian cancer [5], and breast cancer [6, 7]. Recent characterization of the molecule has shown that B7-H4 can inhibit T cell proliferation and IL-2 secretion [8], and thus, it has been hypothesized to protect tumor cells from T cell-mediated destruction. We proposed the development of a humanized antagonistic antibody to B7-H4 that could be used to treat B7-H4 expressing tumors such as breast cancer as a new immunotherapeutic approach for cancer patients.

## Body

***Specific Aim 1***. Generation of an antagonistic humanized B7-H4 antibody.

Aside from characterization of B7-H4 expression in tumor tissues, almost all work with B7-H4 has been done in murine models. For the purpose of our studies with human tissues, we developed a human B7-H4-Fc fusion protein that consists of the extracellular domains of human B7-H4 fused to the Fc region of human IgG1 **(Fig. 1)**. In concurrence with published data in mice [8], human B7-H4-Fc was able to bind to human T cells activated with anti-CD3/CD28 beads, as well as on unstimulated monocytes (CD11c+CD11b+), erythrocytes (glycophorin A+), and B-cells (CD19+) **(Fig. 2)**, as detected by flow cytometry. Interestingly, *in vitro* activation of T cells, but not of the other cell populations, was required for expression of the B7-H4 receptor.

Using APC-labeled or DyLight 488-labeled B7-H4-Fc, we have discovered B7-H4 receptors on several human lymphoma and leukemia cell lines. CEM, Karpas 299, and TLBR-1, cell lines derived from acute T-cell lymphoblastic leukemia, large cell anaplastic lymphoma, and breast implant-associated anaplastic large cell lymphoma, respectively, bound B7-H4-Fc, but Jurkat, an acute T-cell leukemia-derived cell line, did not bind B7-H4-Fc **(Fig. 3)**. Following this finding, we have screened 10 other hematopoietic cell lines, comprised of NK-, B-, T-, and megakaryocyte-like cells, most of which bound B7-H4Fc (data not shown). This novel finding was unanticipated and exciting, as it open new doors to the study and treatment of hematopoietic malignancies. If engagement of the B7-H4 receptor with B7-H4 is able to inhibit lymphocytic proliferation, B7-H4-Fc may be able to be administered as treatment against malignant lymphocytes. Furthermore, B7-H4-Fc can also be used as a targeting molecule to deliver cytotoxic drugs to these malignant cells. This work on T-cell lymphomas was motivated by our need to find B7-H4 receptor expressing cell lines that could be used in subsequent experiments.

In anticipation of developing antagonistic antibodies to B7-H4, we developed an assay to test the suppressive effects of B7-H4 on T cell proliferation isolated from peripheral blood of health donors. Surprisingly, however, our data could not reproduce the *in vitro* suppression achieved with murine B7-H4 on murine T cells [8]. Several different methods of presenting B7-H4-Fc to T cells were tested, including immobilized B7-H4-Fc attached to the surface of 96-well plates, Dynabeads (Invitrogen), or copolymer beads. We also tried different ways of stimulating T cells, including anti-CD3 (clone HIT3a) and anti-CD3/CD28 Dynabeads, both of which stimulated T cell proliferation. In addition to different concentrations of B7-H4, different time points of collection and different readouts, including proliferation measured by CFSE and the presence of activation markers like CD25 and CD69, were tested. In each experiment, T cell proliferation and activation did not differ significantly between plated B7-H4-treated and plated IgG1 or FGFR1Fc-treated cells **(Fig. 4A)**. However, while published reports show inhibition with plated B7-H4Fc and not soluble B7-H4Fc, in our experiments, high amounts of soluble B7-H4Fc (50 μg/mL) was able to moderately inhibit proliferation due

to anti-CD3 antibodies but not to anti-CD3/CD28 Dynabeads. Of our 5 purified B7-H4 antibodies, 2 are able to reverse this suppression **(Fig. 4B, C)**.

Several possibilities may explain the discordance between our findings with human B7-H4 and published work using murine B7-H4. It may be that B7-H4 is an evolutionarily conserved molecule but whose function and signaling may be redundant or easily surmounted in humans versus mice. To elucidate the role of B7-H4 in the human immune system, we shifted our studies to the identification of the B7-H4 receptor on activated T cells. Identification of the receptor may provide important information about the function of B7-H4 in human tumors. It is also possible that antagonistic antibodies may bind either B7-H4 alone, its receptor, or the ligand/receptor complex, and therefore, information about the molecular identity of the receptor is vital for these studies. We have already begun performing pull-down assays using B7-H4-Fc as bait. Initial studies performed by our laboratory have shown that the receptor is protease insensitive, as suggested by digestion and flow cytometry experiments **(Fig. 5).** Therefore, it is possible that B7-H4 binds a non-protein ligand; however, several other experiments seem to contradict this finding. While traditional Western blots was unable to detect a receptor using B7-H4Fc as a probe, a single protein band was detected by a native Western, indicating that a native conformation is required for ligand-receptor engagement **(Fig. 6A)**. Because binding requires native conformation in addition to membrane solubilization, these factors may explain why pull-down assays have proven to be difficult and may require further optimization with non-traditional detergents. Furthermore, we have an estimated protein size for B7-H4R of approximately 70-80 kDa, based on biotin-transfer experiments **(Fig. 6B)**. Based on these protein studies, we can narrow down candidate receptors from the mass spectrometry data.

Even though *in vitro* studies with B7-H4-Fc showed only mild suppression of T cell proliferation, our goal has not changed to produce an antagonist human antibody. The abundance of B7-H4 on the surface of over 95% of breast cancer cases [6], lack of protein expression in normal tissues, and its reverse correlation with prognosis [3,4,5] make B7-H4 an excellent target for antibody-mediated or directed therapy. In addition, intracellular B7-H4 has been shown to have anti-apoptotic effects in biliary epithelial

cells from patients with primary biliary cirrhosis [9]. In biliary epithelial cells acquired from primary biliary cirrhosis, Chen *et al*. [9] showed that silencing of B7-H4 using RNA interference was able to induce apoptosis in those cells without the addition of other cells, like cytotoxic T lymphocytes, or cytotoxic molecules. Furthermore, with our finding of the B7-H4R on other immune cell types, it is conceivable that T cells may not be the primary responder to B7-H4, but are influenced by other B7-H4R+ immune cells in the tumor microenvironment. This would be supported by the fact that other cell types, CD3-CD11c+CD11b+ and subsets of B cells constitutively express the receptor and at higher levels than activated T cells **(Fig. 2)**.

In preparation of producing a human or humanized antibody, studies were undertaken to produce murine hybridomas that may be used to define functional assays. For these studies, monoclonal antibodies against B7-H4 have been generated by immunizing BALB/c and NIH Swiss mice with B7-H4-Fc in Freund's adjuvant and other proprietary immune activators. After 3-5 rounds of immunizations, including a final boost 4 days before sacrificing the mice, splenocytes from immunized mice were fused with mouse myeloma NS-0 cells and culture supernatants were screened for binding to B7-H4-Fc by ELISA. Positive supernatants were then screened against other Fc fusion molecules generated in the laboratory (IL-2-Fc, B7.1-Fc) to identify antibodies that bound to the Fc portion of B7-H4-Fc. A total of 84 antibodies have been generated that recognizes the extracellular domain of B7-H4-Fc by ELISA. Those antibodies were then screened by flow cytometry against the B7-H4 positive human breast adenocarcinoma cell lines SKBR-3 and MDA-MB-468. Twenty-five of the 84 antibodies were found to bind and are therefore suitable for use by flow cytometry **(Fig. 7A)**. By comparison, a commercial anti-B7-H4 antibody (H74, eBiosciences) was not able to bind SKBR-3, possibly due to its lower affinity or to the epitope that it binds. Five of these 25 antibodies have been subcloned and purified for further testing. As previously mentioned, 2 of the antibodies are able to reverse B7-H4's inhibitory effect on T cell proliferation **(Fig. 4B, C)**. Those 2 clones are also able to block B7-H4Fc binding to receptor positive cell lines **(Fig. 7B)**. While further *in vitro* studies are being conducted to test the effects of B7-H4Fc and these antagonistic antibodies on other immune cell types, these two clones are good candidates for further development and humanization.

***Specific Aim 2***. Evaluation of the clinical efficacy of B7-H4 antibodies in 4T1 murine breast cancer model and in human breast tumor-bearing mice.

Cytotoxic effects due to antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytoxocity (CDC), and direct cytoxocity were assessed using CytoTox 96® NonRadioactive Cyotoxicity Assay from Promega. Because the five purified clones are murine IgG1 antibodies, they did not exhibit any ADCC or CDC activity on B7-H4 positive cell lines, as expected (data not shown). However, previously we had shown that our antibody supernatants yielded greater rates of apoptosis than HER-2 antibodies in SK-BR-3 **(Fig. 8)**. Unfortunately, results with purified antibodies could not replicate this result. Repeated experiments using the CytoTox 96® assay as well as an MTT assay confirmed that the anti-B7-H4 antibodies do not inhibit cell proliferation or induce apoptosis. It is likely that previous data shown in Fig. 8 was due to other factors in the media of antibody supernatants, and not due to the antibodies themselves. Because previous studies correlate B7-H4 with metastasis and invasion [4, 10], we are beginning to test the effect of our antibodies on cellular migration. Preliminary data in a wound healing assay showed that one of our clones, #32-14, inhibited cellular migration in the B7-H4+ breast adenocarcinoma cell line, MDA-MB-468 **(Fig. 9).** Migration was not inhibited in SK-BR-3 using the wound healing assay. Results will be repeated in other B7-H4 positive cell lines, as well as confirmed with other types of invasion and migration assays. While the antibodies did not produce a cytotoxic effect, humanization of these candidate antibodies will allow us to study these antibodies for antibody-dependent cell cytotoxicity and further study their therapeutic effects in SKBR-3 xenograft tumor models in mice.

In order to study antibody biodistribution and therapy in murine models, we are currently creating several stable cell lines for B7-H4 expression. We inserted *B7-H4* cDNA into a pIRES-GFP plasmid with a CMV promoter and have sequenced *B7-H4* from transformed bacterial colonies. We are currently stably transfecting human ovarian cell line SK-OV-3, human embryonic kidney cell line 293T, and mouse breast cancer line 4T1. All cell lines are negative for human and murine B7-H4 expression. These stable cell lines will be crucial since we want to analyze the activity of our B7-H4 antibodies in

an immunocompentent host as well as its effect on human tumors in a xenogeneic model. Furthermore, having cell lines that stably overexpress B7-H4 will allow us to investigate how B7-H4 may alter cellular phenotype, especially in the context of survival, proliferation, and metastasis.

***Specific Aim3.*** Analyze human breast cancer biopsies for the expression of B7-H4 and tumor infiltrating lymphocytes.

Antibody clones were screened on paraffin embedded cell pellets by immunohistochemistry **(Fig. 10A-D).** Suitable antibodies were then screened on normal breast tissue and invasive ductal carcinoma, and compared to commercial antibodies from Santa Cruz Biotechnology, Abcam, and Abbiotec. Although all commercial antibodies are commercialized for their use by immunohistochemistry, only Abbiotec showed positive staining on SK-BR-3 and negative staining on SK-OV-3 cell pellets. Compared to the polyclonal antibody from Abbiotec, several of the clones produced in our lab exhibited superior membrane staining on the SK-BR-3 cell pellets. Using these clones, as well as the antibody from Abbiotec for comparison, these antibodies were screened against normal breast tissue, lobular adenocarcinoma, and ductal adenocarcinoma. As has been previously reported [6], B7-H4 exhibited membranous and cytoplasmic staining and is overexpressed in both lobular and ductal cancer of the breast, although more prominent staining is noted in ductal carcinoma **(Fig. 10E-H)**. Apical staining of ductal cells is noted in normal breast tissue **(Fig. 10E)**, confirming reports by Tringler et al. [6], although it had been previously reported by Choi et al. [11] that normal breast tissue did not express B7-H4 protein. Even though staining is positive in normal breast tissue, staining was limited to apical, luminal expression of B7-H4.

Because previous studies already correlate B7-H4 expression with T cell infiltrates as well as pathology in breast cancer [6, 10], we did not continue with Specific Aim 3. Instead, tissue microarrays were acquired of normal tissues, normal adjacent tissues, as well as cancer tissues of different organs to further test whether B7-H4 is a good candidate antigen for immunotherapy. Preliminary results show that B7-H4 membranous expression is generally limited to specific cancers, especially squamous cell, although cytoplasmic staining is noted in renal tubules and membranous staining is noted

in lung epithelium (data not shown). Interestingly, strong membranous staining is observed in the ovarian follicles (data not shown), which has not been reported elsewhere. Staining on more tissue microarrays are being done to shed light on B7-H4's function in normal tissue as well as to anticipate side effects of therapeutic antibodies.

In addition to staining of paraffin embedded tissue, fluorescence microscopy was done to analyze localization of B7-H4 in cell lines. It had been previously reported that B7-H4 can be transported to the nucleus, where it may convey downstream effects on gene transcription [12]. However, the vast majority of cells stained for intracellular B7-H4 stained perinuclear **(Fig. 11B)**. Nuclear staining of B7-H4 was a rare event, occurring in less than 1% of stained cells, and did not increase when adding nuclear transport inhibitor, leptomycin B (data not shown). Interestingly cell surface staining of B7-H4 did not exhibit continuous membranous staining, but exhibited a punctate staining pattern **(Fig. 11A)**. This may indicate that B7-H4 is in a complex at the cell surface. More studies are underway to determine the purpose of this protein in host cell function.

**Key Research Accomplishments**

- Generation of human B7-H4-Fc fusion protein (antigen).
- Discovery of a B7-H4 receptor on several lymphoma and leukemia cell lines, and on primary hematopoietic cells.
- Generation of 84 mouse anti-human B7-H4 monoclonal antibodies for use in the detection of B7-H4 by ELISA.
- Of the 84 monoclonal antibodies suitable for ELISA, 25 mouse anti-human B7-H4 monoclonal antibodies can detect cell surface B7-H4 by flow cytometry.
- Of the 84 monoclonal antibodies suitable for ELISA, 5 are suitable for immunohistochemistry of paraffin embedded tissues, and are superior to 3 popular commercial B7-H4 antibodies.
- Detection of cell surface B7-H4 in organs not reported elsewhere.
- Selection of 2 blocking antibodies to B7-H4 binding T cells.
- Determined approximate size of candidate protein receptor.
- Submitted 4 bands to mass spectrometry for identification of the receptor.

**Publications**

Funding paid for the training of personnel, who presented and published the following:

Peer-Reviewed Scientific Journals:

- Li Z, Jang JK, Lechner MG, Hu P, Khawli L, Scannell CA, Epstein AL. Generation of tumor-targeted antibody-CpG conjugates. *J Immunol Methods*. 2013 Mar 29;389(1-2):45-51. doi: 10.1016/j.jim.2012.12.009
- Jang JK, Khawli L, Park R, Wu B, Li Z, Canter D, Conti P, Epstein AL. Cytoreductive chemotherapy improves the biodistribution of antibodies directed against tumor necrosis in murine solid tumor models. *Mol Cancer Ther.* (Submitted May 14, 2013)

Abstracts:

- Jang JK, Hu P, Li Z, Khawli L, Epstein AL. Antibody targeted CpG for the immunotherapy of solid tumors. In: Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research; 2012 Mar 31-Apr 4; Chicago, IL. *Cancer Res* 2012;72(8 Suppl):Abstract nr 1539. doi:1538-7445.AM2012-1539

- Jang-Wu JK, Hu P, Li Z, Khawli L, Epstein AL. Antibody-directed CPG targets tumor microenvironment and provides active immunotherapy. In: Proceedings of the 27th Annual Scientific Meeting of the Society for Immunotherapy of Cancer; 2012 Oct 26-28; North Bethesda, MD. *J Immunother* 2012; 35, p 35.

Presentations

- Poster presentation. "Antibody targeted CpG for the immunotherapy of solid tumors." AACR 103rd Annual Meeting; Mar 31-Apr 4, 2012; Chicago, IL.
- Poster presentation. "Antibody-directed CPG targets tumor microenvironment and provides active immunotherapy." SITC 27th Annual Meeting; Oct 26-28, 2012; North Bethesda, MD.

**Reportable Outcomes**

- Development of B7-H4 antibodies suitable for immunohistochemistry that are superior to clones available from Abbiotec, Santa Cruz Biotechnology, and Abcam.
- Development of B7-H4 antibodies that block B7-H4 from binding T cells.
- Article published in *Journal of Immunological Methods*.
- Article submitted (in review) to *Molecular Cancer Therapeutics*.

**Other Acheivements**

- Submitted a preliminary application for funding on the humanization of B7-H4 antibodies as well as the study of B7-H4 function in cancer development from CDMRP in June 2013.

**Conclusion**

We have generated a large selection of mouse anti-human B7-H4 monoclonal antibodies that are further being assessed for antagonistic activity, antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity, and direct cytotoxicity. While the antibodies exhibit no ADCC or CDC effect as mouse IgG1 antibodies, humanization of the antibodies may grant these additional benefits. Preliminary results show that several clones are able to block binding of B7-H4 to T cell lines, and are able to increase T cell activation and proliferation in the presence of B7-H4Fc. The

development of a working animal model is underway to test tumor-targeting ability and therapeutic effects of our mouse monoclonal antibodies, prior to their humanization.

However, the suppressive activity of B7-H4 on T cells is not as remarkable in the human setting as it is in murine models. More work needs to be done characterizing the role of B7-H4 in the human setting. For this purpose, we have set out to identify the receptor of B7-H4 on hematopoietic cells, as well as study its role in the host cell. If the receptor for B7-H4 is identified, this would be a major milestone in the field since prior attempts by many groups have failed. It would also open the door to making a human antibody to the B7-H4 receptor which would represent another approach for inhibiting this ligand/receptor interaction on breast cancer cells.

We also report here novel findings not anticipated by our original grant proposal. We have demonstrated the presence and absence of the B7-H4 receptor on several lymphoma and leukemia cell lines, as well as in PBMCs from healthy donors. This may clarify some of the biology behind the different phenotypes and behavior of different hematopoietic malignancies, and also leads the field to study the effect of B7-H4 on other immune cells in addition to T cells. Our work to identify the B7-H4 receptor could shed light on B7-H4's mysterious roles in tumorigenesis and immunology.

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# Supporting Data

## (Figures)

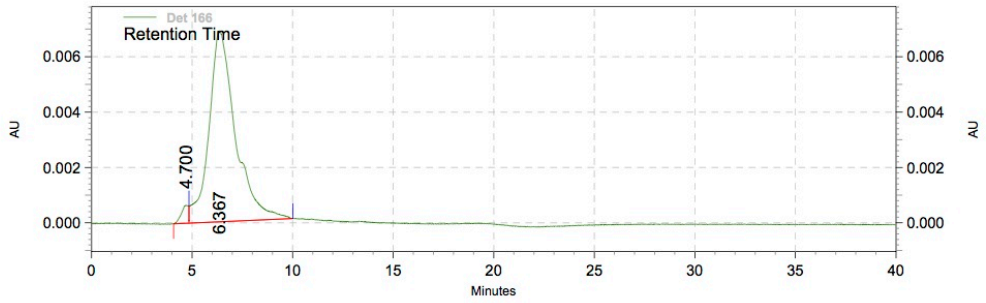
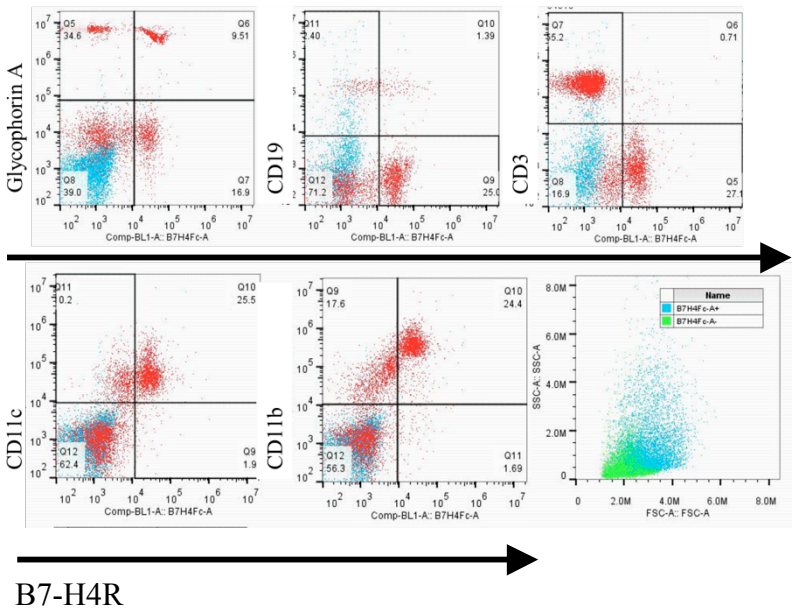



**Figure 1. Generation of human B7-H4-Fc**. cDNA encoding the signal and extracellular domains of human B7H4 was generated by PCR amplification from full-length cDNA purchased from Open Biosystem (Lafayette, CO). Primary PCR of B7H4 was performed with the 5' and 3' primers 5'-TCG ATC AAG CTT GCC GCC ACC ATG GCT TCC CTG GGG CAG ATC-3' AND 5'-TGT GTG AGT TTT GTC AGC CTT TGA CAG CTG-3', respectively. The hinge-CH2-CH3 portion of human IgG1 was PCR amplified with 5' primer 5'-CTA AAC TCA AAG GCT GAC AAA ACT CAC ACA TGC CCA-3' and 3' primer 5'-TGA TTA ATG ATC AAT GAA TTC TCA TTT ACC CGG AGA CAG GGA-3'. The gene encoding huB7H4-Fc was produced by assembling with 5' primer of B7H4 and 3' primer of human, respectively. The B7H4-Fc fusion gene was then digested with *Hind3* and *EcoRI* and inserted into *Hind3* and *EcoRI* sites of pN24 expression vector, resulting in the expression vector pN24/B7H4-Fc. B7H4-Fc fusion protein was expressed in NS0 cell line. Shown is the HPLC profile of B7-H4-Fc.

**A**




**B.**

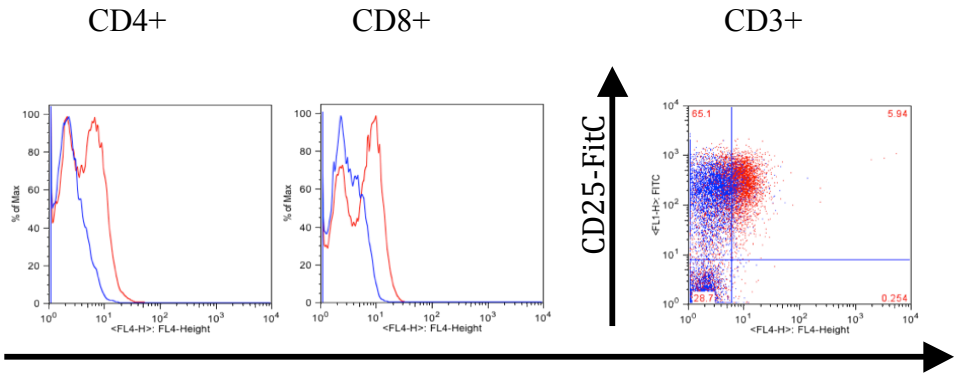



**Figure 2**. **B7-H4 receptor positivity on human lymphoid cells**. **(A)** Unstimulated peripheral blood were isolated from healthy donor peripheral blood and stained with B7-H4-Fc-DL488 or FGFR1-Fc-DL488, and antibodies for immune phenotyping and analyzed by flow cytometry. Unstimulated cells are CD25-, CD80-, and CD69- (data not shown). **(B)** $CD3^{+}$ T cells were isolated from healthy donor peripheral blood using magnetic cell sorting and incubated with anti-CD3/CD28 Dynabeads (Invitrogen) for 3 days. Cells were stained with B7-H4-Fc-APC or IgG1-APC, CD4-PE, CD8-PE, and CD25-FITC and analyzed by flow cytometry. Blue curves and dot plots represent isotype control and red curves and dot plots represent antibodies of interest.

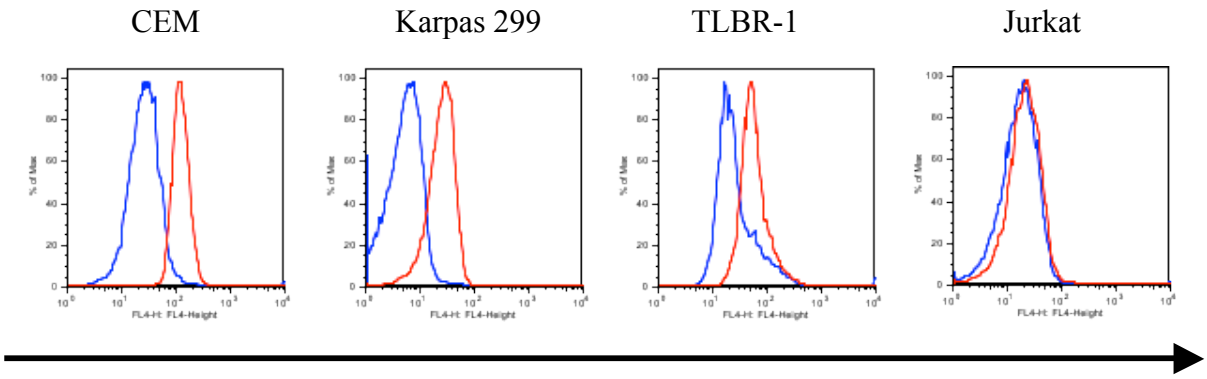
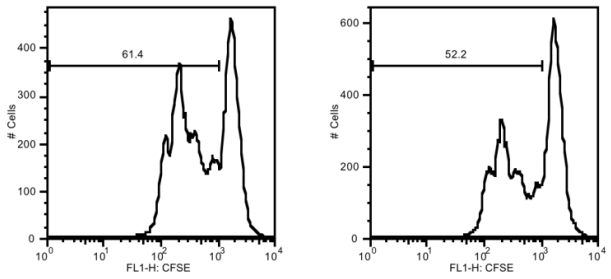


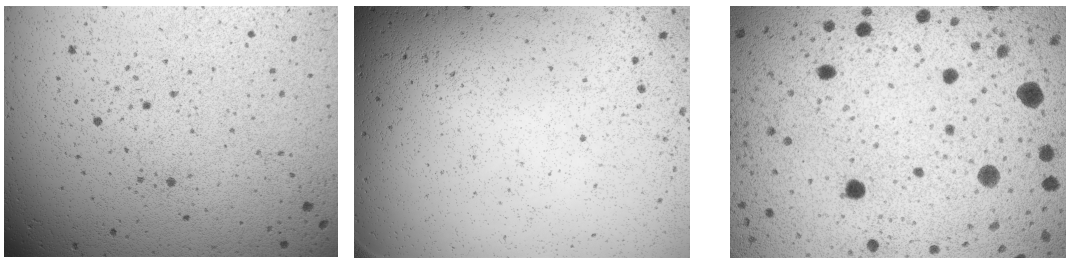

**Figure 3. B7-H4 binding to human T-cell lymphoma cell lines**. Red line represents cells stained with B7-H4-Fc-APC, and blue represents cells stained with IgG1-APC. The above cell lines were analyzed by flow cytometry. Note the positivity in CEM, Karpas 299, and TLBR-1 cell lines.

**A anti-CD3/CD28 Dynabeads, B7-H4Fc/IgG1 plated**



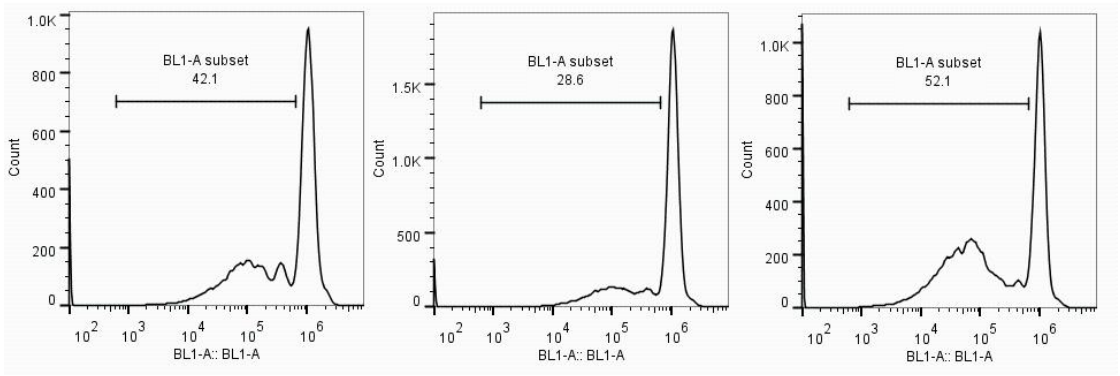

**B7-H4-Fc-treated** **IgG1-treated**

**B**



**FGFR1Fc soluble** **B7-H4Fc soluble** **B7-H4Fc + #35-9 ab soluble**

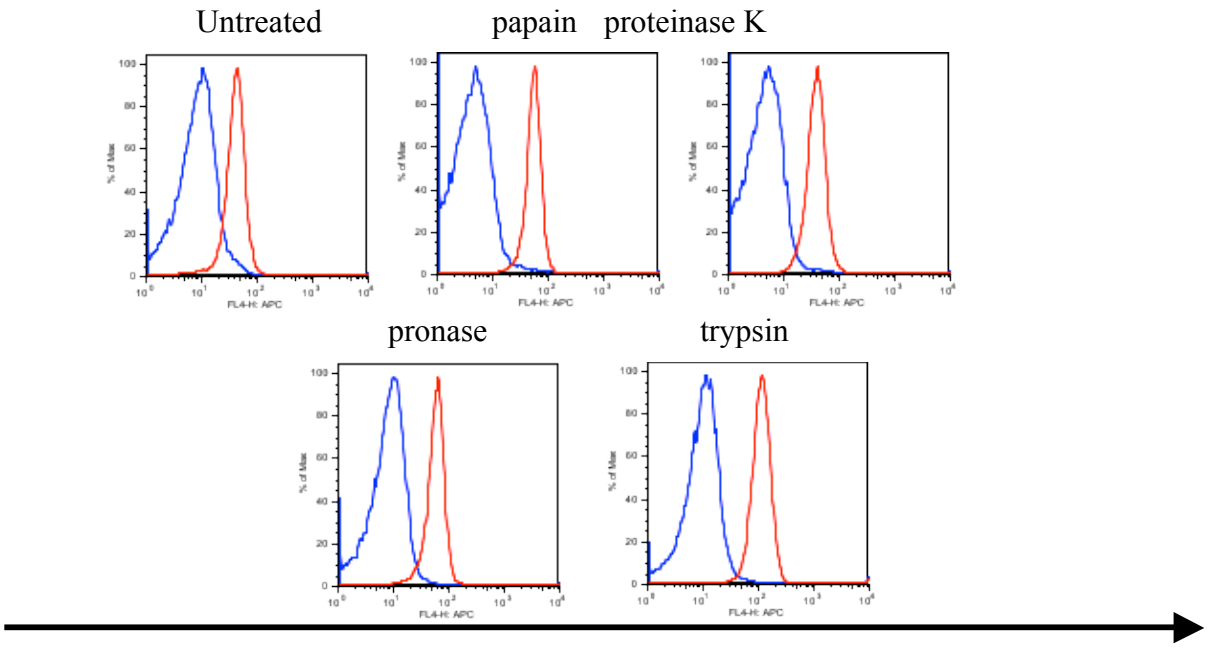
**C anti-CD3 (HIT3a) plated, B7-H4-Fc/FGFR1-Fc soluble**




**FGFR1Fc soluble** **B7-H4Fc soluble** **B7-H4Fc + #35-9 ab soluble**

**Figure 4. Suppression assay data. (A, B, C)** are representatives of several different experiments done trying to demonstrate a suppressive effect of B7-H4-Fc on human T cells. Briefly, in these proliferation experiments, human $CD4^+$ T cells are isolated from healthy donor peripheral blood using magnetic isolation kits from Miltenyi Biotec and stained with CFSE. Cells are treated accordingly and measured for proliferation by flow cytometry. **(A)** Cells were stimulated with anti-CD3/D28 Dynabeads (Invitrogen) and exposed to either B7-H4-Fc or IgG1 plated on 96-well plates for 3 days. **(B,C)** Cells were stimulated with plated anti-CD3 (clone HIT3a) and exposed to 50 µg/mL of soluble B7-H4-Fc or FGFR1Fc soluble on 96-well plates for 3-5 days. **(B)** Images of activated T cell colonies (40x magnification). **(C)** CFSE assay analyzed by flow cytometry.

**A**




**B**

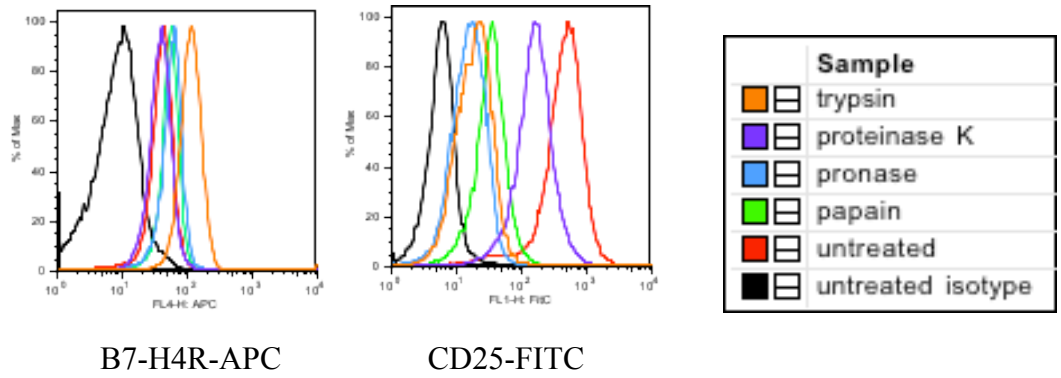
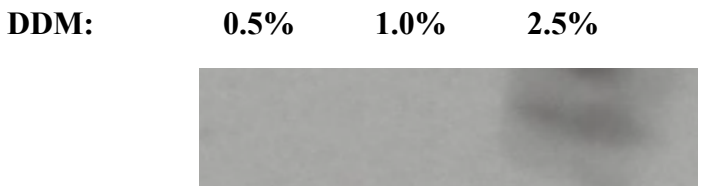



**Figure 5. B7-H4 receptor is protease resistant.** Karpas 299 cells were treated with 0.3 mg/mL papain, 0.025 mg/mL proteinase K, 0.15 mg/mL pronase, or 0.25% trypsin for 45 minutes at 37°C. Cells were assessed for viability using trypan blue under a light microscope, then stained for B7-H4R using B7-H4-Fc and for CD25 as a positive control for protease activity. **(A)** Flow cytometry data for protease-treated cells for B7-H4R. Red line represents cells stained with B7-H4-Fc-APC, and blue represents cells stained with IgG1-APC. **(B)** Overlays of histogram for B7-H4R and CD25 positivity in protease-treated cells. Note that no protease-treated cells are less positive for B7-H4R than untreated cells (in red), but all protease-treated cells show decreased CD25 expression compared to untreated cells (in red). Therefore, B7-H4R expression as detected by flow cytometry is insensitive to the above proteases, or B7-H4R has a rapid turnover rate and its expression is recovered prior to staining.

**A**




**B**

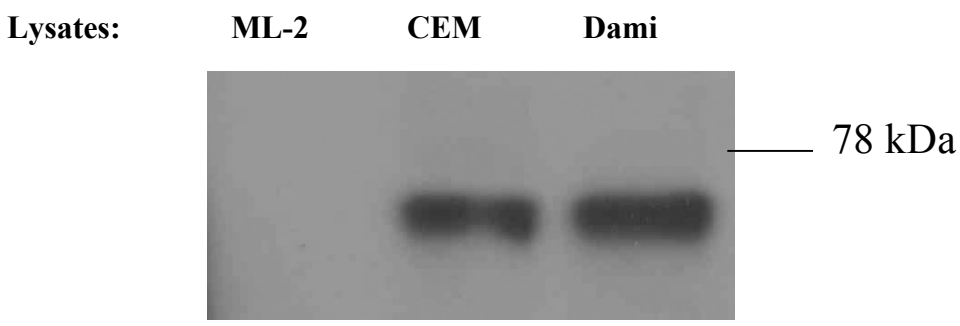
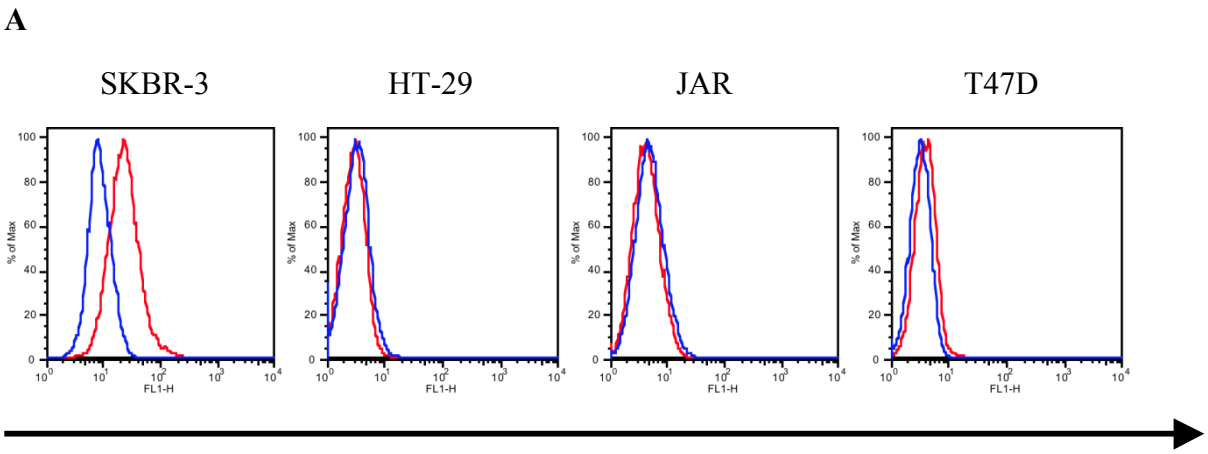



**Figure 6. Western blots on B7-H4R. (A)** CEM lysates were prepared in 0.5, 1.0, or 2.5% DDM (n-dodecyl β-D-maltoside) and separated on a native gel prior to transfer on a PDVF membrane. Membrane was probed with B7-H4Fc followed by anti-human Fc-HRP conjugate. **(B)** Biotin-SSBED-labeled B7-H4Fc was incubated with lysates from ML-2 (B7-H4R-), CEM (B7-H4R+), and Dami (B7-H4+) cell lines. UV light allows transfer of the biotin from the B7-H4Fc molecule to proteins engaging B7-H4Fc. Lysates were reduced prior to gel electrophoresis, which cleaves biotin from B7-H4Fc but leaves biotin attached to the interacting protein. Positive transfer of biotin was detected using streptavidin-HRP and a chemiluminescent substrate.



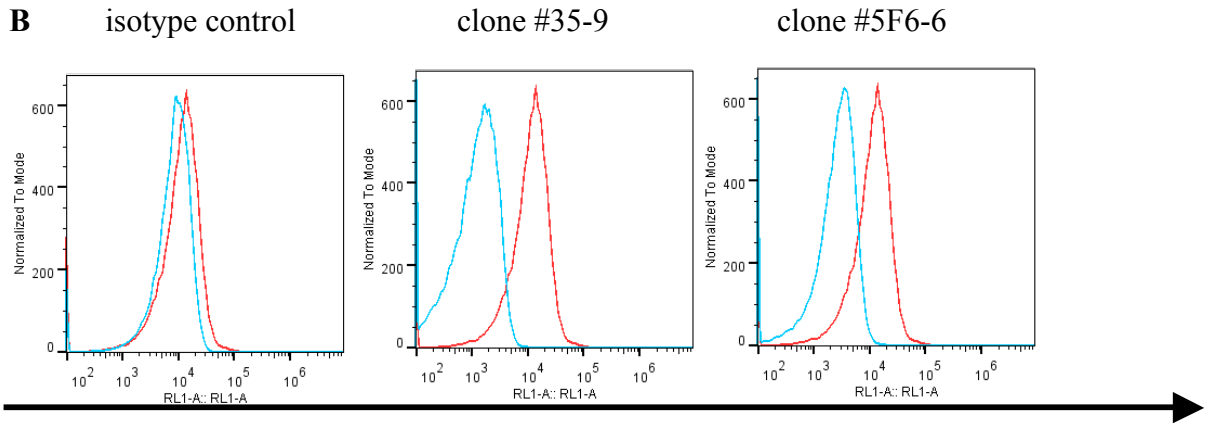




**Figure 7. Representative flow cytometry data for mouse monoclonal anti-human B7-H4. (A)** B7-H4 staining on SKBR-3, HT-29, JAR, and T47D cell lines derived from breast adenocarcinoma, colorectal adenocarcinoma, choriocarcinoma, and breast ductal carcinoma, respectively. Red line represents cells stained for B7-H4, and blue represents cells stained with isotype control. A sheep anti-mouse IgG conjugated to FITC was used as secondary. **(B)** B7-H4 antibodies can block B7-H4Fc from binding putative receptor on cell lines. APC-labeled B7-H4Fc was incubated with B7-H4 antibodies or an isotype control 30 minutes prior to staining cells. Red line represents B7-H4 staining without the

presence of an antibody, and blue line represents staining in the presence of an antagonistic antibody or an isotype control.

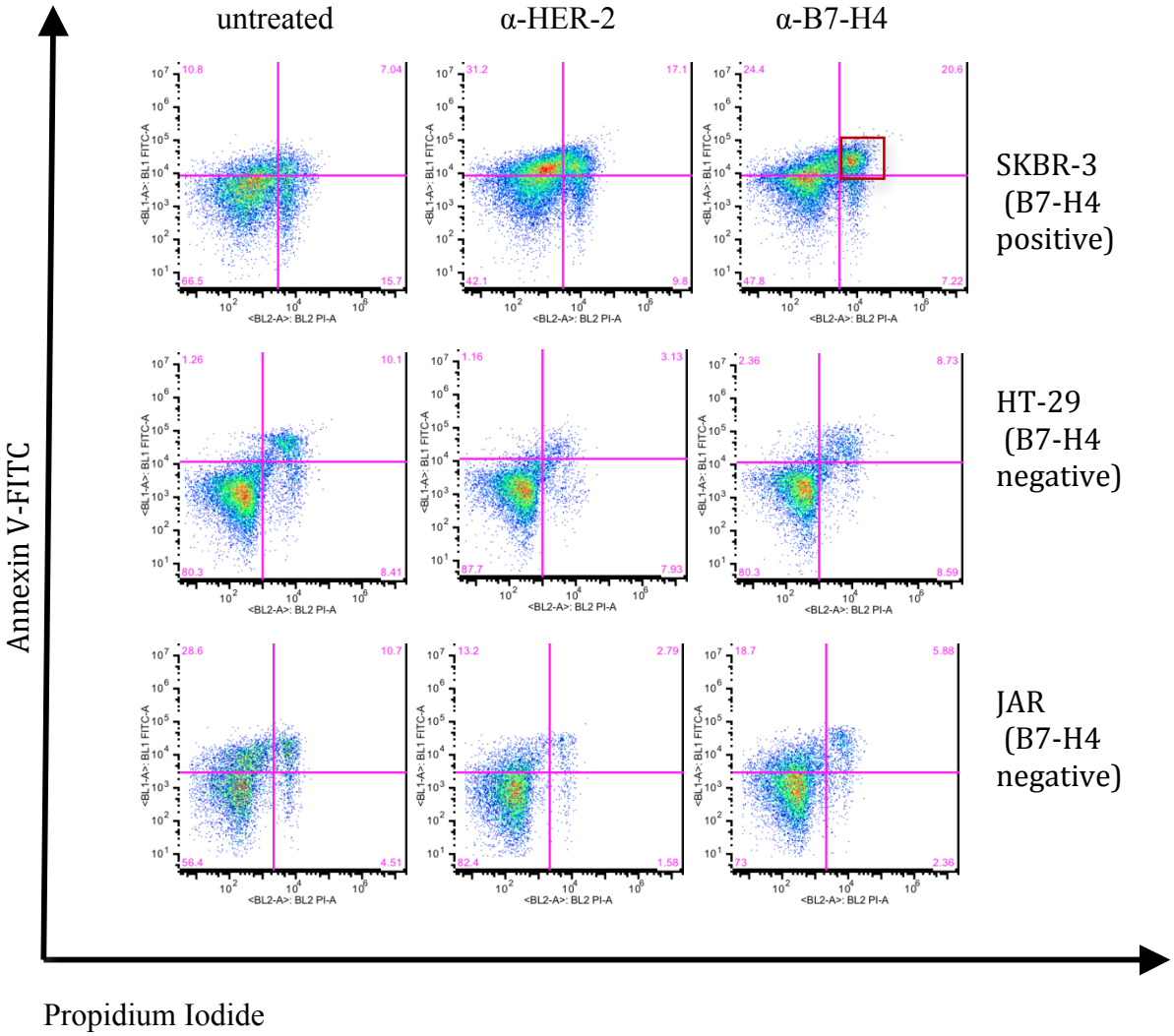
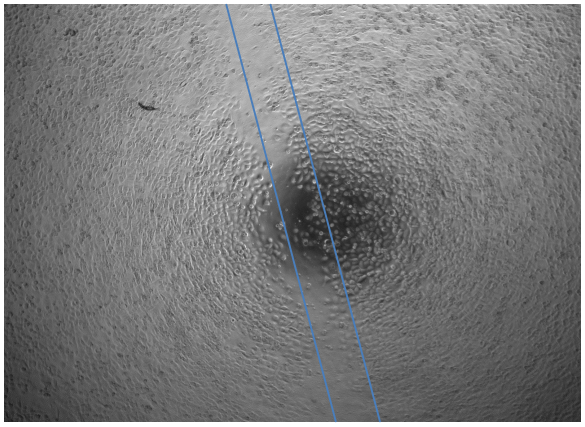
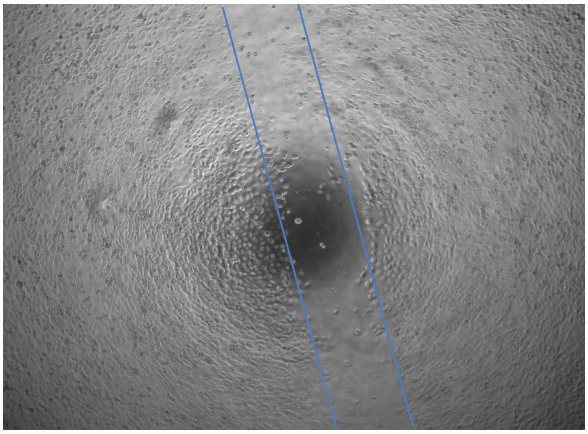



**Figure 8. Apoptotic assay for the B7-H4 antibodies.** Cells were incubated in the presence of newly generated murine B7-H4 antibodies, HER-2 antibody (AHer) as a positive control, isotype control (not shown), or left untreated for 2 days. Apoptosis was measured using annexin V-FITC and propidium iodide. Apoptotic cells are identified as annexin V positive and propidium iodide negative. Late dead cells are identified as propidium iodide positive. Above is a representation of the data using anti-B7-H4 clone 6D8. Note the slight increase in dead cells in anti-B7-H4-treated SKBR-3 cells compared to anti-HER-2 treated and untreated cells. Twenty different antibodies were screened once in this preliminary experiment.

**A**



**C**



**B**

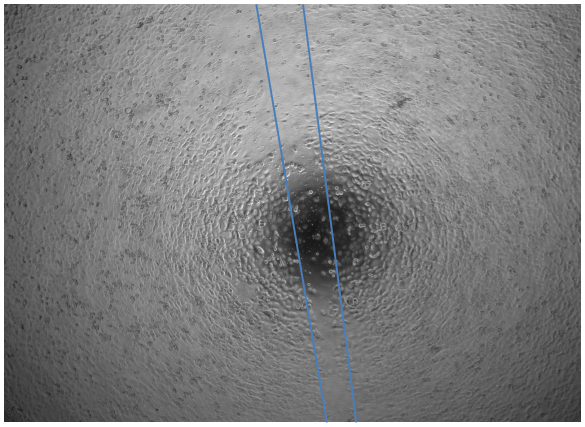


**Figure 9. Wound healing assay over 36 hours.** MDA-MB-468 cells were plated to confluency in a 96-well plate. At time 0, a yellow pipet tip was used to scratch the bottom of the plate. Cell migration was noted over 72 hours. Image shown is taken at 36 hours. **(A)** Untreated cells. **(B)** Cells incubated with 10 µg/mL isotype control. **(C)** Cells incubated with 10 µg/mL anti-B7-H4 antibody, clone #32-14. Wound healing assay was also done in SK-BR-3 and SK-OV-3, but yielded insignificant differences between antibody treatments.

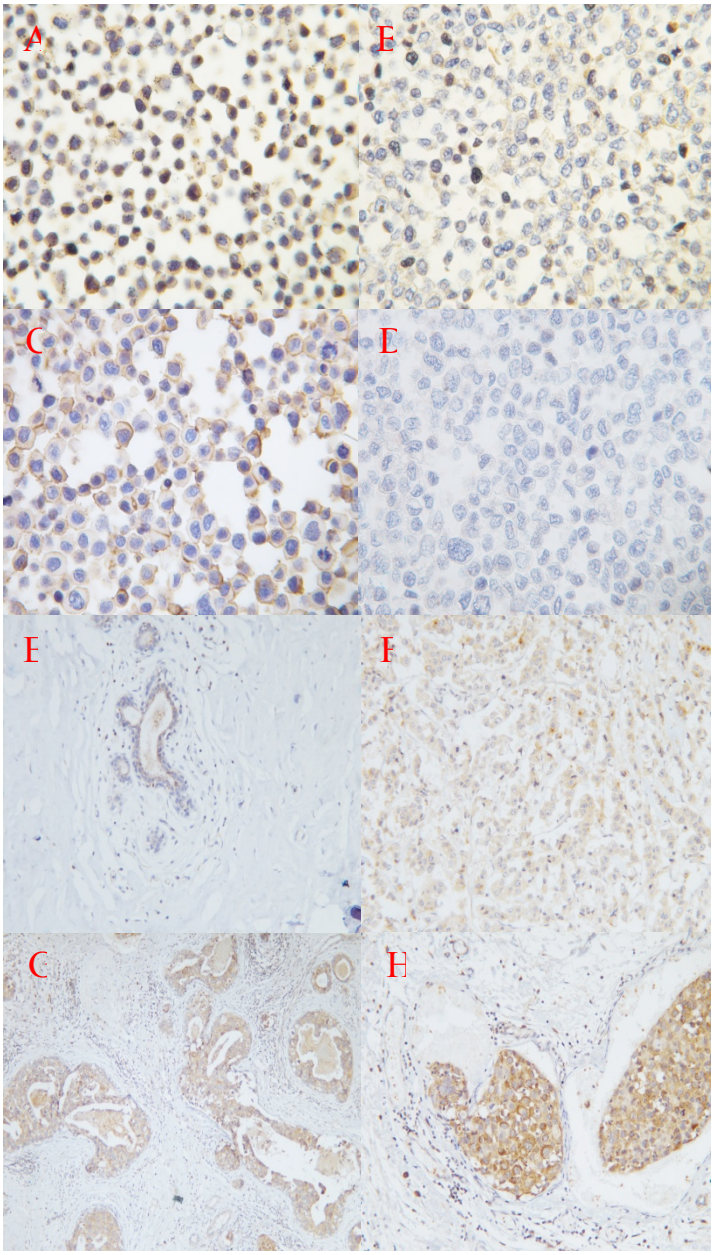
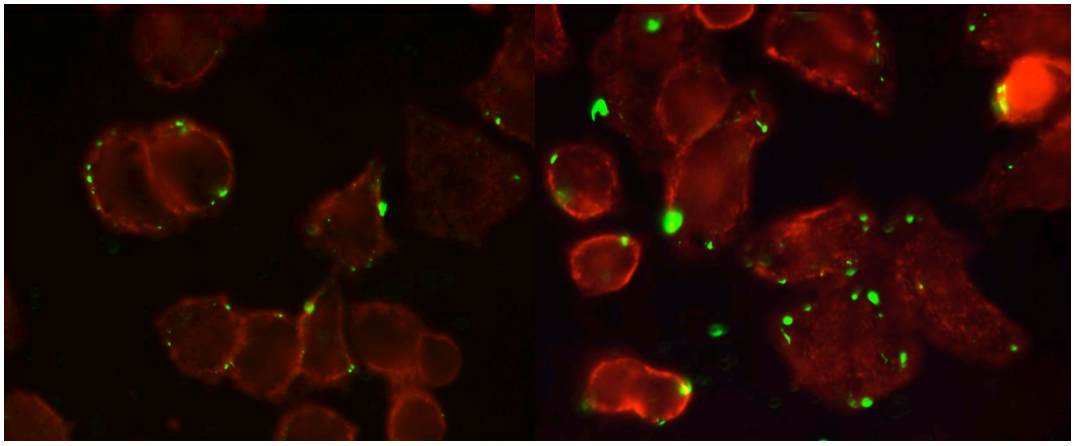



**Figure 10. Immunohistochemistry of B7-H4 expression in paraffin embedded tissues. (A, C)** SK-BR-3 and **(B,D)** SK-OV-3 paraffin embedded cell pellets were used to screen in-house B7-H4 antibodies as well as 4 commercial antibodies (400x magnification). **(A,B)** Stains produced using Abbiotec anti-B7-H4 (rabbit polyclonal, #250473). **(C-H)** Stains produced using clone #35-8 produced in our lab. Clone #35-8 produced **(C)** membranous staining of B7-H4 positive cell line, SK-BR-3, and showed **(D)** little background staining of the B7-H4 negative cell line, SK-OV-3. (E) Membranous, apical staining of B7-H4 in normal breast tissue (100x magnification). **(F)** Overexpression of B7-H4 in breast lobular carcinoma (100x magnification). **(G, H)** Overexpression of B7-H4 in breast ductal carcinoma (40x, 100x magnification, respectively).

**A** B7-H4/membrane **Non-permeabilized**



**B** B7-H4/membrane **Permeabilized**

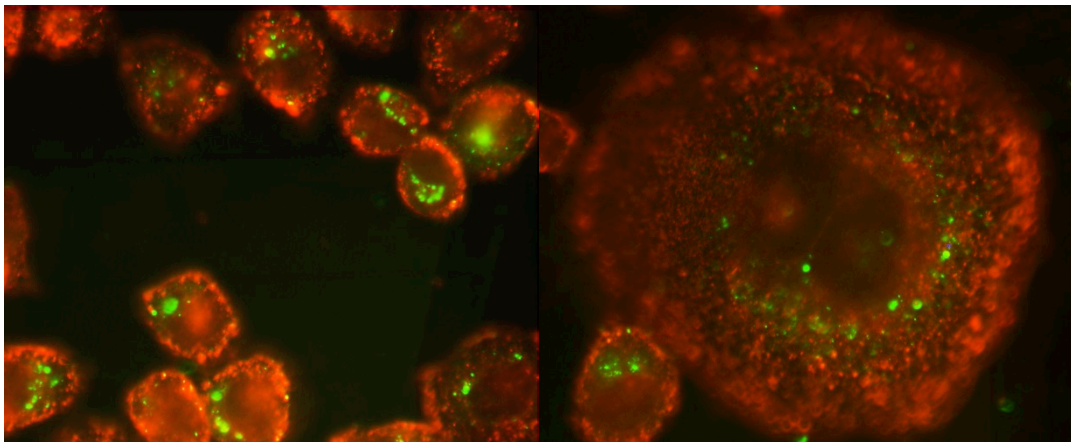


**Figure 11. Fluorescence microscopy of B7-H4 in SK-BR-3.** Cells were seeded on treated coverslips and stained for B7-H4 (Alexa Fluor 488). Counterstaining of membranes was done using wheat germ agglutin (Alexa Fluor 555). **(A)** Cells were fixed after staining for cell surface B7-H4, and exhibited a punctate pattern instead of uniform membaranous expression as expected. **(B)** Cells were fixed and permeabilized with 4% paraformaldehyde and 0.2% Triton X-100 prior to staining for intracellular B7-H4. Intracellular staining exhibits a perinuclear pattern. Fluorescence microscopy stains were also done on SK-OV-3, which were completely negative for both surface and intracellular B7-H4 (data not shown).