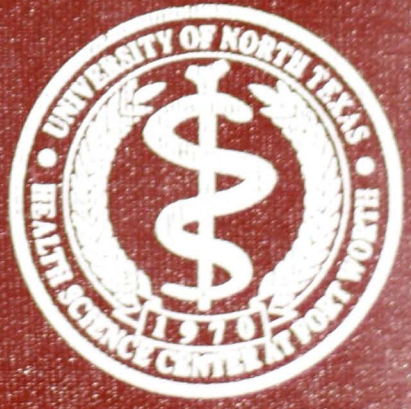
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ABSTRACT

Natural killer cells are a third population of lymphocytes, distinct from T and B cells. NK cells make up approximately 5-8% of the lymphocyte population. NK cells are non-MHC-restricted cytotoxic effector cells which are effective against intracellular pathogens, virally-infected cells and tumor cells. 2B4 is a natural killer cell receptor originally identified in the mouse as a surface molecule involved in non-MHC-restricted killing and enhancement of IFN-γ secretion. The human and rat homologues of 2B4 have recently been cloned in our laboratory. Interferon gamma (IFN-γ) is a cytokine with potent anti-viral and anti-proliferative effects. In addition, this cytokine acts as a global immune regulator by regulating gene expression and serving to attract other immune cells. In this work, we establish the function of human 2B4 in a NK cell line, YT. We have shown that human 2B4 activation induces cytolytic function and enhances IFN-γ release in YT cells. Additionally we show that 2B4's regulation of IFN-γ occurs at the transcriptional level, both through mRNA stability and increased promoter activity. We also demonstrate that several regions in the IFN-γ promoter respond to 2B4 activation through increased activity. Finally we investigate the importance of 2B4 and IFN-γ both separately and together in the rejection of metastatic tumor cells in

C57BL/6 mice. Our results confirm that both 2B4 and IFN-γ are critical in the rejection of metastatic tumor cells. Through the use of activating monoclonal antibodies, our studies indicate that 2B4's anti-tumor activity is through IFN-γ as well as through activation of the cytolytic function of NK cells.

MOLECULAR REGULATION OF INTERFERON GAMMA IN 2B4-ACTIVATED NATURAL KILLER CELLS: FUNCTIONAL ROLE IN TUMOR REJECTION

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MOLECULAR REGULATION OF INTERFERON GAMMA IN 2B4-ACTIVATED NATURAL KILLER CELLS: FUNCTIONAL ROLE IN TUMOR REJECTION

DISSERTATION

Presented to the Graduate Council of the University of North Texas Health Science Center at Fort Worth in Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

By

Lori Ann Johnson, B.A.

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

Immunity

Mammals are protected by two types of immunity – acquired and innate. Acquired immunity is obtained during life following an encounter with an antigen that promotes the development of antibodies against that antigen. Innate immunity is present at birth and is hereditary. Acquired immunity is very specific and innate immunity is more general.

Many different cell types play a role in immunity, among them are lymphocytes. Lymphocytes make up approximately 25% of the leukocyte (white blood cell) population under normal, healthy circumstances, but increase in response to infection. Lymphocytes originate in fetal stem cells and develop in the bone marrow. There are three types of lymphocytes: B cells, T cells and natural killer (NK) cells. T & B cells are involved in specific acquired immunity; NK cells are involved in innate immunity (1).

Immature B cells circulate and produce antibodies as a result of genetic rearrangement and express them on their cell surface. When the cell encounters an antigen specific for the antibody expressed on its surface, it becomes activated and differentiates into either a plasma cell or a memory cell. Plasma cells produce and secrete the antibody.

Memory cells remain and upon a second encounter with the specific antigen further differentiate into plasma cells.

T cells express cell surface receptors specific for a particular antigen. These receptors achieve their specificity through genetic rearrangement. T cells can become activated upon binding of their T cell receptor (TCR) by the antigen for which it is specific.

Only recently have NK cells been classified as the third type of lymphocyte. The lymphoid origin of NK cells was confirmed through FACS analysis identification of a common lymphoid progenitor, which may give rise to NK, T or B cells (2). NK cells are quite different from B and T cells. NK cells are involved in innate immunity rather than acquired. They are not specific for a particular antigen, but are more generalized effector cells that do not undergo genetic rearrangement (3, 4). This work is primarily focussed on the action of NK cells.

Natural Killer Cells

NK cells are large granular lymphocytes making up approximately 5% of the lymphocyte population. NK cells are non-adherent, non-phagocytic, and lack markers specific for either T or B cells (5). These cells are $CD3^-$, sIg^-, $CD16^+$, $CD56^+$ (6). NK cells are part of our first line of defense against pathogens that enter the body. They are also important

defenders against transformed or infected cells that sometimes escape other means of immune surveillance. NK cells have been shown to defend against virally infected cells, tumor cells and parasites (6-8). Unlike T cells, NK cells do not express antigen specific receptors (9).

NK cell effector function consists of cytokine production as well as direct cytotoxicity through perforin and granzyme B (6, 10, 11). NK cells can be activated to kill with more efficiency and specificity by cytokines including IL-2, IFNs, IL-12 and IL-15 (12-15). Expression of NKLAM is also associated with cytokine activation of NK cells (16). NK cell cytotoxicity can involve ADCC (antibody dependent cell-mediated cytotoxicity) via the FcγRIII receptor (CD16, the low affinity receptor for human IgG) (9). Early studies of NK cells led us to believe that NK effector functions was solely through ADCC. However, work that Dr. Goldfarb did in the 1980's failed to confirm that ADCC was solely responsible for NK cell's cytotoxic activity, and suggested that the effector function could be either through ADCC or through separate "NK receptors" (5). Since that time, several NK specific receptors have been identified. Studies of NK cell receptors have shown that effector function is regulated by signals from these receptors (8). Some NK cell receptors have been shown to activate effector function and others to inhibit this function.

In addition to cytotoxic activities, NK cells also contribute to cytokine production and invasiveness (17-19). Cytokines produced by NK cells include IFN-γ, TGFβ, IL-1β, IL-10, GM-CSF. Additionally, NK cells produce MMPs (17, 20-22). These cytokines have an effect on NK cell function, but also serve as immune regulators through the recruitment of other immune system cells. For example, IL-12 is essential for NK cell production of IFN-γ during viral infection, while IFN-α and IFN-β are required for NK cell cytotoxicity (23). Additionally, the release of NKCF (natural killer cytotoxic factor) by PBMC (peripheral blood mononuclear cells) is correlated with NK activity in vitro.

Natural Killer Cell Receptors

Several NK cell receptors have been identified in the recent past (8). There are two types of receptors – activating and inhibitory. Positive signals originate from activating receptors and negative signals from inhibitory receptors. The balance of these signals determines whether the NK cell kills its target or is inhibited from doing so (8, 24-26). Activation of NK cells is independent of MHC expression on the target cell, however inhibition of killing is typically regulated through recognition of MHC Class I (8). The NK cell will lyse a target cell if it lacks the MHC-I molecule for binding of the inhibitory receptor and expresses a specific ligand for an

activating receptor (1). NK receptors belong to one of two gene superfamilies based on their structural homology and chromosomal location (8). The two superfamilies are the immunoglobulin superfamily (IGSF) and the lectin superfamily (LSF) (8). Our lab has identified four novel NK cell receptors. Two of the receptors belong to the CD2 subset of the immunoglobulin superfamily, 2B4 (CD244) and CS1(27-29). The other two receptors belong to the lectin superfamily LLT1 and LLT2 (30).

Activating NK receptors recognize any number of ligands on the surface of target cells initiating a signaling cascade which results in cytolytic action by the NK cell. Members of both the lectin superfamily and the immunoglobulin superfamily participate in the activation of NK cells.

Lectin superfamily activating receptors include NKR-P1A and NKR-P1C, NKG2 family members and LY49 family members. Little is known about the signaling that results from the binding of these receptors. LLT1, a lectin superfamily member identified in our lab, has been mapped to the NK gene complex on chromosome12 and its predicted receptor structure lacks ITIM (immunoglobulin tyrosine inhibitory motif) motifs suggesting it may be involved in NK cell activation (30).

The immunoglobulin superfamily also has several members that participate in the activation of NK cells including 2B4, CD2, CD16, NKp30,

NKp44 and NKp46 (27, 31, 32). CD2 associates with CD58 and may serve as a co-stimulatory interaction in the activation of NK cells (8). 2B4, recently designated CD244, associates with CD48 and results in enhanced cytotoxicity and cytokine secretion (17, 33).

Also contributing to the action of NK cells are inhibitory receptors. Inhibitory receptors typically recognize self-MHC molecules on the surface of target cells and inhibit killing (9, 34-44). Collectively known as the inhibitory receptor superfamily (IRS), inhibitory receptors fall into three distinct families, ly49, CD94/NKG2 and KIR and signal through ITIM (immunoreceptor tyrosine-based inhibitory motif) sequences (8). Ly49 receptors of the C-type lectin superfamily, recognize H-2 class I MHC molecules in mice (41, 45-49). No human homologues to LY49 have been identified (8). KIR receptors of the immunoglobulin superfamily recognize HLA class I molecules in humans and are located on human chromosome 19 (50-54). Self-MHC molecules are often altered in tumor transformation and viral infections presumably to evade T cell recognition. This alteration prevents the recognition by inhibitory receptors allowing the NK signaling balance to shift toward activation (1, 55, 56). Activated NK cells are then able to kill the tumor cell or infected cell that may otherwise have gone undetected. Much remains to be learned about the signaling pathways in NK cells.

An important distinction between activating and inhibitory receptors is that of tyrosine motifs in their cytoplasmic regions. Activating receptors contain ITAM (immunoreceptor tyrosine activating motif) sequences that associate with a molecule called DAP12 (28, 57, 58). Additionally, MAPK (mitogen activated protein kinase) has been shown to be critical in the mobilization of perforin and granzyme B toward the point of contact with a target cell (59). Inhibitory receptors contain ITIM (immunoreceptor tyrosine inhibitory motif) sequences that recruit phosphatases like SHP1 and SHP2 that inhibit NK cell activation through the dephosphorylation of other signaling molecules (8, 60, 61).

While NK cell activity is largely beneficial to the host, there are a few instances where NK cells contribute to pathogenesis. Of particular concern are recipients of bone marrow transplants. If transplanted bone marrow cells do not express all of the host MHC antigens, some subsets of NK cells could become activated to reject the transplanted cells (44). Additionally patients with SLE (systemic lupus erythematosus) are deficient in NK cell activity due to an impaired response to interferon and interferon inducers (62). It has recently been shown that XLP (X-linked lympho-proliferative disorder) is associated with mutations in SAP (SLAM associated protein) which associates with the cytoplasmic tail of activating receptors 2B4 and SLAM (63, 64). It is believed that defective

signaling through 2B4 and SLAM may contribute to the pathogenesis of XLP.

2B4 (CD244)

2B4 was originally identified by Dr. Mathew after generating a panel of monoclonal antibodies against murine NK cells (42). It was found on all resting and IL-2 activated NK cells and a subset of splenic T cells. Binding of surface 2B4 with monoclonal antibody was shown to activate cytolytic activity, IFN-γ secretion and granule exocytosis in IL-2 cultured NK cells. 2B4 was found to contain an Ig C-2 domain suggesting that it belonged to the immunoglobulin superfamily (27). Evidence that 2B4 was involved in non-MHC-restricted killing included its expression only on cell types which participate in this type of killing and its lack of expression on cell types that are not involved in this type of killing (27). Additionally, binding with monoclonal antibody modulates this killing against a variety of FcR+ and FcR- targets (18). Since the discovery of this molecule, our lab group has been devoted to understanding the role 2B4 in the immune system of mice, rats and humans.

The encoded 2B4 protein is 66 kDa with 398 amino acids, a leader sequence of 18 amino acids and an transmembrane domain of 24 amino acids (27). It is predicted to have eight N-glycosylation sites and high

homology to murine and rat CD48 as well as human LFA-3 (27). Three transcripts derived from differential splicing are apparent in Northern blot analysis (27). Southern blot analysis from several mouse strains reveals that 2B4 belongs to a family of closely related genes found on mouse chromosome 1. Originally it seemed as though 2B4 was only present in some inbred mouse strains, but recent work in our lab has shown that it is present in all inbred mouse strains. Murine 2B4 contains polymorphism in the v-domain which restricts recognition by monoclonal antibodies (65-68).

Murine 2B4 contains 4 tyrosine motifs in the cytoplasmic domain (27) which are also present in SLAM and believed to be involved in SHP-2 (69) and SLAM-SAP interactions (70). Activation with mAb also results in the up-regulation of egr-1 and c-fos mRNA expression (18, 71) as well as an increase in surface expression of 2B4 as well (72). Our lab has cloned and sequenced the promoter of m2B4 and found it to contain consensus-binding sequences for several known transcription factors (72).

Our lab has cloned the rat homologue of 2B4 expressed in rat NK cells. The rat homologue contains 395 amino acid residues and two Ig domains in the extracellular region and three tyrosine motifs in the cytoplasmic region. Additionally, northern blot analysis indicates the presence of multiple transcripts of rat 2B4 (73). Also, we have cloned

another rat NK receptor related to 2B4. This related receptor is unique compared to human and mouse 2B4 in that it lacks tyrosine motifs in the cytoplasmic tail. Also, this related receptor exists in both soluble and transmembrane forms (74).

Recently, we cloned the human homologue of 2B4 (28) and found it to be expressed in NK and T cell lines, spleen lymph nodes, and peripheral blood leukocytes. Human 2B4 was localized to chromosome 1 and shown to be a new member of the CD2-subfamily along with SLAM, CD48, CD58, CD84, CS1 and Ly9 (28, 29, 63, 75-79). It is also a member of the immunoglobulin super family as determined by structural similarities, chromosomal location and evolutionary relationship with other members (80).

Human 2B4 contains a 20-aa leader sequence, 201-aa extracellular domain, 24-aa transmembrane domain, and 120-aa cytoplasmic domain (63). Human 2B4 also contains 4 tyrosine-based motifs in the cytoplasmic tail similar to murine 2B4 (63). The molecular weight of h2B4 is ~86 kDa (63).

C1.7 is a monoclonal antibody available through Coulter and originally marketed as one that recognized NK cells. It is now known that C1.7 recognizes human 2B4. C1.7 mAb reacts with all human NK cells and approximately 50% of CD8+ T cells – cells that mediate non-MHC-restricted killing (19). Studies in our lab and others have shown that

activation of PBMC with C1.7 mAb result in enhanced cytotoxic activity and increased IFN-γ secretion (17, 28).

Recently our lab found that the natural high affinity ligand for 2B4 is CD48 (81-83). Studies with a CD48 fusion protein show that it can trigger NK-mediated cytotoxicity and IFN-γ secretion, but does not activate T cells or monocytes (77, 84, 85). This study also shows that the effect varies in different NK cell clones suggesting that other NK receptors may contribute to this action. Along these lines, it was proposed that the lack of activation in T cells is due to their lack of other necessary NK receptors (86).

2B4 has recently been shown to be associated with two diseases. X-linked lymphoproliferative syndrome (XLP) has been defined as a genetic defect in the signaling protein SAP (63). SAP associates with cytoplasmic tail of SLAM and 2B4 in activated NK cells (63, 87, 88). Patients suffering from XLP lack the ability to activate NK cells via surface 2B4 (88-91). This suggests that faulty 2B4 signaling may be instrumental in the pathogenesis of XLP. Additionally, 2B4 surface expression on CD8+ T cells was recently shown to be a better predictor of HIV disease progression than the commonly used CD4+ T cell count (92).

CD48

CD48 is a member of the immunoglobulin superfamily expressed on lymphocytes, dendritic cells and macrophages (93). CD48 was originally described as a counter-structure for CD2 in mice and rats (93) and shown to be involved in the activation of lymphocytes (94-97). Results showing that CD2 knockout mice were phenotypically normal while CD48 knockout mice were impaired in T cell development and activation, suggested the possibility that CD48 may bind another structure. Indeed it was later found that CD48 and 2B4 have high affinity binding which was supported by immunoprecipitation, flow cytometry and western blot analyses (33, 80).

Interferon Gamma

Interferon-gamma (IFN-γ) was one of the first cytokines discovered and was reported by Wheelock in a 1965 issue of *Science* as a substance with anti-viral properties produced by leukocytes. The substance reported by Wheelock was originally described as 'Type II Interferon', and then later called 'Immune Interferon' and 'Macrophage-Activating Factor' interchangeably before finally being designated 'Interferon Gamma' by the International Interferon Nomenclature Committee in 1980 (98). Since its discovery, IFN-γ has been the focus of

many investigations in biomedical research. Studies of IFN-γ, while extensive, still are expanding today.

IFN-γ is a homodimeric protein that is N-glycosylated at two sites in its biologically active form. Its size has been reported between 20 kilodaltons and 34 kilodaltons, the differences due to variations in glycosylation. IFN-γ is made up of six alpha helices and no beta sheet domains. The two subunits of the protein come together in an antiparallel configuration (99). IFN-γ plays a central role in immune regulation via interaction with the IFN-γ receptor found on the surface of a large variety of cell types including fibroblasts, monocytes, macrophages, lymphocytes, epithelial cells, keratinocytes and platelets (100).

IFN-γ has been shown to induce the enzyme indoleamine-2,3-dioxygenase (IDO) which catalyzes the oxidative cleavage of the pyrrole ring in tryptophan resulting in tryptophan depletion (99). Tryptophan metabolism is speculated to contribute to the anti-proliferative and anti-viral effects of IFN-γ through the hampering of protein synthesis. IFN-γ is also known to potentiate respiratory burst and synthesis of nitrogen monoxide (NO) which is important in the defense against bacterial pathogens, molds or protozoa. Additional benefits of this induced NO synthesis may include anti-tumoral activity in that cells that are induced to synthesize NO often undergo apoptosis or arrest of DNA synthesis (99).

The gene coding for IFN-γ is a single copy gene located on human chromosome 12 (101, 102). The gene consists of four exons and three introns that are highly conserved among species with known IFN-γ genes. The gene for IFN-γ has been identified in all mammalian species as well as in chickens. The majority of studies have involved human and murine IFN-γ (103).

Interferon-gamma stimulates signaling cascades resulting in the activation of gene transcription. MHC class I and class II molecule expression is up-regulated in professional antigen presenting cells as well as in other cell types, increasing their ability to present antigen. Also up-regulated is the expression of Fc receptors on neutrophils and mononuclear phagocytes increasing their ability to recognize antibody coated targets (104, 105). Expression of ICAM-1 and B7, the ligand for CD28, is augmented in response to IFN-γ. These molecules serve as co-activators of lymphocytes (106, 107). Tumor associated antigen expression is also modulated by IFN-γ, rendering tumor cells more susceptible to cytotoxic T and NK cells. IFN-γ can also assist in the activation of macrophages, promote T cell differentiation, and promote expression of certain immunoglobulin (Ig) isotypes in B cells.

Interferon-gamma is produced only by activated lymphocytes, particularly T cells and NK cells. It's production is regulated by a variety of

cytokines as well as through the binding of cell surface molecules. IL-2 is a classic stimulator of IFN-γ production. Additionally IL-12 and IL-18 have been shown to enhance the production of IFN-γ both synergistically and independently. Binding of several cell surface molecules has been shown to be followed by increased secretion of IFN-γ including 2B4 (CD244), CD28, CD2 and beta 1 integrins (8, 108, 109). Several other cytokines have been shown to inhibit the production of IFN-γ including IL-10, IL-4, IFN-α, IFN-β and TGF-β1 (99, 110-112). Vitamin A and its derivatives (retinoic acid) have also been shown to down regulate the transcription of IFN-γ (113).

Adequate function of IFN-γ is essential for acquired resistance to infections and cancer. Malfunction of IFN-γ is associated with inflammatory complications and autoimmune disease. Overall the effects of IFN-γ are crucial in anti-viral, anti-tumoral, anti-proliferative and immunomodulatory processes, however the regulation of its production must be tightly controlled.

The regulatory mechanisms controlling the production of IFN-γ in NK cells is not well understood. T cell production has been heavily studied and provides clues to deciphering the mechanisms in NK cells. Regulation of IFN-γ in activated T cells has been shown to occur at the transcriptional level (114). It remains clear that the regulation of IFN-γ is

complex and varies due to cell type as well as the particular stimuli involved in activation.

Only recently has an important pathway in IFN-γ regulation been delineated. In activated T cells stimulated with IL-12, the p38 MAPK pathway is involved in the induction of IFN-γ mRNA and secretion (115). Trotta et al. demonstrated that the p38 MAPK pathway is also involved in the appearance NK cell IFN-γ mRNA following incubation with target cells (116). Recently our lab demonstrated that 2B4 induced secretion of IFN-γ was also through the p38 MAPK pathway (117).

The human IFN-γ gene promoter contains several binding sites for several transcription factors. Each of these transcription factors have been shown to interact with the promoter under some circumstances and not to interact in other circumstances. Many cytokine signals are mediated through STAT transcription factors (118). In activated T cells, STAT4 has been shown to be essential to the transcription of IFN-γ (115). Jun-ATF2 complexes have been shown to increase in level, phosphorylation and promoter binding in IL-12 stimulated T cells (115, 119, 120). CREB-ATF1 proteins compete for binding with Jun-ATF2 complexes and have a negative impact on IFN-γ promoter activity (120).

NFAT and NFκB have several binding sites in the IFN-γ promoter and cooperation between these two factors is necessary for maximal

promoter activity in activated T cells, although both factors do not appear under all stimulators (121, 122). The transcription factor YY-1 has been shown to have both enhancing and silencing effects on IFN-γ expression (122-124). While another study has shown that AP-1 and NKκB play a role in NK cell production of IFN-γ under IL-2 stimulation (125). All things considered, the regulation of IFN-γ in lymphocytes is at least in part at the transcriptional level and involves promoter regions that participate both positively and negatively following lymphocyte activation.

Anti-tumor Activity

Several studies have shown that NK cells mediate anti-tumor activities (126-128). These studies are supported by the finding that spleenocytes isolated from NK deficient mice have reduced cytotoxicity against YAC-1, RMA-S and B16 tumor cells in vitro (129). Additionally NK deficient mice show an impaired ability to reject tumor cells in an induced metastatic tumor model (129).

Evidence that IFN-γ plays a role in the antitumor effect of NK cells comes from several studies. IL-12, an inducer of IFN-γ, has been shown to elicit an antitumor affect against a variety of tumor cells including colon, kidney and lung carcinomas as well as B16F10 melanoma cells (130-138). These effects of IL-12 were believed to be through IFN-γ which is supported

by a finding that IFN-γ and perforin are required for IL-12 mediated anti-tumor immunity (139). In vivo IL-12 was found to be produced following injection with an anti-CD40 antibody which was correlated with increased NK cytolytic activity among PBL and significant antimetastatic effects (140).

Perforin is a molecule produced exclusively by NK cells and CTLs and has been shown to be an important molecule in the elimination of tumor cells (102, 132, 141, 142). Perforin deficient mice display increased tumor growth and metastasis than wild-type mice (143-146). Depletion of NK cells resulted in more metastases than in perforin deficient mice, suggesting that other factors play a role in NK cell protection from tumor metastases (146).

Interferon-gamma has been shown to up-regulate the expression of Fas and FasL by B16 melanoma cells, which led to apoptosis of these cells (147). IFN-γ knockout mice were as susceptible as NK cell depleted mice to lung metastases, although not as susceptible as IFN-γ - Perforin double knockout mice (145). Several in vivo metastases models demonstrated that the role of IFN-γ is distinct from that of perforin (145, 148).

CD48, the counter-receptor for 2B4 has resulted in tyrosine phosphorylation upon binding suggesting that it is involved in lymphocyte activation (149). Binding of CD48 with mAb has shown a strong in vivo

antitumor effect (150, 151). Additionally transfection of CD48 into two poorly immunogenic tumor cell lines enhances their immunogenicity (152). These data originally suggested a role for CD2, the low affinity ligand for CD48. Now that 2B4 has been identified as the high affinity ligand, a role for 2B4 is likely.

The focus of this work involves the elucidation of the relationship between 2B4 and IFN-γ and the functional role of said relationship against tumor metastases. Chapter two herein establishes the function of 2B4 in human cell line, YT. In this cell line, 2B4 activation results in enhanced secretion of IFN-γ. Chapter three establishes the regulation of IFN-γ by 2B4 at the transcriptional level. We found that IFN-γ mRNA stability is enhanced in addition to enhanced IFN-γ promoter activity in response to 2B4 activation. Chapter four identifies the regions of the IFN-γ promoter that respond to 2B4 activation. We have identified 7 of 10 regions of the promoter as responsive to 2B4 activation. Chapter five examines the role of 2B4 and IFN-γ in a murine tumor metastasis model. We found that both 2B4 and IFN-γ are vital in the rejection of metastatic tumor cells. Additionally, we were able to show that 2B4's anti-tumor mechanism is through not only IFN-γ, but other mechanism(s) as well.

CHAPTER TWO

FUNCTIONAL ROLE OF h2B4: 2B4 ACTIVATION OF YT CELLS INDUCES NATURAL KILLER CELL CYTOLYTIC FUNCTION AND IFN-γ RELEASE

SUMMARY

2B4 is a surface molecule found on all human NK cells, a subset of $CD8^+$ T cells, monocytes and basophils. It was originally identified on mouse NK cells and the subset of T cells that mediate non-MHC-restricted killing (18). Recently our lab cloned the human homologue of 2B4 (h2B4) (28) and found h2B4 to also mediate non-MHC-restricted cytotoxicity. In this study, we examine h2B4 in regulating various functions of NK cells using a human NK cell line YT, with mAb C1.7, an antibody that specifically recognizes h2B4. Binding of surface 2B4 with mAb C1.7 increases YT's ability to destroy tumor cells in addition to increased production of IFN-γ. The significance of this data is discussed in chapter six.

INTRODUCTION

NK cells have the ability to recognize and kill certain tumor and virally infected cells in the absence of prior stimulation and without MHC-restriction (3, 7, 8). NK cells provide important mechanisms of primary defense against virus-infected cells and tumor metastases through cytotoxic activities and the production of various cytokines such as IFN-γ, TNF-α, and GM-CSF (21, 153). NK cells express several surface molecules that regulate NK cell function both positively and negatively. It is the sum of these signals that ultimately determines NK cell function and activation (8, 154). Thus it is important that these surface molecules on NK cells are identified and that their roles in regulating NK cell functions are characterized in order to gain a better understanding of the role of NK cells in the immune response.

We have previously identified a surface molecule designated 2B4, which is expressed on all murine NK cells and a subset of T cells that mediate NK-like killing (18, 27). In addition to defining cells capable of non-MHC-restricted killing, the 2B4 molecule is also involved in modulating their function. The lytic activity of cultured NK cells and non-MHC-restricted T cells is greatly enhanced in the presence of a monoclonal

antibody (mAb) against 2B4. We have also characterized the human homologue of 2B4, h2B4 (28). In both mice and humans, 2B4 is the counter-receptor for CD48 (33, 80). It has been reported that mAb C1.7, initially characterized by its ability to activate human cytotoxic lymphocytes, is able to recognize h2B4 (63). In addition to NK cell cytolytic functions, we have also investigated other NK cell activities such as secretion of degradative enzymes including matrix metalloproteinases (MMPs) through collaborative efforts with Dr. Goldfarb's laboratory. NK cells also produce various proteolytic enzymes including MMPs.

Here we have examined h2B4 with mAb C1.7 and its ability to affect NK cell functions including its influence on MMP expression. In this study, we used YT cells as a human NK cell model. The YT cells have a surface phenotype similar to human NK cells and have NK-like killing activity (155). YT cells have a high expression of h2B4 and show increased cytolytic activity upon stimulation with mAb C1.7. Stimulation of YT cells by mAb C1.7 increases production of IFN-γ and modulates the expression of h2B4. We also reported that h2B4 stimulation up regulates the expression of MMP-2. In sum, our results suggest that the NK cell receptor 2B4 participates in many regulatory functions.

MATERIALS AND METHODS

Media

4+RPMI: A one liter package of powdered RPMI media (Life Technologies, Rockville, Maryland, Catalog #31800-022) is combined with 2.0 grams sodium bicarbonate (Life Technologies, Catalog #11810-025) and dissolved in 800 ml. deionized, distilled water. pH is adjusted to 7.4 with HCl and volume adjusted to one liter. Media is then supplemented with 10% standard fetal bovine serum (HyClone Lab, Logan, Utah, Catalog #SH30088.03). Media is further supplemented with Sodium Pyruvate – 1X final concentration, non-essential amino acids – 1X final concentration, antibiotic/antimycotic – 1X final concentration and buffered with HEPES buffer (all Life Technologies, Catalog # 11360-070, 11140-050, 15240-062 and 15630-080 respectively).

Cell lines

The cell lines used in the completion of this project are listed in Table 2.1.

Table 2.1

Cell	***Origin***	***Media***
YT	Human leukemic NK cell line	4+RPMI
K562	Human erythroleukemia cell line	4+RPMI
BW	Murine B cell line	4+RPMI
P815	Murine lymphoma cell line	4+RPMI
RMA	Murine lymphoma cell line	4+RPMI
DB	Human B cell line	4+RPMI
Jurkat	Human T cell leukemic cell line	4+RPMI
722.221	Human MHC class I deficient B cell line	4+RPMI

Cell Culture

All mammalian cell lines were maintained in sterile conditions in Nunc EasYFlasks with canted necks and filtered tops. Cell growth media was 4+ RPMI. Media was completely replaced every 48 hours. Cells were maintained at 37°C with 5% CO_2 saturation.

Antibodies

C1.7 antibody which recognizes h2B4 (63) was purchased from Coulter (Orlando, FL). Isotype mAb control was kindly donated by Dr. V. Kumar, UT Southwestern, Dallas, TX.

Flow Cytometric analysis

YT, DB and Jurkat cells resuspended at 5 X 10^5 cells/100 µl in blocking buffer (PBS, 1 % BSA) were incubated with 2 µg/ml of C1.7 mAb for 40 min on ice. Cells were then washed and resuspended in blocking buffer. Secondary goat $F(ab')_2$ FITC-αmIgG (Coulter, Miami, FL) was added at a final dilution of 1/200 (v/v). After 30 min incubation on ice in the dark, the cells were washed two times in blocking buffer and resuspended in 1 ml. blocking buffer. Routine analysis was performed using a Coulter EPICS XL flow cytometer.

Cell-mediated cytotoxicity assay

Target cells were labeled by incubating 1 X 10^6 cells with 2 MBq of $Na_2{}^{51}CrO_4$ (NEN Research Products, Boston, MA) for 90 min at 37°C under 5 % CO_2 in air. The target cells were then washed three times in culture media. 1 X 10^4 labeled target cells (100 µl) were incubated with effector YT cell suspension (100 µl) under various conditions of IL-2 (20 U/ml conc.), mAb C1.7 (200 ng/ml conc.) and/or isotype mAb control (200 ng/ml conc.). Effector YT cells were resuspended at 1, 2, 5, 10 and 20 times the number of labeled target cells. After incubation for 4 h at 37 °C under 5 % CO_2 in air, the cells were pelleted at 250 X *g* for 5 min, 100 µl of the

supernatants were removed and their radioactivity was measured. The percentage of specific lysis was calculated by the following equation: (*a* – *b*/*c* - *b*) X 100, where *a* is the radioactivity of the supernatant of target cells mixed with effector cells, *b* is that in the supernatant of target cells incubated alone, and *c* is that in the supernatant after lysis of target cells with 1 % Nonidet P-40.

Interferon-γ Release Assay

YT cells were incubated in the presence of K562 cells in 1 ml. culture media and in various conditions including IL-2, C1.7 mAb and isotype mAb control for 16 hours at 37°C under 5 % CO_2 in air. Where indicated, IL-2, mAb C1.7 and isotype mAb control, was added to final concentrations of 20 U/ml, 200 ng/ml and 200 ng/ml, respectively. The cells were then spun down at 250 X *g* for 5 min at 4°C. 100 µl of the supernatant was then extracted and tested for the presence of IFN-γ. IFN-γ protein secretion was quantitated immunologically by IFN-γ human ELISA system (Amersham), as per manufacturer's instructions.

Interferon-γ ELISA

BIOTRAK ELISA system from Amersham-Pharmacia-Biotech was used to quantitate IFNγ in cell free supernatant. Supplied directions were followed.

50 µl of biotinylated antibody reagent was added to each well followed by 50 µl of cell free supernatant or standard. Wells were covered with a clear adhesive and incubated at room temperature for 2 hours. Wells were emptied and washed three times with wash buffer. Wells were aspirated after final wash to ensure removal of all wash buffer. 100 µl of streptavidin-HRP conjugate reagent was added to each well. Wells were covered with an adhesive strip and incubated at room temperature for 30 minutes. Wells were again washed three times with wash buffer and then aspirated to remove all liquid. 100 µl of TMB substrate solution (provided with kit) was added to each well and the plate was incubated uncovered in the dark for 30 minutes. 100 µl stop solution was added to each well and the plate was read at 450 nm within 30 minutes.

RESULTS

Expression of h2B4 is found on YT cells but not DB and Jurkat cells

C1.7 is an antibody shown to recognize h2B4 on NK cells (63). Prior to using the YT cell line to examine the function of h2B4 in NK cell function, we needed to show that the YT cell line expresses h2B4 on its surface and that C1.7 antibody recognizes it. Figure 2.1 is a flow cytometry histogram that shows neither DB (a human B cell line) nor Jurkat (a human T cell line) cells express 2B4, but YT cells do express 2B4 as detected with C1.7 mAb. These results indicate that YT cells are a suitable model for examining the function of NK cell activation through 2B4 and that mAb C1.7 may be used to study 2B4 activation.

Effect of mAb C1.7 on YT Cells Increases Cytotoxic Activity

Our lab has shown that binding of h2B4 by a monoclonal antibody increase cytolytic activity in human polyclonal NK cells (28). C1.7 monoclonal antibody was originally characterized by its ability to increase cytolytic activity of polyclonal NK cells (19). Based on the expression of h2B4 which is recognized by C1.7 on YT cells in conjunction with previous studies in our lab and others, we were interested in determining if C1.7 binding of h2B4 on the surface of YT cells can elicit functional response,

specifically cytolytic activity. Using a chromium release assay, we studied the cytolytic activity of activated YT cells against ^{51}Cr-labeled K562 and P815 target cells. The monoclonal antibody bound both to the FcγR receptor on the target cells and to 2B4 on the NK cells, imitating the receptor-ligand binding. The NK cells lysed the target cells, measured by the release of ^{51}Cr, through redirected lysis. Figures 2.2 and 2.3 show the lysis against these targets. Binding of 2B4 through C1.7 failed to increase cytolytic activity against FcγR- target cells 721.221, RMA and Jurkat cells (data not shown). Additionally, lysis of K562 and P815 targets by C1.7 treated YT cells does not require IL-2, but is enhanced by IL-2 (data not shown).

H2B4 up-regulates IFN-γ production.

IFN-γ is a cytokine shown to stimulate signaling cascades resulting in activation of gene transcription. This activity is important in anti-viral, anti-proliferative, and immunomodulatory processes (158). We wanted to test the effect of h2B4 stimulation on IFN-γ production by YT cells. One million cells were incubated under varying conditions: (a) media alone, (b) IL-2, (c) IL-2 plus mAb C1.7 or (d) isotype antibody control for 16 h and then tested for IFN-γ production by IFN-γ ELISA. While the presence of IL-2

increased the production of IFN-γ by YT cells, IFN-γ release was significantly augmented by the additional presence of mAb C1.7 (Fig. 2.4). The addition of isotype antibody control to IL-2-stimulated cells failed to show any significant change in IFN-γ production. The addition of mAb C1.7 to YT cells alone caused IFN-γ secretion at levels similar to YT cell secretion of IFN-γ when stimulated by IL-2 alone (data not shown).

Stimulation of YT cells through h2B4 increases expression of MMP-2

Additionally, through collaboration with Myoung Kim in Dr. Goldfarb's laboratory, we observed that h2B4 stimulation increased the migration of YT cells through Matrigel invasion chamber (unpublished observation). Dr. Kim found that 2B4 stimulation increased the expression of MMP-2 by 4-5 fold over control level, while the expression of membrane-type (MT)-MMP-1 was not changed significantly. These results suggested that stimulation of 2B4 could lead to other functions of NK cells different from cytolytic activity. Such functions include extracellular matrix degradation by induction of MMP expression. Because this work was done by Dr. Kim, it is not shown here.

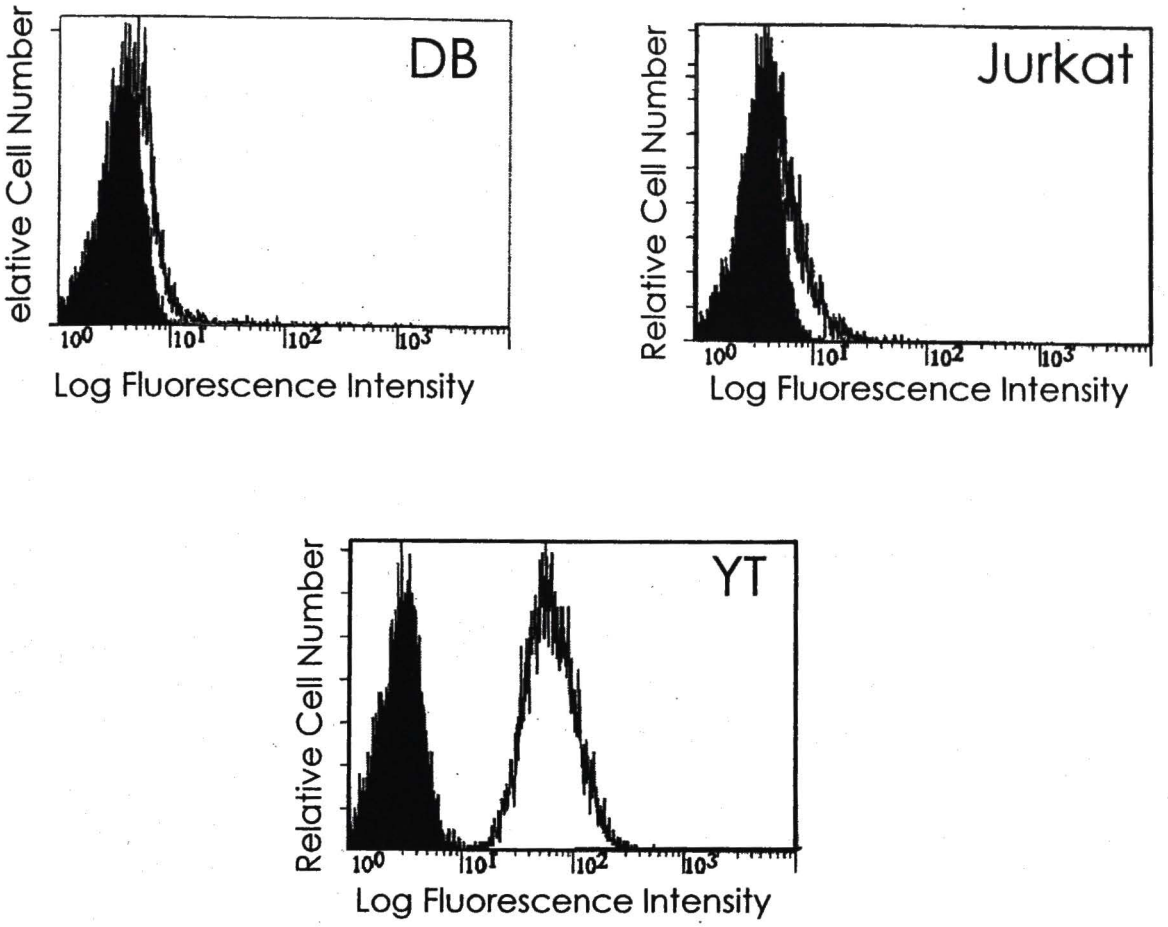


Figure 2.1
C1.7 mAb detects expression of 2B4 on YT cells but not DB or Jurkat cells.
Expression of h2B4 on YT, DB and Jurkat cells. Cells were incubated with mAb C1.7 (open curve), or isotype control (closed curve) as described in "Materials and Methods". A secondary FITC-anti mouse IgG Ab was then used to detect C1.7 staining.

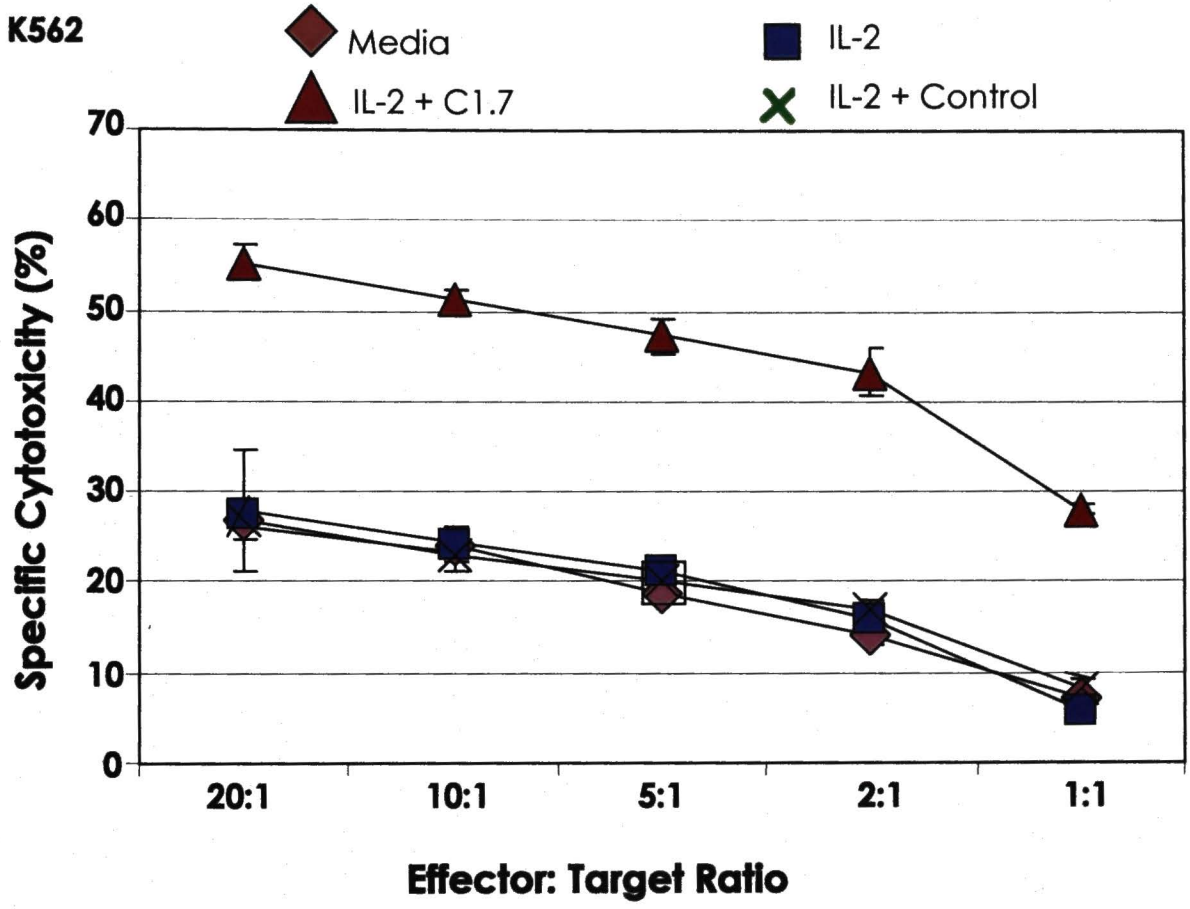


Figure 2.2
2B4-activation results in enhanced killing of K562 target cells through direct cytotoxicity.

mAb C1.7-induced, NK cell-mediated cytotoxicity. YT cells were used as effector cells in standard 4-hr ^{51}Cr release assays against ^{51}Cr-labeled FcγR- K562 target cells. Assays were performed in the presence of culture media alone or with stimulation as indicated. All data points represent the mean of a minimum of 3 independent trials. Vertical bars represent the standard deviation.

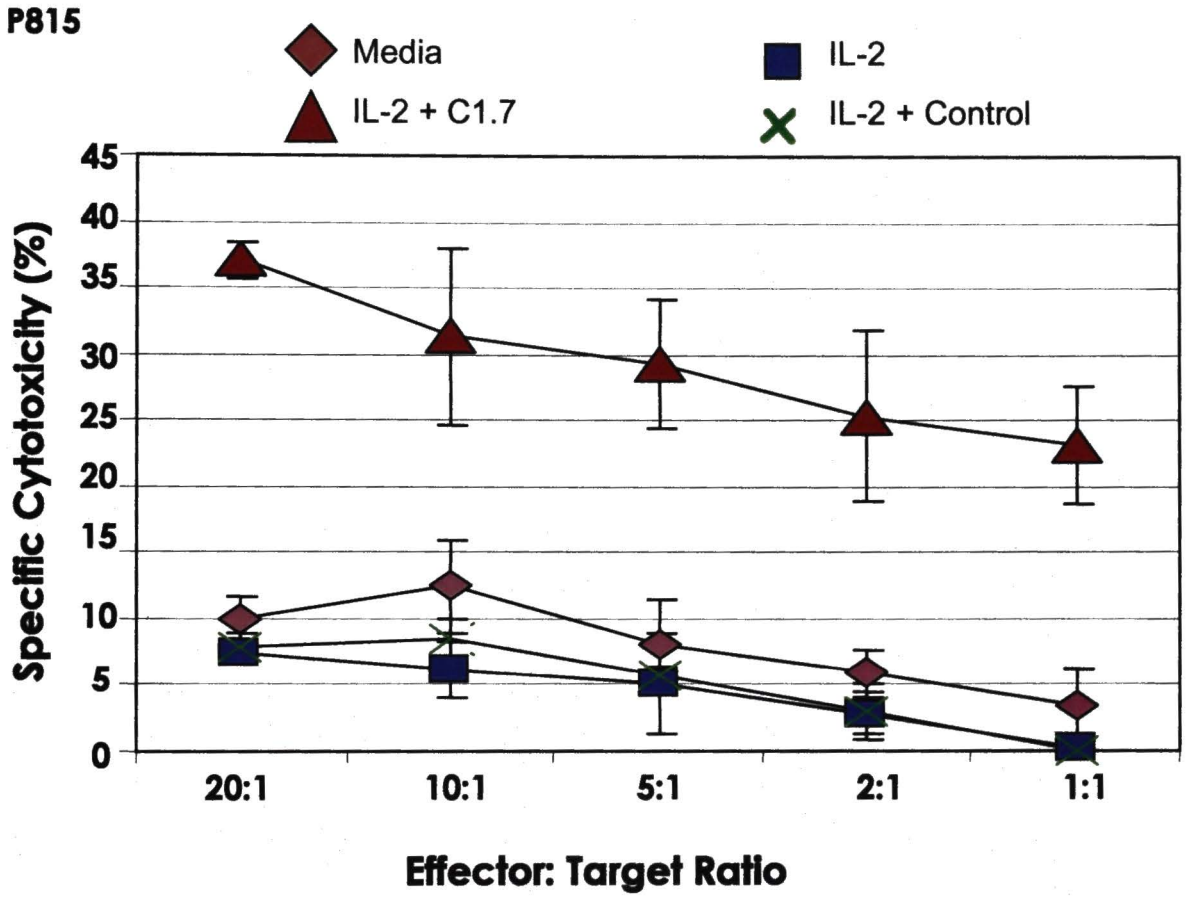


Figure 2.3
2B4-activation results in enhanced killing of P815 target cells through reverse antibody dependent cellular cytotoxicity (rADCC).
MAb C1.7-induced, NK cell-mediated cytotoxicity. YT cells were used as effector cells in standard 4-hr ^{51}Cr release assays against ^{51}Cr-labeled $Fc\gamma R^+$ P815 target cells. Assays were performed in the presence of culture media alone or with stimulation as indicated. All data points represent the mean of a minimum of 3 independent trials. Vertical bars represent the standard deviation.

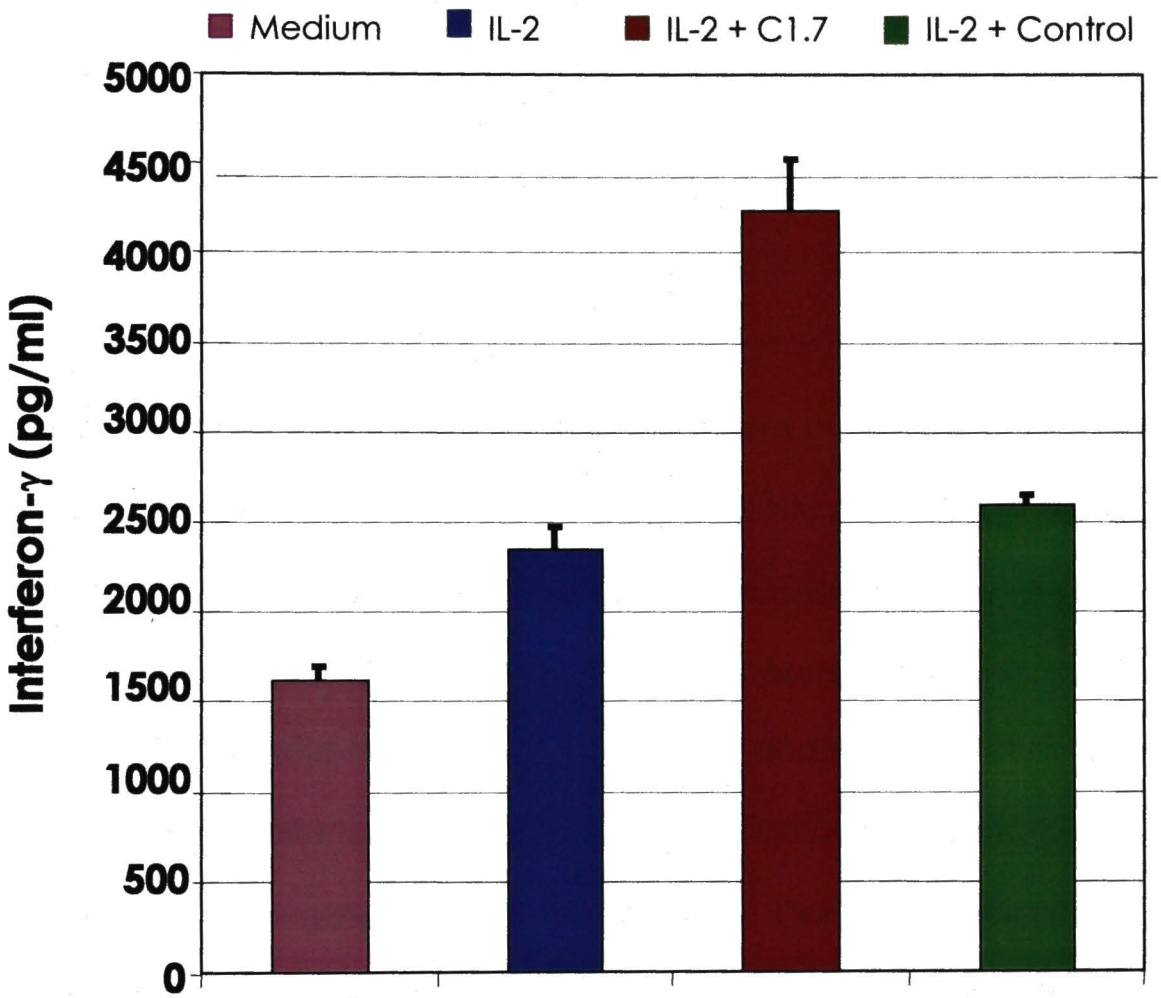


Figure 2.4
2B4-activation results in enhanced secretion of IFN-γ.
YT cells (1 X 10^6) were incubated in the presence of target cells (K562) at a 10:1 ratio. Cells were incubated in culture media alone or with stimulation as indicated. The cells were then spun down and 100 μl of supernatant was assayed for the presence of IFN-γ by ELISA.

CHAPTER THREE

ANALYSIS OF IFN-γ PRODUCTION FOLLOWING 2B4 ACTIVATION IN HUMAN NATURAL KILLER CELLS

SUMMARY

IFN-γ is a cytokine that regulates various functions of the immune system. The major producers of IFN-γ are T cells and NK cells. 2B4 is a novel activating receptor expressed on all human NK cells, a subset of CD8+ T cells, monocytes and basophils. Activation of human NK cells through surface 2B4 enhances NK cell cytolytic function and secretion of IFN-γ. We have examined the regulation of IFN-γ production by the human NK cell line YT upon activation through surface 2B4. Our data indicate that binding of surface 2B4 by mAb C1.7, that specifically recognizes 2B4, induces transcriptional activation of IFN-γ. Partial inhibition of transcription did not prevent the transcriptional up-regulation of IFN-γ. S1 nuclease protection analysis indicated that transcriptional activation as well as mRNA stability may account for the increased production of IFN-γ by human NK cells following 2B4 stimulation. The significance of this data is discussed in chapter six.

INTRODUCTION

Natural killer cells play a major role in early defense against viruses, intracellular bacteria and tumor cells by direct cell mediated cytotoxicity as well as by producing various cytokines (5, 6, 159). Among the cytokines produced by NK cells, IFN-γ may be particularly important as it controls viral replication, activates macrophages, enhances MHC class I and class II antigen presentation, and directs antigen specific immune responses (160). IFN-γ also promotes Th1 immune responses (160). IFN-γ acts via interaction with the IFN-γ receptor, found on most cell types (161). Studies of the IFN-γ protein have determined the receptor binding sites to be located in both the amino and carboxy ends, however macrophage activation is only dependent on the carboxy terminal domain (162). Antiviral activity requires both carboxy and amino terminal domains (163).

IFN-γ is the only interferon that is also a cytokine, and as such has a global effect on immune system regulation (5). IFN-γ enhances function of antigen presenting cells (164, 165), is involved in recruitment of lymphocytes through augmentation of ICAMs (106) and other cytokines (166), activates T cells (167, 168), inhibits proliferation for most cells, and can induce apoptosis (169, 170). Additionally, IFN-γ is associated with inflammation and consequently is tightly regulated (99). While most of IFN-

γ's actions are beneficial to the host, in some circumstances, particularly autoimmune diseases, IFN-γ can contribute to disease (171-173). Because IFN-γ is produced only in instances of trauma or infection, the lymphocytes must be activated to produce the protein (174). NK cells are capable of producing IFN-γ following a single soluble signal, typically IL-2, IL-12 or IL-18 (175, 176). However, combinations of two of these cytokines are potent inducers of IFN-γ production by human NK cells (176). NK cells also produce IFN-γ following contact with target cells (160). T cells produce IFN-γ following interaction of the T cell receptor with its ligand in the context of MHC. For both NK and T cells, optimal production of IFN-γ occurs following a combination of three types of signals; ligand/receptor interaction, cytokine stimulation, and contact with other cells via cell adhesion molecules (172).

We have previously identified a surface molecule designated 2B4, which is expressed on all murine NK cells and a subset of T cells and monocytes (18, 177). Structural characterization identified 2B4 as a novel member of the immunoglobulin superfamily belonging to the CD2 subgroup (27). We have also characterized the human homologue of 2B4 (28). In both mice and humans, 2B4 is the counter-receptor for CD48 (33). Ligation of surface 2B4 by α-2B4 mAb enhanced cytolytic activity of NK cells against various targets (18). Human 2B4 is expressed on all NK cells,

about 50% of CD8+ T cells, monocytes and basophils (77). Recently we have shown that activation of 2B4 with anti-2B4 monoclonal antibody results in enhanced proliferation, enhanced cytotoxicity and elevated secretion of IFN-γ by human NK cell line, YT (17). However, the regulatory mechanism controlling the NK cell IFN-γ production is poorly understood. In the present study, we have examined the regulation of IFN-γ following activation of human NK cells via surface 2B4. We used the human NK cell line, YT that expresses 2B4, as our model system. Our data indicate that activation of human NK cells via surface 2B4 induces transcriptional activation of IFN-γ. Moreover, mRNA stability may also contribute to the enhanced production of IFN-γ by human NK cells following 2B4 activation.

MATERIALS AND METHODS

Media

4+RPMI: A one liter package of powdered RPMI media (Life Technologies, Rockville, Maryland, Catalog #31800-022) is combined with 2.0 grams sodium bicarbonate (Life Technologies, Catalog #11810-025) and dissolved in 800 ml. deionized, distilled water. pH is adjusted to 7.4 with HCl and volume adjusted to one liter. Media is then supplemented with 10% standard fetal bovine serum (HyClone Lab, Logan, Utah, Catalog #SH30088.03). Media is further supplemented with Sodium Pyruvate – 1X final concentration, non-essential amino acids – 1X final concentration, antibiotic/antimycotic – 1X final concentration and buffered with HEPES buffer (all Life Technologies, Catalog # 11360-070, 11140-050, 15240-062 and 15630-080 respectively).

Cell lines

The cell lines used in the completion of this study are listed in Table 3.1.

Table 3.1

Cell Line	Origin	Media
YT	Human leukemic NK cell line	4+RPMI
K562	Human erythroleukemia cell line	4+RPMI

Cell Culture

All mammalian cell lines were maintained in sterile conditions in Nunc EasYFlasks with canted necks and filtered tops. Cell growth media was 4+ RPMI. Media was completely replaced every 48 hours. Cells were maintained at 37°C with 5% CO_2 saturation.

Antibodies

C1.7 antibody which recognizes h2B4 (63) was purchased from Coulter (Orlando, FL). Isotype mAb control was kindly donated by Dr. V. Kumar, UT Southwestern, Dallas, TX.

Reagents and chemicals

Tissue culture media and fetal bovine serum were purchased from Gibco BRL (Gaithersburg, MD). C1.7 (anti-2B4 mAb) was purchased from Coulter (Pittsburgh, PA). Actinomycin D was purchased from Sigma Chemical (St. Louis, MO) in dehydrated form and was resuspended in DMSO to a concentration of 1 mg/ml.

Interferon-γ Release Assay

YT cells were incubated in the presence of K562 cells in 2 ml. culture media and in various conditions including IL-2, C1.7 mAb and isotype control mAb for 16 hours at 37°C under 5 % CO_2 in air. Where indicated, IL-2, mAb C1.7 and isotype mAb control was added to final concentrations of 20 U/ml, 200 ng/ml and 200 ng/ml, respectively. Also where indicated, Actinomycin D (ActD) was added at various time points to a final concentration of 10mg/ml to inhibit new transcription. The cells were then spun down at 250 X *g* for 5 min at 4°C. 100 µl of the supernatant was then extracted and tested for the presence of IFN-γ. IFN-γ protein secretion was quantitated immunologically by IFN-γ human ELISA system (Amersham), as per manufacturer's instructions.

Interferon-γ ELISA

BIOTRAK ELISA system from Amersham-Pharmacia-Biotech was used to quantitate IFNγ in cell free supernatant. Supplied directions were followed. 50 µl of biotinylated antibody reagent was added to each well followed by 50 µl of cell free supernatant or standard. Wells were covered with a clear adhesive strip and incubated at room temperature for 2 hours. Wells were emptied and washed three times with wash buffer. Wells were

aspirated after final wash to ensure removal of all wash buffer. 100 µl of streptavidin-HRP conjugate reagent was added to each well. Wells were covered with an adhesive strip and incubated at room temperature for 30 minutes. Wells were again washed three times with wash buffer and then aspirated to remove all liquid. 100 µl of TMB substrate solution was added to each well and the plate was incubated uncovered in the dark for 30 minutes. 100 µl stop solution was added to each well and the plate was read at 450 nm within 30 minutes.

Cell Stimulation for Transcript Analysis

4 x 10^6 YT cells and 4 x 10^5 K562 cells were needed per reaction condition. The appropriate number of viable cells were harvested by centrifugation at 250 x g, 4°C. Cells were resuspended in fresh 4+RPMI and 1 ml. of each cell type was aliquoted per reaction such that each reaction had a final volume of 2 ml. Where indicated, cells were stimulated with 200 ng/ml C1.7 mAb. Also where indicated, transcription is inhibited by the addition of 10 mg./ml. Reactions are incubated at 37°C with 5% CO_2 saturation for various time points. At appropriate time points, cells were harvested by centrifugation at 250 x g, 4°C. Supernatant was saved for ELISA analysis and RNA was isolated from cell pellet as indicated.

Cytoplasmic RNA Isolation

Cytoplasmic RNA was isolated from 4 – 5 million cells using the Qiagen RNeasy Mini Isolation System according to manufacturer's instructions (Valencia, CA).

S1 Nuclease Analysis

10 μg of cytoplasmic RNA was used to hybridize with γ^{32}P-ATP end-labeled oligos specific for interferon gamma mRNA or β-actin mRNA. Hybridization reactions were heated to 90°C for 10 minutes prior to incubating at 55°C for 16 – 24 hours. Following hybridization, each reaction was digested with S1 nucelase (Gibco BRL, Gaithersburg, MD) in the presence of calf-thymus DNA (Sigma Chemical, St. Louis, MO). Each reaction was ethanol precipitated with carrier tRNA and resuspended in 10 μl 0.1M NaOH. 3 μl were combined with 3 μl formamide loading dye, heated to 90°C before resolution on a 6% denaturing polyacrylamide gel. Gels were exposed to X-ray film overnight at -85°C. Hybridization oligonucleotides were designed for this study and are listed in Table 3.2 (Integrated DNA Technologies, Inc. Coralville, IA).

Table 3.2

Oligonucleotides Used in This Study

Gene	*Sequence*	*Position*
IFN-γ	5'- GCGACAGTTCAGCCATCACTTGGATGA GTTCATGTATTGCTTTGCGTTGGACATTCAAG TCAGTTACCGAATAATTAGTC -3'	+731 to +750
β-actin	5'- GTTGAAGGTCTCAAACATGATCTGGGT CATCTTCTCGCGGTTGGCCTTGGGGTTCAG GGGGGCCT -3'	+482 to +504

Cloning the Interferon-γ Promoter

Various lengths of fragments of the human IFN-γ gene promoter were obtained by PCR from genomic DNA using oligos described in Table 4.2. PCR products of appropriate size were TA cloned into the pGEMT-easy vector (Promega Corp) with T4 DNA ligase (New England Biolabs) overnight at 14°C. Ligation reactions were used to transform *E. coli* as described herein. Positive *E. coli* colonies were procured and cultured for plasmid isolation. IFN-γ promoter fragments were subsequently excised from the pGEMT-easy vector with restriction endonuclease digestion and cloned into the pGL2 luciferase promoter reporter vector (Promega) as

described above. These ligation reactions were also used to transform *E. coli* and positive colonies procured and confirmed by sequence analysis.

Preparation of Electrocompetent E. coli

Five ml. sterile LB broth was inoculated with a single colony of *E. coli* DH5α and grown overnight at 37°C with moderate shaking. 500 ml. LB broth was inoculated with 2.5 ml. of the overnight culture and grown for approximately 6 hours (mid-log phase) at 37°C with 300 rpm rotary aeration. Cultures were then chilled in an ice-water bath for 10 to 15 minutes prior to being harvested by centrifugation at 4000 rpm for 20 minutes at 4°C. The cell pellet was washed twice in 500 ml. ice-cold water and then centrifuged as before. Cell pellet was then washed in 40 ml ice-cold 10% glycerol and centrifuged as before. An equal volume of ice-cold 10% glycerol was used to resuspend the pellet before aliquoting cells into 40 µl. volumes. Electrocompetent cells were stored at -80°C.

Bacterial Transformation by Electroporation

Electroporation apparatus was set to 2.5 kV, 25 µF, 200 ohms. 500 µg plasmid DNA was added to 40 µl electrocompetent *E. coli.* and kept on ice. Cell-DNA mixtures were transferred to an ice-cold 0.1mm electroporation cuvette. Condensation was removed from the cuvette

with a kimwipe immediately prior to pulsing in the electroporator. Cells were immediately recovered in 1 ml. LB broth and incubated for 1 hour at 37°C with 150 rpm rotary aeration. 100 µl was spread on LB agar containing 100 µg/ml ampicillin for the selection of resistant colonies.

Bacterial Culture and Procurement

A desired volume of sterile LB broth was inoculated with a single *E. coli* colony grown on LB agar. The culture was incubated 15 – 18 hours at 37°C with 180 rpm rotary aeration. Cultures that were suspected to contain desired plasmid constructs were used to inoculate fresh LB media containing 100 µg/ml ampicillin and grown under the same conditions for 4 hours. One ml. of four hour cultures were mixed with an equal volume of glycerol and stored at -80°C.

Preparation of Transfection Quality Plasmid DNA

Five ml. of LB broth with 100 µg/ml ampicillin was inoculated with a single colony of *E. coli* containing the desired plasmid. Inoculated cultures were grown at 37°C with 180 rpm rotary aeration for 15 to 18 hours. One ml. of the overnight cultures were used to inoculate 500 ml. of LB broth with 100 µg/ml. ampicillin and grown for approximately 15 hours at 37°C with 180 rpm rotary aeration. Cells were harvested by centrifugation at 6000 x g,

4°C for 10 minutes. Cell pellets were resuspended in 4 ml. glucose/Tris/EDTA solution. Lysozyme (25 mg/ml) was added to cell suspension and incubated at room temperature for 10 minutes. 10 ml. freshly prepared 0.2 M NaOH/1% SDS was added followed by gentle mixing and incubation on ice for 10 minutes. 7.5 ml. 3M potassium acetate solution was added and gently mixed before incubating on ice for 10 minutes. Cell lysate was centrifuged for 10 minutes at 20, 000 x g at 4°C. Supernatant was filtered through kimwipes into a fresh tube. Isopropanol was added at 0.6 volumes of supernatant and mixed by inversion. Plasmid DNA precipitated at room temperature within 10 minutes and collected by centrifugation at 15,000 x g for 10 minutes at room temperature. The plasmid pellet was resuspended in 4 ml. TE buffer:CsCl (1:1.1). 400 µl of 10 mg/ml ethidium bromide was added. The plasmid solution was transferred to a 5 ml. ultracentrifuge tube and volume was adjusted with TE:CsCl to be within 0.1 mg of the weight of the other tubes (counterbalance). A plasmid band was resolved by centrifugation at 500,000 x g for 20 hours at 20°C. An 18 guage needle fitted to a 3 ml. syringe was used to extract the plasmid band from the tube using UV visualization. Plasmid bands were recovered to TE:CsCl solution and the ultracentrifugation was repeated to obtain ultra pure DNA. Ethidium bromide was extracted using CsCl saturated butanol until clear. 2 volumes of ice-cold 100% ethanol were

added. Any floating DNA was collected to a separate tube containing 70% ethanol and washed. Remaining DNA was precipitated overnight at -20°C and then washed with 70% ethanol before being suspended in TE pH 8.0.

Transient Transfection

Transient transfections are carried out in 12-well tissue culture plates purchased from Costar. 6 µl of D-MRIE-C lipid-bases transfection reagent (Life Technologies,) was added to each well along with 500 µl Opti-MEM media (Life Technologies) and gently mixed. 4µg of an IFN-γ promoter containing vector was combined with 400 ng of pRL3-CMV (control vector) were diluted in 500 µl Opti-MEM media and then added to the appropriate well and gently mixed. DNA and lipid mixtures were incubated at room temperature for 45 minutes to allow them to complex. Two million YT cells were required for each reaction. Cells were harvested by centrifugation at 250 x g, 4°C for 5 minutes. Cell pellets were resuspended in a volume of Opti-MEM media that is equivalent to 200 µl per reaction. 200 µl of cell suspension was gently added to each transfection well. Transfection was accomplished during a 4 hour incubation at 37°C with 5% CO_2 saturation. Transfected cells were then stimulated as required and described herein.

Cell Stimulation for Promoter Analysis

2 x 10^5 K562 cells were needed per reaction condition. The appropriate number of viable cells were harvested by centrifugation at 250 x g, 4°C. Cells were resuspended in fresh 4+RPMI at a volume equivalent to 2 ml. per reaction condition. 2 ml. of cell suspension was added to each appropriate well containing transfected YT cells. Where appropriate, 200 ng/ml. C1.7 mAb was added to each well. Reaction plates were incubated for 48 hours at 37°C with 5% CO_2 saturation. Cells were then harvested and assayed for luciferase as an indicator of promoter activity.

Luciferase Assay

The Dual Luciferase Assay System (Promega Corp) was used to detect luciferase levels in transfected cells. Stimulated and unstimulated transfected YT cells were collected into 15 ml. tubes and centrifuged at 250 x g, 4°C for 5 minutes. Cells were washed in 1 ml. PBS before being suspended in Passive Lysis Buffer (provided with assay system). Cells lysed while being rocked in passive lysis buffer for one hour at room temperature. Reactions were centrifuged at maximum speed in a microcentrifuge, 4°C for 3 minutes. 20 µl of luciferase-containing supernatant is used for quantification of luciferase following the addition

of a substrate (LAR II) which permits detection by a luminometer. A second substrate which permits detection of renilla luciferase (control) was added and detected in a luminometer. Luciferase amounts were normalized against renilla luciferase whose production was driven via CMV promoter.

RESULTS

2B4 Activation of YT cells upregulates the production of secreted IFN-γ

In order to establish the time course for the up-regulation of IFN-γ following ligation of surface 2B4, we determined the production of secreted IFN-γ by YT cells over a period of 0 to 8 hrs following incubation with mAb C1.7, which specifically recognizes human 2B4. We stimulated 4 million YT cells, with a 10:1 ET ratio, with 200 ng/ml C1.7 mAb. Cells were incubated at 37°C with 5% CO_2 for up to 8 hours. Cell-free supernatants were collected and analyzed at 0, 2, 4, 6, and 8 hours following 2B4 activation. Figure 3.1 shows a steady rise in the amount of secreted IFN-γ at each time point following activation. Because interaction with target cells is required for optimal production of IFN-γ by NK cells, we have included K562 tumor cells in the experiments. As seen in Figure 3.1, incubation of NK cells with target cells alone also result in the production of INF-γ. However, the difference in the induction of IFN-γ between 2B4 activated NK cells and the control group which contain target cells and an isotype control antibody becomes significant (with respect to one standard deviation) by 8 hours following activation.

Actinomycin D prevents an increase in secreted IFN-γ by 2B4-activated YT cells.

The increase in the induction of secreted IFN-γ as seen in figure 3.1, could be due to several mechanisms, such as transcriptional up-regulation of IFN-γ gene, enhanced mRNA stability or translational up-regulation. To dissect the different pathways, we inhibited the transcription of genes using actinomycin D. The concentration of the actinomycin D was selected based on published studies with similar cell types. Additionally, we experimented with higher concentrations of ActD and found 10 μg/ml to be the highest concentration not toxic to the cells (data not shown). We stimulated 4 million YT cells, with a 10:1 ET ratio, with 100 μg/ml C1.7 mAb. Cells were incubated at 37°C with 5% CO_2 for 4 hours prior to inhibiting transcription with 10 μg/ml actinomycin D. Cell-free supernatants were collected and analyzed at 0, 1.5, 3, 5, and 7 hours following actinomycin D treatment. At least partial inhibition of transcription resulted in a reduction of IFN-γ secretion in 1.5 hrs, and this level of IFN-γ remained almost the same over a period of several hours. The initial inhibition was more prominent in 2B4 stimulated cells compared to the unstimulated control cells as seen in figure 3.2. Figure 3.2 also shows that beyond 1.5 hours following the inhibition, there is no significant

difference in the level of IFN-γ secreted between the 2B4 activated group and the control group, which was not treated with C1.7.

2B4 stimulation of YT cells enhances IFN-γ mRNA stability

Our initial analyses by using reverse-trancsriptase PCR as well as S1 nuclease protection assay indicated transcriptional induction of IFN-γ mRNA following 2B4 stimulation. To determine the effect of partial inhibition of transcription on the expression of IFN-γ mRNA, we utilized S1 nuclease protection assay with RNA isolated at various time points after actinomycin D treatment of YT cells which were incubated with C1.7 or 22B5, the isotype control antibody. We stimulated 4 million YT cells, with a 10:1 ET ratio, with 100 μg/ml C1.7 mAb. Cells were incubated at 37°C with 5% CO_2 for 4 hours prior to partially inhibiting transcription with 10 μg/ml actinomycin D. Cells were harvested and cytoplasmic RNA isolated at 0, 1, 2, 3 and 4 hours following actinomycin D treatment. 10 μg of cytoplasmic RNA was hybridized with single stranded DNA fragments specific for IFN-γ and β-actin. S1 nuclease was used to degrade unhybridized nucleic acid. Products were resolved with 6% denaturing PAGE. The control group in which 2B4 was not activated showed a minor amount of IFN-γ mRNA with a steady decline over the 4 hour period (figure 3.3A). β-actin mRNA levels remained relatively constant. Figure 3.3B shows an increase in the amount of IFN-γ mRNA present in 2B4 activated cells over a period of 4 hours following actinomycin D treatment. This increase

could be due to the processing of hnRNA to mRNA or to partial inhibition of transcription.

2B4 activation results in enhanced IFN-γ promoter activity

The full-length IFN-γ promoter was cloned upstream of a promoterless luciferase gene. The promoter construct was transiently transfected into YT cells as described in Materials and Methods. Transfectants were incubated in the presence of target cells at a ratio of 10:1(both un-stimulated and stimulated) and stimulated with 200 ng/ml C1.7 mAb (stimulated only). A second plasmid containing the renilla luciferase gene driven by a CMV promoter was co-transfected for standardization. Firefly luciferase was normalized to renilla luciferase. Transfectants containing the full-length IFNγ promoter had a four-fold increase in promoter activity when stimulated with C1.7 mAb over unstimulated transfectants (Figure 3.4).

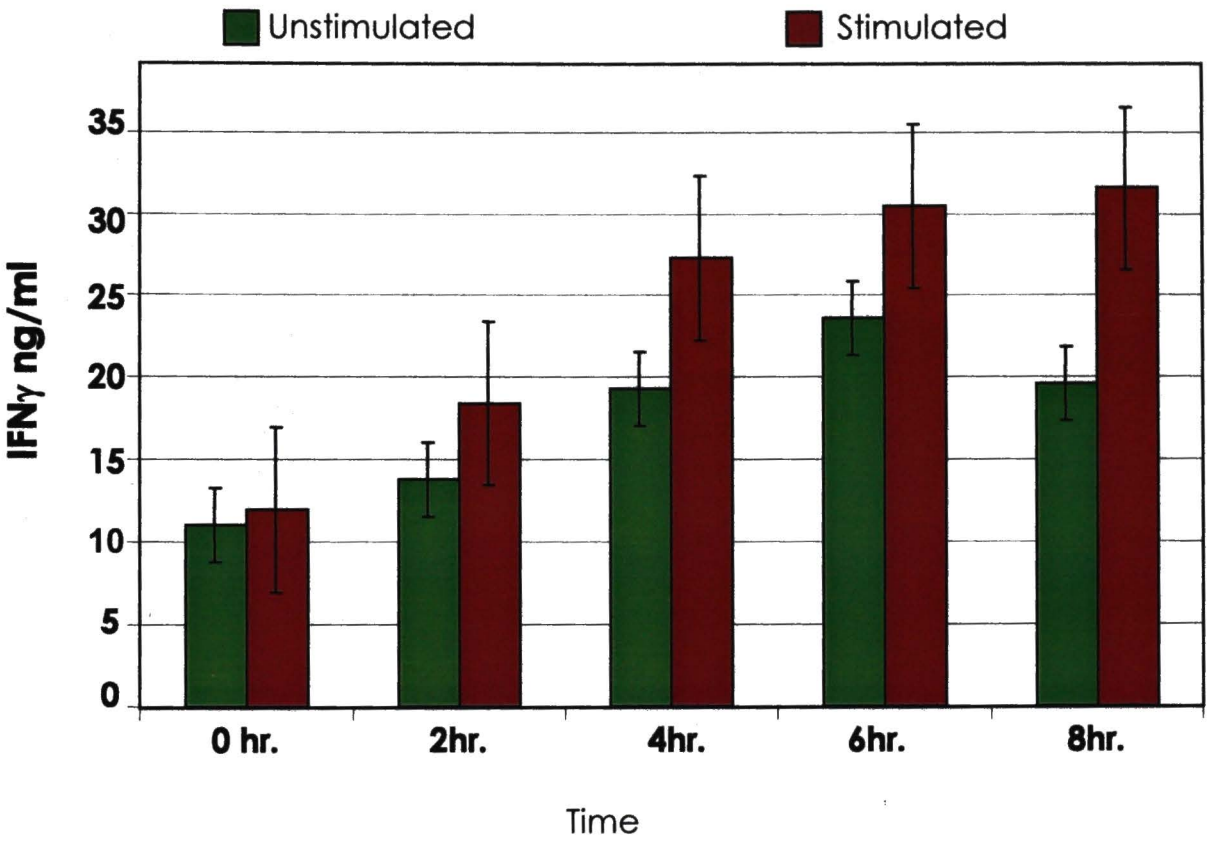


Figure 3.1
2B4-activation results in enhanced IFN-γ within 8 hours.
YT cells were incubated in the presence of target cells (K562) at a 10:1 ratio. C1.7 was used to stimulate at 200 ng/ml where indicated. Cell-free supernatant was analyzed for interferon gamma at 0, 2, 4, 6 and 8 hours by ELISA. All reaction conditions were done in triplicate.

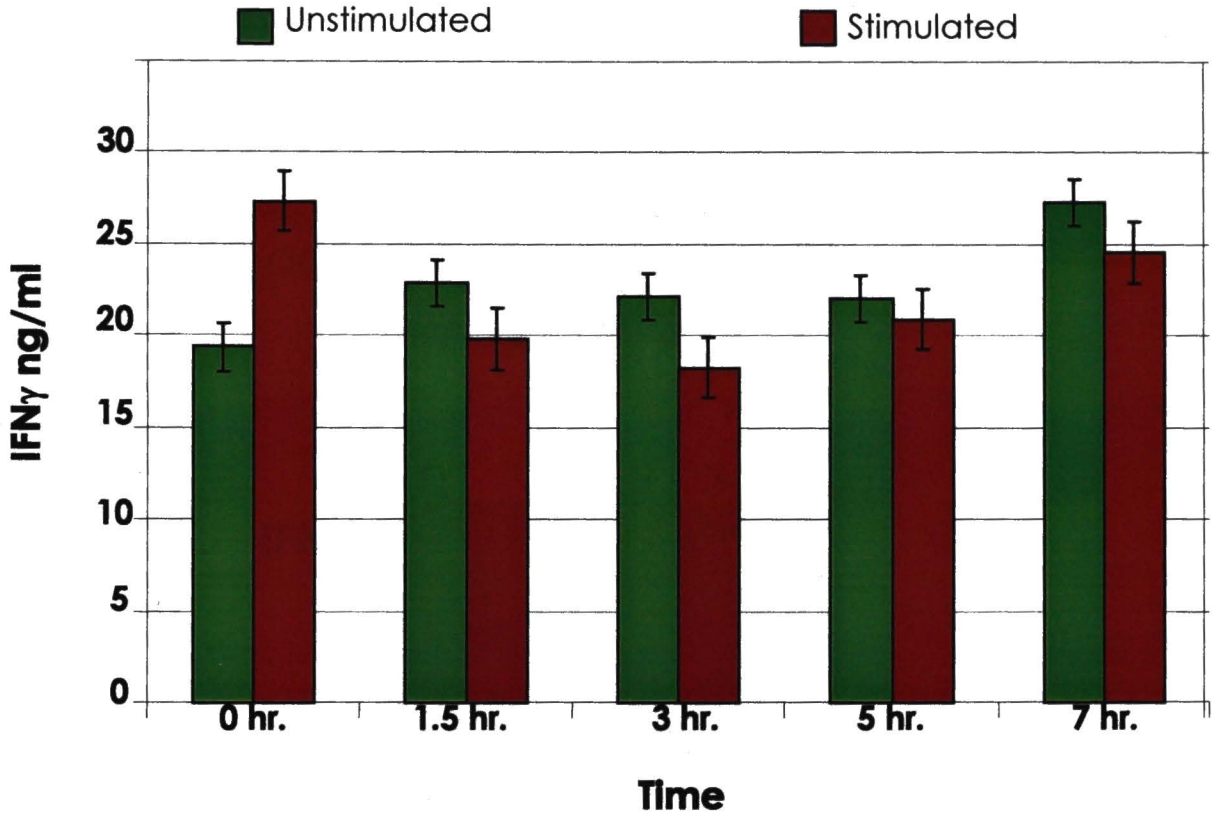


Figure 3.2
Actinomycin D prevents 2B4-activated YT cells from secreting an increased amount of IFN-γ.
YT cells were incubated in the presence of target cells (K562) at 10:1 ratio. C1.7 was used to stimulate at 200 ng/ml (solid bars). Control shown in striped bars. Actinomycin D was added 4 hours after C1.7 stimulation at a concentration that partially inhibited transcription, but was not lethal to the cells (10 μg/ml). Cell-free supernatant was analyzed for interferon gamma at 0, 1.5, 3, 5 and 7 hours following the addition of Actinomycin D (25 μg/ml) by ELISA. All reaction conditions were done in triplicate.

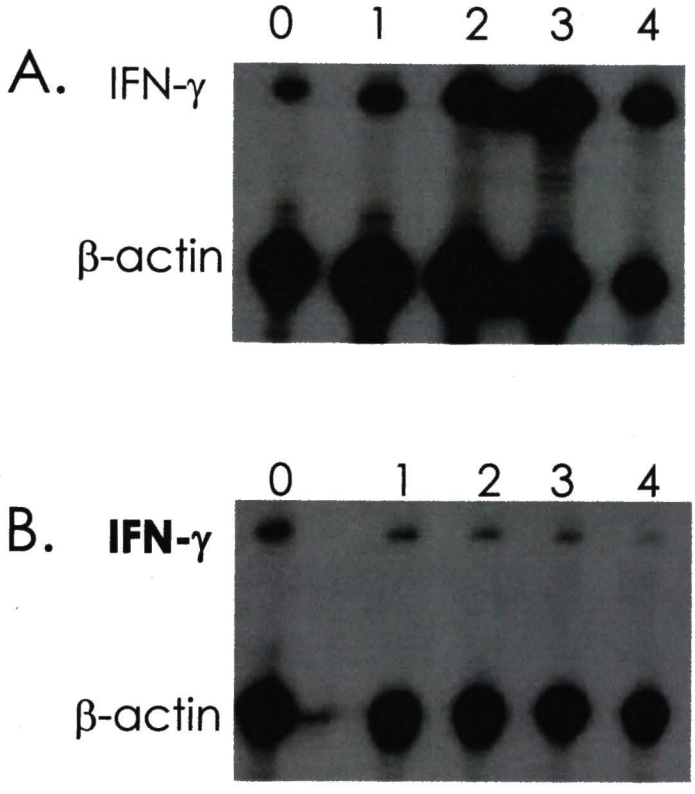


Figure 3.3
2B4-activation results in enhanced IFN-γ mRNA stability.
S1 Nuclease Protection. YT cells were incubated in the presence of target cells (K562) at 10:1 ratio. (A)C1.7 was used to stimulate at 200 ng/ml. (B) No antibody was added. (Both)Actinomycin D was added 4 hours after C1.7 stimulation at a concentration that partially inhibited transcription, but was not lethal to the cells (10 μg/ml). RNA was isolated at 0 hrs (lane 1), 1 hr. (lane 2), 2 hrs. (lane 3), 3 hrs. (lane 4) and 4 hrs. (lane 5). 10 μg of cytoplasmic RNA was hybridized with sequence specific oligos for IFN-γ & β-actin. Unhybridized nucleic acid was digested with S1 nuclease. Products were resolved with 6% denaturing PAGE.

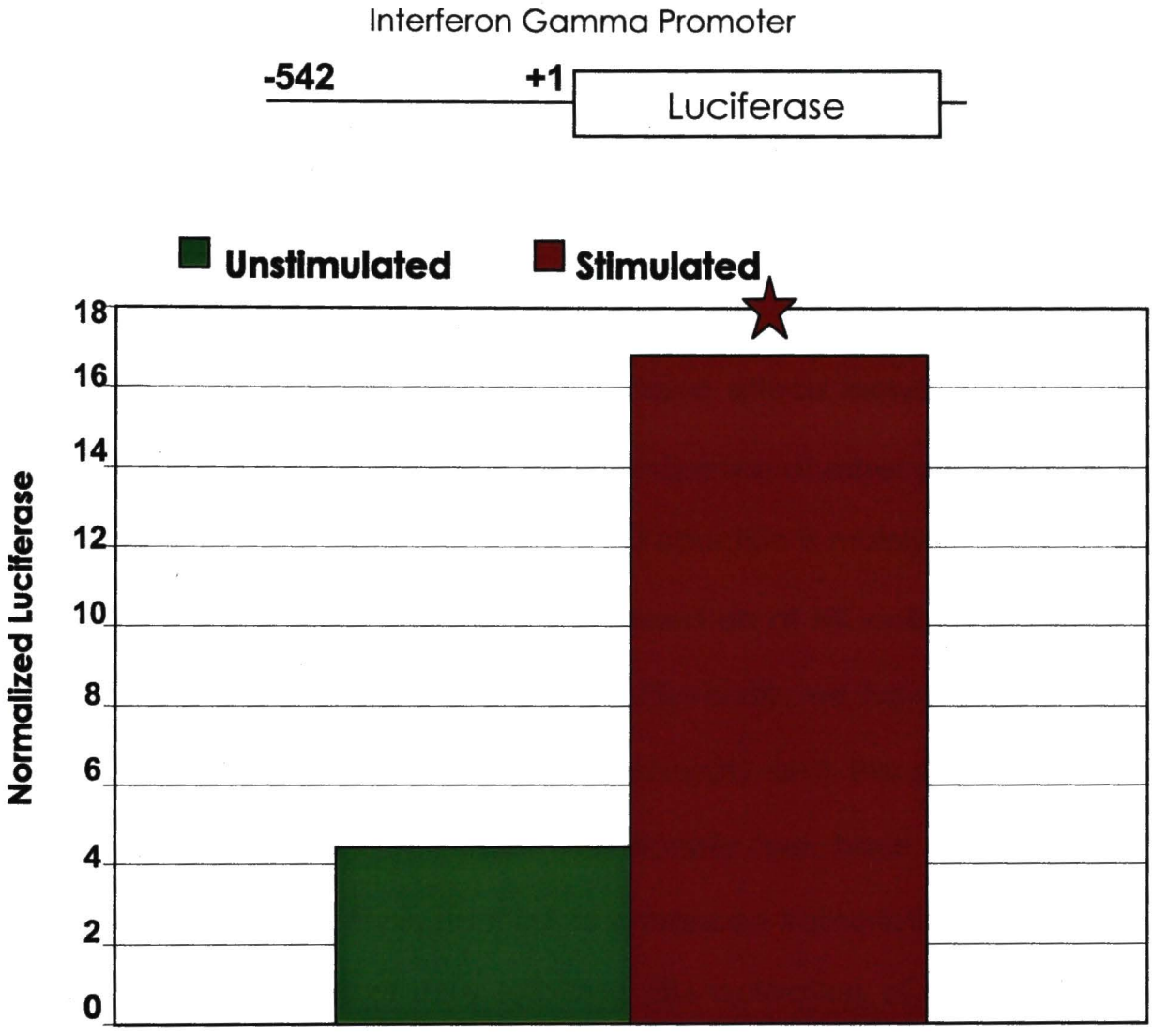


Figure 3.4
2B4-activation results in an up-regulation of the IFN-γ promoter.
The full-length IFN-γ promoter was cloned upstream of a promoterless luciferase gene, vector pGL.542. Vector pGL.542 was transiently transfected into YT cells, which were subsequently cultured in the presence of K562 target cells in media alone or with 200 ng/ml C1.7. The red star indicates statistical sianificance of p = 0.0136 as determined by t-

CHAPTER FOUR

MOLECULAR CLONING AND CHARACTERIZATION OF THE IFN-γ PROMOTER IN 2B4-ACTIVATED NATURAL KILLER CELLS

SUMMARY

IFN-γ is a cytokine with global immune effects including anti-viral and anti-proliferative activities as well as induction of other cytokines and recruitment of other immune cells. IFN-γ production is mainly by NK and T cells. 2B4 is a cell surface receptor expressed on all NK cells, a subset of CD8+ T cells, monocytes and basophils. Previously, we have shown that binding of 2B4 results in enhanced cytotoxicity and the production of interferon gamma by NK cells. Additionally we have shown that enhanced mRNA stability in addition to enhanced transcription contribute to the elevated levels of IFN-γ following 2B4 activation of NK cells. The focus of the current study is to identify key promoter regions involved in the production of IFN-γ by 2B4 activated NK cells. The significance of this data is discussed in chapter six.

INTRODUCTION

Natural killer (NK) cells are an important part of our innate immunity. They provide early defense against viruses, intracellular bacteria and tumor cells. NK cells employ two main methods in defending against these pathogens: direct cell mediated cytotoxicity and production of cytokines (5, 6, 159). NK cells are triggered to respond through binding of activating receptors on the NK cell surface.

2B4 is an activating receptor previously identified on the surface of NK cells and a subset of T cells that mediate NK like killing (18, 177). 2B4 belongs to the immunoglobulin superfamily and is a member of the CD2 subfamily (27). Additionally we have characterized the human homologue of 2B4 and found CD48 to be the counter-receptor in both mice and humans (27, 28). Functional analysis in YT cells (an NK cell line) reveals enhanced proliferation, enhanced cytotoxicity and elevated secretion of IFN-γ upon binding of 2B4 with monoclonal antibody.

Among the cytokines produced by NK cells, IFN-γ may be particularly important as it has a global effect on immune regulation (5). Functions of IFN-γ include the control of viral replication, activation of macrophages, enhancing MHC class I and class II antigen presentation,

and direction of antigen specific immune responses (160). IFN-γ is also involved in recruitment of other lymphocytes through augmentation of ICAMs and other cytokines (166). IFN-γ can inhibit proliferation in most cells and can also induce apoptosis (169, 170).

Due to its global effect, IFN-γ is tightly regulated (99). It is only produced following lymphocyte activation (174). The regulatory mechanisms controlling the production of IFN-γ has been the focus of many studies in T cells. Through the many studies, we can begin to piece together the molecular regulation of IFN-γ production by T cells. Production of IFN-γ by NK cells remains poorly understood. A recurring theme in the discussions of this topic is that the regulation of the IFN-γ gene is very complex and likely differs among cell types as well as in response to different stimuli.

Studies done in T cells give us several clues to how this gene is regulated following lymphocyte activation. However, it must be noted that these are only clues and that each lymphocyte population, under the influence of each activation stimulus should be investigated separately in order to gain a true understanding. Sica, et. al. found multiple NFκB binding sites within the IFN-γ promoter and that the nuclear appearance of NFκB is induced by IL-2 but not IL-12 (121, 122). They were also able to show that a cooperation between NFAT and NFκB induce maximal transcription of the IFN-γ gene in T cells under PMA stimulation

(121). Penix et. al shows that a Jun/ATF-2 heterodimer binds to the proximal element (-73 to –48) to positively affect transcription in PMA activated Jurkat cells, while another group shows the same heterodimer binding to the distal element (-98 to –78) and not the proximal element in IL-12 stimulated cells (119, 120). Barbulescu et. al. showed binding of an AP-1 site (-190) and a STAT4 site (-236) in IL-12/IL-18 activated T cells (178). AP-1 and NFκB bind the IFN-γ promoter in an NK cell line under IL-2 stimulation (125). Transcription factors required for IFN-γ expression during Th1 cell differentiation include AP-1, STAT4, and IRF-1 (119, 179-183). YY-1 has been suggested as both an enhancer and silencer of IFN-γ expression (122, 124).

While production of cytokines by activated lymphocytes is predominantly controlled at the transcriptional level, some studies have reported regulation through transcription and/or enhanced mRNA stability following binding of cell surface receptors (184, 185). Additionally, we have shown that binding of 2B4 may regulate IFN-γ production by the human NK cell line, YT, through mRNA stability and transcription (186).

The focus of the current study is to identify key promoter regions in regulating IFN-γ transcription following 2B4 activation of human NK cells. Results from this study will help narrow the focus on potential transcription factors which may play a role in this regulation.

MATERIALS AND METHODS

Media

4+RPMI: A one liter package of powdered RPMI media (Life Technologies, Rockville, Maryland, Catalog #31800-022) is combined with 2.0 grams sodium bicarbonate (Life Technologies, Catalog #11810-025) and dissolved in 800 ml. deionized, distilled water. pH is adjusted to 7.4 with HCl and volume adjusted to one liter. Media is then supplemented with 10% standard fetal bovine serum (HyClone Lab, Logan, Utah, Catalog #SH30088.03). Media is further supplemented with Sodium Pyruvate – 1X final concentration, non-essential amino acids – 1X final concentration, antibiotic/antimycotic – 1X final concentration and buffered with HEPES buffer (all Life Technologies, Catalog # 11360-070, 11140-050, 15240-062 and 15630-080 respectively).

Cell lines

The cell lines used in the completion of this study are listed in Table 4.1.

Table 4.1

Cell Line	***Origin***	***Media***
YT	Human leukemic NK cell line	4+RPMI
K562	Human erythroleukemia cell line	4+RPMI

Cell Culture

All mammalian cell lines were maintained in sterile conditions in Nunc EasYFlasks with canted necks and filtered tops. Cell growth media was 4+ RPMI. Media was completely replaced every 48 hours. Cells were maintained at 37°C with 5% CO_2 saturation.

Antibodies

C1.7 antibody which recognizes h2B4 (63) was purchased from Coulter (Orlando, FL). Isotype mAb control was kindly donated by Dr. V. Kumar, UT Southwestern, Dallas, TX.

Reagents and chemicals

Tissue culture media and fetal bovine serum were purchased from Gibco BRL (Gaithersburg, MD). C1.7 (anti-2B4 mAb) was purchased from Coulter (Pittsburgh, PA).

Cloning the Interferon-γ Promoter

Various lengths of fragments of the human IFN-γ gene promoter were obtained by PCR from genomic DNA using oligos described in Table 4.2. PCR products of appropriate size were TA cloned into the pGEMT-easy vector (Promega Corp) with T4 DNA ligase (New England Biolabs) overnight at 14°C. Ligation reactions were used to transform *E. coli* as described herein. Positive *E. coli* colonies were procured and cultured for plasmid isolation. IFN-γ promoter fragments were subsequently excised from the pGEMT-easy vector with restriction endonuclease digestion and cloned into the pGL2 luciferase promoter reporter vector (Promega) as described above. These ligation reactions were also used to transform *E. coli* and positive colonies procured and confirmed by sequence analysis.

Preparation of Electrocompetent E. coli

Five ml. sterile LB broth was inoculated with a single colony of *E. coli* DH5α and grown overnight at 37°C with moderate shaking. 500 ml. LB broth was inoculated with 2.5 ml. of the overnight culture and grown for approximately 6 hours (mid-log phase) at 37°C with 300 rpm rotary aeration. Cultures were then chilled in an ice-water bath for 10 to 15 minutes prior to being harvested by centrifugation at 4000 rpm for 20 minutes at 4°C. The cell pellet was washed twice in 500 ml. ice-cold water

and then centrifuged as before. Cell pellet was then washed in 40 ml ice-cold 10% glycerol and centrifuged as before. An equal volume of ice-cold 10% glycerol was used to resuspend the pellet before aliquoting cells into 40 µl. volumes. Electrocompetent cells were stored at -80°C.

Bacterial Transformation by Electroporation

Electroporation apparatus was set to 2.5 kV, 25 µF, 200 ohms. 500 µg plasmid DNA was added to 40 µl electrocompetent *E. coli.* and kept on ice. Cell-DNA mixtures were transferred to an ice-cold 0.1mm electroporation cuvette. Condensation was removed from the cuvette with a kimwipe immediately prior to pulsing in the electroporator. Cells were immediately recovered in 1 ml. LB broth and incubated for 1 hour at 37°C with 150 rpm rotary aeration. 100 µl was spread on LB agar containing 100 µg/ml ampicillin for the selection of resistant colonies.

Bacterial Culture and Procurement

A desired volume of sterile LB broth was inoculated with a single *E. coli* colony grown on LB agar. The culture was incubated 15 – 18 hours at 37°C with 180 rpm rotary aeration. Cultures that were suspected to contain desired plasmid constructs were used to inoculate fresh LB media containing 100 µg/ml ampicillin and grown under the same conditions for

4 hours. One ml. of four hour cultures were mixed with an equal volume of glycerol and stored at -80°C.

Preparation of Transfection Quality Plasmid DNA

Five ml. of LB broth with 100 μg/ml ampicillin was inoculated with a single colony of *E. coli* containing the desired plasmid. Inoculated cultures were grown at 37°C with 180 rpm rotary aeration for 15 to 18 hours. One ml. of the overnight cultures were used to inoculate 500 ml. of LB broth with 100 μg/ml. ampicillin and grown for approximately 15 hours at 37°C with 180 rpm rotary aeration. Cells were harvested by centrifugation at 6000 x g, 4°C for 10 minutes. Cell pellets were resuspended in 4 ml. glucose/Tris/EDTA solution. 25 mg/ml lysozyme was added to cell suspension and incubated at room temperature for 10 minutes. 10 ml. freshly prepared 0.2 M NaOH/1% SDS was added followed by gentle mixing and incubation on ice for 10 minutes. 7.5 ml. 3M potassium acetate solution was added and gently mixed before incubating on ice for 10 minutes. Cell lysate was centrifuged for 10 minutes at 20, 000 x g at 4°C. Supernatant was filtered through kimwipes into a fresh tube. Isopropanol was added at 0.6 volumes of supernatant and mixed by inversion. Plasmid DNA precipitated at room temperature within 10 minutes and collected by centrifugation at 15,000 x g for 10 minutes at room temperature. The

plasmid pellet was resuspended in 4 ml. TE buffer:CsCl (1:1.1). 400 µl of 10 mg/ml ethidium bromide was added. The plasmid solution was transferred to a 5 ml. ultracentrifuge tube and volume was adjusted with TE:CsCl to be within 0.1 mg of the weight of the other tubes (counterbalance). A plasmid band was resolved by centrifugation at 500,000 x g for 20 hours at 20°C. An 18 guage needle fitted to a 3 ml. syringe was used to extract the plasmid band from the tube using UV visualization. Plasmid bands were recovered to TE:CsCl solution and the ultracentrifugation was repeated to obtain ultra pure DNA. Ethidium bromide was extracted using CsCl saturated butanol until clear. 2 volumes of ice-cold 100% ethanol was added. Any floating DNA was collected to a separate tube containing 70% ethanol and washed. Remaining DNA was precipitated overnight at -20°C and then washed with 70% ethanol before being suspended in TE pH 8.0.

Transient Transfection

Transient transfections are carried out in 12-well tissue culture plates purchased from Costar. 6 µl of D-MRIE-C lipid-bases transfection reagent (Life Technologies,) was added to each well along with 500 µl Opti-MEM media (Life Technologies) and gently mixed. 4µg of an IFN-γ promoter containing vector was combined with 400 ng of pRL3-CMV (control

vector) were diluted in 500 µl Opti-MEM media and then added to the appropriate well and gently mixed. DNA and lipid mixture were incubated at room temperature for 45 minutes to allow them to complex. Two million YT cells were required for each reaction. Cells were harvested by centrifugation at 250 x g, 4°C for 5 minutes. Cell pellets were resuspended in a volume of Opti-MEM media that is equivalent to 200 µl per reaction. 200 µl of cell suspension was gently added to each transfection well. Transfection was accomplished during a 4-hour incubation at 37°C with 5% CO_2 saturation. Transfected cells were then stimulated as required and described herein.

Cell Stimulation for Promoter Analysis

2 x 10^5 K562 cells were needed per reaction condition. The appropriate number of viable cells were harvested by centrifugation at 250 x g, 4°C. Cells were resuspended in fresh 4+RPMI at a volume equivalent to 2 ml. per reaction condition. 2 ml. of cell suspension was added to each appropriate well containing transfected YT cells. Where appropriate, 200 ng/ml. C1.7 mAb was added to each well. Reaction plates were incubated for 48 hours at 37°C with 5% CO_2 saturation. Cells were then harvested and assayed for luciferase as an indicator of promoter activity.

Luciferase Assay

The Dual Luciferase Assay System (Promega Corp) was used to detect luciferase levels in transfected cells. Stimulated and unstimulated transfected YT cells were collected into 15 ml. tubes and centrifuged at 250 x g, 4°C for 5 minutes. Cells were washed in 1 ml. PBS before being suspended in Passive Lysis Buffer (provided with assay system). Cells lysed while being rocked in passive lysis buffer for one hour at room temperature. Reactions were centrifuged at maximum speed in a microcentrifuge, 4°C for 3 minutes. 20 μl of luciferase-containing supernatant is used for quantification of luciferase following the addition of a substrate (LAR II) which permits detection by a luminometer. A second substrate which permits detection of renilla luciferase (control) was added and detected in a luminometer. Luciferase amounts were normalized against renilla luciferase whose production was driven via CMV promoter.

Table 4.2

Oligonucleotides Used in This Study

Sequence	***Construct***
5' ATT GCT CTC GAG GCT GCA CCT CCT CTG GCT GCT GGT ATT TAT ACC 3'	542 Reverse
5' ATT GCT GGT ACC TCT AGA GCA ATT TGA AAC TTG TGG TAG ATA 3'	542 Forward
5' ATT GCT GGT ACC GGG CGA AGT GGG GAG GTA C 3'	302 Forward
5' ATT GCT GGT CC GTA AAA GTG CCT CAA AGA ATC 3'	252 Forward
5' ATT GCT GGT ACC GAA TGG CAC AGG TGG GCA TAA TG 3'	222 Forward
5' ATT GCT GGT ACC CAT AAT GGG TCT GTC TCA TC 3'	209 Forward
5' ATT GCT GGT ACC CTG TCT CT CGT CAA AGG AC 3'	199 Forward
5' ATT GCT GGT ACC CTA AAG GAA ACT CTA ACT AC 3'	170 Forward
5' ATT GCT GGT ACC CCT TAG TTA TTA ATA CAA AC 3'	130 Forward
5' ATT GCT GGT ACC CCT ATC TGT CAC CAT CTC ATC 3'	99 Forward
5' ATT GCT GGT ACC ACT TGT GAA AAT ACG TAA TCC 3'	71 Forward

RESULTS

Cloning the Interferon-γ Promoter

The full-length human (542-bp) IFN-γ promoter was PCR amplified from genomic DNA isolated from the Jurkat cell line (Fig 4.1). This PCR product was TA cloned in the pGEMT-easy vector as described in Materials and Methods. This cloned plasmid was used as template to amplify several deletion fragments of the IFN-γ promoter (Fig. 4.2). Each of these promoter fragments was subcloned upstream of a promoter luciferase gene for use in transient transfection studies of promoter activity under 2B4 stimulation. All constructs were confirmed by sequence analysis.

Promoter Construct Analysis

The smallest promoter construct examined was from –71 to +1 cloned upstream of the luciferase gene. Promoter constructs were transiently transfected into YT cells as described in Materials and Methods. Transfectants were incubated in the presence of target cells at a ratio of 10:1(both unstimulated and stimulated) and stimulated with 200 ng/ml C1.7 mAb (stimulated only). A second plasmid containing the renilla

luciferase gene driven by a CMV promoter was co-transfected for standardization. Firefly luciferase was normalized to renilla luciferase. The minimal promoter construct (pGL.71) produced very little luciferase in unstimulated cells, but when stimulated with C1.7 a 12-fold increase in promoter activity was observed (Fig 4.3).

The next largest construct (pGL.99) showed promoter activity at a 2.5-fold increase over pGL.71 in unstimulated cells while C1.7 stimulated cells promoter activity was at a 1.5-fold increase over unstimulated pGl.99 transfectants (Fig 4.4).

pGL.130 transfectants displayed a more than 5-fold increase in promoter activity over pGL.99 transfectants, but failed to show a difference between unstimulated and stimulated pGL.130 transfectants (Fig. 4.5).

Promoter activity in unstimulated transfectants containing the pGL.170 vector increased more than 1.5-fold over activity in pGL.130 unstimulated transfectants. Stimulated pGL.170 transfectants had a more than 2.5-fold increase in promoter activity over unstimulated pGL.170 transfectants (Fig. 4.6).

Unstimulated transfectants containing the pGL.199 vector showed decreased promoter activity compared to pGL.170 transfectants by

nearly 6.5-fold. Stimulated transfectants with this vector showed no significant difference from unstimulated transfectants (Fig. 4.7).

Unstimulated transfectants with pGL.209 a nearly 3-fold in promoter activity over unstimulated pGL.199 transfectants. C1.7 stimulated pGL.209 transfectants showed a 16-fold increase in promoter activity over stimulated pGL.199 transfectants and double the activity of unstimulated pGL.209 transfectants (Fig. 4.8).

pGL.222 transfectants unstimulated showed an increase in promoter activity over unstimulated pGL.209 transfectants by 3-fold. Stimulated pGL.222 transfectants had a 5-fold increase over unstimulated pGL.222 transfectants (Fig. 4.9).

Unstimulated transfectants containing the pGL.252 vector showed promoter activity at nearly double that of unstimulated pGL.222 transfectants. Stimulated pGL.252 transfectants displayed promoter activity four times greater than unstimulated pGL.252 transfectants (Fig. 4.10).

pGL.302 transfectants displayed decreased promoter activity when unstimulated compared to pGL.252 transfectants when unstimulated by half. Stimulated pGL.302 tranfectants showed no significant difference compared to unstimulated (Fig. 4.11).

Transfectants containing the full-length IFNγ promoter showed relatively the same promoter activity as pGL.302 transfectants when unstimulated, however when stimulated with C1.7 mAb, full-length transfectants had a four-fold increase in promoter activity (Fig. 4.12).

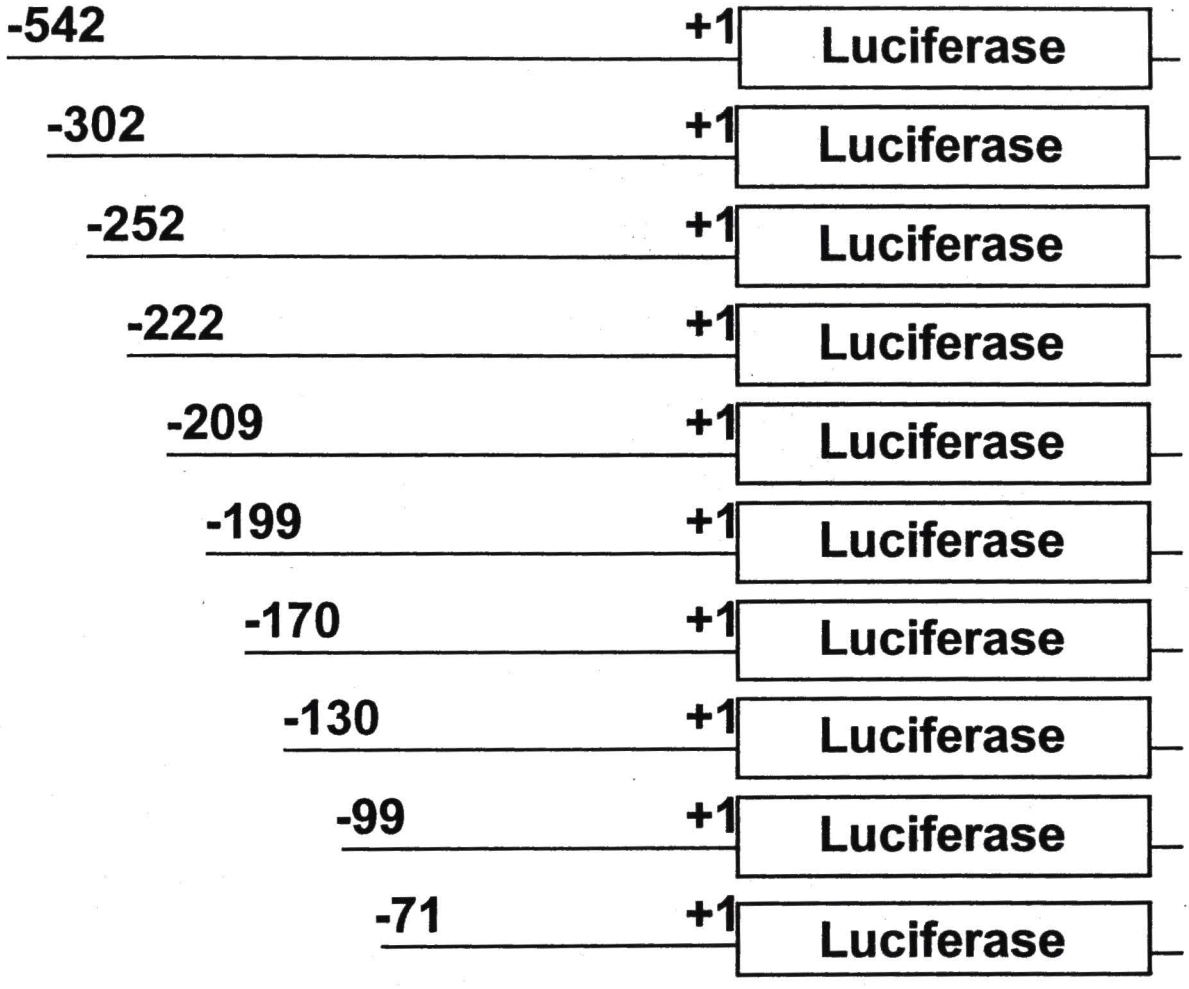


Figure 4.1
The human IFN-γ promoter cloning schematic.
The full-length IFN-γ promoter and nine deletion constructs were cloned upstream of a promoterless luciferase gene by PCR. Each construct was designed to eliminate a potential transcription factor binding site. This figure is a schematic of the promoter-reporter construct design.

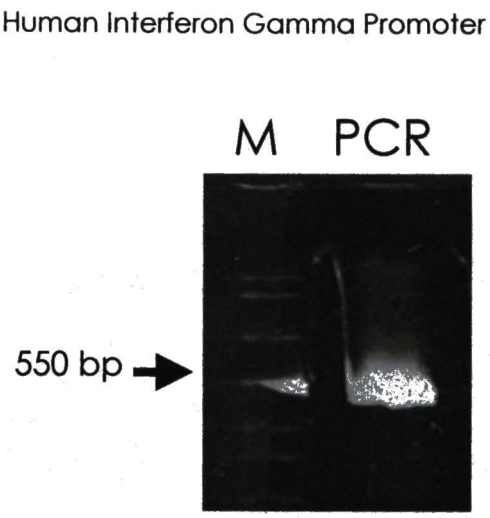


Figure 4.2
The human IFN-γ promoter was cloned by PCR from genomic DNA.
PCR product amplified from human genomic DNA using primers specific for the human IFN-γ gene promoter as listed in Table 4.2. PCR product was TA cloned into the pGEM T-easy vector (pGEM.542) and used as template for the amplification of deletion fragments of the promoter shown in Figure 4.3.

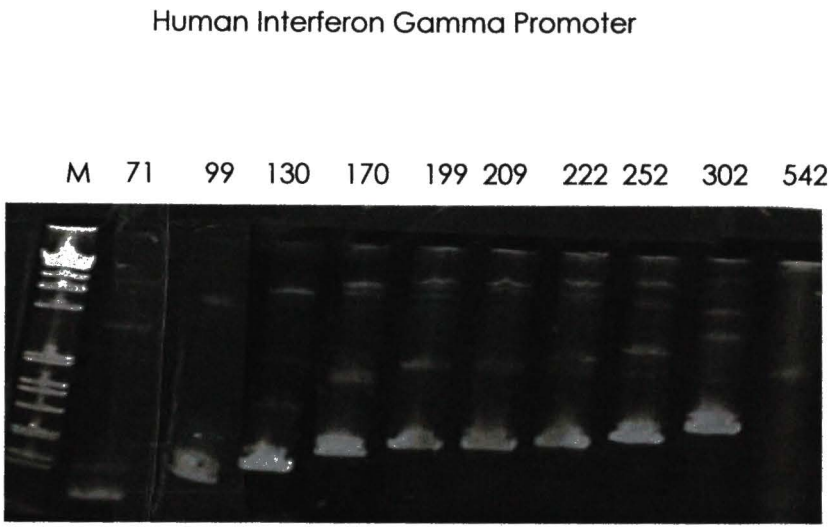


Figure 4.3
Nine deletion fragments of the IFN-γ promoter were cloned by PCR.
Each of the IFN-γ promoter deletion fragments shown here as PCR products. Deletion fragments were amplified from pGEM.542 (Figure 4.1) using sequence specific primers listed in Table 4.2. Each deletion fragment was designed to eliminate a potential transcription factor binding site located within the promoter.

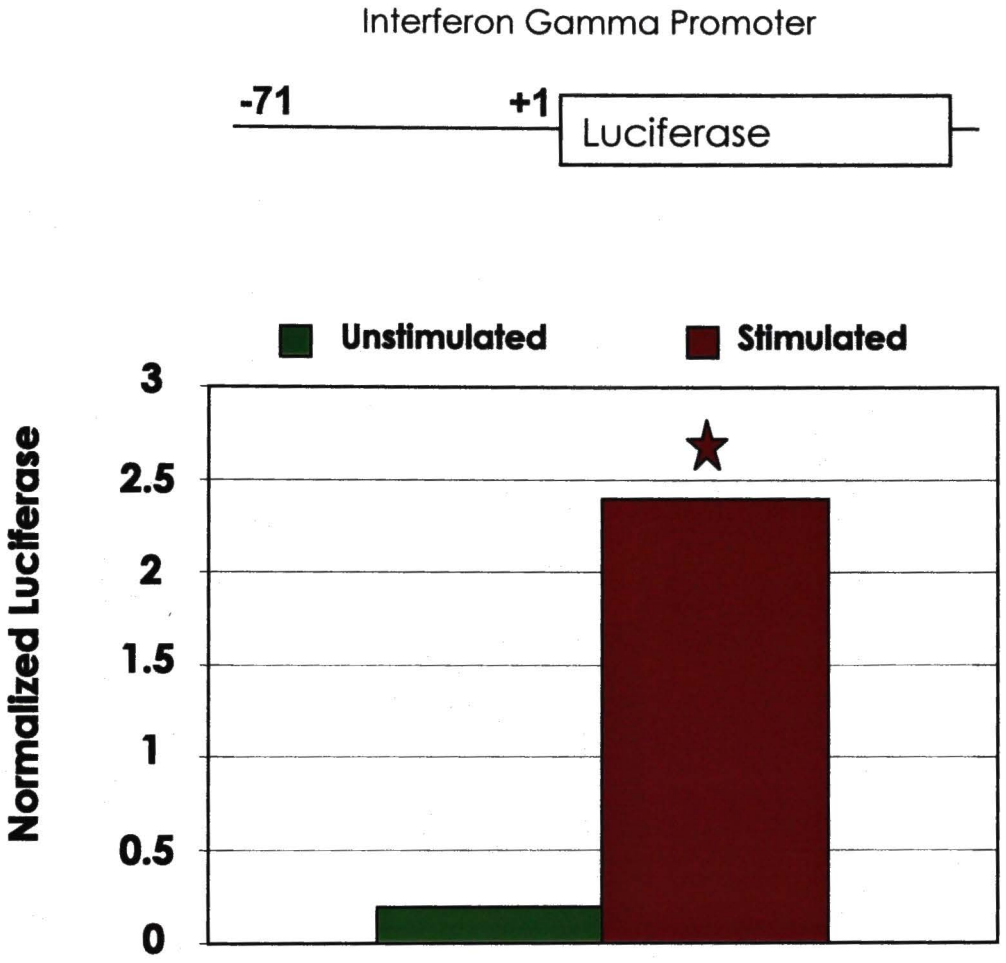


Figure 4.4
2B4-activation results in an up-regulation of the IFN-γ promoter region from –71 to +1.
A deletion fragment spanning from –71 to +1 of the interferon gamma promoter was cloned upstream of a promoterless luciferase gene, vector pGL.71. Vector pGL.71 was transiently transfected into YT cells, which were subsequently cultured in the presence of K562 target cells in media alone or with 200 ng/ml C1.7. The red star indicates statistical significance at p = 0.024 as determined by t-test.

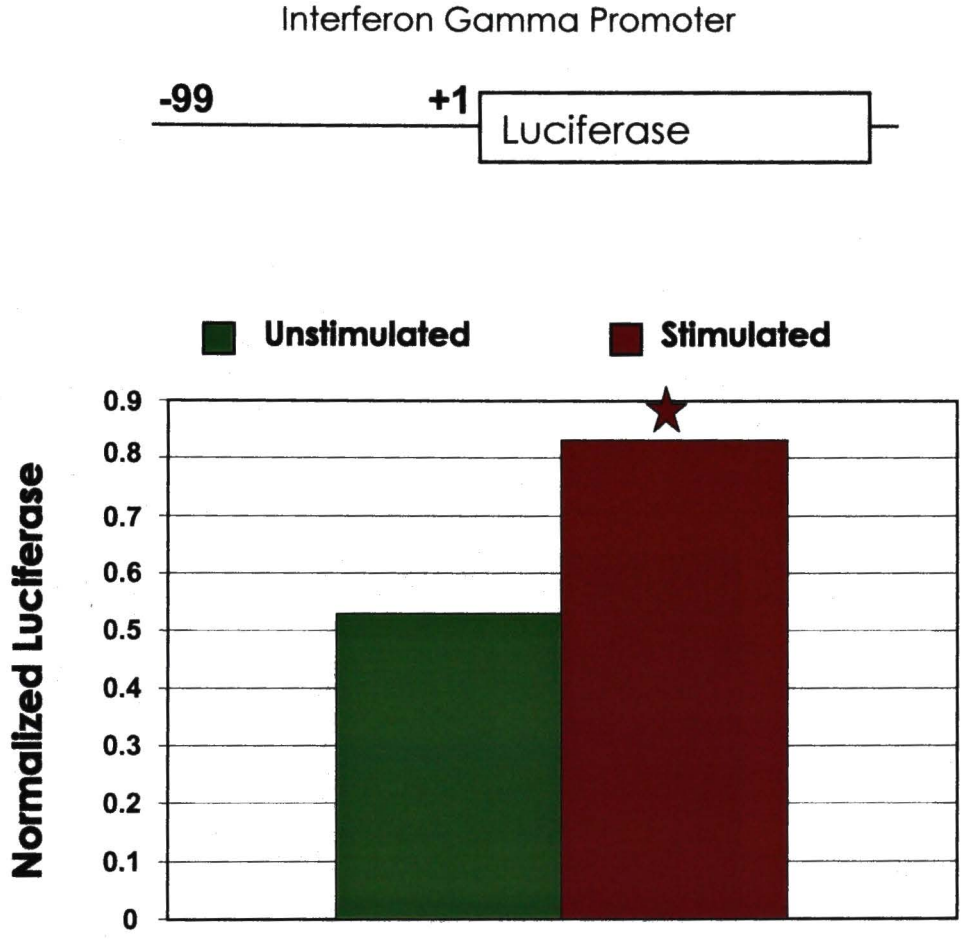


Figure 4.5
2B4-activation results in an up-regulation of the IFN-γ promoter region from –99 to +1.
A deletion fragment spanning from –99 to +1 of the interferon gamma promoter was cloned upstream of a promoterless luciferase gene, vector pGL.99. Vector pGL.99 was transiently transfected into YT cells, which were subsequently cultured in the presence of K562 target cells in media alone or with 200 ng/ml C1.7. The red star indicates statistical significance at $p = 0.024$ as determined by t-test.

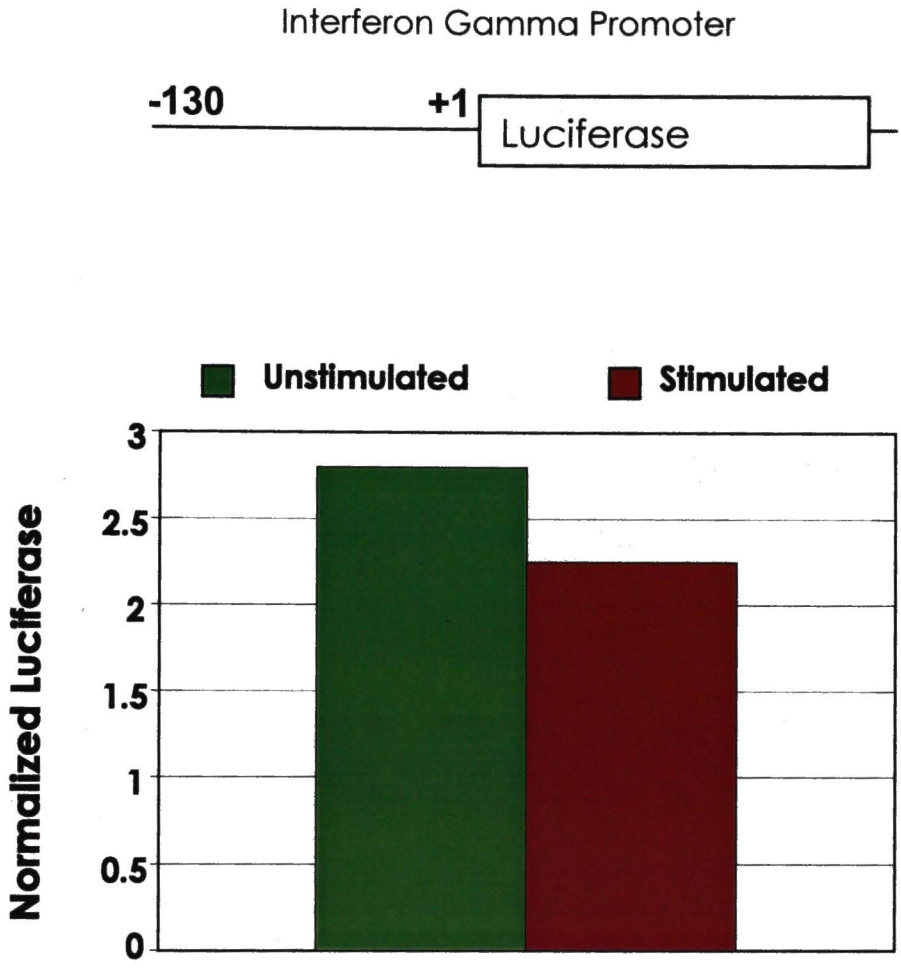


Figure 4.6
2B4-activation does not affect the IFN-γ promoter region from –130 to +1.
A deletion fragment spanning from –130 to +1 of the interferon gamma promoter was cloned upstream of a promoterless luciferase gene, vector pGL.130. Vector pGL.130 was transiently transfected into YT cells, which were subsequently cultured in the presence of K562 target cells in media alone or with 200 ng/ml C1.7. No significant difference was found between YT cells and 2B4-activated YT cells.

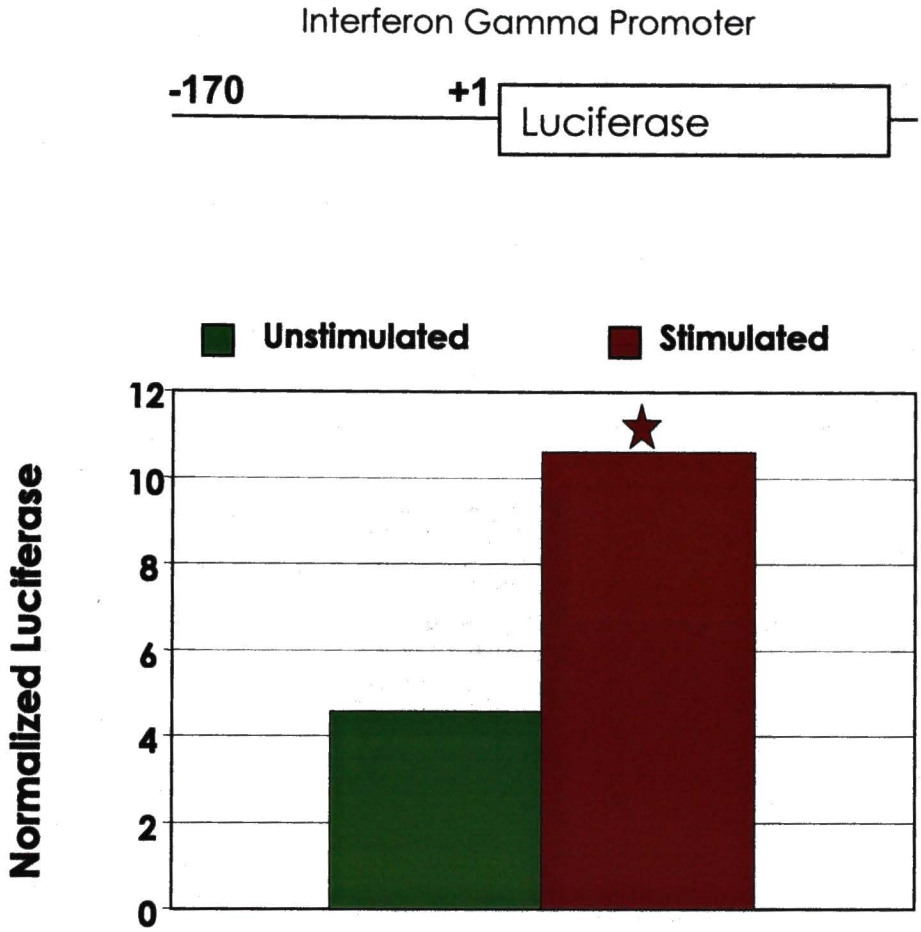


Figure 4.7

2B4-activation results in an up-regulation of the IFN-γ promoter region from –170 to +1.

A deletion fragment spanning from –170 to +1 of the interferon gamma promoter was cloned upstream of a promoterless luciferase gene, vector pGL.170. Vector pGL.170 was transiently transfected into YT cells, which were subsequently cultured in the presence of K562 target cells in media alone or with 200 ng/ml C1.7. The red star indicates statistical significance at p = 0.006 as determined by t-test.

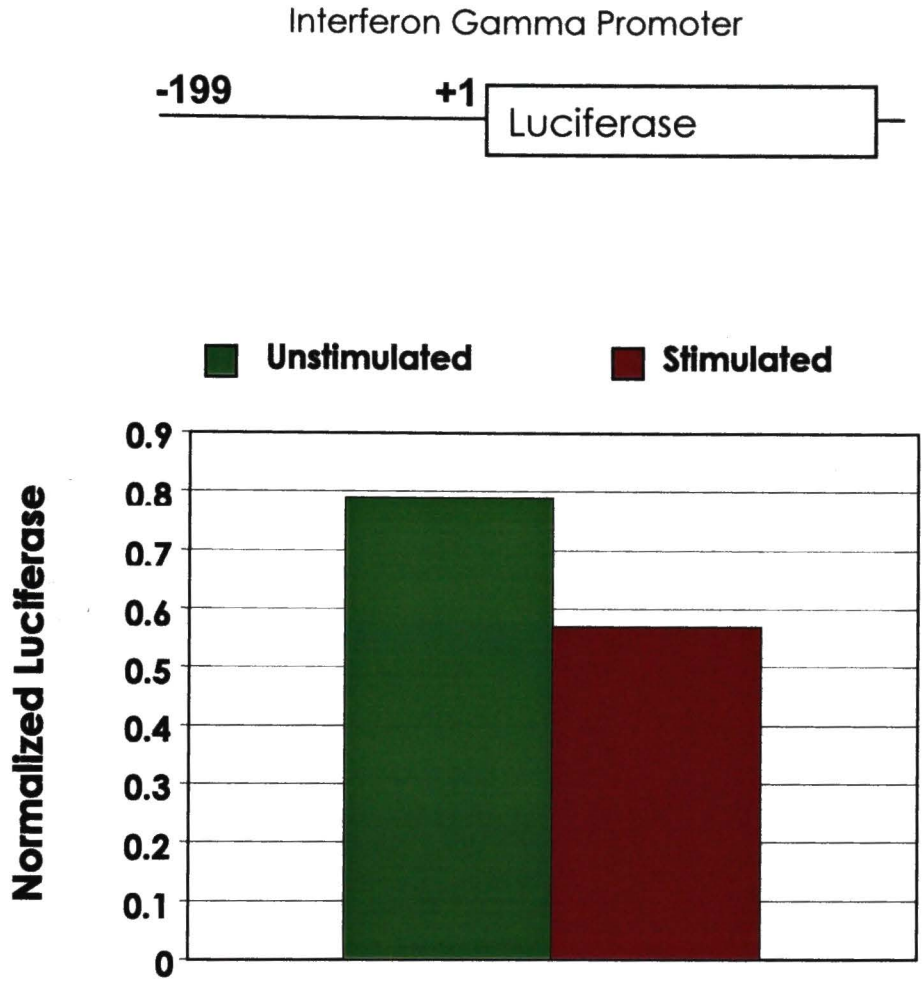


Figure 4.8
2B4-activation does not affect the IFN-γ promoter region from –199 to +1.
A deletion fragment spanning from –199 to +1 of the interferon gamma promoter was cloned upstream of a promoterless luciferase gene, vector pGL.199. Vector pGL.199 was transiently transfected into YT cells, which were subsequently cultured in the presence of K562 target cells in media alone or with 200 ng/ml C1.7. There was no significant difference between YT cells and 2B4-activated YT cells.

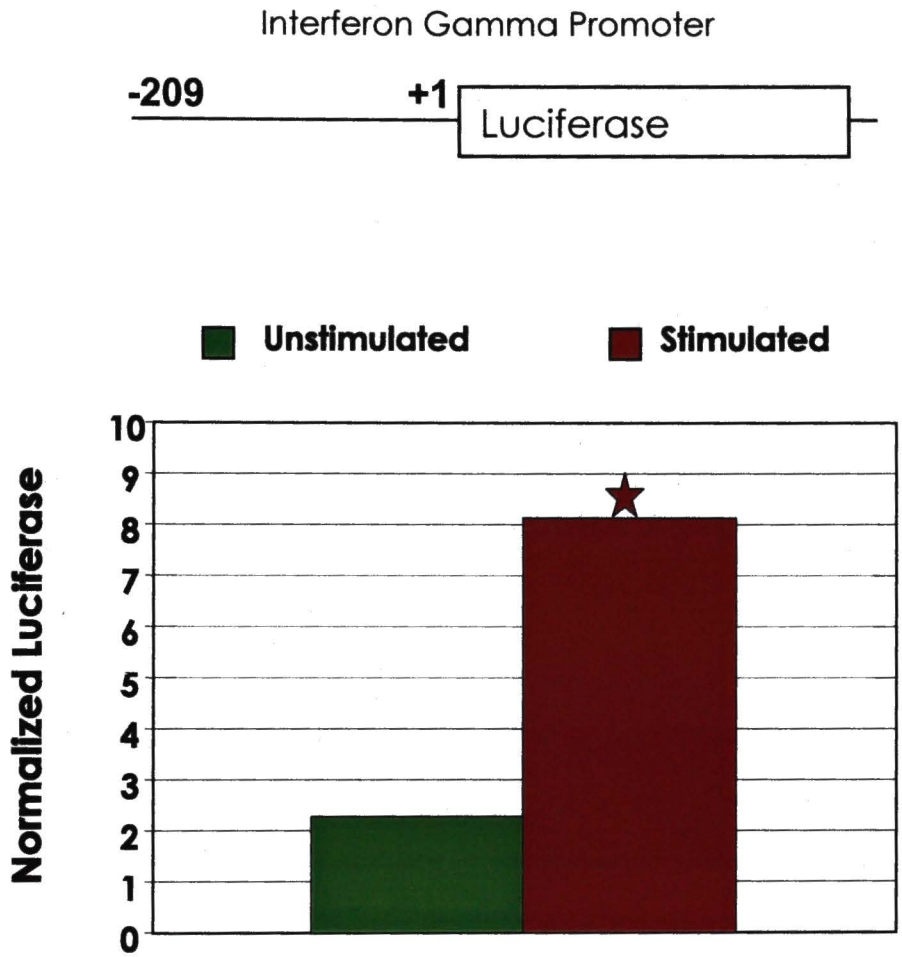


Figure 4.9

2B4-activation results in an up-regulation of the IFN-γ promoter region from –209 to +1.

A deletion fragment spanning from –209 to +1 of the interferon gamma promoter was cloned upstream of a promoterless luciferase gene, vector pGL.209. Vector pGL.209 was transiently transfected into YT cells, which were subsequently cultured in the presence of K562 target cells in media alone or with 200 ng/ml C1.7. The red star indicates statistical significance at $p = 0.0000028$ as determined by t-test.

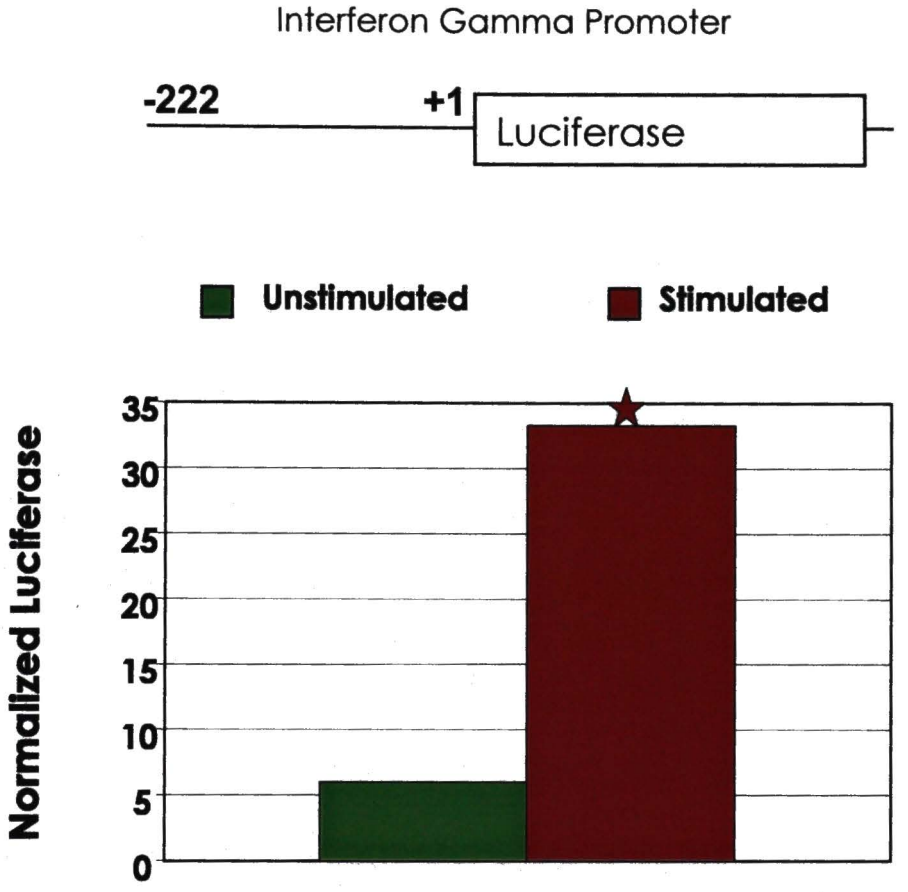


Figure 4.10

2B4-activation results in an up-regulation of the IFN-γ promoter region from –222 to +1.

A deletion fragment spanning from –222 to +1 of the interferon gamma promoter was cloned upstream of a promoterless luciferase gene, vector pGL.222. Vector pGL.222 was transiently transfected into YT cells, which were subsequently cultured in the presence of K562 target cells in media alone or with 200 ng/ml C1.7. The red star indicates statistical significance at p = 0.0426 as determined by t-test.

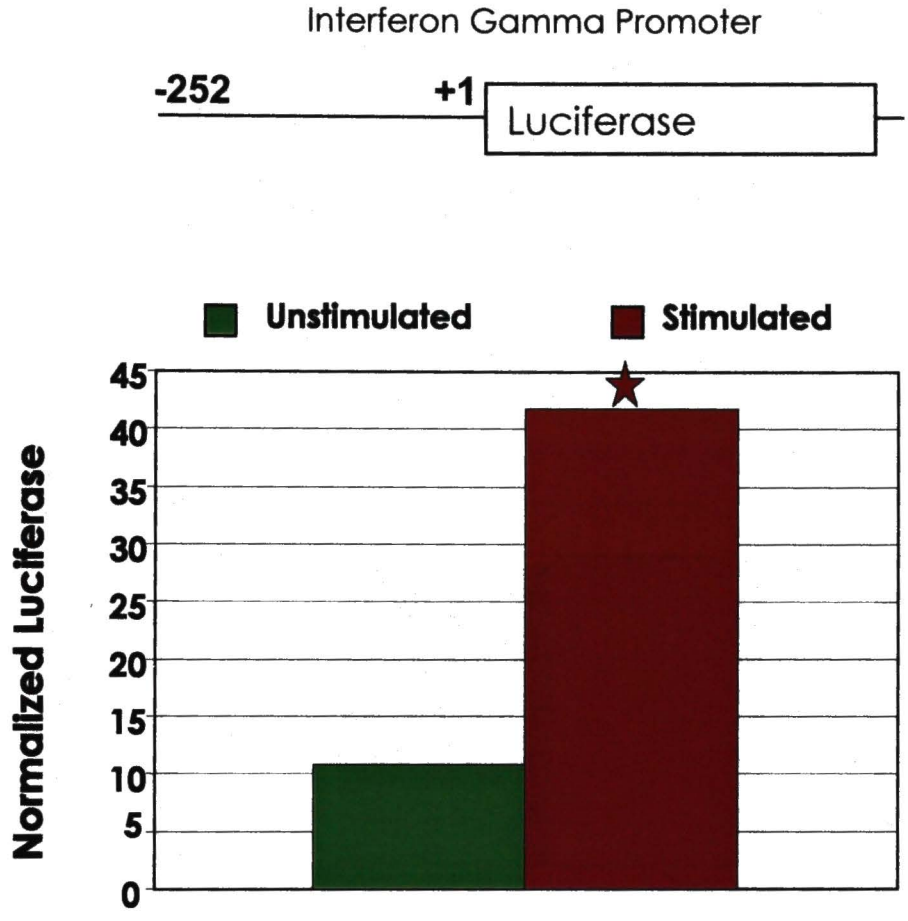


Figure 4.11

2B4-activation results in an up-regulation of the IFN-γ promoter region from –252 to +1.

A deletion fragment spanning from –252 to +1 of the interferon gamma promoter was cloned upstream of a promoterless luciferase gene, vector pGL.252. Vector pGL.252 was transiently transfected into YT cells, which were subsequently cultured in the presence of K562 target cells in media alone or with 200 ng/ml C1.7. The red star indicates statistical significance at $p = 0.00037$ as determined by t-test.

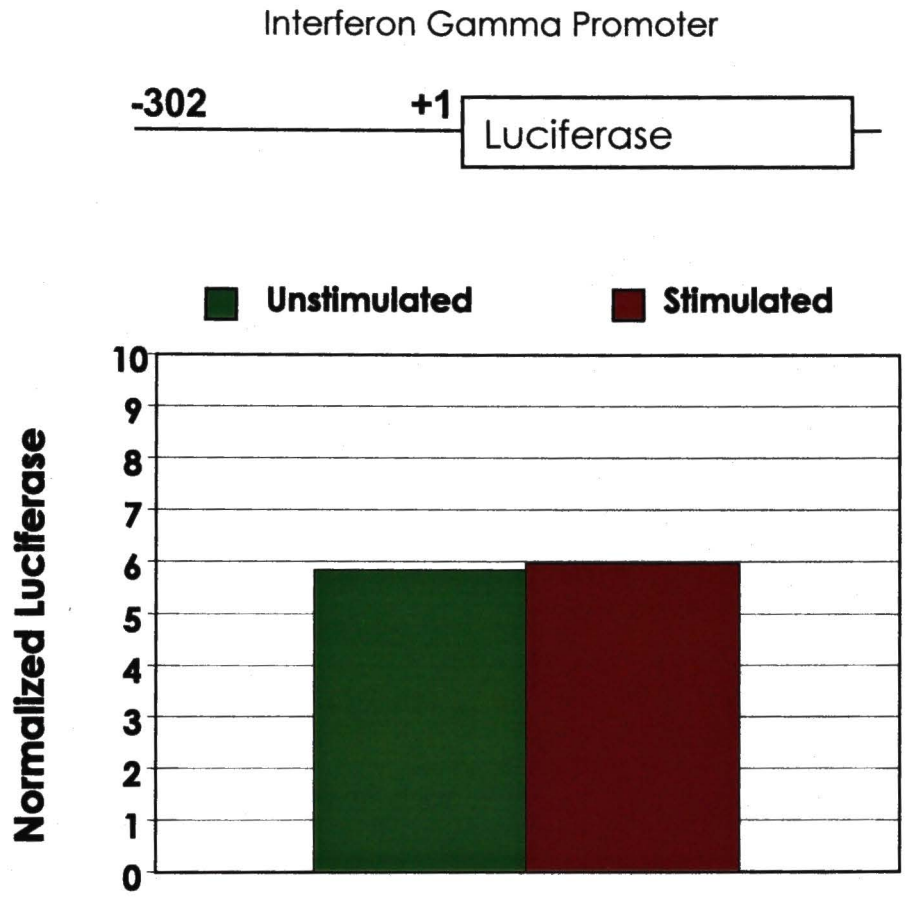


Figure 4.12

2B4-activation results in an up-regulation of the IFN-γ promoter region from –302 to +1.

A deletion fragment spanning from –302 to +1 of the interferon gamma promoter was cloned upstream of a promoterless luciferase gene, vector pGL.302. Vector pGL.302 was transiently transfected into YT cells, which were subsequently cultured in the presence of K562 target cells in media alone or with 200 ng/ml C1.7. There was no significant difference between YT cells and 2B4-activated YT cells.

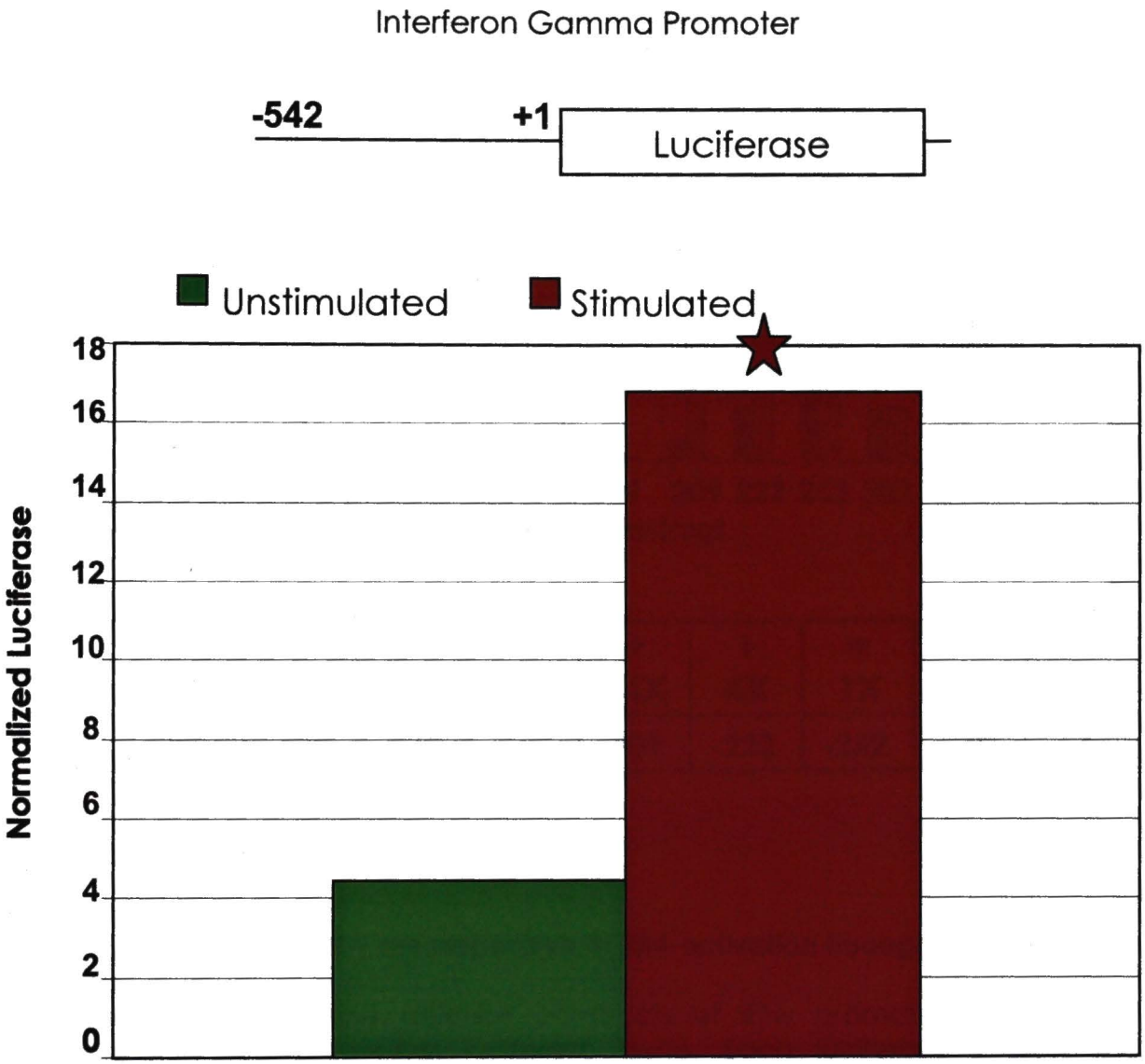
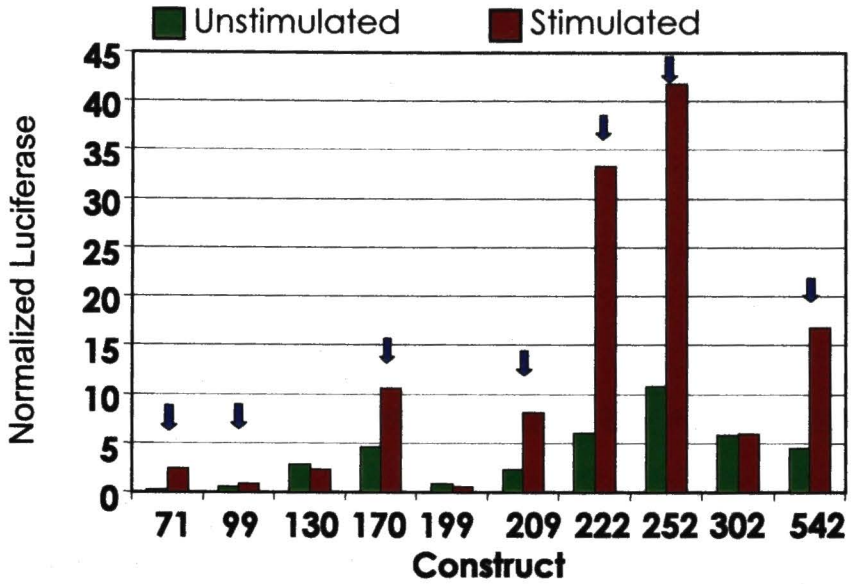


Figure 4.13
The full-length IFN-γ promoter was cloned upstream of a promoterless luciferase gene, vector pGL.542. Vector pGL.542 was transiently transfected into YT cells, which were subsequently cultured in the presence of K562 target cells in media alone or with 200 ng/ml C1.7. The red star indicates statistical significance at $p = 0.0136$ as determined by t-test.

A.



B

	- 3X	+ 3X	+ 5X	- 19X	+ 14X	+ 4X	+ 1X	- 7X	+ 3X
-71	-99	-130	-170	-199	-209	-222	-252	-302	-542

Figure 4.14

Several areas of the IFN-γ are responsive to 2B4-activation through increased activity.

The full-length and nine deletion constructs of IFNγ promoter was cloned upstream of a promoterless luciferase gene. Each luciferase vector was transiently transfected into YT cells along with a renilla luciferase vector under CMV promoter. Transfectants were subsequently cultured in the presence of K562 target cells in media alone or with 200 ng/ml C1.7. Luciferase was normalized to renilla luciferase as a measure of transfection efficiency. Blue arrows identify regions of the promoter that are responsive to 2B4 activation (A). Each construct in 2B4-activated YT cells was compared to the next smallest construct. The fold increase or decrease reported. A plus sign indicates a positive regulation and a minus sign indicates a negative regulation (B).

CHAPTER FIVE

FUNCTIONAL ROLE OF 2B4 AND IFN-γ IN THE REJECTION OF METASTATIC TUMOR CELLS

SUMMARY

2B4 is a natural killer cell receptor originally identified in the mouse as a surface molecule involved in non-MHC-restricted killing and enhancement of IFN-γ secretion. Recently, we have shown that human 2B4 activation induces cytolytic function and enhanced IFN-γ release in YT cells (a human NK cell line). 2B4's regulation of IFN-γ has been found to occur at the transcriptional level, both through mRNA stability and increased promoter activity. In the present study, we investigate the importance of 2B4 and IFN-γ both separately and together in the rejection of metastatic tumor cells in C57BL/6 mice. The significance of this data is discussed in chapter six.

INTRODUCTION

Natural killer (NK) cells are an important part of our innate immunity. Several studies have shown that NK cells mediate anti-tumor activities (126-128). These studies are supported by the finding that spleenocytes isolated from NK deficient mice have reduced cytotoxicity against YAC-1, RMA-S and B16 tumor cells in vitro (129). Additionally NK deficient mice show an impaired ability to reject tumor cells in an induced metastatic tumor model (129). NK cells are triggered to respond through binding of activating receptors on the NK cell surface.

Among the cytokines produced by NK cells, IFN-γ may be particularly important as it has a global effect on immune regulation (5). IFN-γ is involved in recruitment of other lymphocytes through augmentation of ICAMs and other cytokines (166). IFN-γ can inhibit proliferation in most cells and can also induce apoptosis (169, 170).

Evidence that IFNγ plays a role in the antitumor effect of NK cells comes from several studies. IL-12 – an inducer of IFNγ has been shown to elicit an antitumor affect against a variety of tumor cells including colon, kidney and lung carcinomas as well as B16F10 melanoma cells (130-137)

IFN-γ has been shown to up-regulate the expression of Fas and FasL by B16 melanoma cells which leads to apoptosis of these cells (147). IFNγ

knockout mice were shown to be as susceptible as NK cell depleted mice to lung metastases, although not as susceptible as IFNγ-Perforin double knockout mice (145). Several in vivo metastases models demonstrated that the role of IFN-γ is distinct from that of perforin (145, 148).

CD48, the counter-receptor for 2B4 has resulted in tyrosine phosphorylation upon binding suggesting that it is involved in lymphocyte activation (149). Binding of CD48 with mAb has shown a strong in vivo antitumor effect (150, 151). Additionally transfection of CD48 into two poorly immunogenic tumor cell lines enhances their immunogenicity (152). These data originally suggested a role for CD2, the low affinity ligand for CD48. Now that 2B4 has been identified as the high affinity ligand, a role for 2B4 is likely.

2B4 is an activating receptor previously identified on the surface of NK cells and a subset of T cells that mediate NK like killing (18, 177). 2B4 belongs to the immunoglobulin superfamily and is a member of the CD2 subfamily (27). Additionally we have characterized the human homologue of 2B4 and found CD48 to be the counter-receptor in both mice and humans (27, 28). Functional analysis in YT cells (an NK cell line) reveals enhanced proliferation, enhanced cytotoxicity and elevated secretion of IFN-γ upon binding of 2B4 with monoclonal antibody.

The focus of the current study is to examine the role of IFN-γ and 2B4 in the rejection of B16F10 metastases. Studies employ the use of anti-2B4 monoclonal antibody as well as antibodies against CD48, the natural ligand for 2B4.

MATERIALS AND METHODS

Media

4+RPMI: A one liter package of powdered RPMI media (Life Technologies, Rockville, Maryland, Catalog #31800-022) is combined with 2.0 grams sodium bicarbonate (Life Technologies, Catalog #11810-025) and dissolved in 800 ml. deionized, distilled water. pH is adjusted to 7.4 with HCl and volume adjusted to one liter. Media is then supplemented with 10% standard fetal bovine serum (HyClone Lab, Logan, Utah, Catalog #SH30088.03). Media is further supplemented with Sodium Pyruvate – 1X final concentration, non-essential amino acids – 1X final concentration, antibiotic/antimycotic – 1X final concentration and buffered with HEPES buffer (all Life Technologies, Catalog # 11360-070, 11140-050, 15240-062 and 15630-080 respectively).

Cell lines

Adherent B16F10 cells were maintained in 4+RPMI and passed when 90% confluency was reached.

Cell Culture

All mammalian cell lines were maintained in sterile conditions in Nunc EasYFlasks with canted necks and filtered tops. Cell growth media was 4+ RPMI. Media was completely replaced every 48 hours. Cells were maintained at 37°C with 5% CO_2 saturation.

Antibodies

C1.7 antibody which recognizes h2B4 (63) was purchased from Coulter (Orlando, FL). Isotype mAb control was kindly donated by Dr. V. Kumar, UT Southwestern, Dallas, TX. αCD48 mAb was purchased from BD Pharmingen (San Diego, CA) and was prepared with no azide and low endotoxin for in vivo applications.

Reagents and chemicals

Tissue culture media and fetal bovine serum were purchased from Gibco BRL (Gaithersburg, MD). C1.7 (anti-2B4 mAb) was purchased from Coulter (Pittsburgh, PA).

Cell Preparation

Adherent B16F10 were grown to ~90% confluency in a T-75cm^2 flask in 4+RPMI. Cells were released from the flask surface using Trypsin-EDTA from

Gibco-BRL (Life Technologies). Growth media was removed from cells. Ten ml Trypsin-EDTA was added to cells and flask was rocked back and forth to force the trypsin over the entire growth surface of the flask for ~1 min or until cells detached from flask. The cells were then collected by centrifugation at 250 x g for 5 minutes. Cells were washed with 5 ml of RPMI – no additives. Cells were re-suspended in 1 ml of RPMI – no additives. Cells were counted and concentration was adjusted to 1 million cells per ml with RPMI – no additives. Cells were placed on ice. Cells were drawn into a 1 ml syringe which was subsequently fitted with a 30 gauge needle.

Tail Vein Injection

C57BL/6 (B6) mice, 2B4 knock-out mice and IFN-γ knock-out mice all share the C57BL/6 strain background to ensure that and differences were not due to genetic advantages or disadvantages among different mice strains. Mice were removed from cages and secured in a restraint with their tails exposed. Mice were positioned on their side and secured. Warm water and pressure was applied to the tail to cause the vein to stand out. Cells were delivered intravenously in a 200 µl volume using a 30 gauge ½ inch needle fitted to a 1 ml. syringe. Bleeding was suppressed by applying

pressure at the site of injection before returning mice to the cage. The number of mice in each study condition is shown in Table 5.1.

Euthanasia and Tissue Harvest

Mice were euthanized by CO_2 asphyxiation 14 days after tumor challenge. Lungs were removed and placed in 10% formalin-PBS solution. Tumors were visualized and enumerated as black spots on the surface of the lungs.

Table 5.1

Mouse Strain	***Antibody Treatment***	***Number of Mice***
B6		11
IFN-γ Knock-out		8
2B4 Knock-out		8
B6	Anti-2B4 mAb	10
IFN-γ Knock-out	Anti-2B4 mAb	9
B6	Anti-CD48 mAb	9
IFN-γ Knock-out	Anti-CD48 mAb	8

RESULTS

We used an experimental metastasis model to examine the role of IFNγ, 2B4 and antibodies against 2B4 and its natural ligand CD48. 10^5 metastatic B16F10 melanoma cells were injected intravenously into C57BL/6 (B6) mice and lung metastases were enumerated 14 days later. Knock-out mice were also of the C57BL/6 strain background.

B6 vs. IFN-γ knock-out

IFN-γ knock-out mice are far more susceptible to establishment of tumor metastases than wild type. Wild type mice developed an average of 128 metastases in the lung as compared to an average of 300 metastases in the lungs of IFNγ knock-out mice (Fig 5.1).

B6 vs. 2B4 knock-out

2B4 knock-out mice were also more susceptible to tumor metastases developing an average of 240 tumors in the lungs fourteen days after tumor challenge (Fig 5.2).

B6 vs. B6 + α2B4

We also examined the effect of 2B4 activation in vivo on the establishment of lung metastases. B6 mice were given 100 μg anti-2B4 monoclonal antibody two days before tumor challenge and again on the day of the tumor challenge for the purpose of activating NK cells via surface 2B4. In mice that received antibody treatment, far fewer metastases established in the lungs, an average of 24 compared to an average of 128 metastases in mice not given the antibody (Fig 5.3).

B6 vs. B6 + αCD48

Antibodies against the natural ligand of 2B4, CD48 also showed in vivo protection against the establishment of tumor metastases, supporting the idea that CD48 is actually a counter receptor also involved in activation. Mice receiving anti-CD48 mAb had an average of 15 tumors as compared to 128 in mice not receiving the antibody (Fig. 5.4).

IFN-γ* knock-out *vs. IFN-γ* knock-out + *α2B4

We also examined the effects of these antibodies in IFN-γ knock-out mice and found that 2B4 activation by monoclonal antibody protects against tumor metastases in these knock-out mice as well. IFN-γ knock-out mice given antibody treatment developed an average of 40 tumors

compared with an average of 300 tumors in the lungs of IFN-γ knock-out not given antibody (Fig 5.5).

IFN-γ knock-out *vs.* *IFN*-γ knock-out + *αCD48*

Antibody against CD48 was also able to confer protection against lung metastases in IFN-γ knock-out mice developing an average of 35 tumors compared to 300 in knock-out mice not receiving antibody (Fig. 5.6).

B6 + α2B4 vs. B6 + αCD48

We also compared the effect of 2B4 activation and that of CD48 activation and found that B6 mice receiving anti-2B4 mAb developed an average of 24 lung metastases while mice receiving anti-CD48 developed an average of 15 lung metastases. This difference was not statistically significant (Fig. 5.7).

IFN*-γ knock-out + *α2B4 vs. IFN*-γ knock-out + *αCD48

The effect of 2B4 and CD48 activation was also compared in IFN-γ knock-out mice. A statistical difference was also not apparent in this comparison where knockout mice that received anti-2B4 mAb developed

an average of 40 lung tumors compared to an average of 35 lung tumors in knock-out mice receiving anti-CD48 mAb (Fig 5.8)

B6 + α2B4 vs. IFN-γ knock-out + α2B4

We also examined the level of protection afforded by mAb treatment by comparing wild-type mice given antibody to knock-out mice given antibody. We found that B6 mice given anti-2B4 mAb were not as susceptible to tumor metastases (average 24) as IFN-γ knock-out mice given anti-2B4 mAb (average 40) despite the significant protection afforded by this antibody (Fig 5.9).

B6 + αCD48 vs. IFN-γ knock-out + αCD48

Results observed with anti-CD48 mAb were similar to what was seen with anti-2B4 mAb. Despite the significant protection of this antibody in both wild-type mice and IFN-γ knock-out mice, the knock-out mice were still more susceptible to lung metastases, developing an average of 35, than B6 mice which developed an average of 15 (Fig. 5.11).

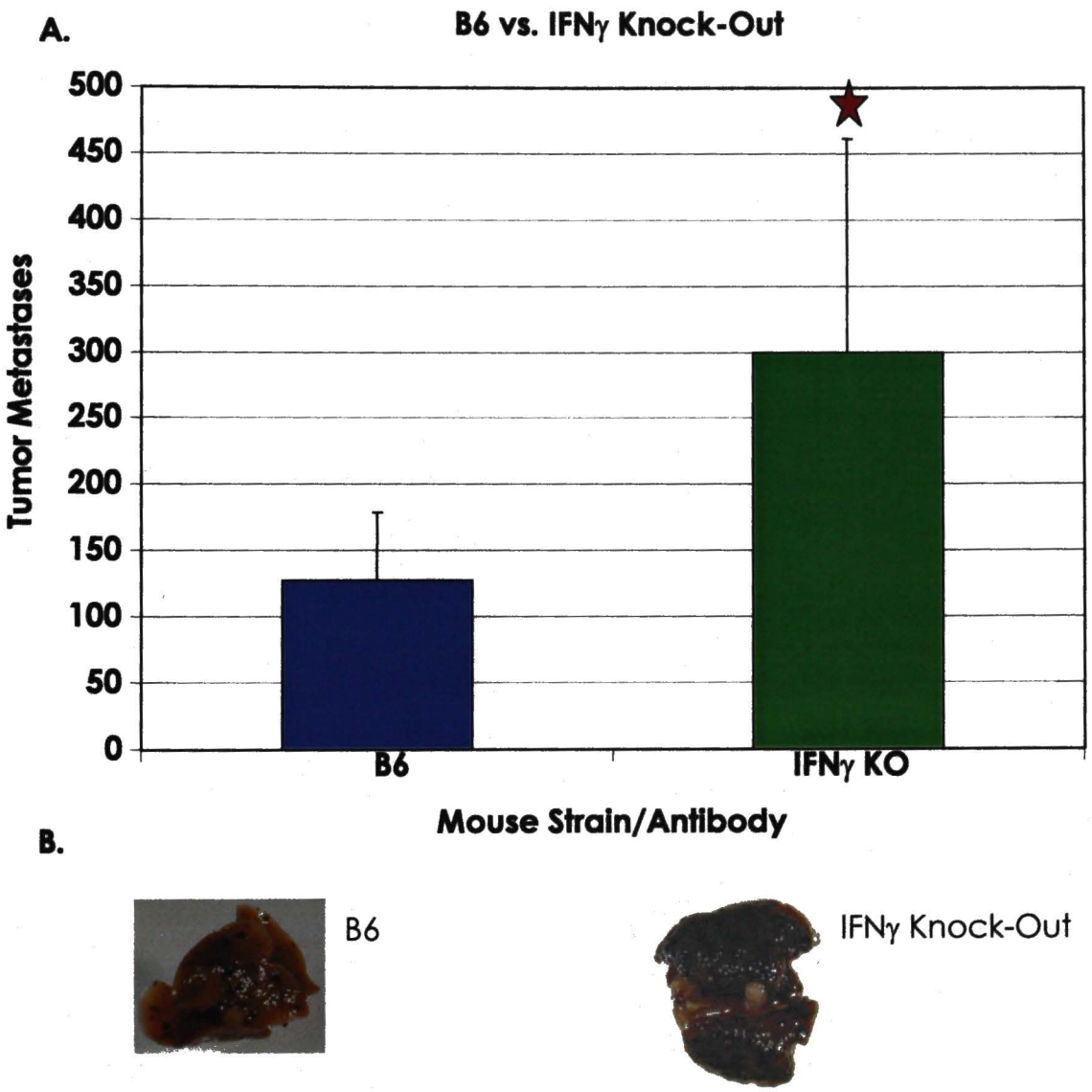
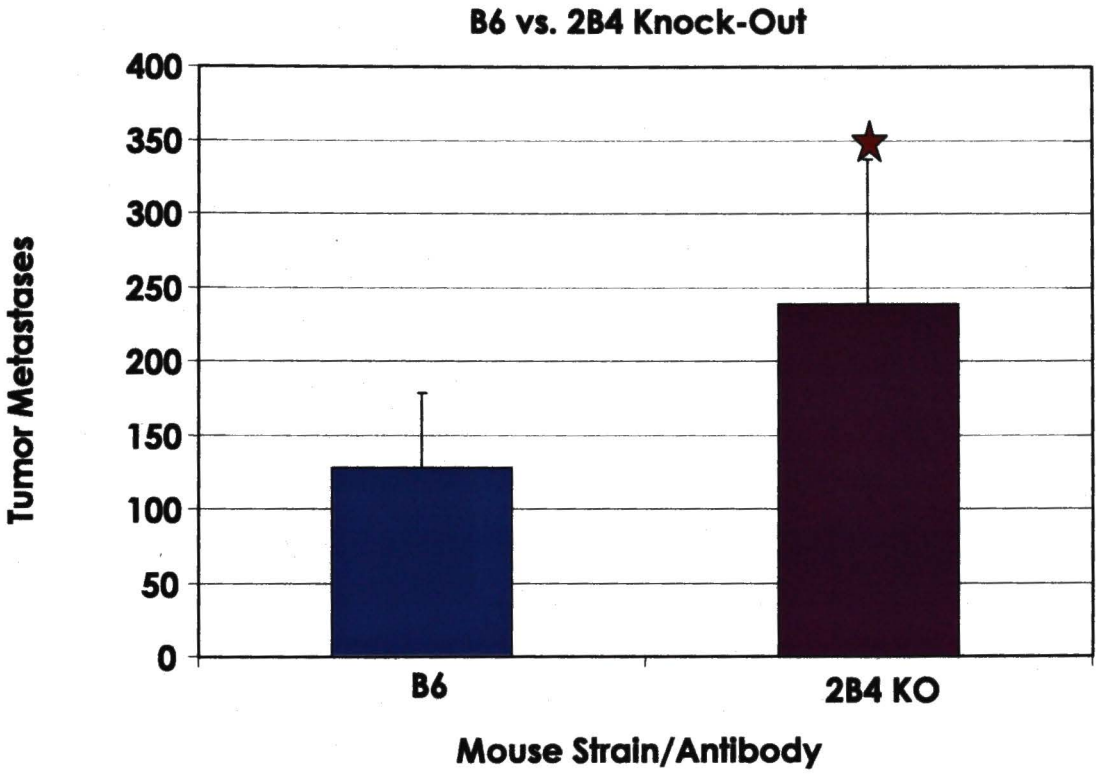


Figure 5.1
IFN-γ knock-out mice are more susceptible to the establishment of B16 melanoma metastases in the lungs of mice.
Mice were challenged intravenously with 10^5 metastatic B16F10. Lungs were harvested day fourteen after tumor challenge and established metastases were counted (A). Vertical bars represent standard deviation. Red star indicates statistical significance at $p = 0.03$ as determined by t-test. Representative photographs of lungs bearing metastases visible as black spots (B).

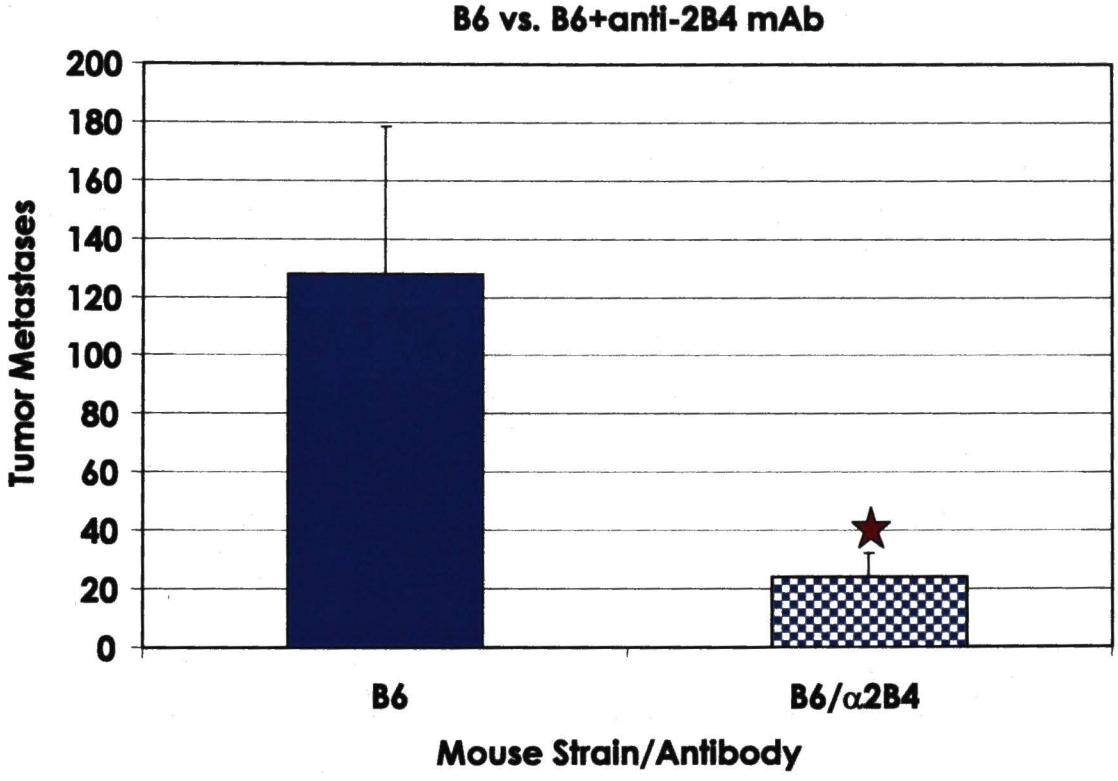
A.



B.

Figure 5.2
IFN-γ knock-out mice are more susceptible to the establishment of B16 melanoma metastases in the lungs of mice.
Mice were challenged intravenously with 10^5 metastatic B16F10. Lungs were harvested day fourteen after tumor challenge and established metastases were counted (A). Vertical bars represent standard deviation. Red star indicates statistical significance at p=0.02 as determined by t-test. Representative photographs of lungs bearing metastases visible as black spots (B).

A.



B.

Figure 5.3

Anti-2B4 mAb treatment reduces the number of metastases that establish in the lungs of B6 mice.

Mice were challenged intravenously with 10^5 metastatic B16F10 cells and given 100μg anti-2B4 mAb on day –2 and day 0 as indicated. Lungs were harvested day fourteen after tumor challenge and established metastases were counted (A). Vertical bars represent standard deviation. Red star indicates statistical significance at p=0.00016. Representative photographs of lungs bearing metastases visible as black spots (B).

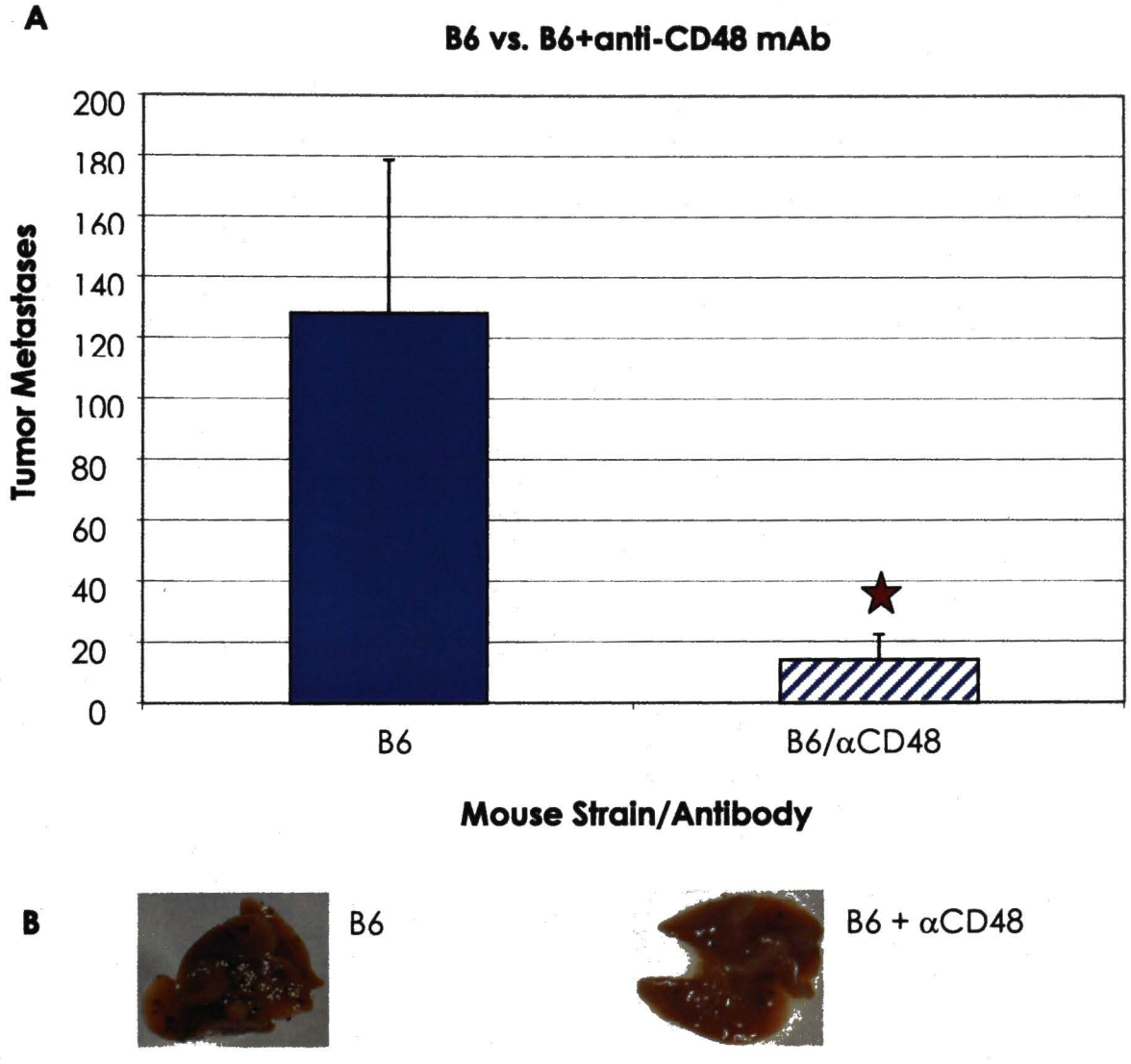


Figure 5.4

Anti-2B4 mAb treatment reduces the number of metastases that establish in the lungs of B6 mice.

Mice were challenged intravenously with 10^5 metastatic B16F10 cells and given 100μg anti-CD48 mAb on day –2 and day 0 as indicated. Lungs were harvested day fourteen after tumor challenge and established metastases were counted (A). Vertical bars represent standard deviation. Red star indicates statistical significance. Representative photographs of lungs bearing metastases visible as black spots (B).

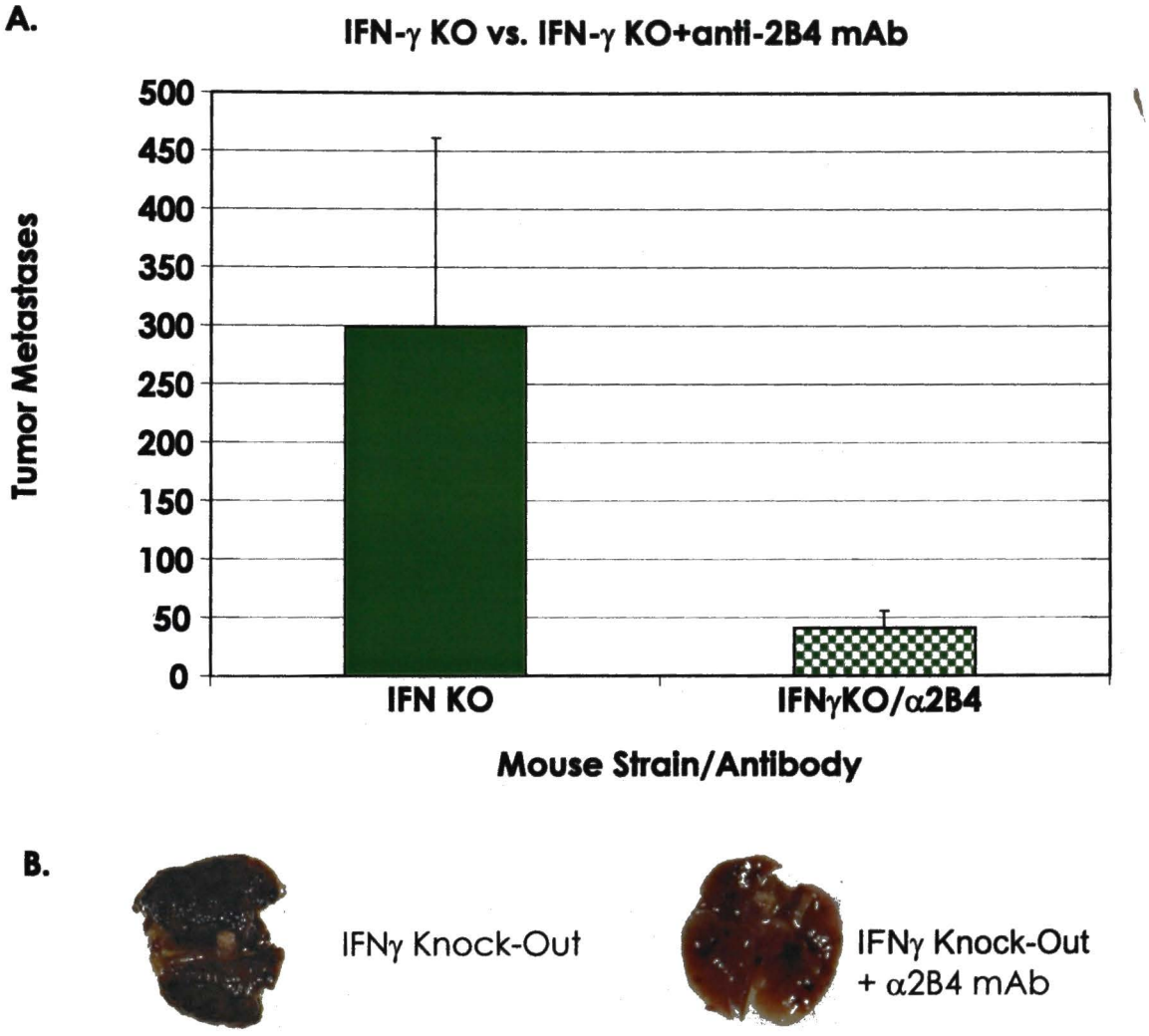
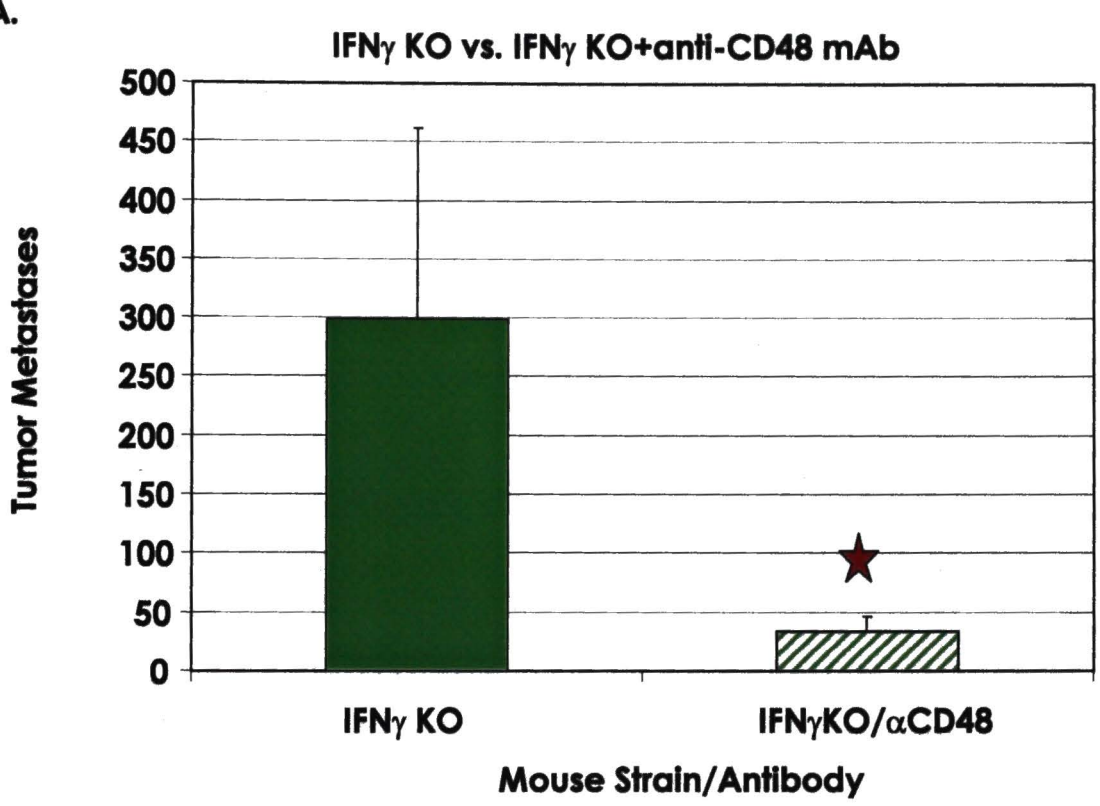


Figure 5.5

Anti-2B4 mAb treatment reduces the number of metastases that establish in the lungs of IFN-γ knock-out mice.

Mice were challenged intravenously with 10^5 metastatic B16F10 cells and given 100 μg anti-2B4 mAb on day –2 and day 0 as indicated. Lungs were harvested day fourteen after tumor challenge and established metastases were counted (A). Vertical bars represent standard deviation. Red star indicates statistical significance at p=0.006 as determined by t-test. Representative photographs of lungs bearing metastases visible as black spots (B).

A.



B.

IFNγ Knock-Out

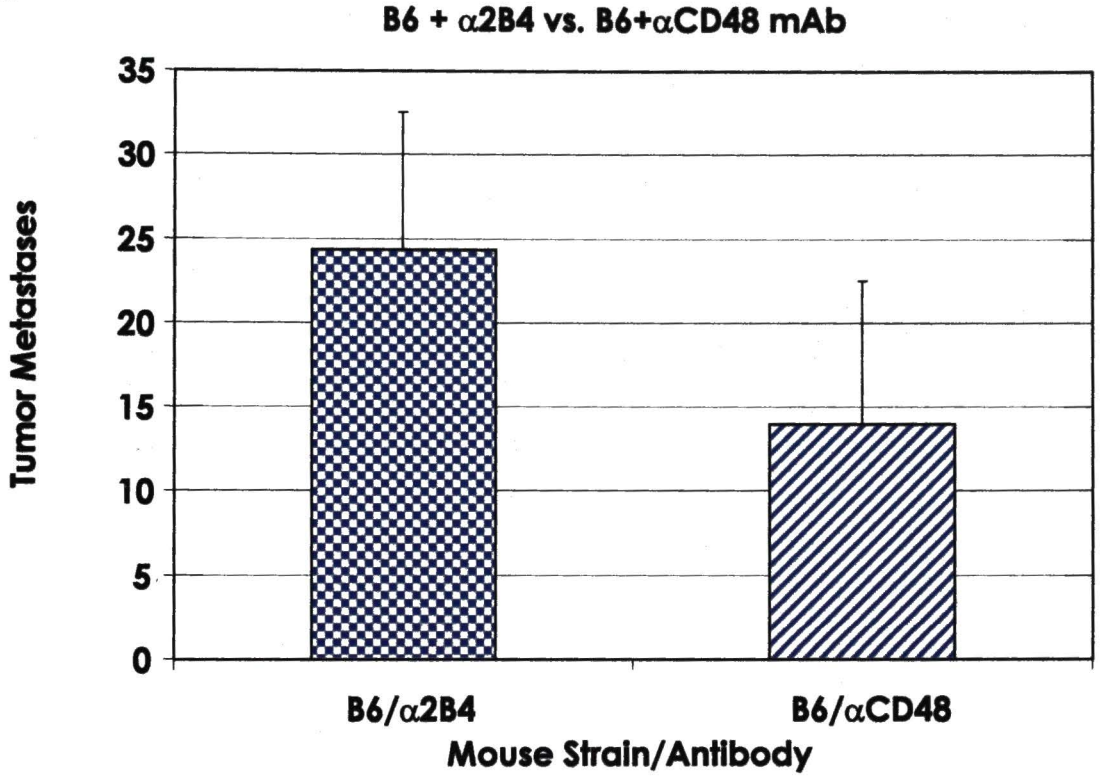
IFNγ Knock-Out
+ αCD48 mAb

Figure 5.6

Anti-CD48 mAb treatment reduces the number of metastases that establish in the lungs of IFN-γ knock-out mice.

Mice were challenged intravenously with 10^5 metastatic B16F10 cells and given 100 μg anti-CD48 mAb on day –2 and day 0 as indicated. Lungs were harvested day fourteen after tumor challenge and established metastases were counted (A). Vertical bars represent standard deviation. Red star indicates statistical significance at p=0.005 as determined by t-test. Representative photographs of lungs bearing metastases visible as black spots (B).

A.



B.

B6 + α2B4 mAb

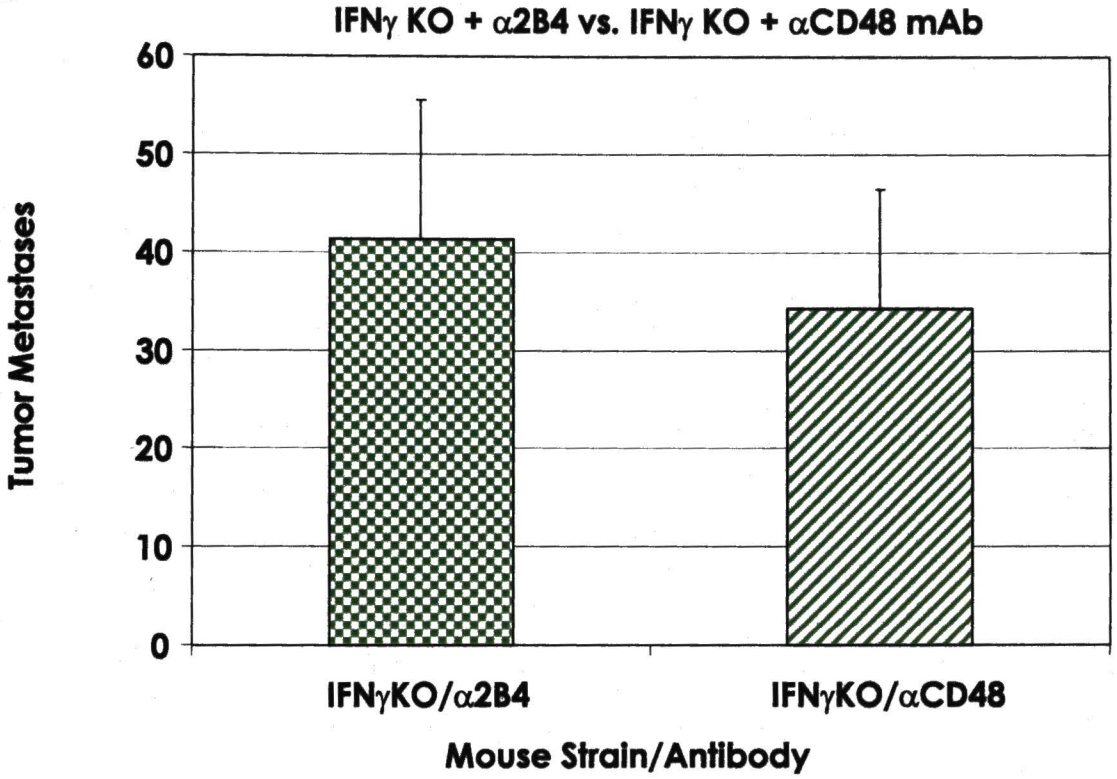
B6 + αCD48 mAb

Figure 5.7

The effect of anti-2B4 mAb treatment is similar to the effect of anti-CD48 mAb in the number of metastases that establish in the lungs of B6 mice.

Mice were challenged intravenously with 10^5 metastatic B16F10 cells and given 100 μg α2B4 or αCD48 mAb on day –2 and day 0 as indicated. Lungs were harvested day fourteen after tumor challenge and established metastases were counted (A). Vertical bars represent standard deviation. Representative photographs of lungs bearing metastases visible as black spots (B).

A.



B.

IFNγ Knock-Out
+ α2B4 mAb

IFNγ Knock-Out
+ αCD48 mAb

Figure 5.8

The effect of anti-2B4 mAb treatment is similar to the effect of anti-CD48 mAb in the number of metastases that establish in the lungs of IFN-γ knock-out mice.

Mice were challenged intravenously with 10^5 metastatic B16F10 cells and given 100 μg α2B4 or αCD48 mAb on day –2 and day 0 as indicated. Lungs were harvested day fourteen after tumor challenge and established metastases were counted (A). Vertical bars represent standard deviation. Representative photographs of lungs bearing metastases visible as black spots (B).

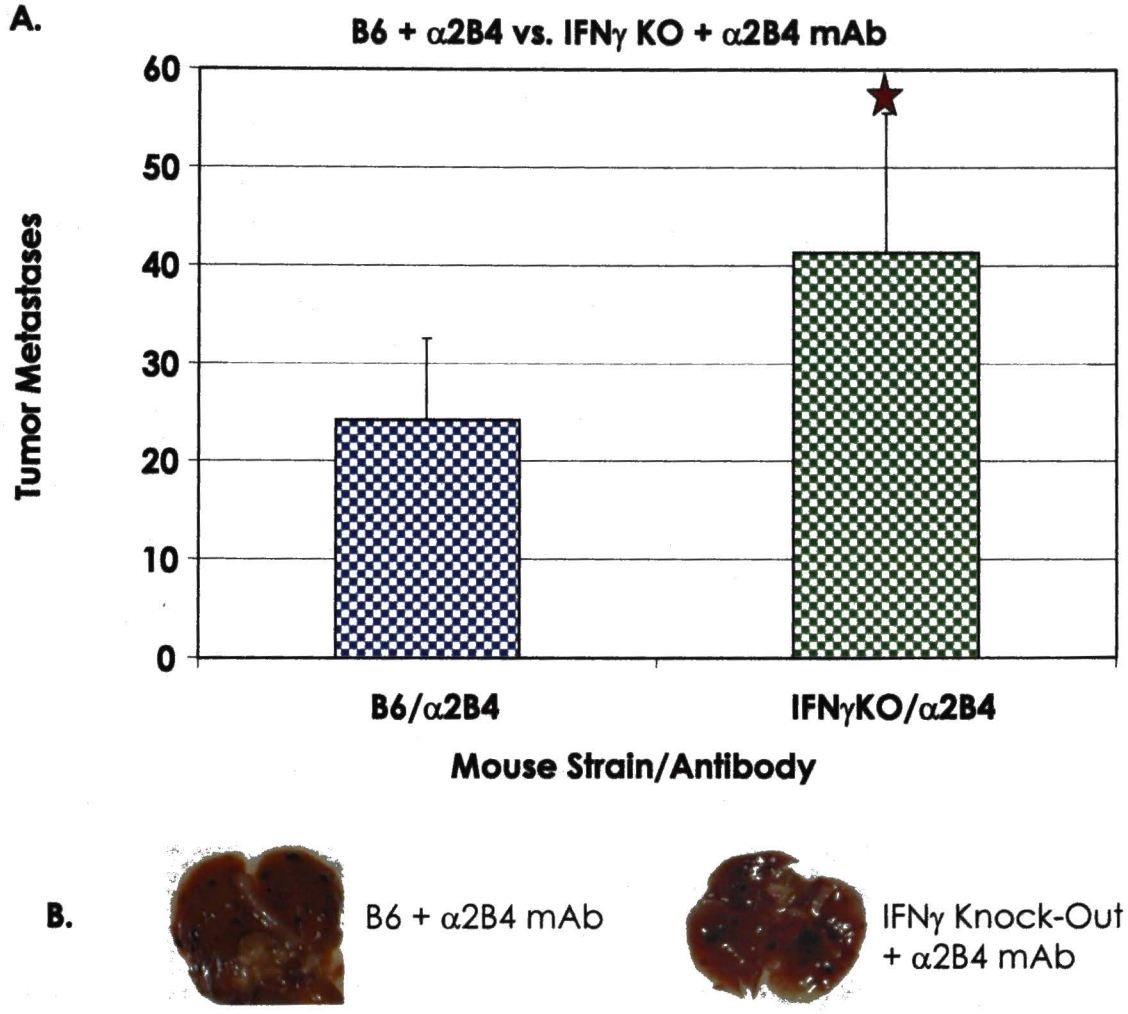


Figure 5.9

IFN-γ knock-out mice are more susceptible to the establishment of B16 melanoma metastases when both receive anti-2B4 mAb.

Mice were challenged intravenously with 10^5 metastatic B16F10 cells and given 100 μg α2B4 mAb on day –2 and day 0 as indicated. Lungs were harvested day fourteen after tumor challenge and established metastases were counted (A). Vertical bars represent standard deviation. Red star indicates statistical significance at p=0.008 as determined by t-test. Representative photographs of lungs bearing metastases visible as black spots (B).

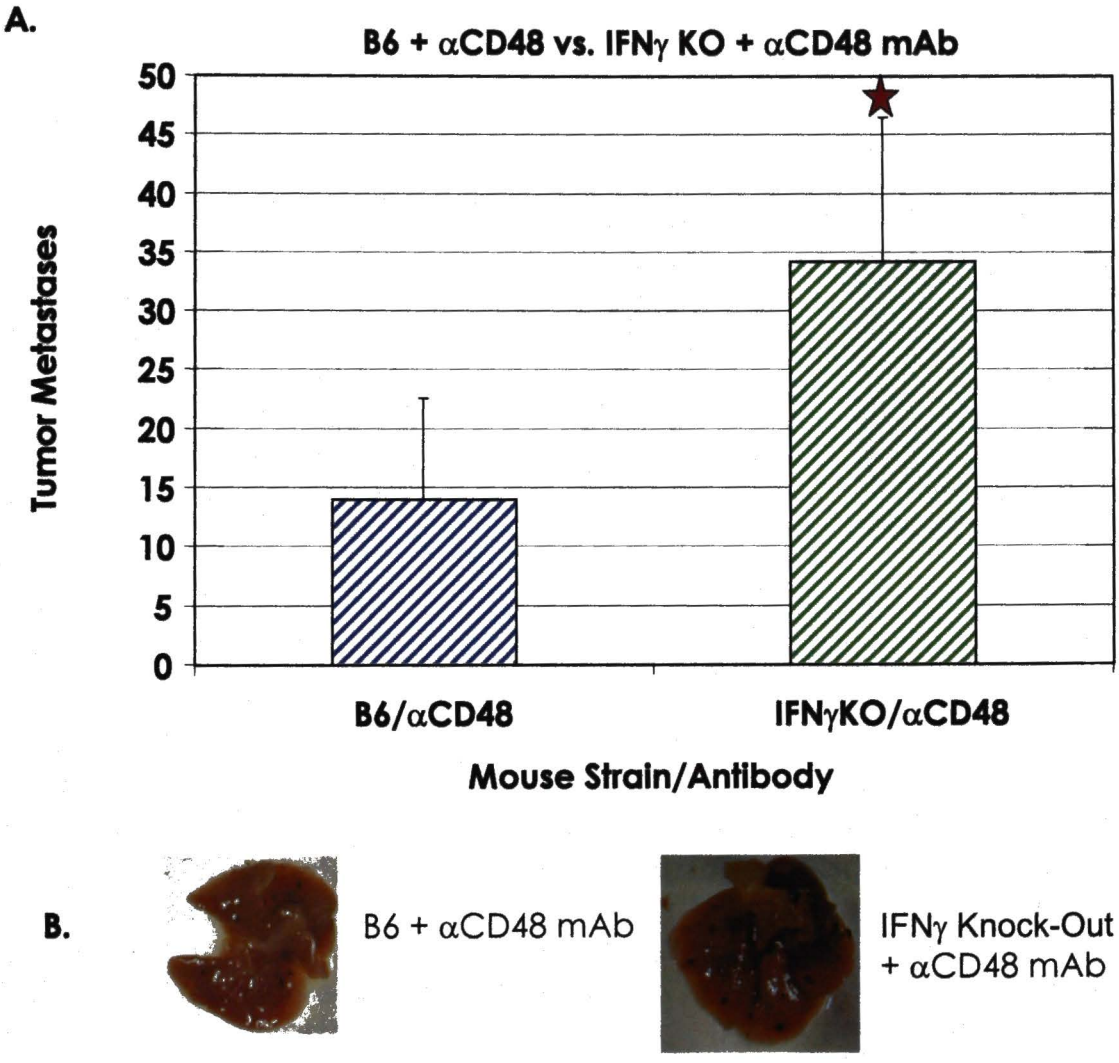


Figure 5.10

IFN-γ knock-out mice are more susceptible to the establishment of B16 melanoma metastases when both receive anti-CD48 mAb.

Mice were challenged intravenously with 10^5 metastatic B16F10 cells and given 100 μg αCD48 mAb on day –2 and day 0 as indicated. Lungs were harvested day fourteen after tumor challenge and established metastases were counted (A). Vertical bars represent standard deviation. Red star indicates statistical significance at p=0.016 as determined by t-test. Representative photographs of lungs bearing metastases visible as black spots (B).

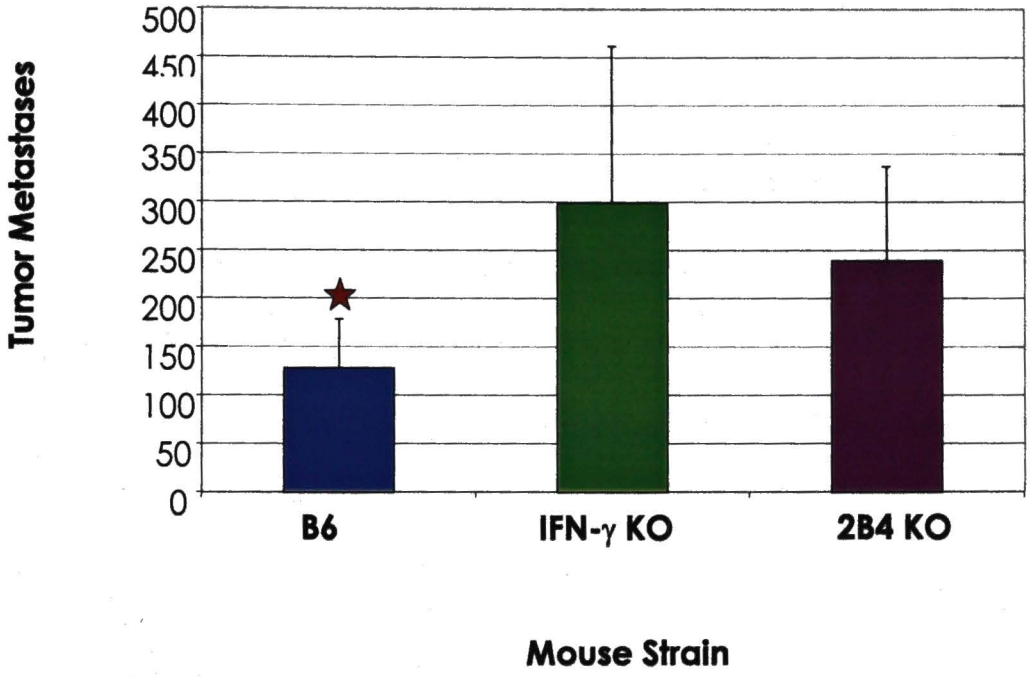


Figure 5.11

IFN-γ knock-out mice and 2B4 knock-out mice are more susceptible to the establishment of B16 melanoma metastases than B6 mice.

Mice were challenged intravenously with 10^5 metastatic B16F10 cells and given 100 μg α2B4 or αCD48 mAb on day –2 and day 0 as indicated. Lungs were harvested day fourteen after tumor challenge and established metastases were counted (A). Vertical bars represent standard deviation. Red star indicates statistical significance at $p \leq 0.05$.

CHAPTER SIX

DISCUSSION

This work is the first to show the regulation of IFN-γ, a global immune regulator by NK cell activating receptor, 2B4. The significance of this regulation as well as the importance of IFN-γ and 2B4 independently has been assessed in vivo for anti-metastatic activity. Additionally, we have established that YT, an NK cell line is a good model for studying 2B4 activation and its effects in vitro.

Chapter two of this work describes the YT cell line as a good model for the study of h2B4 activation in vitro. A cell line model is a valuable tool due to the difficulty in obtaining approval for the use of human subjects and the availability of volunteers for studies. Additionally, a cell line allows all of the work to be done with the same cells rather than with samples pooled from numerous donors and eliminates the need for the purification of NK cells from whole blood samples.

The 2B4 receptor is a member of the immunoglobulin superfamily and belongs to the CD2 subgroup which includes SLAM, CD48, CD58, CD84 and Ly9. We have recently identified CD48 as the high affinity counter-receptor of 2B4 in both mice and humans (33). Early studies found that CD48 binds CD2 with low affinity in the mouse (187), whereas CD58 is

the low affinity counter-receptor for CD2 in the human (93). We have found the 2B4-CD48 interaction is six to nine-fold times stronger than that of CD2-CD48 interaction. The 2B4-CD48 interaction may be more physiologically significant and shed new light on conflicting observations surrounding CD48 ligand studies. Moreover, it has also been reported that CD48-2B4 interactions enhance the lytic function of human NK cells (77). This study shows that binding of surface 2B4 on YT cells induces NK cytolytic function and cytokine production. Additionally, through collaboration with Dr. Myoung Kim we have shown that 2B4 activation also regulates the production of MMP–2, a member of matrix-degrading proteinases whose expression is also modulated by TGF-β, IFN-γ, IL-1β, TNF-α, PMA, and PGE_2 (188).

The signaling pathway of h2B4 is still unknown. h2B4 contains four tyrosine-based motifs that resemble immunoreceptor tyrosine-based inhibitory motifs on its cytoplasmic tail (28). These motifs are similar to SLAM, and have been shown to interact with protein tyrosine phosphatase, SHP-2 and the SH-2 containing adaptor molecule, SAP (69). Defective SAP in patients suffering from X-linked lymphoproliferative-disease leads to the inability to control B-cell proliferation caused by Epstein-Barr virus infections. However, it is still unclear what signal transduction pathway h2B4 utilizes to send its activating message. h2B4

may associate with another signal transduction molecule as suggested by its activating signal.

This study reveals that h2B4 leads to the induction of NK cell functions including both cytotoxicity and cytokine release. These findings therefore argue well for further investigation of 2B4 and related molecular structures in the regulation of NK cell function.

Chapter two of this work examines the regulation of IFNγ by 2B4 activation. IFNγ has been shown to be regulated by many agents, including cell surface molecules, cytokines and chemical agents (114, 125, 178, 185, 189-192). Each inducing agent appears to regulate IFNγ in a unique way. As with many cytokines, regulation is often at the transcript level (193, 194). We set out to decipher the regulation under 2B4 activation with respect to mRNA stability and promoter activity.

Numerous reports have shown IFN-γ to be an important cytokine involved in global immune regulation, as well as a source of anti-viral and anti-tumor protection. A major source of secreted IFN-γ is the natural killer cell population. Activated lymphocytes secrete IFN-γ in response to soluble factors, contact with target cells, or by receptor ligand interaction. Some combination of these factors is usually necessary for initiation of IFN-γ production, however NK cells have been shown to secrete IFN-γ in response to a single soluble factor **(75, 151, 195)**. Here we examined the

production of IFN-γ by the human natural killer cell line YT, in response to 2B4 activation (ligand/receptor interaction) following contact with the K562 cell line, a prototypical target for human NK cell killing.

Previously we reported an increase in the overall amount of IFN-γ secreted by YT cells following 2B4 activation with C1.7 mAb at 18 hours following stimulation **(17)**. In this report we show a significant difference in the amount of IFN-γ secreted by 2B4-activated YT cells compared to control as early as 8 hours following 2B4 activation. Throughout this report we examine the regulatory mechanism for this increase in IFN-γ. Because YT cells are already activated (IL-2 independent), we were able to observe an increase in secreted IFN-γ immediately, simply by incubating the YT cells with K562 cells (Figure 2.4). As early as 8 hours following 2B4 activation, we observed a significantly higher amount of secreted IFN-γ by YT cells stimulated with C1.7 over those that were not stimulated with C1.7. This along with previously reported data, provides strong evidence for the augmentation of IFN-γ production as a result of 2B4 activation.

We were curious to see if mRNA stability played a role in the increase in secreted IFN-γ. We were able to partially inhibit transcription in the YT cells with a sub-lethal dose of Actinomycin D. Again we looked at secreted IFN-γ levels over a period of 7 hours following the inhibition of transcription. There was an initial drop in the amount of secreted IFN-γ

(Figure 3.2), but the overall level of secreted IFN-γ remained stable over the entire 7-hour period. This data suggests that message stability may play a role in the level of IFN-γ production following a combination of stimulating signals, 2B4 activation and target cell contact in this case. Further evidence of IFN-γ message stability following a combination of signals is reported by Chan, et. al (175).

To examine this possibility further, we isolated cytoplasmic RNA from C1.7 stimulated YT cells and unstimulated YT cells that underwent Actinomycin D treatment. S1 nuclease protection was used to determine relative amounts of IFN-γ mRNA as compared to β-actin mRNA. Figure 3.3 demonstrates a far greater amount of IFN-γ mRNA present in YT cells stimulated with C1.7 than in cells that were unstimulated. Additionally, there is actually an increase in the amount of IFN-γ mRNA present over the course of 4 hours. This may be due to the fact that the sub-lethal dose of Actinomycin D only partially inhibits transcription or the increase of mRNA as it was processed from hnRNA. Additionally, this data provides evidence that mRNA stability is at least partially responsible for the increase in production of IFN-γ following 2B4 activation.

We have cloned the full-length IFN-γ promoter into a luciferase expression vector in order to examine promoter activity following 2B4 activation. Transient transfection studies with this construct demonstrate a

four-fold increase in promoter activity when transfected YT cells are stimulated via surface 2B4 (Figure 3.4). In addition to mRNA stability, transcriptional induction of IFN-γ seems to play a predominant role in the increased production of secreted IFN-γ.

Numerous studies have investigated treatment possibilities of IFN-γ, directly and indirectly, in several types of cancers. One group demonstrates enhanced inhibition of tumor growth by activated dendritic cells following stimulation by IFN-γ (196). Additionally, the inclusion of IFN-γ in first-line chemotherapy resulted in prolonged progression-free survival among patients with ovarian cancer (197). Another study reports success in driving a Th1 type response in renal cell carcinoma patients following the administration of autologous tumor cell vaccines in combination with IFN-γ (198). Finally, a coinjection of vectors encoding IFN-γ and IL-12, an IFN-γ inducer, provided enhanced resistance to tumor challenge in mice (199).

In addition to many exciting anti-cancer treatment possibilities, IFN-γ is also being investigated for treatment applications in viral infections. Important links between IFN-γ and HIV progression have been made. McMichael et. al. (200) have recently reported that a reduction in IFN-γ producing CD8+ T cells may play a major role in AIDS development. Another group found that vaccines co-expressing IFN-γ provided safe

enhanced protective responses from HIV-1 infection in macaques (201). A link between 2B4 and HIV disease progression has recently been shown. Higher expression of 2B4 on CD8+ T cells correlated with AIDS progression more closely than a reduction in CD4+ T cells (92). Unlike in NK cells, T cell 2B4 ligation does not result in enhanced cytolytic function (77). However, the role of 2B4 on the IFN-γ production by T cells has not been investigated.

At present, the molecular mechanism underlying the increased transcription of IFN-γ upon 2B4 stimulation remains to be fully determined. Further studies must be done to elucidate the molecular mechanism that is responsible for this phenomenon. Studies examining the transcriptional mechanism are currently underway in our laboratory. Understanding the mechanism of IFN-γ production in response to 2B4 activation could provide valuable information for the development of novel treatment strategies for cancer and virally-infected patients.

Chapter four of this work was focused on further deciphering the regulatory mechanism involved in IFNγ transcription in 2B4 activated NK cells. Work presented in chapter three revealed a four-fold increase in promoter activity when cells were stimulated with mAb C1.7. In this chapter we have cloned ten serial deletion fragments of the IFNγ

promoter in order to identify which regions of the promoter are responsive to 2B4 activation.

Each of ten serial deletion promoter fragments were cloned from the full-length promoter construct by PCR (Figure 4.2) and subcloned upstream of a promoterless luciferase gene. Each of these constructs were transiently transfected into YT cells in order to examine their activity in response to 2B4 activation. Eight of the ten promoter regions were found to respond to 2B4 activation by an increase in activity. The remaining two regions showed decreased promoter activity even in cells that were not activated with C1.7 (Figure 4.13). Based on the data presented here, it appears that 2B4 regulation results in a generalized increase in promoter activity. It will be interesting to investigate the precise binding proteins involved in 2B4 regulation of the IFN-γ promoter, particularly in the region between –252 and –209 where promoter activity is strongly up-regulated in response to 2B4-activation.

IFNγ is a tightly regulated cytokine due to its global effect on the immune system. IFNγ has been implicated in several studies as an anti-tumor agent (145, 202-204). Additionally, IFNγ has an anti-proliferative effect which could contribute to the anti-tumoral effect as well as the anti-viral activity that this cytokine possesses (145, 202-204). 2B4 has also been speculated to have anti-tumoral activity based on in vitro

cytotoxicity assays (see Chapter 2) as well as a role in viral infections. Recently it has been shown that 2B4 expression on CD8+ T lymphocytes is a better indicator of HIV disease progression than the commonly used CD4+ T cell count (92). In light of the data obtained in this chapter, it is tempting to speculate that 2B4 action may be largely due to its regulation of IFN-γ.

Chapter five examines the in vivo role of 2B4 and IFN-γ in a B16F10 tumor metastasis model. Because 2B4 has been shown to up-regulate the transcription of IFN-γ, an important cytokine controlling tumor growth and metastasis (145), it was hypothesized that 2B4 may contribute to the rejection of tumor metastases via IFN-γ.

C57BL/6 mice as well as 2B4 gene knockout mice and IFNγ knockout mice in the C57BL/6 strain background were challenged with 10^5 metastatic B16F10 melanoma cells via tail vein injection. The fact that all mice shared the same strain background ensures that differences in tumor susceptibility are not due to genetic advantages or disadvantages due to strain differences. This ensures that the only genetic advantages or disadvantages that are playing a role are the specific genes knocked out. Our data shows that both 2B4 and IFNγ knockout mice are significantly more susceptible to the establishment of B16F10 tumors in the lungs following this tumor challenge. It was also found that 2B4 knockout

mice and IFNγ knockout mice were statistically similar in the amount of established lung metastases (Figure 5.11). These findings alone suggested that 2B4's anti-tumor activity may be due to its effect on IFNγ. However, when we activated 2B4 with the use of a monoclonal antibody, we found that the parent strain mice as well as IFNγ knockout mice developed far less lung metastases than those not given the antibody. B6 mice given the 2B4 antibody still developed significantly less tumors than IFNγ knockout mice given the antibody. This data reveals that IFNγ indeed plays a role in protection against metastases under 2B4 activation, but that 2B4 protects via other mechanisms as well. This finding supports the recent find by Dr. Chuang in our lab group that 2B4 regulates cytotoxic activity and IFN-γ production through separate signaling pathways.

Activation through mAb directed against CD48 (the counter-receptor of 2B4) was also found to confer protection against lung metastases in both B6 mice and IFNγ knockout mice with greater protection for B6 (Figure 5.10). This data suggests that the 2B4-CD48 interaction is very important in the protection against metastatic tumor cells, both downstream of 2B4 as well as downstream of CD48. Recently, a member of our lab found that 2B4 activation regulates cytotoxicity and cytokine release via separate pathways (117). This recent finding is

supported by the anti-metastatic activity seen in vivo in B6 and IFNγ mice under 2B4 activation.

The data in presented in chapter five illustrates the importance of 2B4 and IFN-γ in the fight against tumor metastases. The role of 2B4 is particularly interesting in that 2B4-activation enhances the anti-tumor activity of NK cells in vivo even in IFN-γ knock-out mice. Further studies should be planned immediately to investigate the effectiveness of 2B4 activation against other tumor cell types. These studies were done with the highly metastatic B16F10 tumor cell line. Perhaps 2B4-activation in studies using the F1 or F5 cell lines, which are less metastatic, would result in even fewer metastases. With the recent discovery that 2B4 is present in all inbred mouse strains, studies investigating 2B4 activation in other mouse strain backgrounds. If studies with other tumor cells and other mouse strains provide data as exciting as the current study, anti-2B4 mAb treatment could be developed for use in humans to help prevent the spread of primary tumors.

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