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AWARD NUMBER DAMD17-94-J-4156

TITLE: Novel Genetherapy for Enhancing Anti-tumor Immunity

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REPORT DATE: November 1998

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. AGENCY USE ONLY *(Leave blank)* | 2. REPORT DATE | 3. REPORT TYPE AND DATES COVERED |
|---|---|---|
| | November 1998 | Final (01 Jul 94 - 30 Jun 98) |

4. TITLE AND SUBTITLE
Novel Genetherapy for Enhancing Anti-tumor Immunity

5. FUNDING NUMBERS
DAMD17-94-J-4156

6. AUTHOR(S)
Sivasubramanian Baskar, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland Baltimore County
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This report describes the immunogenicity of the transfected tumor cells that co-express I-Ad and B7.1 molecules and this was investigated in different ways. Experiments with both 66.1/Ad/B7 and 410.4/Ad/B7 clones demonstrated that while higher levels of I-Ad expression than B7.1 expression induced tumor rejection in 80% of the mice, in the converse situation the tumors were malignant. The double transfectants expressing higher levels of B7.1 molecules than I-Ad molecules, were clearly able to activate allogenic T cells and were susceptible to lysis by allogenic cytotoxic T cells. Their failure to be rejected by the syngeneic host is not understood. Nonetheless, the transfectants expressing both I-Ad and B7.1 molecules induced a complete abrogation of the lung metastasis compared to the transfectants that express either one of these molecules. It seems unlikely that the failure to reject the potentially immunogenic but malignant transfectants is due to polarization towards the development of Th2 type T cells. Finally, the immunogenic double transfectants by themselves, or with additional help from IL-12 co-administration, are able to significantly reduce metastasis from a previously established wild type tumor. This clearly underscores the therapeutic efficacy of the vaccine tumor cells.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
35

16. PRICE CODE

| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | Unlimited |

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Barker 12/18/98

PI - Signature Date

# TABLE OF CONTENTS

## (5) INTRODUCTION:

The identification and characterization of many tumor antigens and the parallel explosion of knowledge in understanding the cellular and molecular mechanisms of antigen recognition by the immune system have given renewed hopes to envisage immunotherapy as a promising modality to treat certain tumors (1-5). Initial trials of immunotherapy of cancer in general, breast cancer in particular, used rather non-specific immunostimulators ranging from Bacillus Calmette-Guerin (BCG) and levamisole to interferon, interleukins and monoclonal antibodies (mAb) (6-8). The purpose of the present study is to genetically modify the tumor cells so as to facilitate their recognition by the host's tumor-specific T lymphocytes and induce efficient and vigorous anti-tumor immunity. Such gene-modified tumor cells can be used as cellular vaccine to treat human cancers.

As known with nominal antigens, it is reasonable to speculate that anti-tumor immune response will involve recognition of the "tumor antigen" by $CD4^+$ helper T cells (Th) and $CD8^+$ cytotoxic T cells (Tc or CTL) in the context of major histocompatibility (MHC) class II and class I molecules, respectively (3-5). Clonal expansion of these T cells and their subsequent functional maturation is governed by cytokines and other accessory molecules present on antigen presenting cells (APC) (9). Some of these cytokines besides being autocrine growth factors, also enhance the cytotoxicity potency of the effector cells (10, 11).

### Cytokines and tumor immunity:

A variety of cytokines including IL-2, IL-4, IL-10, IL-12, TNFa, IFNg and GM-CSF have been used to augment tumor regression *in vivo* (12). In some cases the therapeutically effective concentrations of cytokines are accompanied by toxic side effects. This problem was overcome by cytokine gene therapy, in which tumor cells were transfected with cytokine genes and sufficient amount of cytokine was released at the tumor site with out leading to high systemic levels (13-16). However, the anti-tumor immunity induced was relatively short-lived and the efficacy varied depending on the tumor model under study (17). In some cases a combination of cytokine administration and vaccine tumor cells genetically engineered to express MHC class I, class II and/or costimulatory molecule such as B7-1 has been shown to offer a synergistic effect that was not seen with either procedure alone. Besides, cytokines such as IL-12 and IL-10 have been shown to be antagonistic in promoting the expansion of Th1 and Th2 type helper T cells, respectively. The impact of such regulatory control on the balance between the Th1 and Th2 cells populations and its effect on tumor immunity is not well understood.

**Tumor immunity by expression of MHC class II:**

While $CD8^+$ T cells are the major effector cells in killing the target tumor cells, their proliferation and functional maturation requires help from $CD4^+$ T cells. Since MHC class II gene expression is tightly regulated and restricted to professional APCs, majority of the tumor cells do not express MHC class II proteins and can not stimulate $CD4^+$ T cells. As a consequence, in the absence of $CD4^+$ T cell help, the $CD8^+$ T cells are not sufficiently matured or activated. We and others have shown that constitutive expression of MHC class II genes in tumor cells resulted in rejection of the tumor cells by syngeneic host (18-21). Rejection of class $II^+$ tranfectants resulted in the induction of protective immunity against wild type tumor cells (18-21). These findings strongly suggest that constitutive expression of MHC class II molecules on tumor cells enable them to directly present the tumor peptides to $CD4^+$ tumor-specific Th cells leading to potent anti-tumor immunity (22).

**Costimulation of T cells and tumor immunity:**

Although the engagement of T cell antigen receptor (TCR) with the antigen/MHC complex is a necessary primary signal for T cell activation, maximal activation of T cells requires additional costimulatory signal(s) (23-25). In fact, in the absence of a costimulatory signal T cells have been shown to enter a state of antigen-specific anergy or deletion (26-28). A variety of cell surface molecules have been shown to deliver costimulatory signals. One of the well studied costimulatory molecules is the B7 family of proteins. Two independently regulated gene products have been identified as members of the B7 family, B7-1 (CD80) and B7-2 (CD86) and these proteins do not share any significant sequence homology (29-31). Both B7-1 and B7-2 have been shown to bind two different receptors on T cells namely CD28 and CTLA-4 (32, 33). Upon receiving the antigen-specific and costimulatory signals both $CD4^+$ and $CD8^+$ T cells undergo further differentiation, enhanced proliferation and secrete elevated levels of cytokines (34-36). We and others have shown that constitutive expression of B7-1 and B7-2 molecules on tumor cells resulted in the rejection of these gene-modified tumor cells and prior injection of $B7^+$ tumors induced protective immunity against subsequent wild type tumor challenges in syngeneic hosts (37-39). In addition, a synergistic effect was observed when tumor cells were gene modified to express both MHC class II and B7-1 molecules; these cells are potent vaccine capable of inducing complete regression of previously established tumors (40).

**Tumor metastasis:**

Metastasis of the primary tumor to the various organs in the body of the host poses one of the difficult challenges to cancer treatment modalities. Therefore, one of the goals of the immunotherapy of cancer is to prevent metastasis if the treatment was initiated early enough, eliminate the existing metastatic nodules if the cancer is already disseminated or at least control the proliferation and rate of metastasis when the cancer relapses.

Based on this knowledge, the goal of the present study is to investigate the effect of constitutive expression of MHC class II, costimulatory molecules, B7-1 or B7-2 as well as coexpression of MHC class II and B7-1 molecules in enhancing anti-tumor immunity against murine mammary carcinoma. The previous annual reports have described the investigations on the immunogenicity/tumorigenicity of the mammary tumor cell transfectants expressing either syngenic MHC class II (IA-d) or the B7 molecules (please see Annual Report -1995, -1996 and -1997). This report describes studies performed with the double transfectants that coexpress I-Ad and B7.1 molecules. The immunogenicity of the double transfectants, their metastatic potential and ability to function as therapeutic vaccine are analyzed.

**Tumor Model:**

Murine mammary carcinoma cell lines, 66.1 (metastatic and non-immunogenic) and 410.4 (non-metastatic and weakly immunogenic) were used in the current studies. These tumor cell lines were derived from a parental spontaneous tumor in Balb/c mice (41, 42). They both express normal levels of MHC class I molecules but do not express MHC class II molecules or the costimulatory molecules B7-1 and B7-2. These criteria bring these two tumor cell lines closer to human breast cancer and serve as a model system to study.

The wild type tumor cell lines 410.4/WT and 66.1/WT were transfected with plasmid vectors containing cDNA encoding murine B7-1, B7-2 proteins or cDNAs encoding syngeneic MHC class II alpha and beta polypeptides which form the I-$A^d$ molecule. For the derivation of tumor cells expressing any one of the proteins, cotransfection with plasmid containing neomycin resistance gene was performed in order to allow selection of transfectants from the wild type tumor cells. For the derivation of tumor cells expressing both MHC class II and B7-1, 410.4/Ad and 66.1/Ad clones were transfected with plasmid containing murine B7-1 cDNA and another plasmid containing cDNA for zeocin resistant gene. All transfections were performed using Lipofectine or Lipofectamine as previously described (18, 39). Transfectants were grown in medium containing predetermined concentrations of the selection drug(s) G418 or G418 plus zeocin, depending on the

transfectants. Surface expression of B7-1, B7-2 and I-$A^d$ proteins was analyzed by flow cytometry using appropriate primary mAb against the particular protein and a fluorescine isothyocyanate (FITC) conjugated secondary antibody. Stably transfected tumor cell lines were established, and clones were generated by limiting dilution. Cloned transfected tumor cell lines were periodically monitored for the surface expression of the protein(s). The tumorigenicity of the transfected tumor cells was determined by in vivo tumor challenge experiments using syngeneic Balb/c mice.

**(6) BODY: Experimental Results**

**Co-expression of MHC class II and B7.1:**

Previous reports documented that both 66.1 and 410.4 transfectants expressing either B7 or I-Ad molecules resulted in a delayed tumor growth compared to the progressive growth of wild type tumor cells in naive Balb/c mice (Annual Reports 1995, 1996 and 1997). A small fraction of mice exhibited complete rejection of the "immunogenic" transfected tumor cells. In all these situations, it was suggested that the transfectants are able to promarily activate the CD4+ helper T cells or the CD8+ effector T cells depending upon whether they express I-Ad molecules or B7 molecules. Therefore, it was envisaged that co-expression of IA-d and B7 molecules will provide an opportunity to simultaneously stimulate both CD4+ and CD+8 T cells leading to a more aggressive anti-tumor immunity. Our previous study in the sarcoma tumor model supports this contention in that tumor cells co-expressing MHC class II and B7 molecules constituted a better vaccine (40).

Towards this goal, clones of 410.4/Ad and 66.1/Ad transfectants were used to super transfect the B7-1 gene. Since there was no significant difference between the B7.1 and B7.2 expressing tumor cells (Annual Reports 1996, 1997), only co-expression of I-Ad and B7.1 molecules was performed. In the previous report (Annual Report 1997) some of the 410.4/Ad/B7.1 clones and 66.1/Ad/B7.1 clones were described. Some of these clones lost the I-Ad and/or the B7.1 molecule rapidly. Therefore, additional clones were derived (in some cases recloning was performed) to obtain stable double transfectants, i.e.,410.4/Ad/B7 and 66.1/Ad/B7 clones. However, the double transfectants of both sub lines expressed stable but different levels of I-Ad and B7 molecules. For example, Figure 1 shows the flow analysis of six stable 410.4/Ad/B7 clones. All of them constitutively expressed I-Ad protein on the cell surface, and uniformly expressed much higher levels of B7.1 protein. The transfectants derived from 66.1 cell line were of three different types (Figure 2): a) clones that expressed relatively more I-Ad than B7.1 molecules (e.g. clones A4, B4), b) clones that expressed more of B7.1 than I-Ad molecules (e.g.

clones 35 and 49), and c) clones that expressed approximately the same amounts of I-Ad and B7.1 molecules (e.g. clone A3). These phenotypes were maintained, under drug selection, for a long period of time.

The immunogenicity of the tumor cells co-expressing MHC class II and B7-1 molecules have been studied by injecting the transfectants into syngeneic Balb/c mice and following the tumor growth in vivo. The anticipation was that, because these transfectants could potentially activate both CD4+ T cells and CD8+ T cells, they will induce a strong anti-tumor immunity resulting in tumor rejection. In addition, experiments have been performed to study the metastatic potential of the tumor cells expressing both MHC class II and B7-1. Furthermore, these transfectants have been used in therapeutic experiments to treat pre-existing tumors. All these studies are described below.

**Immunogenicity of transfectants co-expressing MHC class II and B7.1:**

To test the effect of constitutive co-expression of syngeneic MHC class II (I-Ad) and B7.1 molecules on the growth/rejection of 410.4 tumor cells, groups of naive Balb/c mice were injected s.c. with 410.4/Ad/B7 transfectants (clones 12, 31 and 41). The control group of mice received 410.4/Neo transfectants. The growth of 410.4/Neo transfectants was not significantly different from that of 410.4/wt tumor cells as observed in earlier experiments (Annual Report 1996). This indicates that the expression of Neomycin resistant gene alone did not influence the growth of these tumor cells in vivo. All the 410.4/Ad/B7 clones tested grew progressively similar to the control transfectants (Table 1). All these clones had much higher levels of B7.1 molecules than I-Ad molecules on the surface at the time of injection into mice. There was no significant change in the expression of these molecules when these clones were cultured in vitro without the addition of selective drugs (data not shown). Whether the same was true during in vivo growth (in the absence of selective drugs) was not determined. Nonetheless, it was surprising to see that the delayed growth pattern and occasional rejection observed with the single transfectants (either I-Ad or B7) (Annual Reports 1996, 1997) has been compromised in these (410.4/Ad/B7) double transfectants co-expressing both the molecules. It is unclear whether or not the relatively high levels of B7.1 expression (overexpression) caused this negative effect. This will be discussed further below.

Similar experiments were carried out with the 66.1/Ad/B7 clones. The 66.1/wt and 66.1/Neo control transfectants exhibited progressive growth as observed earlier (Annual Reports 1996, 1997). The 66.1/Ad/B7 transfectants exhibited different growth properties in vivo (Table 2). Clones A4 and B4, both expressing comparable levels of I-Ad and B7.1, were immunogenic

in that 80% of the mice challenged s.c. with live tumor cells completely rejected the tumors. In the remaining 20% of mice there was a substantial delay in the growth of the tumors, and the tumors remained small (<5 mm) even after 90 days. By contrast, in the control group 100% of mice challenged with 66.1/Wt cells showed progressive growth reaching >1.5 cm in about 60 days. The clones A4 and B4 grew progressively in Balb/c nude mice, suggesting that functional T cells are required to mediate the rejection of these tumor cells observed in the immunocompetant Balb/c mice. On the other hand, two additional 66.1/Ad/B7 clones (35 and 49) after s.c. injection in to naive Balb/c mice, after an initial delay, progressively grew in100% of the mice (Figure 3). Interestingly, these two clones had significant and stable expression of both I-Ad and B7.1 molecules, although the pattern of expression was converse to that seen in the immunogenic clones, A4 and B4 (Figure 2).

**Immunogenicity of double transfectants: Relationship between I-Ad and B7.1 expression:**

Based on the results obtained with 66.1/Ad/B7 and 410.4/Ad/B7 clones, it is suggested that greater levels of I-Ad expression correlates with tumor immunogenicity and rejection by the syngeneic host (Table 3). It is conceivable that expression of high levels of I-Ad would facilitate increased presentation of potentially immunogenic tumor antigenic peptide(s) resulting in the induction of tumor immunity. The provision of B7.1-mediated costimulation seems to be critical because in the absence of B7.1, higher levels of I-Ad alone did not result in significant rejection (Annual Reports 1996 and 1997). Moderate levels of B7.1 expression is sufficient to provide enough costimulatory activity. On the other hand, higher levels of B7.1 expression seem to deliver a negative effect as the tumor cells (410.4/Ad/B7 clones and 66.1/Ad/B7 clones 35, 49) are not rejected even though they expressed moderate amounts of I-Ad. The two receptors, CD28 and CTLA-4 on T cells, both of which can bind B7.1, have been shown to have opposing effects in terms of T cell activation. Studies using anti-CTLA-4 antibody have described that blocking the negative regulation mediated by CTLA-4 engagement could promote tumor immunity (). It is not clear whether expression of high levels of B7.1 by the transfected tumor cells has a dampening effect through the CTLA-4 pathway. Nonetheless, it is likely that the quantitative difference between the molecules that have stimulatory and inhibitory effects (I-Ad and B7.1, respectively) would have an impact on the outcome of the tumor cell vaccine.

**MHC class I expression in the transfected tumor cells:**

The level of MHC class I proteins on the surface of the target tumor cells (e.g. Kd and Dd molecules in the case of Balb/c-derived tumors) is critical both in the CTL induction phase and the effector phase (susceptibility to lysis) of the anti-tumor immune response. To address this issue, flow cytometric analysis was performed to determine the relative levels of expression of Kd and Dd molecules in the wild type (66.1/Wt) and transfected (66.1/Ad/B7) tumor cells (Figure 4). First, the level of Kd molecules expressed was almost identical to the level of Dd molecules expressed by any of the tumor cells tested. Second, the levels of Kd and Dd expression in clones A4 and B4 were similar to that observed in the wild type tumor cells. Third, clones 35 and 49 showed relatively reduced levels of Kd and Dd expression. It is likely that this is just a clonal variation. However, this observation opened another possibility that the clones 35 and 49 are not immunogenic because they may not induce a good CTL activity and/or may not serve as good targets.

Two sets of experiments were carried out to analyze this possibility. First, the ability of transfected tumor cells to activate T cells was tested using a standard primary allogenic mixed lymphocyte response (MLR) in vitro. Splenic T cells from an allogenic mice (A/J) were used as responders and growth arrested 66.1-derived tumor cells as stimulators. A number of different tumor cell clones were tested at different stimulator/responder ratios, and the results from a representative experiment are depicted in Figure 5. The primary allogenic MLR stimulated by the 66.1/Ad/B7 clones was equal to or better than that stimulated by the 66.1/wt tumor cells. This enhancement may be in part due to the B7.1-mediated costimulation. Interestingly, at the optimal stimulator/responder ratio, there was no significant difference in the ability to induce the MLR response between the double transfectants, clones B4, 35 and 49. To further substantiate this in vitro observation, additional in vivo tumor rejection experiments were carried out using the same allogenic set up (Table 4). As observed before, the 66.1/Ad/B7 clones 35 and 49 (unlike the clone B4) grew progressively in syngenic naive Balb/c mice. However, both clones (35 and 49) were readily rejected by the allogenic host, A/J mice. The Balb/c mice challenged with 66.1/wt tumor cells showed numerous metastatic nodules in lungs, whereas there were no or a few lung mets following challenge with 66.1/Ad/B7 clones in Balb/c mice. The lungs of the A/J mice were devoid of any lung mets. The metastatic abilities of the transfected tumor cells will be discussed further below.

The experiments described above demonstrate that the clones 35 and 49 are, at least, able to activate allogenic CTL and are not inherently resistant to lysis by the allogenic CTL. Further

experiments are required to find out other regulatory mechanisms that are involved which prevent the rejection of the B7 overexpressing transfectants by the syngenic host.

**Is there a Th2 cell bias during immunization with double transfectants?**

Activation of T cells both in vitro and in vivo, has been shown to be influenced by the cytokines present in the milieu. For example, interleukin 4 (IL-4) has been shown to promote activation of Th2 T cells whereas IL-12 facilitates the activation of Th1 T cells. A number of studies have shown that preferential activation of Th1 T cells helps generate a good CTL response and anti-tumor immunity. Administration of neutralizing anti-IL-4 antibody has been shown to promote the activation of Th1 T cells and confer protection from parasitic infections (), and elicit anti-tumor immunity in a renal carcinoma model (). Based on these reports, two parallel experiments were performed to study the influence of IL-4 on the ensuing anti-tumor immune response.

First, Balb/c mice received either the diluent (PBS) or anti-IL-4 antibody and were challenged with the 410.4/Ad/B7 tumor cells. Both groups of mice exhibited similar pattern of tumor growth (Figure 6). Next, IL-4 gene knock out mice (IL-4 -/-) were used to confirm the above results. The growth of 66.1/Ad and 66.1/Ad/B7 tumor cells in IL-4 -/- mice was compared to their growth in IL-4 +/+ mice (Figure 7). As observed before, both 66.1/Ad and 66.1/Ad/B7 tumor cells exhibited delayed growth/rejection compared to the control group which received 66.1/Neo tumor cells in the IL-4 +/+ mice (Annual Report 1997 and the present report). Contrary to the expectation, there was no advantage in tumor rejection in the IL-4 -/- mice. In fact, more mice were succumbed to tumor growth in the IL-4 -/- groups (panels C and E) than the corresponding control groups (panels B and D). This suggests that IL-4 may not have a negative effect in the induction of anti-tumor immunity in this tumor model.

**Co-expression of I-Ad and B7.1 molecules abrogates spontaneous metastasis.**

Next, it was of interest to investigate whether the expression of I-Ad and/or B7.1 molecules will influence the metastatic properties of these tumor cells. Groups of naive Balb/c mice were challenged s.c. with either1 x 10e5 or 3 x 10e5 different 66.1-derived tumor cells. Mice were sacrificed when the tumor size reached about 2 cm or when the tumors begin to ulcerate and the number of metastatic nodules in lungs were enumerated (see Methods, Figure 8). Mice challenged with 66.1/wt and 66.1/Neo tumor cells, at both doses, had numerous metastatic nodules in lungs indicating spontaneous metastasis from the primary injection site. The

mice challenged with 66.1/B7 tumor cells (clone ) showed similar numbers of lung mets as the wild type cells, although there was a moderate reduction with a lower challenge dose. The mice challenged with 66.1/Ad trasfectants showed significantly reduced lung mets with both challenge doses. By contrast, mice challenged with 66.1/Ad/B7 cells (clone B4) had no visible lung mets. It is unlikely that there could have been micromets which may be difficult to detect because some of these mice were examined several months after the primary challenge and did not show any lung mets. Interestingly, even the 66.1/Ad/B7 clones that progressively grew (clones 35 and 49) showed significant reduction in the number of lung mets (0-10 mets/mouse) compared to the 66.1/wt tumor cells (>200 mets/mouse). These results show that co-expression of I-Ad and B7.1 molecule on tumor cells not only resulted in complete rejection (clone B4) but also abrogation of spontaneous metastasis. This superior immunogenicity of these double transfectants prompted the use of these cells as vaccine to treat pre-established wild type tumors in a therapeutic set up.

**Treatment of pre-established tumors:**

The ultimate goal of all cancer treatment modalities is to cure or at least control the malignant disease. Towards this aim, the therapeutic efficacy of the double transfectants was tested as follows. Balb/c mice were challenged s.c. with wild type tumor cells. After the tumor has established, the mice were subjected to different treatment strategies (Table 5). The growth of the primary tumor at the site of injection was not significantly altered by the different treatment procedures, although in certain groups the growth was retarded. However, there was a significant difference in the metastasis to lungs. The untreated mice or mice treated with irradiated wild type tumor cells showed numerous lung mets (100-200 or more) and also become moribund within 30-45 days. By contrast, treatment with the immunogenic 66.1/Ad/B7 clone B4 significantly reduced the number of lung mets. These mice were healthy and did not become moribund. They had relatively smaller primary tumors and begin to ulcerate in about 90 days at which time they were sacrificed. Similar treatment with the sister clone (35) also showed a moderate reduction in metastasis. With 410.4 sub line, the treatment was performed with a mixture of 410.4/Ad/B7 clones, and there was a moderate reduction in the lung mets. Similar results were also obtained when treated with IL-12 alone. However, when both 410.4/Ad/B7 cells and IL-12 were given as treatment there was a significant reduction in the number of lung mets.

Another set of experiments was carried out to substantiate the therapeutic efficacy. In this approach, the single transfectants were mixed and used as vaccine. Figure 9 shows the results of the experiment. Balb/c mice bearing pre-established 66.1/wt tumor cells were either untreated or treated with a mixture of 66.1/Ad plus 66.1/B7.1 tumor cells, IL-12 alone or a combined treatment

with the mixture of 66.1/Ad plus 66.1/B7 tumor cells and IL-12. Only the combined treatment group showed significant reduction in the metastatic load.

Together, these results established that the transfected tumor cells co-expressing I-Ad and B7.1 molecules are certainly capable of functioning as potent vaccine and induce a strong anti-tumor immunity necessary for a therapeutic application. IL-12 provides a synergistic effect and enhances the efficacy of the vaccine particularly when single transfectants were used.

## (7) CONCLUSIONS:

The results presented in this report demonstrate that co-expression of I-Ad and B7.1 molecules on tumor cells has useful advantages from the vaccine standpoint compared to the expression of either molecules alone. However, there is an important quantitative aspect relating to the levels of expression of I-Ad and B7.1 molecules and the vaccine efficacy. There seems to be a threshold amounts of I-Ad molecules required in order to sufficiently present the tumor antigenic peptides to activate the CD4+ T cells. One could presume that the more I-Ad/peptide complexes are generated on the vaccine tumor cell surface, the better will be the induction of helper T cells. On the other hand, similar quantitative relationship seems to apply, in a converse manner, for the costimulatory signal provided by the B7.1 molecule. While B7.1 expression on the vaccine tumor cells is certainly required to induce tumor immunity (), over expression of B7.1 may impart a negative effect and facilitate tumor progression. A recent study described that CTLA-4, unlike CD28 also a co-receptor for B7.1, delivers a negative signal which inhibits T cell activation resulting in tumor growth. This inhibitory effect was overcome by administration of anti-CTLA-4 antibodies. Further experiments are required to understand whether B7.1 over expression by the transfectants facilitates the CTLA-4-mediated inactivation pathway.

It is not clear whether or not the observed differences in the endogenously encoded MHC class I expression (Kd and Dd) on the double transfectants play a role in the rejection of the tumors by the syngenic host. There was no significant difference in the ability of the transfectants expressing relatively low versus high levels of Kd/Dd molecules in stimulating an allogenic MLR response in vitro, and they were equally susceptible to lysis by the allogenic CTL in vivo. However, it is hard to compare the allogenic tumor rejection with the syngenic tumor rejection because the CTL precursor frequency in the former is expected to be much higher. Nonetheless, it is clearly evident that the transfectants expressing I-Ad and B7.1 are superior in stimulating the allogenic T cells than their wild type counterparts.

With the reference to the role of cytokines in modulating the Th1 versus Th2 responses, IL-4 does not seem to play a significant role in this tumor model. Both the treatment with neutralizing anti-IL-4 antibody and the use of IL-4 gene knock out mice supports this contention. On the other hand, IL-12, the cytokine known to promote Th1 function, certainly has a positive impact on the induction of anti-tumor immunity. This is particularly evident in the therapeutic situation where fast activation of T cells is required.

Another important finding is that the co-expression of I-Ad and B7.1 molecules, even though does not always reduce the growth of the primary tumor, it consistently and completely abrogates the spontaneous metastasis from the primary injection site. In addition, these transfectants are clearly a superior vaccine in significantly reducing the metastasis from a pre-existing wild type tumor.

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## FIGURE LEGENDS

**Figure 1:**

Flow cytometric analysis of six 401.4/Ad/B7 representative clones: The tumor cells were stained with a mouse mAb against murine I-$A^d$ (MKD6) and a rat mAb against murine B7.1 (1G10). Appropriate FITC conjugates were used as secondary antibodies. In each histogram, the solid lines on the left depict staining with the FITC conjugated antibodies alone. Transfection control cells (410.4/Neo and 410/Neo/Zeo) did not show any positive staining with the anti-I-Ad and anti-B7.1 antibodies (data not shown).

**Figure 2:**

Flow cytometric analysis of five 66.1/Ad/B7 representative clones: The tumor cells were stained with a rat mAb against murine I-$A^d$ (M5.114) and a rat mAb against murine B7.1 (1G10). FITC conjugated mouse anti-rat Ig was used as a secondary antibody for both I-Ad and B7.1 staining. In each histogram, the thin solid line depicts staining with the FITC conjugated antibody alone, the thick solid line depicts staining for I-Ad and the dotted line depicts staining for B7.1. Transfection control cells (66.1/Neo and 66.1/Neo/Zeo) did not show any positive staining with the anti-I-Ad and anti-B7.1 antibodies (data not shown for the latter).

**Figure 3:**

Tumorigenicity of 661./Ad/B7 positive tumor cells (clones 35 and 49): Naive Balb/c mice received 3 x 10e5 (e = exponential) live tumor cells s.c. on day 0. Tumor growth was monitored as described in the before (Annual Report 1995). Each line represents an individual mouse.

**Figure 4:**

Flow cytometric analysis of MHC class I expression: The 66.1/Neo and 66.1/Ad/B7 (clones A4, B4, 35 and 49) tumor cells were stained with FITC conjugated goat anti-mouse Ig alone (thin solid line). Alternatively these tumor cells were stained with mouse anti-Kd mAb, 31-3-4S (thick solid line) or mouse anti-Dd mAb, 34-5-8S (thin dotted line) in combination with the FITC conjugate.

**Figure 5:**

Primary mixed lymphocyte response (MLR): Nylon wool non-adherent spleen cells from A/J mice were used as responders (6 x 10e5 cells/well). The tumor cells (66.1/wt, 66.1/Ad/B7 clones B4, 35 and 49) were growth arrested by treatment with mitomycin C (50 ug/ml) and used as stimulators. The indicated numbers of stimulators were used. After 5 days, the cultures were

pulsed with 3H-Thymidine (1 uCi/well) and the radioactivity incorporated (cpm) was measured using a beta liquid scintillation counter.

**Figure 6:**

In vivo treatment with anti-IL-4 antibody: Balb/c mice were given three weekly i.p. injections of anti-IL-4 antibody (1 mg/injection, purified 11B11 antibody). The antibody treatment was from day –7 through day 21 (panel B). The control group received PBS (panel A). On day 0, the 410.4/Ad/B7 tumor cells (3 x 10e5/mouse) were injected s.c. and the tumor growth was monitored.

**Figure 7:**

Tumor growth in IL-4 knock out mice: Naive wild type (IL-4 +/+) Balb/c challenged s.c. with 1 x 10e5 live tumor cells, 66.1/Neo (panel A), 66.1/Ad (panel B) or 66.1/Ad/B7 (panel D). IL-4 gene knock out
(IL-4 -/-) mice were obtained from the Jackson Labs, Bar Harbor.
The IL-4-/- mice were challenged s.c. with 1 x 10e5 the 66.1/Ad (panel C) or 66.1/Ad/B7 (panel E) tumor cells. The tumor growth was monitored.

**Figure 8:**

Co-expression of I-Ad and B7.1 abrogates spontaneous metastasis: Groups of Balb/c mice were challenged s.c. with either 1 x 10e5 or 3 x 10e5 live tumor cells indicated in the y axis. The growth of the primary at the injection site was monitored. Mice were sacrificed when the primary tumor begin ulcerate or when the mice become moribund. Majority of the mice that received 66.1/Ad/B7 (clone B4) did not develop primary tumors. Therefore, these mice were sacrificed at $\geq$60 days. The number of mets in lungs were too many (except the 66.1/Ad/B7 group) and was often difficult to accurately count. Therefore, the approximate number of metastatic nodules in lungs is depicted and should be considered only with the relative differences.

**Figure 9:**

IL-12 enhances the therapeutic potential: Naïve Balb/c mice received s.c. 3 x 10e5 live 66.1/wt tumor cells on day 0. Twenty days later they were treated with either three weekly i.p. injections (1 ug/injection) of murine recombinant IL-12, weekly i.p. injection of 2 x 10e6 irradiated transfectants (a 1:1 mixture of 66.1/Ad and 66.1/B7) or both IL-12 and transfectants. The treatments were continued for 4 – 6 weeks. The control group did not receive any treatment following 66.1/wt challenge. Mice were sacrificed and the lung metastasis was measured as mentioned in Figure 8.

Table 1

B7 high double transfectants are not rejected

| Tumor | Challenge dose | Tumor Incidence |
|---|---|---|
| 410.4/Neo | 1 x 10e5 | 5/5 |
| 410.4/Ad/B7 | | |
| clone 12 | 3 x 10e5 | 5/5 |
| clone 31 | 1 x 10e5 | 5/5 |
| clone 41 | 1 x 10e5 | 5/5 |
| Mixture | 1 x 10e5 | 5/5 |

Groups of Balb/c mice were challenged s.c. with the indicated numbers of individual 410.4/Ad/B7 transfectants or a mixture of 410.4/Ad/B7 transfectants (clones 12, 14, 19, 25 and 31). The control group was challenged with 410.4/Neo cells. The tumor growth was measured as described in previously.

## Table 2

### I-Ad high double transfectants are immunogenic

| Tumor Challenge | Mice | Tumor Incidence | Lung Metastasis |
|---|---|---|---|
| 66.1/Neo | Balb/c | 5/5 | >200 |
| 66.1/Ad/B7(B4) | Balb/c | 2/10* | 0 |
| 66.1/Ad/B7(A4) | Balb/c | 2/10* | 0 |
| 66.1/Ad/B7(A4) (s.c.) | Nude | 2/2 | 100-200 |
| 66.1/Ad/B7(B4) (s.c.) | Nude | 3/3 | 100-200 |

Mice were challenged s.c. with live tumor cells (1 x 10e5). The tumor growth and metastasis into lungs were monitored as described previously. * These mice showed a small tumor (< 5 mm) compared to the Balb/c nude mice which had large tumors (>1.5 cm) at the time of sacrifice.

**Table 3**

**Immunogenicity of double transfectants: Relationship between B7.1 and MHC class I and class II expressions**

| Tumor | Response in vivo[a] | Mean fluorescence intensity[b] I-Ad | B7.1 | Kd | Dd |
|---|---|---|---|---|---|
| 66.1/wt | Malignant | 0 | 0.1 | 73 | 56 |
| 66.1/Neo | Malignant | 0 | 0 | 44 | 10 |
| 66.1/Ad/B7 | | | | | |
| clone A3 | Malignant | 9 | 10 | ND | ND |
| clone A1 | Rejected | 134 | 8 | 65 | 63 |
| clone A4 | Rejected | 53 | 4 | 21 | 16 |
| clone B4 | Rejected | 111 | 7 | 80 | 67 |
| clone 35 | Malignant | 5 | 45 | 24 | 19 |
| clone 49 | Malignant | 6 | 61 | 15 | 12 |
| 410.4/wt | Malignant | 0 | 0 | 51 | 48 |
| 410.4/Ad/B7 | | | | | |
| clone 12 | Malignant | 5 | 16 | 35 | 28 |
| clone 14 | Malignant | 4 | 41 | ND | ND |
| clone 19 | Malignant | 8 | 38 | ND | ND |
| clone 25 | Malignant | 10 | 55 | ND | ND |
| clone 31 | Malignant | 6 | 67 | ND | ND |
| clone 41 | Malignant | 7 | 130 | ND | ND |

[a] The cumulative data from a number of different experiments are represented here.

[b] The mean fluorescence intensity is derived by subtracting the values obtained with the appropriate FITC conjugate alone.

Table 4

Low I-Ad and high B7.1 expressing double transfectants are rejected by an allogenic host

| Tumor | Host | Tumor Incidence | Lung Metastasis |
|---|---|---|---|
| 66.1/wt | Balb/c | 5/5 | >200 |
| 66.1/Ad/B7(B4) | Balb/c | 0/5 | 0 |
| 66.1/Ad/B7(35) | Balb/c | 5/5 | 10-50 |
| 66.1/Ad/B7(49) | Balb/c | 3/3 | 0-10 |
| 66.1/Ad/B7(35) | A/J | 0/5 | 0 |
| 66.1/Ad/B7(49) | A/J | 0/4 | 0 |

Balb/c and A/J mice were challenged s.c. with 2 x 10e5 indicated tumor cells. The tumor growth and metastasis into lungs were monitored as described previously.

Table 5

Treatment of Pre-established Tumors

| Primary Tumor Challenge[a] | Treatment[b] | Primary Tumor Growth | Lung Metastasis |
|---|---|---|---|
| 66.1/wt | None | 5/5 | >200 |
| 66.1/wt | 66.1/wt | 5/5 | >200 |
| 66.1/wt | 66.1/Ad/B7(B4) | 5/5 | 0-10 |
| 66.1/wt | 66.1/Ad/B7(35) | 5/5 | 50-100 |
| 410.4/wt | None | 15/15 | 100-200 |
| 410.4/wt | 410.4/Ad/B7 | 16/16 | 50-100 |
| 410.4/wt | IL-12 | 5/5 | 50-100 |
| 410.4/wt | 410.4/Ad/B7 + IL-12 | 5/5 | 0-10 |

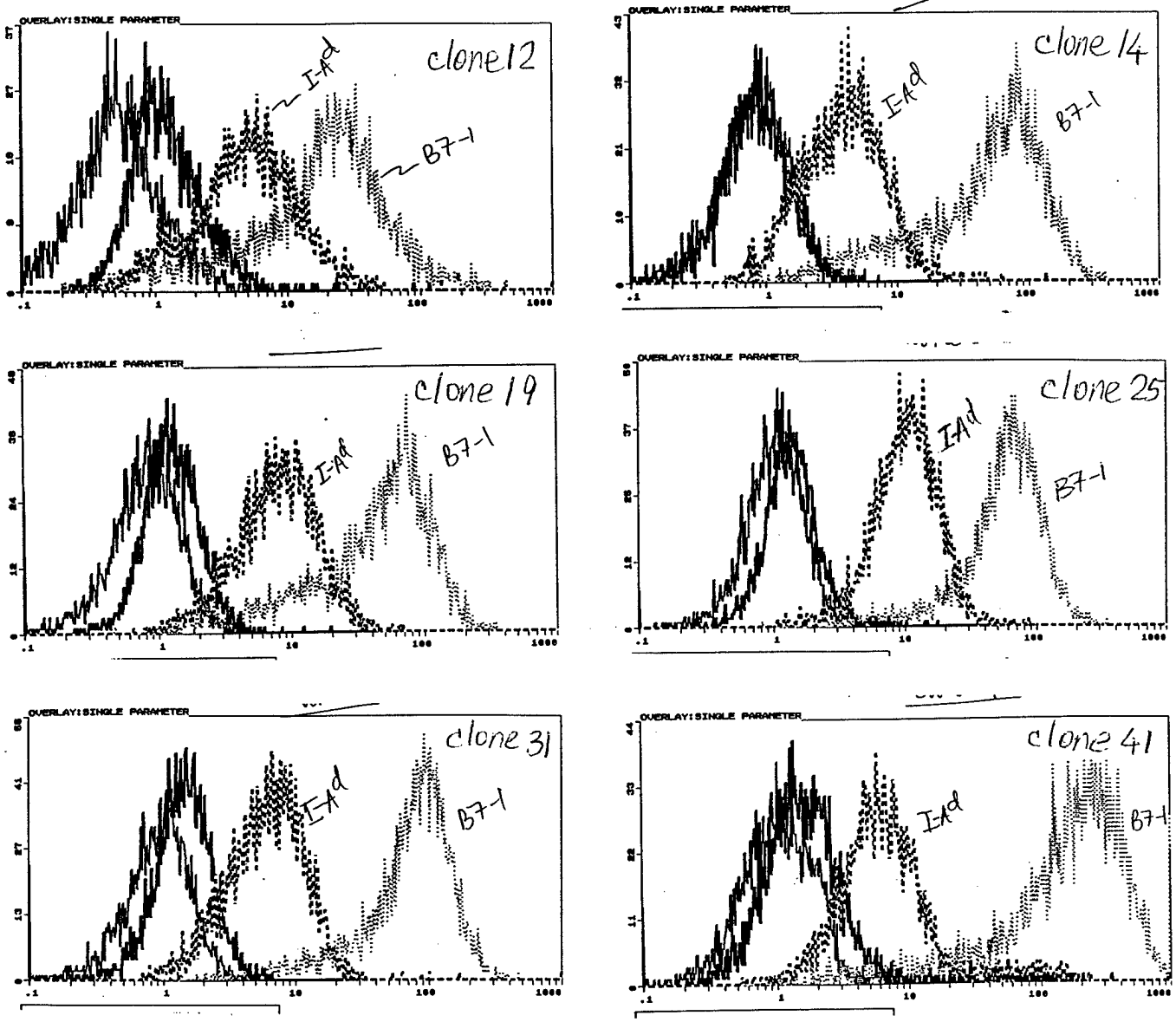
[a] Balb/c mice were challenged s.c. with 1-3 x 10e5 live indicated tumor cells.

[b] Twenty days later, they were either left untreated or treated i.p. with 4-6 weekly injections of irradiated tumor cells (2 x 10e6 per injection), IL-12 (1 ug per injection) or both.

The tumor growth at the primary injection site and metastasis into lungs were assessed at the time of sacrifice.

# Figure 1

410.4/Ad/B7.1 clones



# Figure 2

66.1/$A^d$/B7.1 clones

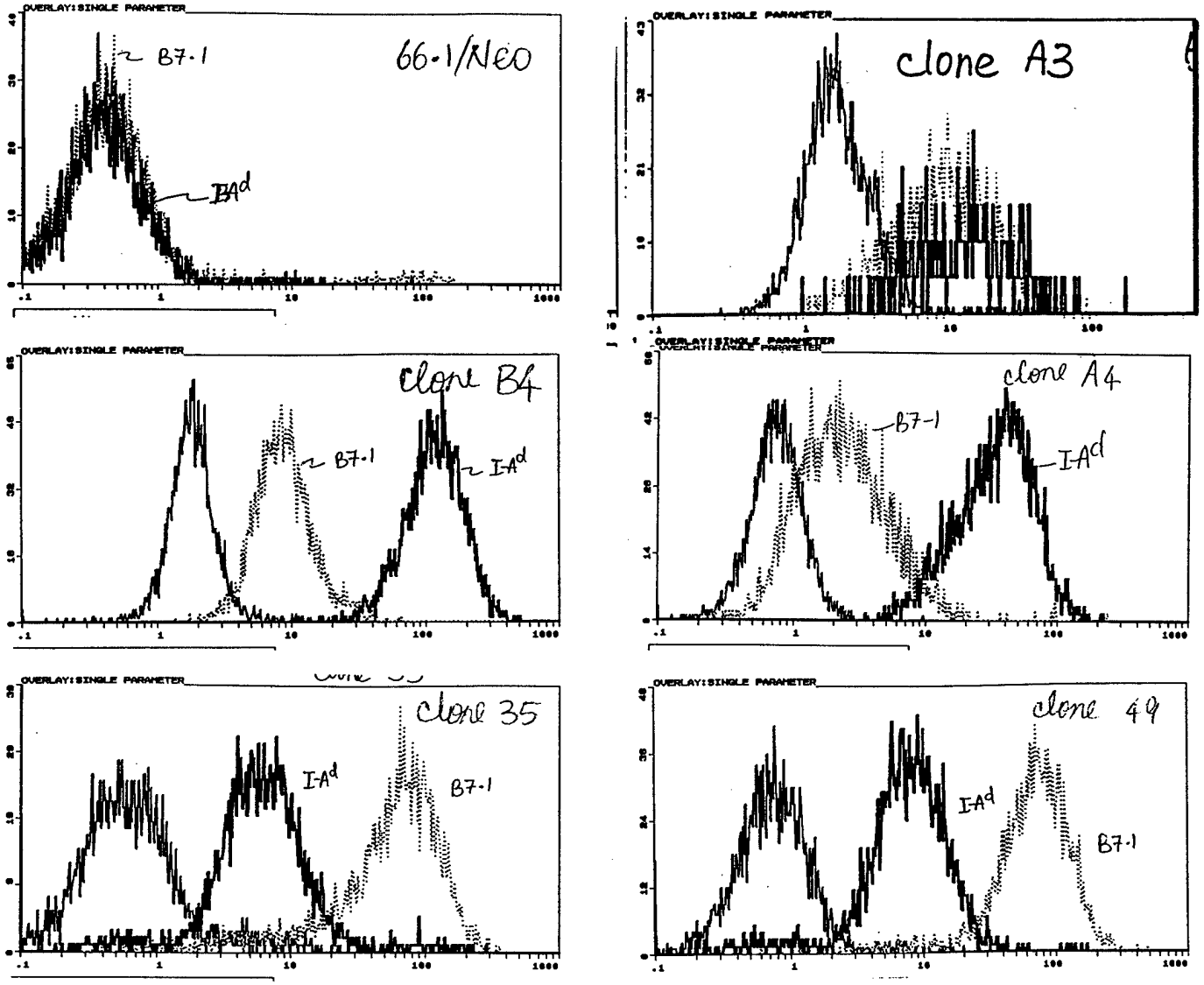
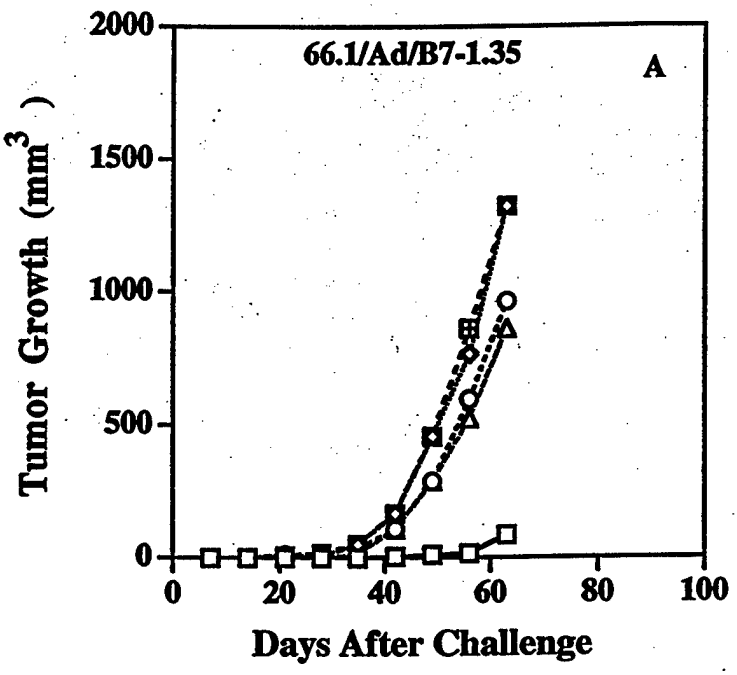
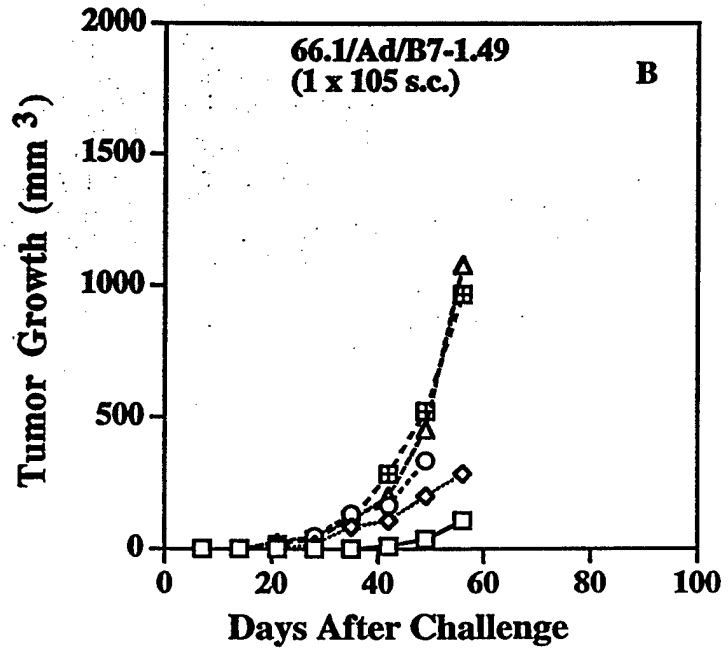


Figure 3


66.1/Ad/B7-1.35
A
Tumor Growth (mm3 )
2000
1500
1000
500
0
0
20
40
60
80
100
Days After Challenge


66.1/Ad/B7-1.49
(1 x 105 s.c.)
B
Tumor Growth (mm 3)
2000
1500
1000
500
0
0
20
40
60
80
100
Days After Challenge

# Figure 4

66.1/Ad/B7.1 clones MHC I

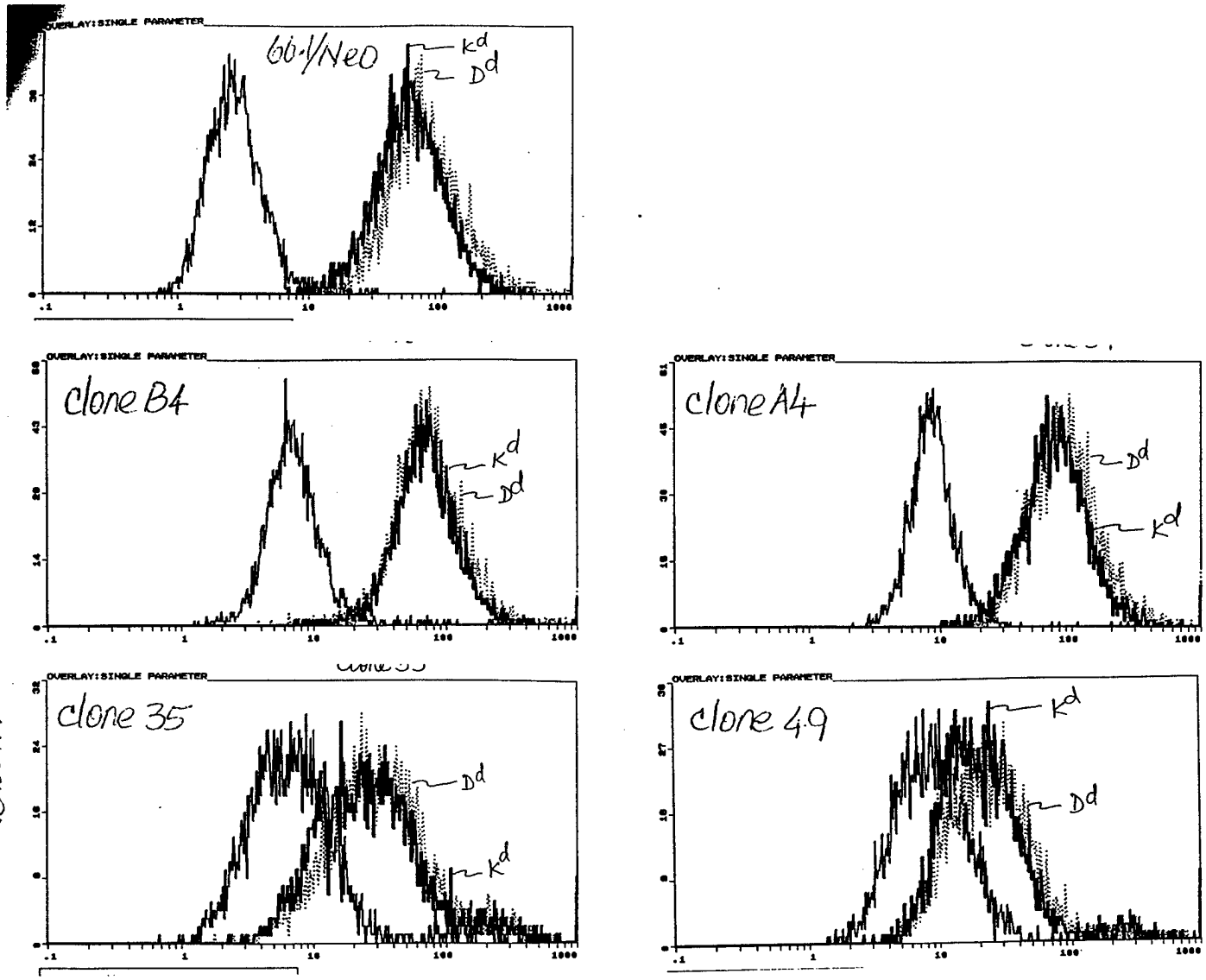


Figure 5

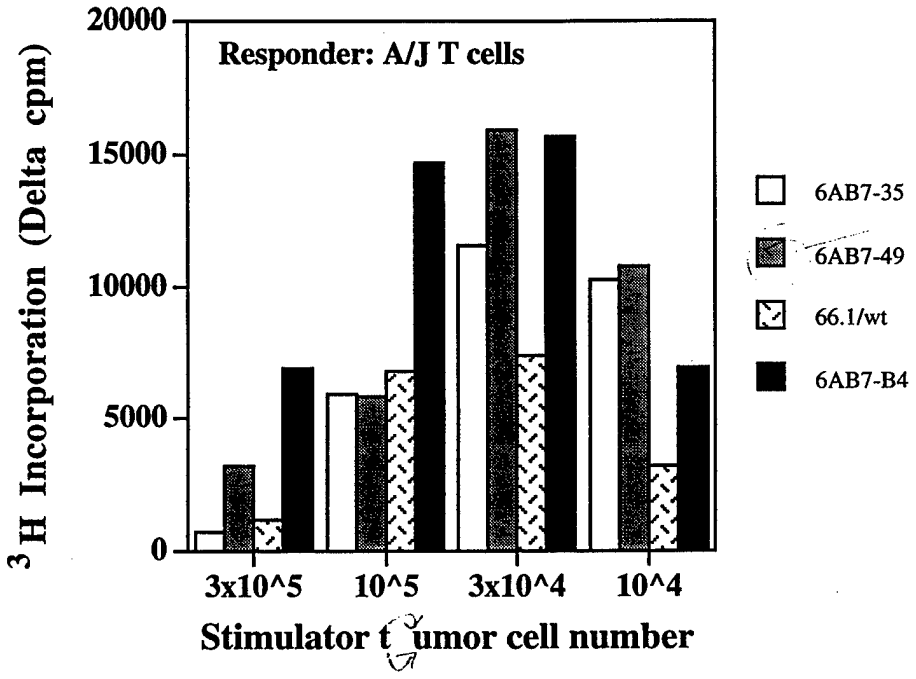
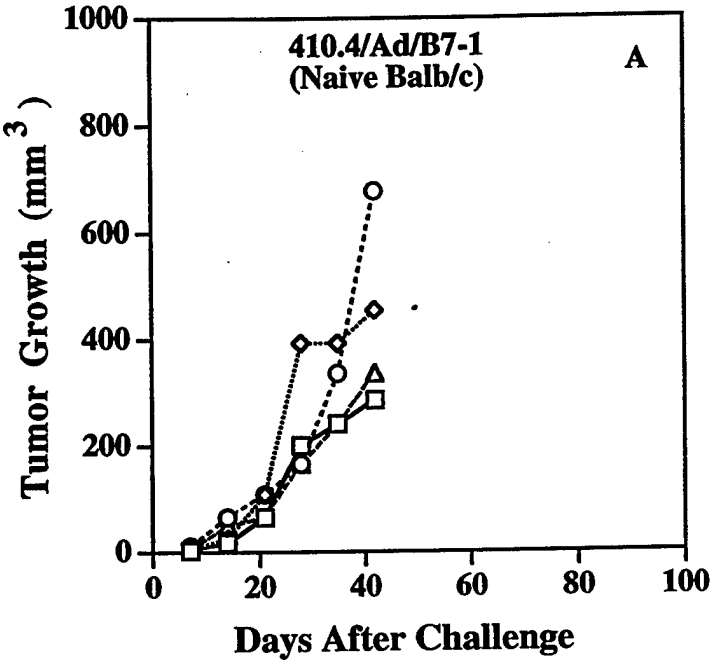

Responder: A/J T cells
20000
15000
10000
5000
0
$^3$H Incorporation (Delta cpm)
3x10^5
10^5
3x10^4
10^4
Stimulator tumor cell number
6AB7-35
6AB7-49
66.1/wt
6AB7-B4

Figure 6


410.4/Ad/B7-1
(Naive Balb/c)
A
Tumor Growth (mm 3 )
1000
800
600
400
200
0
0
20
40
60
80
100
Days After Challenge

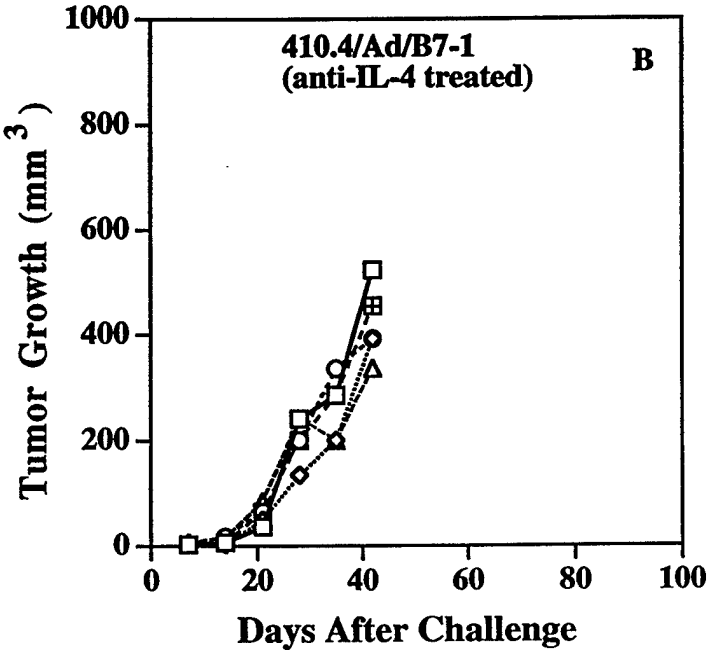

410.4/Ad/B7-1
(anti-IL-4 treated)
B
Tumor Growth (mm 3 )
1000
800
600
400
200
0
0
20
40
60
80
100
Days After Challenge

Figure 7

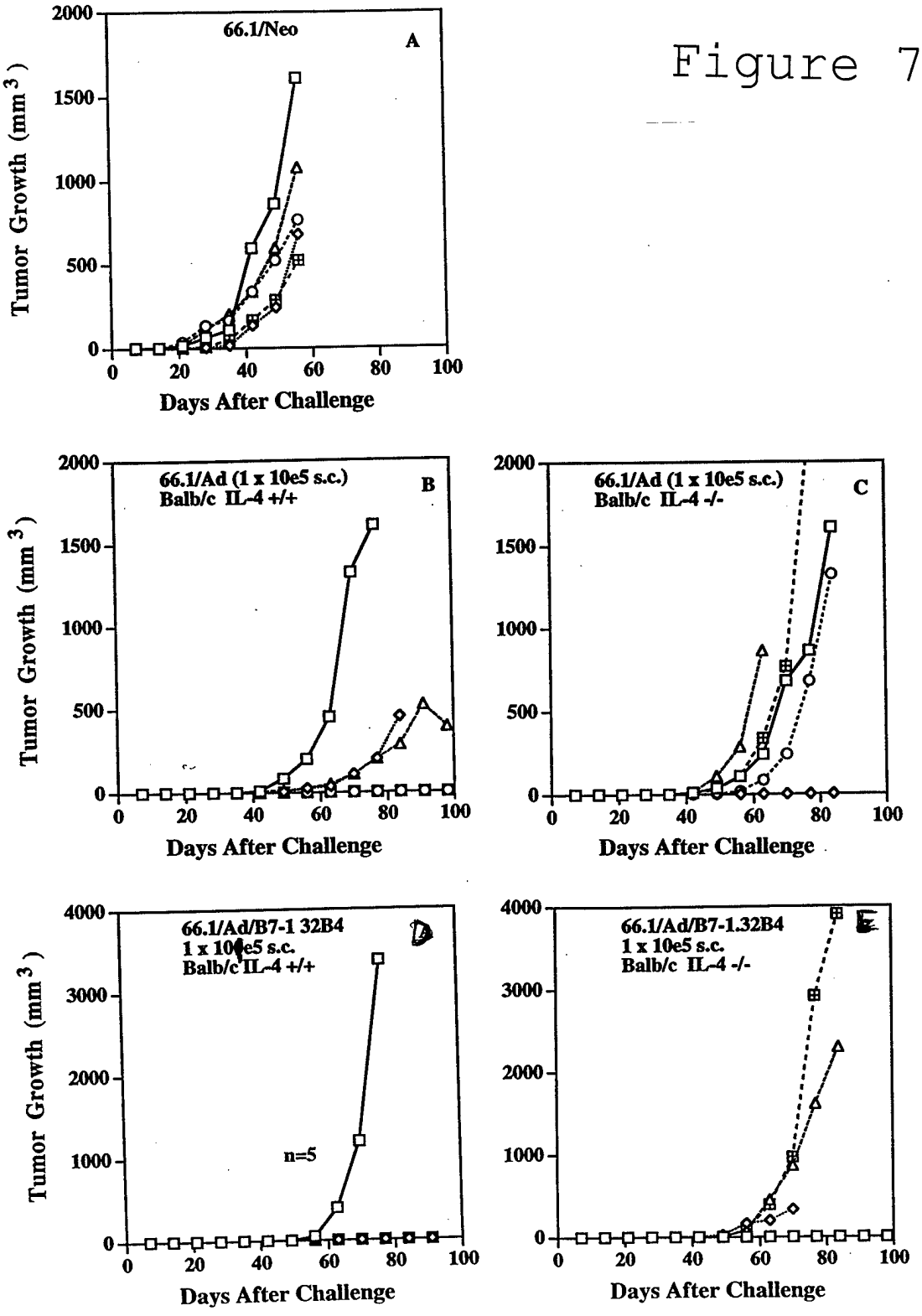

66.1/Neo
A
Tumor Growth (mm 3 )
Days After Challenge
66.1/Ad (1 x 10e5 s.c.)
Balb/c IL-4 +/+
B
66.1/Ad (1 x 10e5 s.c.)
Balb/c IL-4 -/-
C
66.1/Ad/B7-1 32B4
1 x 10e5 s.c.
Balb/c IL-4 +/+
n=5
66.1/Ad/B7-1.32B4
1 x 10e5 s.c.
Balb/c IL-4 -/-

Figure 8

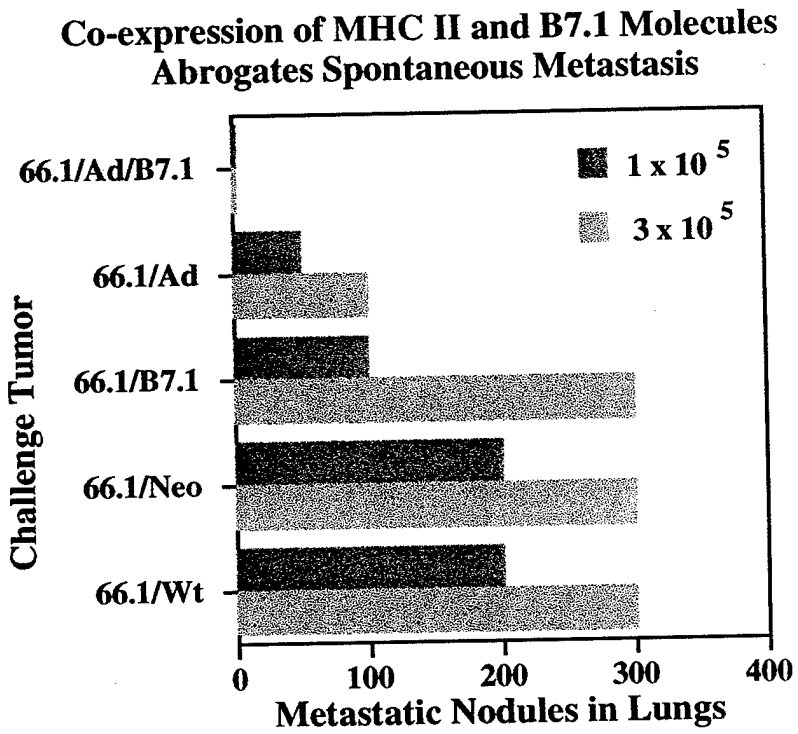
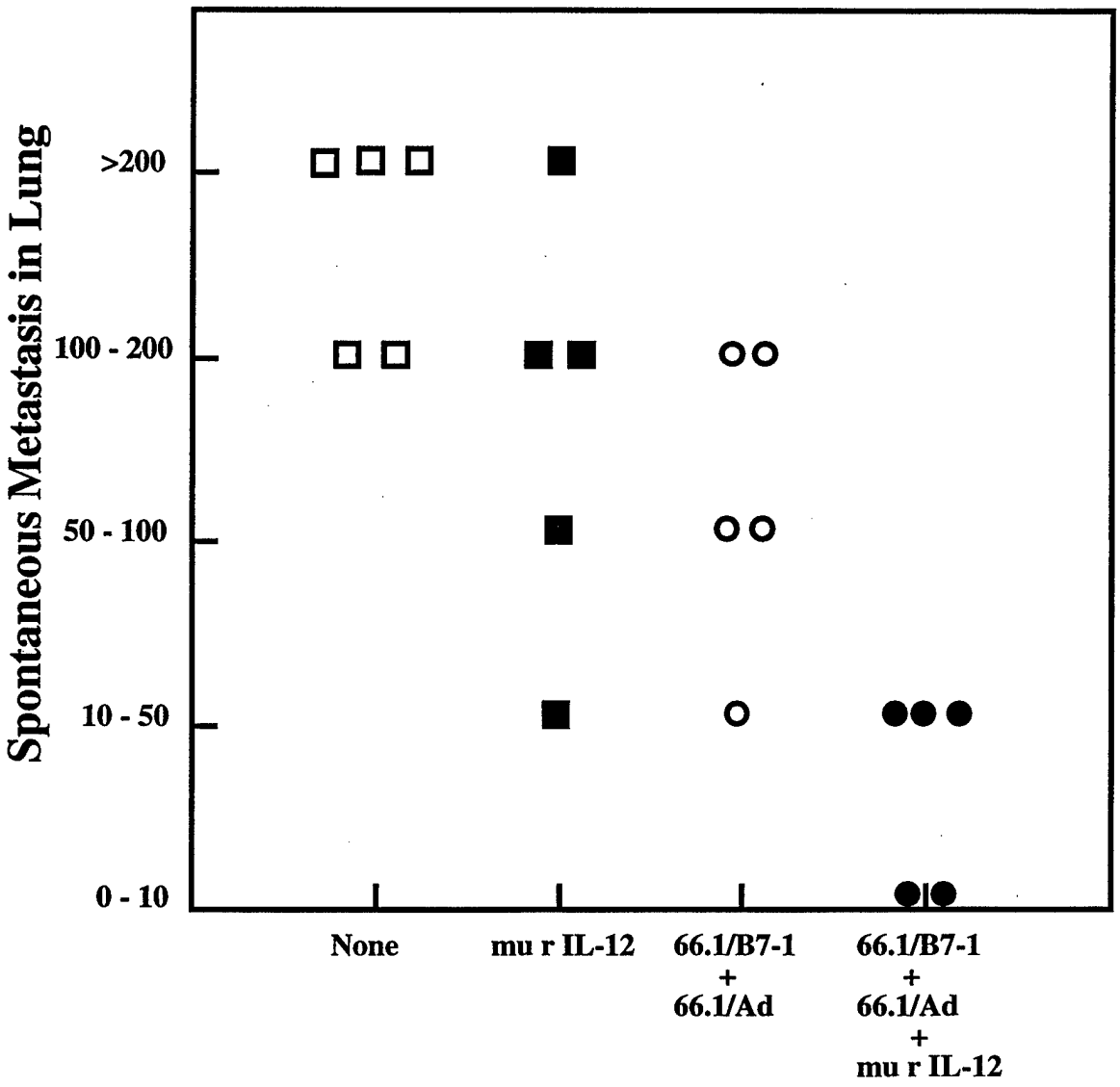

Co-expression of MHC II and B7.1 Molecules
Abrogates Spontaneous Metastasis
66.1/Ad/B7.1
66.1/Ad
66.1/B7.1
66.1/Neo
66.1/Wt
Challenge Tumor
1 x 10 5
3 x 10 5
0
100
200
300
400
Metastatic Nodules in Lungs

Figure 9

**Murine r IL-12 Enhances the Therapeutic Potential of I-Ad and B7-1 Transfected Mammary Tumor Cells**



Reports on Scientific Meetings:

1. S. Baskar (1997) Interleukin-12 enhances the immunogenicity of the major histocompatiblity complex class II and/or B7 transfected mammary tumor cells and helps control metastasis. *The Department of Defence Breast Cancer Research Program Meeting: Era of Hope. Washington D.C. Oct. 31 - Nov. 04. Proceedings Vol. III, pp. 955-956.*

2. S. Baskar (1998) Murine mammary carcinoma: Control and treatment of primary and metastatic tumors. *4th International Symposium on Predictive Oncology and Therapy. Nice, France. Oct. 24 – 27.* Session 703, Immunotherapy II. Oral presentation.

Personnel:

None (other than the P.I.)