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13. ABSTRACT (Maximum 200 Words) When vaccination fails to protect the host from a subsequent challenge with a tumor, that tumor is generally characterized as nonimmunogenic. This designation suggests that the host has not recognized, or is tolerant of the tumor antigens. Our recent studies suggest that this is not true. We have demonstrated that progressively growing subcutaneous tumors sensitize tumor-specific T cells; however, the antigen-reactive T cells are polarized to secrete type 2 (T2) cytokines (e.g. IL-4 and IL-10), and lack therapeutic activity upon adoptive transfer. Conversely, immunogenic tumors induce predominantly type 1 (T1) antitumor responses, exhibiting highly polarized tumor-specific IFN- γ secretion. This proposal examines issues that are critical to understanding the mechanism for tumor regression following vaccination/immunotherapy. The first issues is whether tumor-specific T2 T cells, induced by progressively growing tumor, can inhibit the therapeutic efficacy of tumor-specific T1 T cells in our 4T1 mammary tumor model. If T2 T cells can inhibit therapeutic T cells it offers an explanation for the failure of tumor vaccine strategies and a possible approach to circumvent the inhibitory effect of T2 T cells. Aim 2 will test whether promoting a T1 response to vaccination will convert the nonimmunogenic 4T1 mammary tumor into a therapeutic vaccine				
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INTRODUCTION

This proposal addresses several issues that are critical to understanding the mechanism for tumor regression following vaccination/immunotherapy. The first issue is whether tumor-specific T2 T cells, induced by progressively growing tumor, can inhibit the therapeutic efficacy of tumor-specific T1 T cells in our 4T1 mammary tumor model. If T2 T cells can inhibit therapeutic T cells it offers a possible explanation for the failure of tumor vaccine strategies in both non immunogenic animal models and patients with cancer. It also offers a possible approach to circumvent the inhibitory effect of T2 T cells. Aim 2 will test whether promoting a T1 response to vaccination will convert the nonimmunogenic 4T1 mammary tumor into a therapeutic vaccine. Successful completion of these studies will provide insight into whether it might be beneficial to polarize a tumor-specific or HER-2/neu-specific T1 cytokine response in breast cancer patients. If these preclinical studies document a significant therapeutic advantage it could lead to the initiation of a new clinical trial for women with breast cancer.

This page contains unpublished data that should be protected.**BODY****Hypothesis/Purpose:**

It is our hypothesis that lymphocytes in lymph nodes draining therapeutic vaccines exhibit superior anti-tumor activity because the tumor-specific T cells contained within produce T1 cytokines which mediate tumor regression. In contrast, nontherapeutic T cells produce T2 cytokines which do not contribute to tumor destruction and may interfere with the antitumor response. Our purpose is to test this hypothesis in the 4T1 mammary tumor model and evaluate the therapeutic efficacy of strategies that drive the development of a tumor-specific T1 cytokine response.

Technical Objectives:

Our preliminary studies suggested that we could generate T cells polarized to secrete T1 or T2 cytokines in response to stimulation with specific tumor cells. These studies also suggested that the therapeutic activity resides in the L-selectin^{Lo} T cells that exhibit a T1 profile. The hypothesis of this objective is that a T1 cell mediates tumor regression through antigen-stimulated release of IFN- γ or other T1 cytokines, and that a tumor-specific T2 cell can inhibit this therapeutic activity by the secretion of T2 cytokines. To test this hypothesis, we proposed to first generate tumor-specific T1 and T2 T cells and test their antitumor efficacy against 3-day established pulmonary metastases. We will then be able to determine whether the addition of tumor-specific T2 T cells can inhibit the antitumor activity of T1 T cells.

1. To generate tumor-specific T1 and T2 T cells and test their antitumor efficacy against 3-day established pulmonary metastases

Initial studies with 4T1 made it clear that we had to improve our vaccine strategy, so we transduced a clone of 4T1 (4T1-9) with GM-CSF (E10-9) since other tumor models in our lab have proven the strategy to be effective (Hu et al., J. Immunology, Oct. 15, 2000). Vaccinating wt BALB/c mice with E10-9 demonstrated that E10-9 tumor-vaccine draining lymph node (TVDLN) cells activated with α CD3 and expanded in low dose IL-2, secreted more tumor-specific IFN- γ than TVDLN from mice vaccinated with 4T1-9 (Fig. 1). These more highly polarized T1 cells from E10-9 TVDLN were also more therapeutic than 4T1-9 TVDLN when adoptively transferred into mice that had 3-day established pulmonary metastases (Table 1).

We obtained STAT6^{-/-} mice in to examine this technical objective with T cells that are incapable of becoming T2 cells. STAT6^{-/-} mice are deficient in their ability to mount a T2 response due to the lack of STAT6 which is an important signal transducer for the IL-4 receptor. The activation of STAT6 leads to its translocation into the nucleus of the cell where it acts to upregulate IL-4 inducible genes. We injected 4T1 subcutaneously into the flank of STAT6^{-/-} mice and WT BALB/c mice to examine the tumorigenicity of 4T1 in each of these mice. Interestingly, STAT6^{-/-} mice were able to reject the tumor at

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doses of 4T1 tumor as high as 10^5 cells (Fig. 2). This is a log higher than the dose of 4T1 that causes progressively growing tumor in 100% of wt BALB/c mice. E10-9 TVDLN from STAT6^{-/-} mice are highly polarized toward a T1 phenotype secreting greater than 20ng/ml IFN- γ when stimulated with 4T1 (Fig. 3). However, the adoptive transfer of these cells were not more therapeutic than E10-9 TVDLN from wt BALB/c mice that are not as highly polarized toward a T1 phenotype (Table 2). We also performed these experiments using 4T1-9 TVDLN in the two strains of mice and found that once again even though T cells from STAT6^{-/-} mice were more highly polarized to a T1 phenotype there was no significant difference in the therapeutic efficacy when the cells were adoptively transferred.

An important observation was made when E10-9 TVDLN cells from STAT6^{-/-} mice were adoptively transferred into wt BALB/c recipients bearing 3-day established pulmonary metastases. Within 48 hours after the adoptive transfer of T cells the recipient mice lost up to 15% of their body weight (Fig. 4), were moribund and depending on the amount of cells transferred would die within 72 hours. Autopsy of these recipient mice revealed inflamed lower intestines and bowel. This pathology was dependent on E10-9 vaccination since the adoptive transfer of naïve T cells from STAT6^{-/-} mice did not cause detrimental effects (data not shown). The T cells had to be primed in a STAT6^{-/-} mouse and transferred into a wt BALB/c recipient. These data suggested that the reason for these pathological effects was that E10-9 was priming T cells in STAT6^{-/-} mice to an antigen that was present in the lower intestine and bowel of wt BALB/c mice but not STAT6^{-/-} mice. It was our hypothesis that this antigen could be a peptide from the STAT6 protein so we examined 4T1 for expression of STAT6 which could serve to prime the T cells in STAT6^{-/-} mice. Both mammary adenocarcinomas, 4T1 and EMT6, as well as the colon carcinoma CT26 express STAT6 (data not shown). We scanned the STAT6 protein for peptides that would bind to MHC class I using a computer algorithm that determines theoretical binding affinities. We chose three peptides based on their theoretical binding affinities and used them to peptide-pulse STAT6^{-/-} splenocytes in order to test their ability to stimulate 4T1 TVDLN cells from STAT6^{-/-} mice. Of the peptides tested, STAT6₅₃₁₋₅₃₉ stimulated 4T1 TVDLN cells from STAT6^{-/-} mice to secrete large amounts of IFN- γ (Fig. 5). 4T1 TVDLN cells from wt BALB/c mice were not stimulated to secrete IFN- γ by this peptide. Since T cells from STAT6^{-/-} mice could be primed by E10-9 to the STAT6₅₃₁₋₅₃₉ peptide, we wanted to determine if the deletion of these T cells in STAT6^{-/-} T cell repertoire would cause 4T1 to grow progressively in STAT6^{-/-} mice. We reconstituted wt BALB/c mice with STAT6^{-/-} bone marrow to cause the deletion of STAT6-reactive T cells while keeping intact the propensity of the lymphocyte population to mount a T1 immune response. We also reconstituted wt BALB/c mice with wt BALB/c bone marrow and STAT6^{-/-} mice with STAT6^{-/-} bone marrow as controls. These mice were subcutaneously injected with 10^4 4T1 tumor cells in the flank and followed for tumor growth. STAT6^{-/-} mice reconstituted with STAT6^{-/-} bone marrow rejected 4T1, and 4T1 grew progressively in wt BALB/c mice reconstituted with wt BALB/c bone marrow (Fig. 6). wt BALB/c mice reconstituted with STAT6^{-/-} bone marrow did not reject 4T1. TVDLN cells from these mice were stimulated with 4T1 as well as peptide-pulsed STAT6^{-/-} splenocytes to verify if the STAT6₅₃₁₋₅₃₉-reactive T cells were deleted in the WT BALB/c mice reconstituted with STAT6^{-/-} bone marrow. T cells from WT BALB/c mice reconstituted with STAT6^{-/-} bone marrow were not stimulated by STAT6₅₃₁₋₅₃₉ pulsed STAT6^{-/-} splenocytes, and interestingly they also lost their reactivity to 4T1 (Fig. 7). These data strongly suggest that rejection of 4T1 in STAT6^{-/-} mice is due to the presence of high affinity T cells that react against STAT6 peptides including STAT6₅₃₁₋₅₃₉. With this additional insight we can now use the

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STAT6^{-/-} system to address the antitumor efficacy of a T1 immune response by performing the vaccination in wt BALB/c mice reconstituted with STAT6^{-/-} bone marrow.

STAT4^{-/-} mice are deficient in mounting a T1 immune response since they lack STAT4, a key signal transducer of the IL-12 receptor. We have generated wt BALB/c mice reconstituted with STAT4^{-/-} bone marrow and current studies are addressing the therapeutic efficacy of a polarized T2 immune response against 4T1.

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KEY RESEARCH ACCOMPLISHMENTS

- Confirmation that L-selectin^{Lo} TVDLN T cells contain the population with therapeutic activity
- Generation and characterization of 4T1 clones that exhibit strikingly different and immunologically interesting phenotypes
- STAT6^{-/-} mice vaccinated with a GM-CSF secreting 4T1 vaccine generate tumor-reactive T cells with capacity to secrete high levels (25 ng/ml/24 hrs) of IFN- γ .
- Adoptive transfer of tumor-reactive STAT6^{-/-} mice effector T cells that are polarized toward a T1 phenotype are not more therapeutic than wt effector T cells.
- Adoptive transfer of tumor-reactive STAT6^{-/-} mice effector T cells in wt recipients cause severe loss of body weight that can result in death of the recipient mice.
- Mouse mammary adenocarcinoma cell lines, 4T1 and EMT6, express the STAT6 protein
- A strong T cell response is primed against the STAT6₅₃₁₋₅₃₉ peptide in STAT6^{-/-} mice when they are vaccinated with 4T1.
- The ability of STAT6^{-/-} mice to reject higher subcutaneous doses of 4T1 than wt BALB/c mice correlates with the presence of STAT6₅₃₁₋₅₃₉-reactive T cells in the T cell repertoire of STAT6^{-/-} mice.

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REPORTABLE OUTCOMES

Manuscripts:

Shawn M. Jensen, Hong-Ming Hu, Bernard A. Fox. Regression of a Stat6-expressing mammary adenocarcinoma in Stat6^{-/-} mice is dependent on the presence of T cells that recognize a Stat6 epitope. (manuscript in preparation).

Abstracts:

S.M. Jensen, H-M. Hu, B.A. Fox. Priming Tumor Vaccine Draining Lymph Node T cells by GM-CSF Transduced 4T1. *Abstract #55.6 FASEB/AAI*, May 12-16, 2000.

S.M. Jensen, H.-M. Hu, B.A. Fox. Increased IFN- γ Secretion by Adoptively Transferred T cells Does Not Correlated with Enhanced Anti-Tumor Therapy. *Abstract #2702 AACR*, March 24-28, 2001.

S Jensen, H-M Hu, B.A. Fox. T cells from Stat6^{-/-} mice vaccinated with a 4T1 tumor vaccine recognize a Stat6 epitope. *Abstract #3.13b/535 International Congress of Immunology*, July 22-27, 2001.

Shawn M. Jensen, Hong-Ming Hu, Bernard A. Fox. Regression of 4T1 in Stat6^{-/-} mice is dependent on Stat6-reactive T cells. *FASEB/AAI 2002* (submitted).

Presentations:

Shawn M. Jensen. 2001. T cells from Stat6^{-/-} mice vaccinated with a 4T1 tumor vaccine recognize a Stat6 epitope. Forschungszentrum fuer Umwelt und Gesundheit (GSF), Klinikum Grosshadern, Ludwig-Maximilians-University, Munich, Germany

patents and licenses applied for and/or issued;

- None

degrees obtained that are supported by this award;

- None

development of cell lines, tissue or serum repositories;

- A series of 4T1 clones have been developed with distinctly different morphologic and phenotypic characteristics. Two of these cell lines have already been provided to Dr. J. Mule' (University of Michigan) and to Dr. Robert Kurt (LaFayette College, Easton, Pennsylvania) for use in collaboration.

informatics such as databases and animal models, etc;

- Characterization of the 4T1 clones is continuing. They may provide advantages over existing 4T1 model.

funding applied for based on work supported by this award;

- None – However, it is expected with the completion of the current studies a new proposal for support will be submitted

employment or research opportunities applied for and/or received on experiences/training supported by this award.

- The training of Shawn Jensen, PhD candidate at Oregon Health Sciences University has been supported by this award

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CONCLUSIONS

Our results suggest that using a GM-CSF transduced breast cancer vaccine increases the priming of tumor-specific T cells in the TVDLN, as determined by an increase in tumor-specific IFN- γ secretion in vitro and increased therapeutic efficacy in vivo. Furthermore, we have identified that downregulation of L-selectin expression can be used as a marker for T cells with therapeutic potential in this model of breast cancer (AAI 2000 Abstract).

Using this GM-CSF secreting tumor vaccine model we have generated effector T cells from wt BALB/c mice and STAT6^{-/-} mice. Vaccination of STAT6^{-/-} mice which are deficient in IL-4 signaling, have consistently given us the most highly T1 polarized T cells. However, it was surprising that the adoptive transfer of these T cells were not more therapeutic than the adoptive transfer of T cells from wt BALB/c mice that were vaccinated with the GM-CSF secreting tumor vaccine. It is puzzling why the STAT6^{-/-} T cells, which exhibit more than a log increase in IFN- γ secretion, don't exhibit greater therapeutic efficacy in vivo. Our recent work in the B16BL6 melanoma model suggests that IFN- γ ko mice (GKO) can still generate effector T cells with therapeutic activity. These GKO effector cells don't exhibit a T2 cytokine profile but do retain expression of LT- $\alpha_1\beta_2$, another T1 cytokine (Winter et al., 2001). The observation that infusion of large numbers of highly polarized T1 T cells from STAT6^{-/-} mice resulted in substantial toxicity and death of some animals suggests that we have reached a dose limiting toxicity with these highly polarized T cells. This appears to be the result of STAT6-reactive T cells from STAT6^{-/-} mice being stimulated by normal STAT6 expressing cells within the wt BALB/c mice. These findings will need to be considered as we begin to translate novel strategies to develop highly polarized tumor-specific T1 T cells for patients with breast cancer since most tumor antigens are self antigens and thus a highly polarized T1 response against a self antigen could lead to substantial toxicity.

Our results have shown that the ability of STAT6^{-/-} mice to reject high doses of the poorly immunogenic tumor, 4T1, correlated with the presence of STAT6-reactive T cells that could presumably recognize STAT6 epitopes on 4T1. This demonstrates the necessity of a tumor antigen that can be recognized sufficiently by the T cell repertoire to provide primed T cells. Although mice which had STAT6-reactive T cells deleted were unable to reject a tumor challenge, it is possible that vaccinating these mice with irradiated tumor prior to tumor challenge might prime lower affinity T cells which could provide protection since they are T1 polarized. Additionally, it is not clear from our results if the rejection of 4T1 in STAT6^{-/-} mice is solely due to the STAT6-reactive T cells or is a combination of STAT6-reactive T cells that are highly polarized toward a T1 phenotype.

Related work in my laboratory is evaluating the immune response of patients on vaccine trials at our Institution (melanoma, renal, breast and non-small cell lung cancer). In breast cancer patients, that received an allogeneic breast cancer vaccine transfected with CD80, we found that 50% of the patients that responded in vitro to the vaccination exhibited a T1 response, while the other 50% exhibited a T2 response (Dols et al. Manuscript submitted). While these are preliminary studies with small numbers of patients they suggest that a substantial proportion of the patients studied had what we would consider an ineffective T2 response. We are in the process of further evaluating if the adjuvant given with the vaccine did cause a polarization. Preliminary observations show that when BCG is given as an adjuvant the tumor-specific cytokine secretion has a T2 profile (IL-5), while when GM-CSF is given as an adjuvant tumor-specific cytokine secretion has a T1 profile (IFN- γ). The goal of this DOD proposal is

to determine whether strategies which polarize the antitumor immune response in vivo and in vitro will augment the antitumor immune response to breast cancer. This goal is still important and future studies may provide important insights that could be rapidly translated to augment existing immunotherapy strategies for patients with breast cancer or other malignancies.

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- A. Dols, S. Meijer, H-M. Hu, P. Wisner, T. Doran, L. Justice, W.J. Urba, J.W. Smith, B.A. Fox. Vaccination of women with metastatic breast cancer using a costimulatory gene (CD80)-modified HLA-A2-matched allogeneic breast cancer cell line: Clinical and Immunological results. (to be submitted).

APPENDICES

AAI 2000 *Abstract* #55.6

Priming Tumor Vaccine Draining Lymph Node T Cells by GM-CSF Transduced 4T1

S.M.Jensen, H.-M.Hu, B.A.Fox

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Recent studies in our lab have shown that using the poorly immunogenic mammary adenocarcinoma cell line, 4T1, as a tumor vaccine are ineffective at generating therapeutic tumor-vaccine draining lymph node (TVDLN) cells. In an effort to improve the priming of T cells by 4T1 we have used a retrovirus encoding the GM-CSF gene (MFG-GM-CSF) to transduce 4T1. It is our hypothesis that the local secretion of GM-CSF by the transduced tumor vaccine will enhance host antigen-presenting cell function thereby enhancing the priming of naïve T cells within the lymph node. We have observed that 4T1 is comprised of a heterogeneous cell population based on morphological characteristics and cell surface marker expression. A clone of 4T1, 4T1-9, was transduced with MFG-GM-CSF, and shown to produce 15ng/10⁶ cells/24hrs. Here we report that L-selectin^{Lo} T cells, representing activated T cells, from TVDLN of mice vaccinated with the GM-CSF secreting clone, E10-9, exhibited tumor-specific IFN-γ release. The level of IFN-γ secretion was 2 fold higher than the L-selectin^{Lo} T cells from TVDLN of mice vaccinated with the 4T1-9 parental clone. There was not detectable IFN-γ secretion within the L-selectin^{Hi} cell populations. Additionally, the frequency of tumor-specific IFN-γ secreting L-selectin^{Lo} T cells was 2-3 fold higher from the E10-9 TVDLN compared to 4T1-9 TVDLN as determined by ELISPOT. Current studies are examining the *in vivo* therapeutic efficacy of these cell populations.

AACR 2001 *Abstract* #2702

Increased IFN- γ Secretion by Adoptively Transferred T cells Does Not Correlate with Enhanced Anti-Tumor Therapy

S.M.Jensen, H.-M.Hu, B.A.Fox

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The poorly immunogenic mammary adenocarcinoma cell line, 4T1, is inefficient in priming therapeutic tumor-vaccine draining lymph node (TVDLN) T cells. One possible explanation for the inability of tumors to prime therapeutic immune responses may be due to immune deviation. This concept suggests that the tumor-primed T cells are polarized towards either a nonproductive type 1 or type 2 phenotype. Previous work in our lab has demonstrated a correlation between therapeutic efficacy of TVDLN T cells and their polarization towards a type 1 phenotype. In an effort to enhance the type 1 polarization of TVDLN T cells from mice vaccinated with 4T1 we used Stat6^{-/-} mice as hosts of 4T1 tumor vaccines. The key role of Stat6 as a signal transducer of the IL-4 receptor and therefore its importance in the differentiation of T cells towards a type 2 phenotype has recently been demonstrated. It is our hypothesis that by disrupting this type 2 differentiation pathway using Stat6^{-/-} mice will cause TVDLN T cells primed against 4T1 tumor antigens to differentiate towards a type 1 phenotype, and result in their increased therapeutic efficacy. Indeed, T cells from Stat6^{-/-} TVDLN exhibited significantly higher tumor-stimulated IFN- γ secretion as compared to control BALB/c TVDLN (25.8 ng/ml versus 1.2 ng/ml, $p=0.02$). This suggested that in the absence of Stat6 T cells from tumor-vaccinated mice polarized toward the type 1 phenotype. However, 4 out of 5 adoptive transfer experiments did not demonstrate a statistically significant increase in the efficacy of T cells from Stat6^{-/-} TVDLN versus control BALB/c TVDLN to eradicate 3-day experimentally established pulmonary metastases (experiments exhibiting both high and low 4T1 tumor burden). These data suggest that increased tumor-stimulated IFN- γ secretion does not correlate with improved therapeutic efficacy by TVDLN, thus questioning the role of excess IFN- γ within the anti-tumor immune response.

International Congress of Immunology 2001 *Abstract* #3.13b/535

T CELLS FROM STAT6^{-/-} MICE VACCINATED WITH A 4T1 TUMOR VACCINE RECOGNIZE A STAT6 EPITOPE

S Jensen, H-M Hu, B.A. Fox,

Earle A. Chiles Research Institute, R. W. Franz Cancer Research Center, PPMC, OGI, OHSU, Portland OR USA

Our recent work suggests that a tumor-specific Type 1 response, but not Type 2, is critical for T cell-mediated tumor regression. Stat6 is an essential signal transducer of the IL-4 receptor. Since Stat6^{-/-} mice are unable to generate a Type 2 response we examined their antitumor response to 4T1, a mammary adenocarcinoma. Stat6^{-/-} mice were significantly more resistant to challenge with 4T1 tumor, rejecting doses a log higher than that which causes tumors in BALB/c (wt) mice. To further examine the antitumor immune response generated in Stat6^{-/-} mice animals were vaccinated with a GM-CSF transduced clone of 4T1. Tumor-vaccine draining lymph node T cells were isolated, activated with anti-CD3 and expanded in IL-2 to generate effector T cells (T_E). Stat6^{-/-} T_E cells recognized 4T1 in vitro, secreting significantly higher IFN- γ compared to control wt T_E (p=.02). However, adoptive transfer of Stat6^{-/-} or wt T_E cells into tumor-bearing wt mice were equally therapeutic. Interestingly, the transfer of Stat6^{-/-} T_E cells caused significant acute loss of body weight in wt recipient mice as compared to transfer of wt T_E cells (p<.001). Analysis of the Stat6^{-/-} T_E cells revealed that they recognize an epitope within the Stat6 protein. Since 4T1 expresses Stat6 current studies are examining whether Stat6-reactive T cells could be responsible for the regression of 4T1 tumors in Stat6^{-/-} mice.

This page contains unpublished data that should be protected.

AAI 2002 (submitted)

Regression of 4T1 in Stat6^{-/-} mice is dependent on Stat6-reactive T cells.

Shawn M. Jensen, Hong-Ming Hu, Bernard A. Fox

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Stat6^{-/-} mice are deficient in their ability to generate a Type 2 response and respond with an enhanced Type 1 response. We examined the antitumor response of Stat6^{-/-} mice to 4T1, a mammary adenocarcinoma. Stat6^{-/-} mice were significantly more resistant to challenge with 4T1 tumor, rejecting doses a log higher than that which causes tumors in BALB/c (wt) mice. A 4-fold increase in the frequency of 4T1-specific T cells, determined by intracellular staining for IFN- γ , was observed in the tumor-draining lymph nodes of Stat6^{-/-} mice compared to wt mice. Analysis of the Stat6^{-/-} T cells revealed that they recognized a Stat6 peptide potentially presented by 4T1 since 4T1 expresses Stat6. To determine the role of Stat6-reactive T cells in 4T1 tumor regression we generated Stat6^{-/-} bone marrow chimeras to delete Stat6-reactive T cells from the T cell repertoire. The Stat6^{-/-} bone marrow chimeras failed to recognize the Stat6 peptide and were as susceptible as wt mice to 4T1 challenge suggesting that Stat6-reactive T cells are necessary for 4T1 regression in Stat6^{-/-} mice.

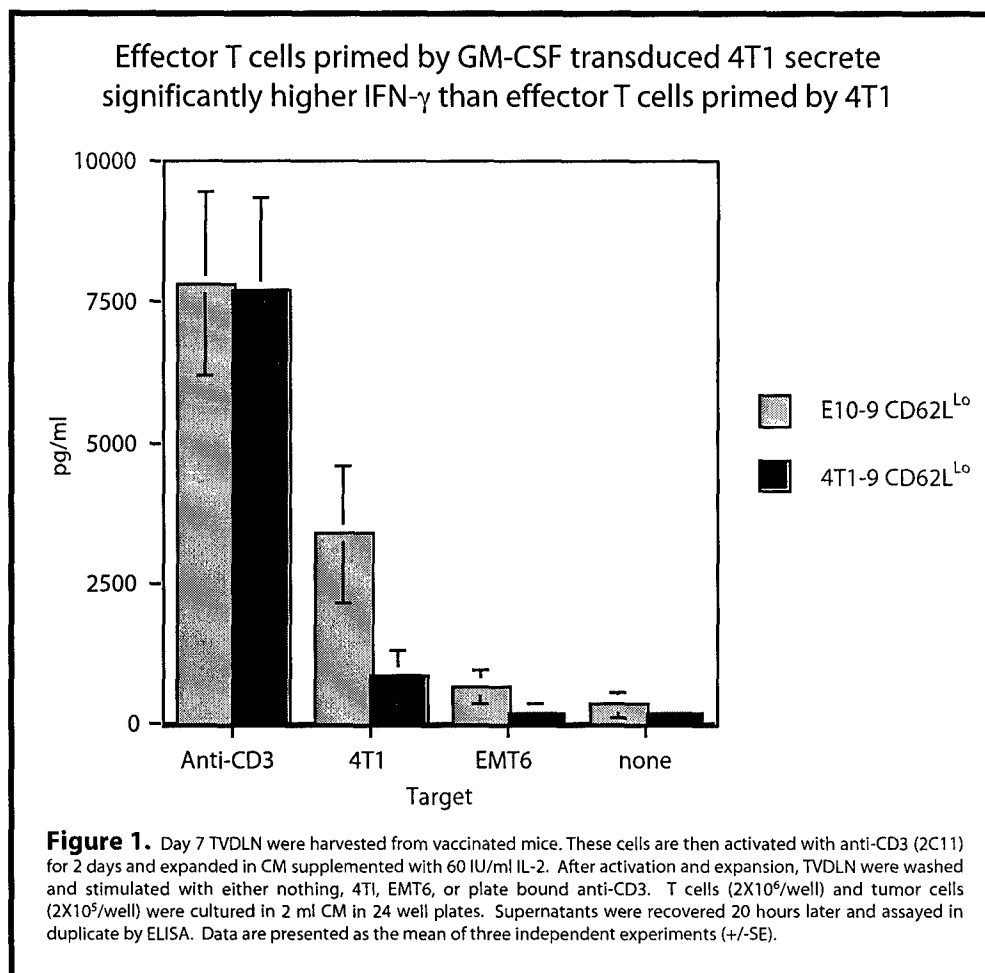


Table 1

Effector T cells primed by GM-CSF transduced 4T1 are more therapeutic than effector T cells primed by 4T1

Tumor Vaccine ^a	T cells ^b	IL-2 ^c	# of pulmonary metastases	Mean pulmonary metastases (SEM)
None	None	+	25, 250, 250, 250, 250	205 (45)
4T1-9	30×10^6 CD62L ^{Lo}	+	181, 250, 250, 250, 250	236 (14)
E10-9	30×10^6 CD62L ^{Lo}	+	3, 3, 7, 10, 12	7 (2)*

* $p < 0.05$ between E10-9 group and 4T1-9 group or IL-2 control group

^a Mice were vaccinated subcutaneously with a dose of 7.5×10^5 indicated tumor cells

^b Single cell suspensions from day 8 tumor vaccine-draining lymph nodes were stimulated in vitro at 2×10^6 cells/ml with aCD3 (2C11) for 2 days and then expanded for 3 days in CM supplemented with 60 IU/ml IL-2. Cells were harvested and adoptively transferred into animals with established 3-day 4T1 pulmonary metastases.

^c IL-2 (90,000 IU) was administered i.p daily for 3 consecutive days following adoptive transfer

4T1 is less tumorigenic in Stat6^{-/-} mice than wt mice

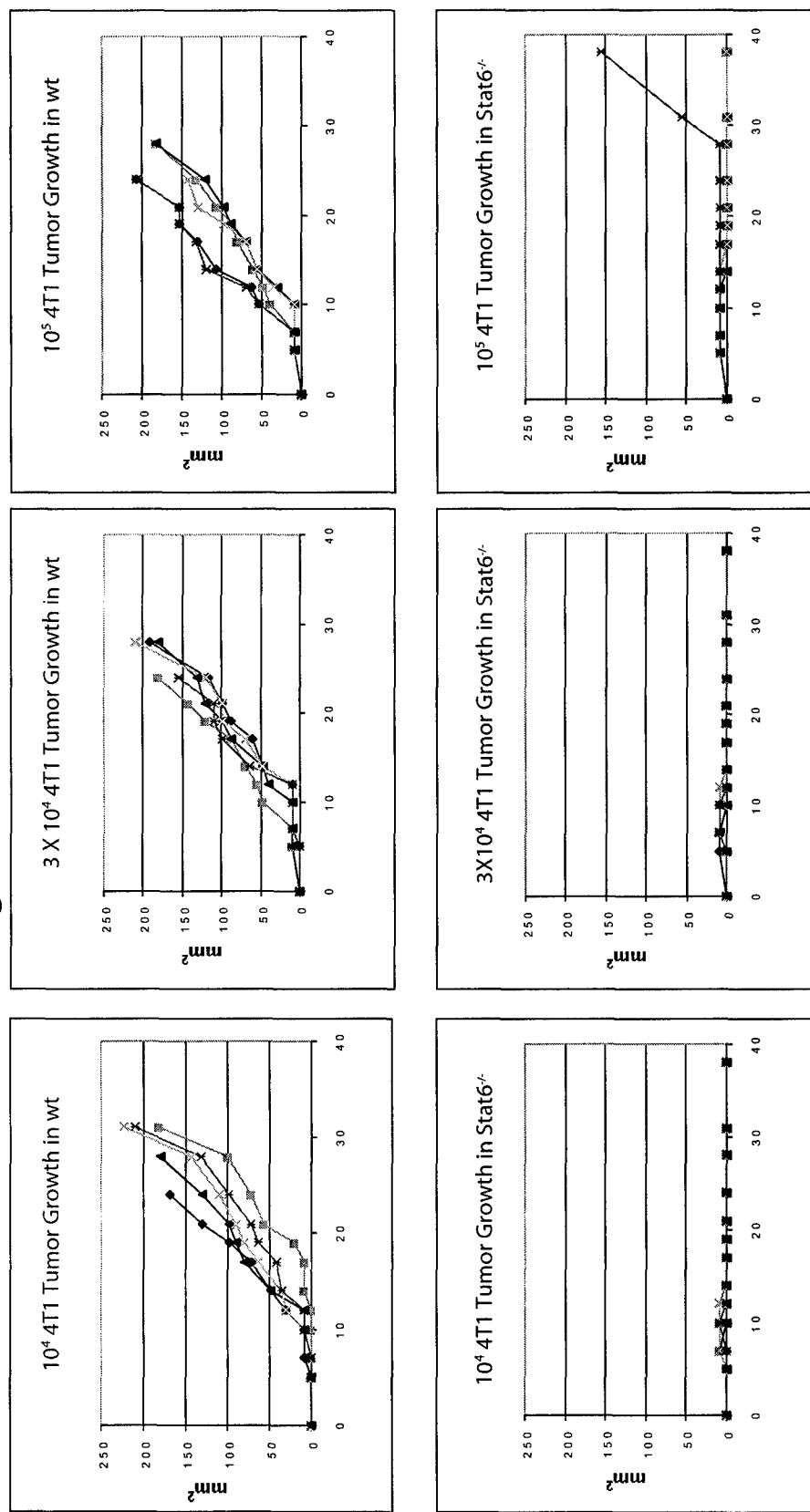


Figure 2. wt or Stat6^{-/-} mice were subcutaneously injected in the left flank with the indicated doses of 4T1. Tumor growth was determined by multiplying the longest diameter of the tumor mass by the perpendicular diameter. Mice were sacrificed when the tumor mass was larger than 150 mm².

Stat6^{-/-} Effector T cells exhibit a significantly higher IFN- γ response to 4T1 than wt Effector T cells

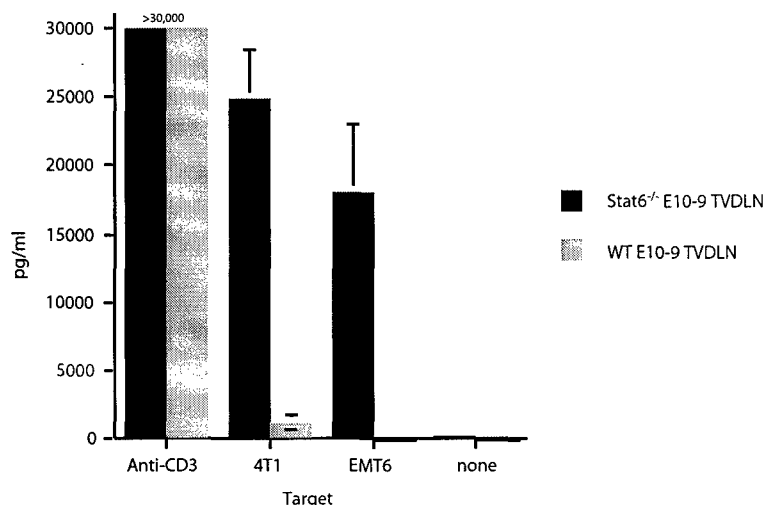


Figure 3. Day 7 TVDLN were harvested from vaccinated mice. These cells are then activated with anti-CD3 (2C11) for 2 days and expanded in CM supplemented with 60 IU/ml IL-2. After activation and expansion, TVDLN were washed and stimulated with either nothing, 4T1, EMT6, or plate bound anti-CD3. T cells (2×10^6 /well) and tumor cells (2×10^5 /well) were cultured in 2 ml CM in 24 well plates. Supernatants were recovered 20 hours later and assayed in duplicate by ELISA. Data are presented as the mean of three independent experiments ($\pm$ SE).

Table 2

Effector T cells generated from Stat6^{-/-} mice by vaccination with a GM-CSF modified 4T1-9, E10-9, are not more therapeutic than wt Effector T cells

Tumor vaccine ^a	T cells ^b	IL-2 ^c	Mean of Pulmonary Metastases (SEM)			
			Exp #1	Exp #2	Exp #3	Exp #4
None	None	+	250 (0)	246 (4)	250 (0)	23 (3)
E10-9	50 X10 ⁶ wt TVDLN	+	250 (0)	18 (5)	250 (0)	9 (3)
E10-9	50 X10 ⁶ Stat6 ^{-/-} TVDLN	+	34 (7)	35 (7)	212 (30)	15 (2)

Tumor vaccine ^a	T cells ^b	IL-2 ^c	Mean of Pulmonary Metastases (SEM)		
			Exp #1	Exp #2	Exp #3
None	None	+	250 (0)	250 (0)	250 (0)
4T1-9	50 X10 ⁶ wt TVDLN	+	194 (16)	250 (0)	164 (15)
4T1-9	50 X10 ⁶ Stat6 ^{-/-} TVDLN	+	250 (0)	231 (19)	216 (15)

^a Mice were vaccinated subcutaneously with a dose of 7.5×10^5 E10-9 tumor cells

^b Single cell suspensions from day 8 tumor vaccine-draining lymph nodes were stimulated in vitro at 2×10^6 cells/ml with aCD3 (2C11) for 2 days and then expanded for 3 days in CM supplemented with 60 IU/ml IL-2. Cells were harvested and adoptively transferred into animals with established 3-day 4T1 pulmonary metastases.

^c IL-2 (90,000 IU) was administered i.p daily for 3 consecutive days following adoptive transfer

The adoptive transfer of Stat6^{-/-} Effector T cells causes acute weight loss in wt recipients

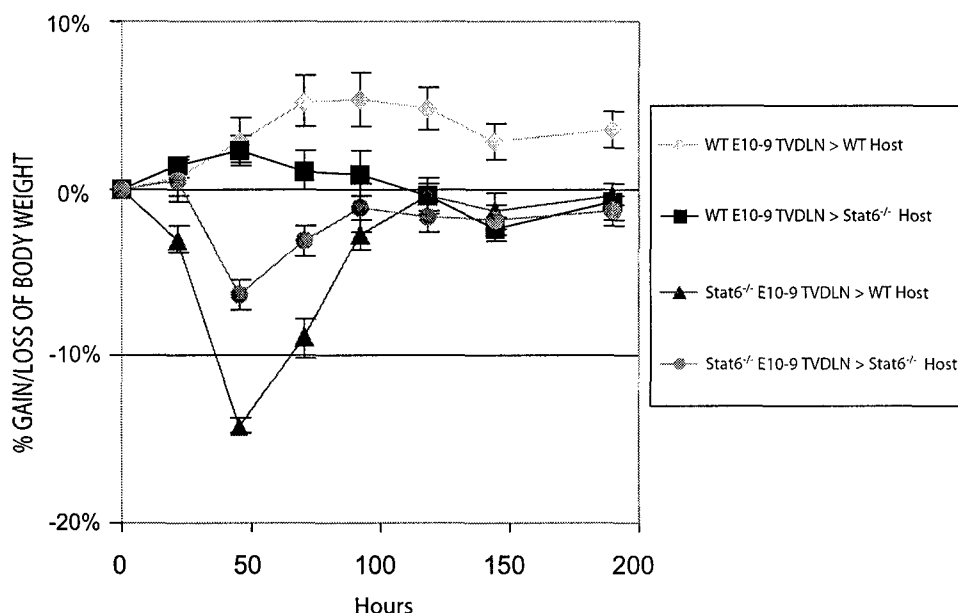


Figure 4. Day 8 TVDLN were harvested from 4T1-vaccinated Stat6^{-/-} or E10-9-vaccinated wt mice. These cells were then activated with anti-CD3 (2C11) for 2 days and expanded in CM supplemented with 60 IU/ml IL-2. After activation and expansion, 5 X 10⁷ TVDLN cells were adoptively transferred into either Stat6^{-/-} or wt mice. Data are presented as the mean of five mice (+/-SE).

T cells from 4T1-vaccinated Stat6^{-/-} mice respond to the Stat6 peptide₅₃₁₋₅₃₉

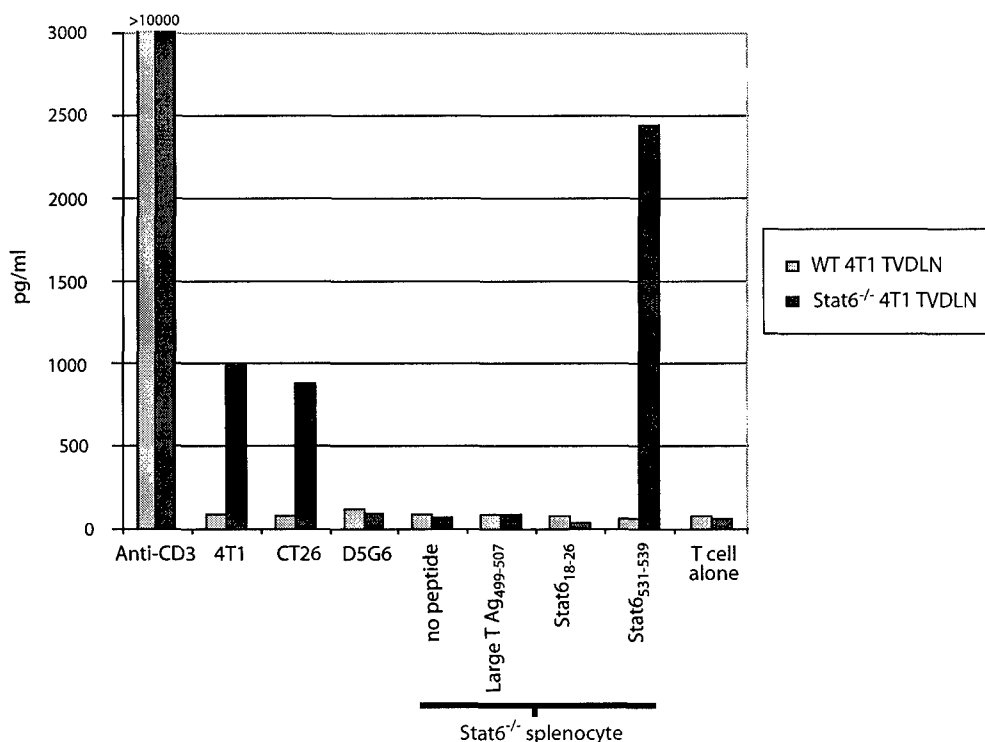


Figure 5. Day 8 TVDLN were harvested from vaccinated mice. These cells were then activated with anti-CD3 (2C11) for 2 days and expanded in CM supplemented with 60 IU/ml IL-2. After activation and expansion, TVDLN were washed and stimulated with either plate bound anti-CD3, 4T1, CT26, or peptide-pulsed Stat6^{-/-} splenocytes. Peptide-pulsed Stat6^{-/-} splenocytes were prepared by pulsing Stat6^{-/-} splenocytes with 10ug of the indicated peptide for 30 min and then washing the splenocytes. T cells (2X10⁶/well) and tumor cells (2X10⁵/well) or splenocytes (2X10⁶/well) were cultured in 2 ml CM in 24 well plates. Supernatants were recovered 6 hours later and assayed in duplicate by ELISA.

4T1 grows in WT mice reconstituted with Stat6^{-/-} bone marrow

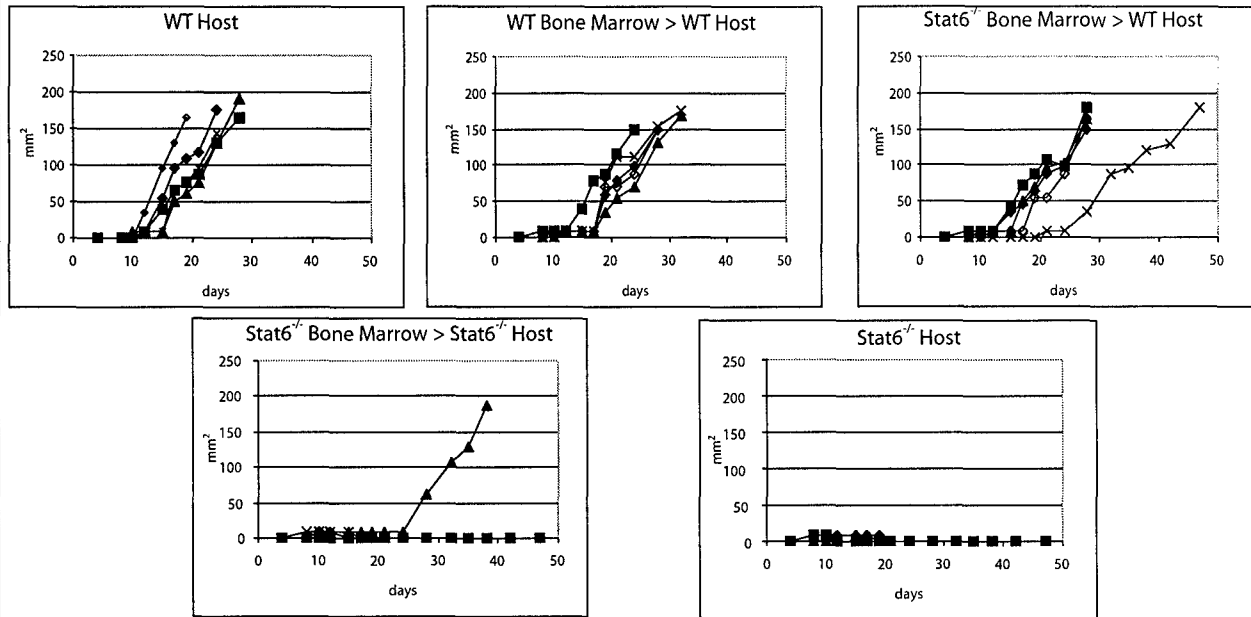


Figure 6. Indicated mice were subcutaneously injected in the left flank with 10^4 4T1. Tumor growth was determined by multiplying the longest diameter of the tumor mass by the perpendicular diameter. Mice were sacrificed when the tumor mass was larger than 150 mm².

Stat6 peptide_C-reactive T cells are depleted when Stat6^{-/-} bone marrow is transferred into WT host

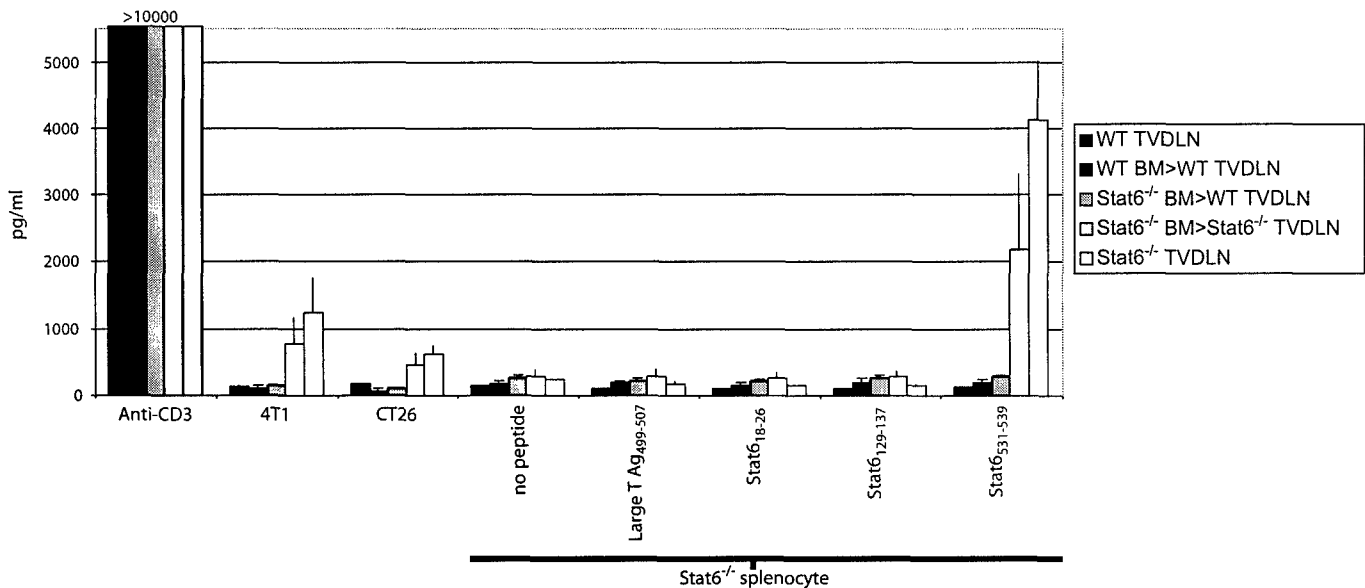


Figure 7. Day 8 TVDLN were harvested from vaccinated mice. These cells were then activated with anti-CD3 (2C11) for 2 days and expanded in CM supplemented with 60 IU/ml IL-2. After activation and expansion, TVDLN were washed and stimulated with either plate bound anti-CD3, 4T1, CT26, or peptide-pulsed Stat6^{-/-} splenocytes. Peptide-pulsed Stat6^{-/-} splenocytes were prepared by pulsing Stat6^{-/-} splenocytes with 10ug of the indicated peptide for 30 min and then washing the splenocytes. T cells (2×10^5 /well) and tumor cells (2×10^5 /well) or splenocytes (2×10^6 /well) were cultured in 2 ml CM in 24 well plates. Supernatants were recovered 6 hours later and assayed in duplicate by ELISA. Data are presented as the mean of two independent experiments ($\pm$ SE).



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REPLY TO
ATTENTION OF:

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28 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information
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2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


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