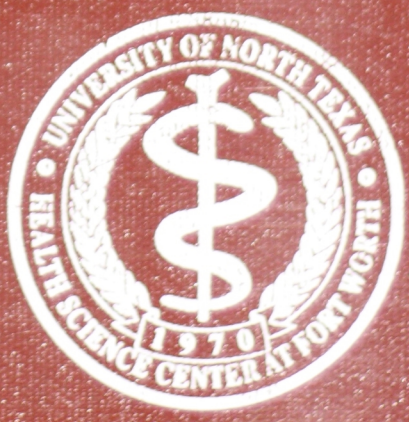
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Natural Killer (NK) cells are large granular lymphocytes of the innate immune system that constitute the first line of defense against viral pathogens and cancer. Unlike cells of the adaptive immune response, NK cells do not recognize specific antigens expressed on MHC receptors, rather they recognize tumorgenic and virally infected cells through a complex balance of activating and inhibiting receptors expressed on both cell surfaces. Lectin-like transcript 1 (LLT1) is a receptor expressed on the surface of human NK cells. LLT1 is expressed on numerous immune cells and subsequent functional analysis indicates that LLT1 plays an activating role on NK cells by way of stimulating interferon-gamma (IFN-G) secretion. LLT1 has also been shown to have a role on non-immune cells, inhibiting the formation and function of osteoclasts. Additionally, the natural ligand of LLT1 has been identified as NKR-P1A (CD161), an NK cell inhibitory receptor known to play an important role in immune regulation. We hypothesize that LLT1 employs multiple signaling pathways to accomplish its activating functions on human NK cells, and may be associated with one of four known transmembrane accessory proteins associated with NK cell activating receptors. We activated LLT1 on NK92 cells with target cells expressing its natural ligand CD161 and analyzed IFN-G production in the presence of pharmacological inhibitors specific for various signaling

mechanisms. These results indicate that LLT1 employs Src-PTK, p38 and ERK signaling pathways, but not PKC, PI3K or calcineurin. These results were followed up with phosphorylation analysis, which confirmed that the ERK signaling pathway is associated with LLT1 IFN-G production. Finally, by analyzing IFN-G mRNA we found that LLT1 activation is not associated with any detectable change in IFN-G mRNA levels, suggesting that LLT1 stimulates NK IFN-G production by modulating post-transcriptional or translational events. Identification of the signaling pathways associated with LLT1 is of great medical significance as this may provide us with novel insights into activating NK cells to counter infection and cancer.

SIGNALING IN NATURAL KILLER CELLS:

NK CELL ACTIVATION BY LLT1 RECEPTOR

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My work is dedicated to my grandmother Hilda C. Bambard. Born three weeks after *Titanic* foundered, the first born child of immigrants; she survived scarlet fever, three influenza pandemics, and two World Wars, with her husband serving in the Second World War. Although her life spanned the development of the automobile and aviation industries, with men walking on the moon, her proudest achievement was her family. Both of her sons earned college degrees, as have half of her grandchildren, with one soon to receive his Ph.D.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Mammalian Immune System

The mammalian immune system is a highly adaptive gathering of cells, antibody and cellular products that collectively protect the host from foreign pathogens and cancerous cells. It is delineated into the innate immune response and the adaptive immune response. The innate immune response is characterized by phagocytic cells and NK cells capable of targeting pathogens and infected or cancerous cells without any prior exposure to foreign antigen. The adaptive immune response is characterized by potent antibody, CD4+ T cell and cytotoxic T lymphocyte (CTL) targeting of foreign antigens that have been previously presented to the immune system by antigen presenting cells. Repeated exposure to a foreign antigen results in a more potent immune response with each additional exposure. This is the more advanced system associated with higher orders of the animal kingdom. Through recombination of the genes associated with antigen receptors, the adaptive immune response is capable of recognizing theoretically every antigenic combination that could be generated. The innate immune response is not modulated by the presence of specific foreign antigen, but by ligation of various receptors on the cell surface. This lack of a pre-sensitization requirement enables the innate immune response to respond rapidly at the first sign of infection or tumor formation and

both directly target pathogens and infected cells as well as stimulate the adaptive immune system via secretion of cytokines (1).

The cells of the immune system are commonly referred to as white blood cells, or leukocytes, differentiating them from red blood cells or platelets also present in whole blood. Leukocytes fall into two broad categories based upon visible morphology, granulocytes named for their granular appearance and agranulocytes named for their lack of visible granules. Granulocytes include neutrophils, eosinophils and basophils whereas the agranulocytes include lymphocytes, monocytes and macrophages. Granulocytes, monocytes/macrophages and NK cells largely potentiate the innate immune response while the adaptive immune response is mostly associated with B and T lymphocytes. Lymphocytes consist of three main cell types, B cells, T cells and NK cells.

B lymphocytes originate in the bone marrow and develop via assistance from marrow stromal cells (2). B cells are characterized by the expression of an antigen receptor on their cell surface. Through a complicated process of immunoglobulin gene expression and rearrangement B cells express antigen-specific surface receptors and upon stimulation are capable of secreting a variety of immunoglobulins specific for their respective antigen. An individual B cell is capable of recognizing one specific antigen and the process of B cell maturation and gene rearrangement occurs in various peripheral lymphoid organs. The antigen receptor on B cells prior to their encountering their specific antigen is surface IgD and IgM. Mature B cells that have not yet encountered their specific antigen are termed naïve B cells and they will continue to circulate through peripheral blood and lymphoid organs until they encounter their specific antigen (3, 4).

When naïve B cells encounter their specific antigen they become activated and will differentiate into either immunoglobulin secreting plasma cells or to memory B cells that can rapidly differentiate into plasma cells upon re-exposure to the same antigen. Note that immunoglobulin secreted by plasma cells will recognize the same antigen as the IgD and IgM receptor present on the naïve B cell/activated plasma cell from which it came.

T lymphocytes also develop from precursor cells found in bone marrow. T cell precursors migrate to the thymus where T cell maturation occurs and precursors differentiate into CD4 and CD8 T cells. In addition to the CD4 and CD8 molecules that give the cells their names, both CD4 and CD8 T cells express an antigen specific T cell receptor (TCR) generated through a process of gene re-arrangement not unlike that associated with B cell antigen receptor development (5). TCRs complex with CD4 and CD8 molecules on the surface of their respective cells and this antigen binding complex is capable of recognizing specific foreign antigen presented to it by Major Histocompatability Complex (MHC) receptors on antigen presenting cells (6). The CD8 TCR complex recognizes antigen presented by MHC class I molecules, which are expressed on all nucleated cells, whereas the CD4 TCR complex recognizes antigen presented by the MHC class II molecule, expressed on antigen presenting cells (APC) (7). Traditional models of mammalian immunology state that MHC-II receptors present antigens associated with extracellular pathogens derived from endosomes after their uptake by the three types of APCs, dendritic cells, macrophages and B cells (8, 9). Alternatively, MHC-I receptors present antigens associated with intracellular pathogens, predominantly viruses and intracellular bacteria, degraded by proteosomes within the

cytoplasm (10, 11). The CD4 T cells stimulated by their specific antigen expressed on MHC-II are termed helper T cells as their primary function is to further potentiate the immune response via cytokine secretion stimulating functions such as immunoglobulin production by plasma cells and activation of macrophages (12). The CD8 T cells stimulated by their specific antigen expressed on MHC-I are termed cytotoxic T lymphocytes (CTL), as their primary function is to kill any nucleated cell expressing in their MHC-I receptors the antigen for which the CTL is specific, as said cell is likely infected with the pathogen from which the antigen is derived (13, 14). Many intracellular pathogens and tumors have evolved mechanisms to escape CTL killing by altering or downregulating the MHC-I receptors of the host cell they infect so they are incapable of activating CTLs (15-18). Such mechanisms include CMV downregulation of MHC-I expression by inhibiting transport of new MHC-I molecules to the cell surface (19), HPV induced degradation of MHC-I within the golgi apparatus (20), melanoma mutation of beta-2-microglobulin resulting in complete loss of MHC-I expression (21), and carcinoma downregulation of Transporter-associated with antigen-processing (TAP) resulting in decreased antigen presentation to MHC-I (22). It is important to note that while such MHC-I avoidance strategies do result in decreased CTL targeting and are in some instances associated with increased disease progression (23-26) in certain circumstances the loss of MHC-I expression by tumors is associated with *improved* clinical outcome (27-29). This apparent contradiction is the result of natural killer cell killing of virally infected cells and tumors that, by altering MHC-I expression to escape

CTL killing have now made themselves susceptible to NK cell killing (30). This phenomenon of natural cytotoxicity and NK cell function will be reviewed shortly.

The CD4 T-helper immune response is generally categorized into the Th1 immune response that targets intracellular pathogens and the Th2 immune response that targets extracellular pathogens (31). The specificity of these two responses is mediated by the differing patterns of cytokine secretion exhibited by the two cell types. Th1 cells secrete IL-2 and IFN-G that stimulate intracellular immune response effector cells including NK cells, CD8+ T cells, and macrophages whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 that stimulate IgE, IgA and IgG1 production as well as mast cell and eosinophil growth (31, 32).

While the MHC-I and MHC-II models of antigen presentation previously described are not incorrect, recent studies have demonstrated that in certain circumstances MHC-I receptors are capable of presenting antigen phagocytosed from the extracellular space and degraded within endosomes, a source of antigen previously associated only with the MHC-II antigen presentation pathway (33-36). Furthermore, a small proportion of T lymphocytes do not express the traditional TCR complex associated with the CD4 and CD8 molecules. These T cells are termed gamma-delta T cells and their gamma-delta-TCR complex is generated through a process of gene rearrangement similar to that of traditional T cells. However, the overall role of gamma-delta T cells in immune function remains unclear (37).

Cytokines

Cytokines are soluble proteins generated by various cell types that produce an effect on target cells expressing a specific cytokine receptor (38). Cytokines can exert their effects on the cell that secreted them (autocrine activity) or on other target cells adjacent to or distal from the secreting cell (paracrine activity), though due to their limited half life in the circulation most cytokines perform their actions within local tissue (38). Though the functional properties of cytokines, hormones and growth factors tend to overlap, cytokines generally refer to those factors that act influence the immune response by acting with leukocytes and growth factors refer to factors that act upon other non-immune cells (38).

Interferon-gamma (IFN-G)

Interferon-gamma (IFN-G) is a cytokine of much importance to proper innate and adaptive immune function. IFN-G functions as a heterodimer and is produced by CD8+ T cells, Th1 CD4+ T cells and NK cells. The specific receptor for IFN-G is expressed on all cell types save erythrocytes (39). IFN-G plays an important role in the early response to intracellular infection and consequently IFN-G is the major cytokine produced by NK cells upon their detection of infected or cancerous cells (40). As NK cells do not store pre-synthesized IFN-G protein for rapid secretion, NK cells must constitutively express a quantity of IFN-G mRNA to facilitate rapid translation of IFN-G upon stimulation (41-43). NK cell release of IFN-G occurs within hours of detecting infection. While T cells

do not begin IFN-G secretion as quickly as NK cells, T cells become the predominant source of IFN-G in the later stages of the immune response (40). The nature of NK cell IFN-G secretion clearly illustrates how NK cells function as the first line sentinels of the immune system.

IFN-G directly inhibits viral replication and activates the innate and adaptive immune responses via multiple mechanisms (44). IFN-G upregulates both MHC-I and MHC-II antigen presentation pathways, induces naïve CD4+ T cells to differentiate into Th1 CD4+ T cells, and activates macrophages to increase phagocytosis, cytokine secretion and production of antimicrobials such as superoxide, nitric oxide and hydrogen peroxide (44, 45). An extensive body of evidence demonstrates the importance of IFN-G in proper immune function. The lack of NK cells or IFN-G is clearly linked with a loss of infection control (40, 46). Mice deficient in IFN-G mediated responses or lacking NK cells are highly susceptible to babesiosis compared to non-deficient mice (47). IFN-G inhibits the early promoter of CMV (48). IFN-G is a potent stimulator of nitric oxide which inhibits viral replication (49). IFN-G inhibits HIV replication during early infection (50). The importance of IFN-G to control of infection is plainly demonstrated when one considers all the various mechanisms that pathogens have evolved to counteract the effects of IFN-G. Inhibition of IFN-G production via neutralization of IFN-G stimulators is one such strategy. Ectromelia virus (EV) produces an IL-18 Binding Protein (IL18BP) which effectively sequesters a large amount of free IL-18, thereby preventing its ability to bind the natural IL-18 receptor and stimulate IFN-G production. Strains of EV deficient in IL18BP are more susceptible to control by the immune system

(51). Direct binding of circulating IFN-G before it is able to bind its natural receptor is another viral strategy to evade IFN-G. Several members of the poxvirus family, including vaccinia, coxpox and rabbitpox produce a soluble IFN-G receptor homologue which acts to physically bind IFN-G and prevent its interaction with authentic IFN-G receptor, thereby reducing the overall level of IFN-G activity. Several of these homologues are capable of recognizing IFN-G from multiple species such as human, rabbit and mouse, which underscores the importance of IFN-G to mammalian immunology (52, 53). *Mycobacterium tuberculosis* undermines the effects of IFN-G by inhibiting the production of IFN-G associated IFN regulatory factor 1 (IRF-1) via interfering with post-transcriptional up-regulation, a function that is likely associated with elevated pathogenicity (54).

Natural Killer (NK) cells

Natural Killer cells are so termed because their initial discovery in both humans and mice centered around their ability to kill tumor cells without any prior exposure to the target (55-57). Though they have been traditionally classified with agranulocytes they contain granules in their cytoplasm and are also termed large granular lymphocytes (LGL) (58). NK cells comprise 5% to 20% of lymphocytes circulating in human blood and are phenotypically differentiated from other lymphocytes by their lack of sIgD and CD3, and the presence of CD16 and CD56 (59). CD16 is the immunoglobulin Fc receptor that mediates NK cell antibody dependent cellular cytotoxicity (ADCC) (60-62). The importance of NK cells to proper immune function, particularly against viral infections

and cancer has been demonstrated experimentally and through clinical observations. Multiple studies of human patients have associated NK cell dysfunction with Hodgkin's lymphoma (63), leukemia (64, 65), and elevated cancer rates (66). NK cell defects are also associated with impaired response to and inability to clear common viral pathogens such as human herpes virus and Epstein-Barr virus (EBV) (67, 68) In mouse models the depletion of NK cells with monoclonal antibodies results in enhanced growth of tumor cell lines despite intact antibody and cellular immune responses (69, 70).

NK cells are capable of responding to signs of infection within hours of detection and constitute the first line of defense against tumors and viral pathogens (71). NK cells potentiate the immune response by exhibiting direct cytotoxicity against infected host cells and pathogens and by secreting cytokines that further activate the immune system (72). NK cells exhibit this protective effect by constitutively analyzing host cells and inducing apoptosis of any cell that exhibits signs of viral infection or cancer (73). NK cells can also directly target pathogens coated with host immunoglobulin by recognizing the Fc region in a process called antibody-dependent cellular cytotoxicity (ADCC) (74). Cell death is mediated by release of intracellular granules containing perforin and granzymes which directly impact the target cell and initiate apoptosis. NK cells analyze individual host cells through an interaction of surface receptors on the NK and target cell. Unlike T-lymphocytes, NK cells do not recognize a specific antigen but rather detect changes in the surface expression of various receptors which may be indicative of infection or cancer. Alteration or downregulation of MHC class I receptors is often a sign of infection that is recognized by NK cells and sufficient to stimulate killing of cells that

otherwise would escape targeting by MHC class I dependent cytotoxic T-cells. The ability of tumor cells to be killed by NK cells is inversely proportional to MHC class I receptor expression by the tumor cells and this has formed the basis for the "missing self hypothesis" describing the interactions between NK cells and their targets (75, 76).

NK cells function to modulate the immune response via cytokine secretion, particularly IFN-G. *Cryptococcus neoformans* is a fungal pathogen cleared by NK cells via IFN-G mediated activation of macrophages rather than by direct cytotoxicity (77). The presence of high levels of IFN-G stimulate CD4+ T cells to differentiate into mature Th1 effector cells, which mediate the immune response against intracellular pathogens (44). In addition to their direct role in targeting pathogens and infected cells, through cytokine secretion NK cells play a direct role in stimulating and guiding the subsequent adaptive immune response.

Inhibitory and stimulatory NK receptors

NK cell activity is regulated by a complex balance between inhibitory and activating receptors on their cell surface. These receptors are associated with a very diverse population of ligands in addition to the traditional MHC class I ligands (78). Inhibitory receptor ligation counters the signals from ligated stimulatory receptors and it is in the absence of inhibitory signals that NK activation occurs. Multiple families of NK inhibitory and activating receptors exist, and some receptors such as 2B4/CD244 may function as an activating or inhibitory receptor under different conditions (79, 80). Activating receptors may regulate cytotoxicity, cytokine secretion or a combination of

both (81, 82). Early study of NK surface receptors indicated that expression of MHC-I receptors imparted target cells with immunity from NK cell cytotoxicity, and the absence of MHC-I receptors was associated with susceptibility to NK cell cytotoxicity (76, 83). This theory suggested that NK cells must express a ligand or ligands for MHC-I that act to inhibit NK cells upon their ligation by MHC-I. Subsequent research identified multiple NK cell ligands for MHC-I that act to inhibit NK cells, adding much credence to the missing-self hypothesis (84-86). The overall population of NK inhibiting receptors has recently been categorized into the 'inhibitory receptor superfamily' (IRS) (87, 88).

As NK cells are not in a constitutively activated state, in addition to predicting the existence of NK inhibiting receptors the missing-self hypothesis suggested that NK cells must also express an activating receptor that is capable of initially detecting the presence of a target cell and activating the NK cell if appropriate (89). Multiple NK cell activating receptors have subsequently been identified since the missing-self hypothesis was first postulated. The NK cell activating receptors NKp30, NKp44 and NKp46 were among the first identified and have been designated as natural cytotoxicity receptors (NCR) (90-92). These NCRs play an important role in stimulating NK cell cytotoxicity as their blocking with monoclonal antibodies reduces cytotoxicity (93), and expression of higher levels of NCRs is associated with increased natural cytotoxicity (94). Note however that they are incapable of binding MHC-I (95) and the precise identity of their ligands on target cells remains unclear (96-98). Additionally, the NK activating receptor NKG2D has also been identified (99).

Analysis of the amino acid sequences of the various NK activating and inhibiting receptors quickly shows an obvious difference between the two groups. Inhibitory receptors exhibit a long intracellular region containing common tyrosine motifs whereas activating receptors exhibit a short intracellular region that lack such tyrosine motifs (100, 101). In inhibitory receptors this is termed the immunoreceptor tyrosine-based inhibitory motif (ITIM). The associated tyrosine residues act to transmit the receptor inhibition signal by recruiting inhibitory phosphatases such as SHP-1, SHP-2 and SHIP-1 (102-105). It is these various signaling molecules that direct the inhibition of NK cell activation (106). On the other hand, activating receptors do not exhibit any such tyrosine motifs in their intracellular regions. To transmit the intracellular activating signal, activating receptors associate with accessory proteins via interactions within their transmembrane regions. It is these accessory proteins that exhibit cytoplasmic tyrosine motifs, termed immunoreceptor tyrosine-based activatory motifs (ITAM) that function to relay the activating signal into the cell (93, 100). In addition to activating and inhibiting function, NK cell receptors can be categorized into two additional groups based upon structural characteristics. The immunoglobulin-like receptors contain Ig domains and the C-type lectin like receptors contain carbohydrate recognition-like domains (87, 107).

C-type lectin receptors

'C-type lectin', 'carbohydrate recognition domain' (CRD) and 'C-type lectin/lectin-like domain' (CTLD) all refer to calcium-dependent carbohydrate-binding eukaryotic proteins that contain a conserved amino acid motif associated with

carbohydrate binding (107). Despite the name, not all proteins containing the motif are calcium-dependent or capable of binding carbohydrates and the term CTLDcp is more accurately used to describe any protein containing the CRD or homologous amino acid motif (108, 109). CTLDcps have been classified into multiple groups based upon domain architecture, including a distinct group of non-calcium binding type-2 transmembrane proteins classified as 'CLTDcp Group V - NK cell receptors' (107, 110).

Killer cell lectin-like receptors (KLR)

Group V NK cell receptors – generally termed KLR – are mostly encoded within the 'NK gene complex' on human chromosome 12 and mouse chromosome 6 (111-113). Although KLRs generally fall into closely related gene groups such as *NKG2, Ly49, NKR-P1* and *Clr,* receptors within these groups do not always exhibit the same functional role (114, 115).

Lectin-like transcript 1 (LLT1)

LLT1 was identified by our laboratory through analysis of unidentified open reading frames in an NK cell cDNA library. DNA sequence analysis of LLT1 indicates that it is a KLR as it contains a CTLD, it is a type-2 transmembrane protein and genomic analysis shows LLT1 is located in the NK gene complex between the CTLDcps CD69 and CD161, both of which play a role in immune regulation (114, 116). Based upon this it was assumed that LLT1 would have a regulatory function in the immune system. A monoclonal antibody was developed by Dr. Mathew's laboratory and used to conduct

functional and expression assays on LLT1. LLT1 was found to be a potent stimulator of IFN-gamma secretion in NK cells. However, it was not found to have any effect on NK cytotoxicity. In addition to expression on NK cells, LLT1 was found to be present on B cells, T cells and monocytes/macrophages (117). Our knowledge of LLT1 has been furthered by additional studies conducted by independent groups. Functional study of the murine homologue of LLT1, Clr-b determined that it plays a role in novel, non-MHC-I based missing self recognition by inhibiting killing of target cells when binding to its ligand NKR-P1B/D (118, 119). In two separate studies the natural ligand of human LLT1 was identified as NKR-P1A (CD161) (120, 121). Both human LLT1 and murine Clr-b were independently cloned from osteoblasts and their ligation was found to inhibit osteoclast formation and function (122, 123). Recently, human glioblastoma has been shown to increase LLT1 surface expression, presumably to inhibit NK cell killing by ligating the inhibitory CD161 receptor, thereby facilitating escape from the immune system (124). When considering these findings we predict that in addition to functional roles on non-immune cells, LLT1 plays a wider role in immune regulation than previously thought and warrants further study.

Intracellular signaling pathways

The ability of cell surface receptors to convert external stimuli into cellular function is dependent upon a complex system of intracellular signaling mechanisms. Inhibitory NK cell receptors generally transmit inhibiting signals via immunoreceptor tyrosine-based inhibitory motifs (ITIM) present in their cytoplasmic tail. Activating NK

cell receptors frequently transmit activating signals via immunoreceptor tyrosine-based activation motifs (ITAM) present not within their cytoplasmic tails, but in the cytoplasmic domain of dimeric transmembrane accessory proteins noncovalently associated with the intracellular region of the activating receptor (125). Activating NK cell receptors employing this strategy typically express a short cytoplasmic tail lacking ITIMs or other tyrosine signaling motif and possess a basic residue within their transmembrane sequence for association with an aspartic acid residue present in transmembrane accessory proteins (116, 126-128). LLT1 exhibits all these properties. Presently, four transmembrane accessory proteins have been identified in NK cells, TCR-zeta, FceRI-gamma, DAP10 and DAP12 (125). Preliminary evidence obtained by our laboratory indicates that LLT1 associates with the DAP10 transmembrane accessory protein. The signaling pathways downstream of ITAMs are very complex and have not been thoroughly elucidated. Generally, upon receptor ligation membrane bound Src family protein tyrosine kinases (Src-PTK) are recruited to the vicinity of the receptor complex where they phosphorylated the tyrosine residues present on the accessory protein ITAMs. These phosphorylated ITAMs subsequently phosphorylate spleen tyrosine kinase PTKs (Syk-PTK) which in turn may activate numerous downstream signaling mechanisms via phosphorylation including PI3K associated with the p38 MAPK pathway, PLC-gamma associated with the PKC pathway, and Grb2 associated with the MEK/ERK pathway. Note that phosphorylation is not required to activate all signaling mechanisms. Transcription factor NF-AT must be de-phosphorylated before it can stimulate transcription (125). In NK cells there is often a degree of redundancy in

signaling pathways, and different receptors can employ different pathways to affect the same result (78, 125).

Medical Significance

NK cells play an important role in control of various intracellular pathogens and cancer. By determining the mechanisms associated with the function of NK cells, specific causes of immune dysfunction and targets for potential new therapies may be suggested. X-Linked Lymphoproliferative Disease (XLP) is a hereditary combined immunodeficiency associated with fulminant EBV infection and lymphoma (129, 130). Recent studies of signaling mechanisms of NK cell receptors identified a mutation in the signaling protein SLAM-associated protein (SAP) associated with loss of receptor function. These findings demonstrated the SAP mutation as the underlying genetic cause of XLP (131-133). Recent findings have also demonstrated that Systemic Lupus Erythematosus (SLE) is associated with NK cell deficiency, possibly associated with NK cell inability to regulate antibody production via cytokine secretion (134). Additionally, SLE disease progression is associated with abnormally high levels of IFN-G mRNA expression and production compared to healthy control PBMC donors, suggesting dysfunction in IFN-G transcriptional or post-transcriptional regulation may be associated with SLE pathogenesis (135). This correlates with previous research indicating progressing SLE disease is associated with overexpression of the IFN-G gene and may be treated by modifying cellular suppression and post-transcriptional control of IFN-G (136). Additional examples of IFN-G dysfunction or pathogen subversion of IFN-G

function and associated disease states have already been discussed. The study of the mechanisms of NK cell activity have demonstrated the cause of multiple disease conditions, and suggested novel pathways for modulating the immune system in the hope of achieving better clinical outcomes. There is every reason to believe that the further study of NK cell function will continue to provide additional medical benefits in the future. Furthermore, LLT1 has recently been shown to be up-regulated by glioblastoma to facilitate tumor escape from NK cytotoxicity, presumably via the inhibitory effects of CD161 activation on the targeting NK cells (124). LLT1 is expressed by osteoblasts and functions to inhibit the formation and function of osteoclasts (122). Identifying the signaling mechanisms associated with LLT1 may enable us to counter the protective effect LLT1 exerts on glioblastoma, thereby treating brain tumor patients with immunotherapy as opposed to radiation and surgical means. Conversely, knowledge of the signaling mechanisms of LLT1 in relation to osteoblast function may enable us to up-regulate their bone-formation activity to treat conditions associated with bone loss such as osteoporosis.

Objectives

This work focused on elucidating the intracellular signaling mechanisms of human LLT1 on natural killer cells. To stimulate LLT1 we developed an anti-LLT1 monoclonal antibody using bacterial and mammalian expressed LLT1 in addition to synthesized extracellular LLT1 peptides. Furthermore, we generated soluble CD161 and CD161 expressing target cells as an alternative LLT1 activation strategy. Once we

established an effective LLT1 activation strategy that stimulated NK IFN-G production we conducted various inhibition assays using specific pharmacological inhibitors to determine which signaling mechanisms are required for LLT1 associated IFN-G production. These indicated that the p38 and ERK signaling pathways were required for LLT1 induced IFN-G secretion. These results were followed up with phosphorylation assays that further confirmed the role of ERK in LLT1 signaling. Finally, to evaluate the specific end mechanism of LLT1 signaling in relation to IFN-G production we evaluated IFN-G mRNA levels in the presence and absence of LLT1 stimulation. These assays indicated that LLT1 stimulated IFN-G production is not associated with detectable changes in IFN-G mRNA levels and we conclude that LLT1 stimulated IFN-G secretion is presumably associated with post-transcriptional regulation of IFN-G expression.

CHAPTER II

GENERATION OF ANTI-LLT1 MONOCLONAL ANTIBODY

SUMMARY

Monoclonal antibodies offer a highly efficacious means of studying the expression, distribution and function of surface receptors *in vitro* (137). Presently, there is no commercially available anti-LLT1 monoclonal antibody capable of binding and activating the functional region of LLT1. Therefore, the generation of a functional monoclonal antibody would provide an excellent tool for the study of LLT1 function. We designed various fusion proteins and synthetic peptides to act as immunogens for murine immunization and subsequent hybridoma generation.

INTRODUCTION

A monoclonal antibody is an antibody produced by a hybridoma that recognizes a specific target. They are important immunological tools in that they are capable of both recognizing their target and in the case of many surface receptors capable of mimicking the effect of the natural ligand of the targeted receptor. A hybridoma secreting monoclonal antibody is produced by the *in vitro* fusion of a splenocyte from an animal immunized with the target of interest and an immortalized myeloma cell. The resulting hybridoma is an immortalized cell secreting the specific antibody produced by the original splenocyte. The advantage of this system is the hybridoma acts as a permanent source of the antibody of interest (138).

Immunized mice are commonly used as a source of splenocytes, including this project. Animals to be immunized undergo multiple rounds of immunization with immunogen before spleen is removed and splenocytes are harvested. As the monoclonal antibody generated will recognize the immunogen with which the animal was immunized, proper immunogen selection is essential to effective monoclonal antibody development. Prior to splenic harvesting, animal serum is often analyzed for presence of polyclonal antibodies against immunogen to confirm the animal is responding to immunizations. After splenocyte and myeloma fusion hybridomas undergo screening to identify clones that exhibit most desirable properties. These generally include ability to recognize immunogen in various conditions such as western blot, ELISA, and flow

cytometry, ability to bind target and stimulate function and the isotype of the secreted monoclonal antibody (137).

For the study of LLT1 a monoclonal antibody will be an indispensable tool. An anti-LLT1 antibody is necessary for immunoprecipitation of LLT1, a prerequisite for identifying the ITAM containing accessory protein with which LLT1 associates. In addition to rapidly identifying the presence of LLT1 on the surface of various cell types, anti-LLT1 will allow us to select LLT1+ or LLT1- cells for further study from a heterogeneous cell population via cell sorting. Additionally, a functional LLT1 monoclonal antibody will provide us with a consistent method of stimulating LLT1 on any cell type so that we may observe downstream effects including employed signaling pathways.

MATERIALS AND METHODS

Peptide immunogen selection and hybridoma generation:

Selection of peptides for immunization was accomplished by analyzing the extracellular region of LLT1 with Invitrogen PeptideSelect Online Designer. By analyzing hydrophillicity and structural availability of the amino acid sequence, peptides of appropriate antigenic potential were selected and synthesized by UT Southwestern Protein Chemistry Technology Center. Peptide sequences were CAYLNDKGASSARHYTERK, SQRFCDSQDADLAQVESF, and YFSDDTKNWTSSQRFCDSQ. SCID mice were initially immunized with peptides and subsequently boosted with soluble pSec-LLT1ext mammalian expressed fusion protein. Our laboratory employed a similar immunization strategy to generate an anti-human CS1 monoclonal antibody for which a patent was subsequently awarded (139, 140). Spleens were removed via hemisplenectomy, splenocytes were harvested and fused with SP2/0 cells. Immunizations and splenocyte harvest and fusion were conducted by Dr. Wayne Lai of UT Southwestern. Hybridoma clones were initially screened at UTSW based upon their ability to recognize LLT1 immunogen peptide bound to an ELISA plate. Polyclonal sera were analyzed in our laboratory via western blot and monoclonal antibody was analyzed in our laboratory via flow cytometry. Although I generated a pGex-LLT1ext vector for bacterial fusion protein expression, this fusion protein proved to be highly

difficult to purify from bacterial lysate under all tested conditions. Consequently, only mammalian expressed pSec-LLT1ext fusion protein was employed for immunizations.

Generation of pSec-LLT1ext expression vector and fusion protein:

Full length human LLT1 was previously cloned into pCi-neo mammalian expression vector (Promega, Madison WI) by Dr. Stephen Mathew. PCR oligonucleotide primers were designed to amplify only the extracellular portion of LLT1 while simultaneously inserting BamHI and XhoI restriction endonuclease sites. BamHI FP 5' TGT GGA TCC GTT GCT GCT TTA AGC GC 3' XhoI RP 5' CAC TCG AGC ATG TAT ATC TGA TT 3' These primers were synthesized (IDT, Coralville IA) and extracellular LLT1 was amplified from pCi-neo-LLT1. PCR product was restriction digested with BamHI and XhoI, as was empty pSectag2/HygroB vector (Invitrogen, Carlsbad CA). Both digested PCR product and pSec vector were run on 1.2% agarose gel, imaged via ethidium bromide fluorescence and gel extracted using Qiaquick Gel Extraction Kit (Qiagen, Valencia CA). Digested PCR product was ligated into opened pSec vector using T4 DNA ligase (Promega, Madison WI) (Figure 2.4). Ligation product was transformed into Invitrogen Subcloning Efficiency DH5alpha Competent Cells per manufacturer's instructions. Transformants were screened on LB plate with ampicillin, six clones were selected, grown overnight in 5 ml LB broth and plasmid DNA was isolated via mini-prep. Plasmids were restriction digested using BamHI and XhoI (New England Biolabs, Beverly MA) and run on 1.2% agarose gel, imaged via ethidium bromide fluorescence to confirm correct LLT1 insert was present. Multiple clones

exhibited single DNA insert of ~450 base pairs, the correct size of the LLT1 extracellular region amplified by PCR. One clone with correct insert was selected and intact plasmid was sent for sequencing by Seqwright (Houston TX). Sequencing confirmed LLT1 extracellular region was correctly inserted in frame with pSec vector. Sequence confirmed pSec-LLT1ext plasmid DNA was prepared via CsCl gradient maxi prep following standard protocols. CsCl prepared plasmid was used to transiently transfect mouse B16 melanoma cells. Transient transfection was carried out in Opti-MEM I reduced serum medium (Gibco, Invitrogen Corp) using FuGENE 6 Transfection Reagent (Roche, Indianapolis IN) per manufacturer protocol. Translated pSec-LLT1ext fusion protein was secreted into medium, harvested, centrifuged to remove cell debris and concentrated ~40X using Amicon Ultra 10000MW centrifugal filter device (Millipore, Billerica MA). Prior to concentration, PMSF was added to 0.1 mM to prevent protease degradation. After concentration total protein was estimated using Coomassie Plus Protein Assay Reagent Kit and Albumin Standard (Pierce, Rockford IL). Protein was subsequently analyzed via SDS-PAGE and western blot via standard protocols. pSec-LLT1ext fusion protein was stained for western blot using 1:2500 dilution of mouse anti c-myc tag monoclonal antibody (Serotec, Oxford UK), 1:10000 dilution of goat anti-mouse HRP conjugate monoclonal antibody (KPL, Gaithersburg MD) and imaged using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica MA).

Generation of pGex-LLT1ext expression vector and fusion protein:

Full length human LLT1 was previously cloned into the pCi-neo mammalian expression vector (Promega, Madison WI) by Dr. Stephen Mathew. PCR oligonucleotide primers were designed to amplify only the extracellular portion of LLT1 while simultaneously inserting BamHI and XhoI restriction endonuclease sites. BamHI FP 5' TGT GGA TCC GTT GCT GCT TTA AGC GC 3' XhoI RP 5' CAC TCG AGC ATG TAT ATC TGA TT 3' These primers were synthesized (IDT, Coralville IA) and extracellular LLT1 was amplified from pCi-neo-LLT1. PCR product was ligated into a TA vector using pGEM-T Easy Vector system (Promega, Madison WI). Ligation product was transformed into Subcloning Efficiency DH5alpha Competent Cells (Invitrogen, Carlsbad CA) per manufacturer's instructions. Transformants were screened on LB plate with ampicillin, clones were selected, grown overnight in 5 ml LB broth and plasmid DNA was isolated via mini-prep. TA vector-LLT1 DNA and pGex-4T-1 bacterial expression vector (GE Healthcare, Piscataway NJ) are restriction digested with BamHI and XhoI and run on 1.2% agarose gel and imaged via ethidium bromide fluorescence. Multiple clones exhibit single DNA insert of ~450 base pairs, the correct size of the LLT1 extracellular region amplified by PCR. Both LLT1 DNA insert and opened pGex vector were gel extracted using Qiaquick Gel Extraction Kit (Qiagen, Valencia CA) and ligated together using T4 DNA ligase (Promega, Madison WI) (Figure 2.1). Following the same protocol used for pSec-LLT1ext, the ligation product was transformed into DH5alpha, screened on LB plates, restriction digested and analyzed via agarose gel and confirmed by sequencing. For expression of pGex-LLT1ext fusion protein, sequence

confirmed pGex-LLT1ext DNA was transformed into electrocompetent BL21 *E. coli* via electroporation. Ampicillin resistant colonies were screened by restriction digestion to confirm plasmid with correct insert was present. Translation of the pGex vector is under control of the lac operon. BL21 containing the correct pGex-LLT1ext plasmid was grown overnight, 37c with shaking. Overnight culture was added into 9X volume of LB with 20 mM Glucose plus ampicillin. This was allowed to grow until OD600 0.5-0.8 (~1 hour) before translation was induced with 1 mM IPTG. This was allowed to grow for 6 hours until the cells were collected via centrifugation (5000G, 4c, 10 min) and washed once with PBS, pH 7.3. Cell pellets were resuspended in sonication buffer (PBS, pH 7.3 with 10% 25 mM Tris pH 8.0/lysozyme 10 mg/ml), 1:20 of the initial culture volume for a 20X reduction in volume (500 ml IPTG induced culture is resuspended in 25 ml sonication buffer). Cells were subsequently sonicated using appropriate settings for sonicator, volume of sample and cell type. Sonicated cells were mixed with an equal volume of 6 M Guanidine HCl and incubated at room temperature for 30 min to facilitate fusion protein solubility. Cells were subsequently centrifuged to collect supernatant containing soluble pGex fusion protein. Supernatant was filtered through four layers of cheesecloth/kim wipes to remove cellular debris. PMSF was added to 0.1 mM to inhibit protease degradation of fusion protein. To purify pGex-LLT1ext fusion protein, supernatant was incubated with 1 ml 50% Glutathione Sepharose 4B (GE Healthcare, Piscataway NJ) for every 100 ml of sonicate at room temperature with gentle agitation for 30 min. Mixture was then centrifuged 500G, 5 min and pellet was washed three times with PBS. After third wash the pellet was resuspended with 1 ml elution buffer (50 mM

Tris-HCL, 10 mM reduced glutathione, pH 8.0) for every 100 ml of sonicate and incubated 10 min at room temperature with gentile agitation. Mixture was then centrifuged 500G, 5 min and the supernatant containing eluted fusion protein was carefully decanted. Two additional elutions were conducted to ensure no additional fusion protein remains bound to the Glutathione Sepharose. Eluted pGex-LLT1ext was concentrated ~40X using Amicon Ultra 30000MW centrifugal filter device (Millipore, Billerica MA). After concentration total protein was estimated using Coomassie Plus Protein Assay Reagent Kit and Albumin Standard (Pierce, Rockford IL). Protein was subsequently analyzed via SDS-PAGE and western blot per standard protocols. pGex-LLT1ext was stained for western blot using 1:4000 dilution of mouse anti-Glutathione-S-Transferase monoclonal antibody (Sigma, St. Louis MO), 1:10000 dilution of goat anti-mouse HRP conjugate monoclonal antibody (KPL, Gaithersburg MD) and imaged using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica MA). pGex-LLT1ext fusion protein was estimated to be approximately 43 kDa based upon the amino acid sequence of extracellular LLT1 and the associated Glutathione-S-Transferase. pGex-4T-1 vector containing no insert was also transformed into BL21 to serve as a control for fusion protein expression and isolation. This empty pGex produced a fusion protein of approximately 29 kDa and consists mostly of Glutathione-S-Transferase.

Isolation of PBMCs from Peripheral Blood:

PBMCs were isolated from the whole blood of healthy donors via ficoll gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO).

Flow Cytometry:

Freshly isolated PBMCs were analyzed by flow cytometry using a Beckman Coulter Cytomics FC 500 Flow Cytometer operated with CXP Analysis 2.1 software. Briefly, PBMCs were harvested and counted by trypan blue exclusion assay. Fc receptors were blocked with 3 ug human Fc fragment (Rockland Immunochemicals, Gilbertsville, PA) for every one million cells in PBS/1% BSA for 30 min on ice. Cells were washed with PBS/1% BSA and resuspended in wells on a 96-well, round bottom plate for staining with $1.0X10^6$ cells/well. To screen LLT1 monoclonal antibodies cells were resuspended in 100 ul hybridoma supernatant or supernatant from a non-specific hybridoma to serve as an isotype control. To screen murine polyclonal LLT1 sera cells were resuspended in 50 ul PBS/1% BSA with 4 ul of polyclonal sera or non-immune mouse sera to serve as an isotype control. After 40 min primary incubation on ice, cells were washed and resuspended with a FITC or PE conjugated goat anti-mouse IgG polyclonal secondary antibody. After 20 min incubation on ice, protected from light the cells were washed, transferred to flow cytometry tubes and analyzed via flow cytometry. C1.7 anti-2B4 monoclonal antibody was used as a positive control reaction.

RESULTS

pGex-LLT1ext fusion protein generation:

Following the protocols described above I was able to generate pGex-LLT1ext fusion protein in BL21 transformed with my expression vector and induced with IPTG (Figure 2.2). However, I was not successful in obtaining soluble pGex-LLT1ext in the supernatants, nor was I ever able to solubilize pGex-LLT1ext from the precipitated bacterial debris pellet (Figure 2.3). Multiple lysis protocols were attempted, including lysozyme alone, sonication alone, and lysozyme with sonication. Additionally, I attempted to solubilize LLT1 from the bacterial debris pellet post lysis using various sonication buffers containing combinations of triton-X-100, guanidine HCL, and DTT. In no instance was I able to obtain pGex-LLT1ext in soluble form. Alternatively, empty pGex fusion protein was readily isolated from lysed BL21 supernatant (Figure 2.3). I hypothesize that the lectin-like nature of LLT1 is not conducive to existing in soluble form and was the cause of my inability to obtain soluble pGex-LLT1ext. It was directly because of this failure that LLT1 peptides and soluble pSec-LLT1ext were generated and employed as immunogens.

pSec-LLT1ext fusion protein generation:

Following the protocols described above I was able to generate pSec-LLT1ext fusion protein in B16 mouse melanoma cells transiently transfected with my expression

vector (Figure 2.5). As open reading frames cloned into the pSec vector are translated with an N-terminus Ig kappa chain secretion signal, pSec-LLT1ext was readily secreted into the transfectant media with no need to lyse cells for harvesting. The presence of secreted pSec-LLT1ext fusion protein was confirmed by western blotting (Figure 2.6).

Peptide-based Monoclonal Antibody:

As the data provided in Figure 2.7 shows, none of the hybridomas generated from LLT1 peptide-immunized mice were capable of binding LLT1 on the surface of human PBMCs via flow cytometry. As a result of this, I initiated a second round of monoclonal antibody generation using only the mammalian expressed pSec-LLT1ext fusion protein for initial immunization and subsequent boosting.

Fusion Protein-based Monoclonal Antibody:

The polyclonal sera of mice immunized five times with pSec-LLT1ext fusion protein exhibited high specificity for immunogen LLT1 fusion protein via ELISA and western blot analysis (not shown). Furthermore, when analyzed via flow cytometry polyclonal sera exhibited much greater MFI when binding LLT1 expressing NK92 compared to non-immune sera (Figure 2.8). Based upon this data, a mouse was selected for hybridoma generation.

Figure 2.1 pGex-LLT1ext Vector Map. The extracellular region of LLT1 is approximately 450 base pairs long. It was ligated into the pGex-4T-1 vector at the BamHI and XhoI restriction endonuclease cut sites.

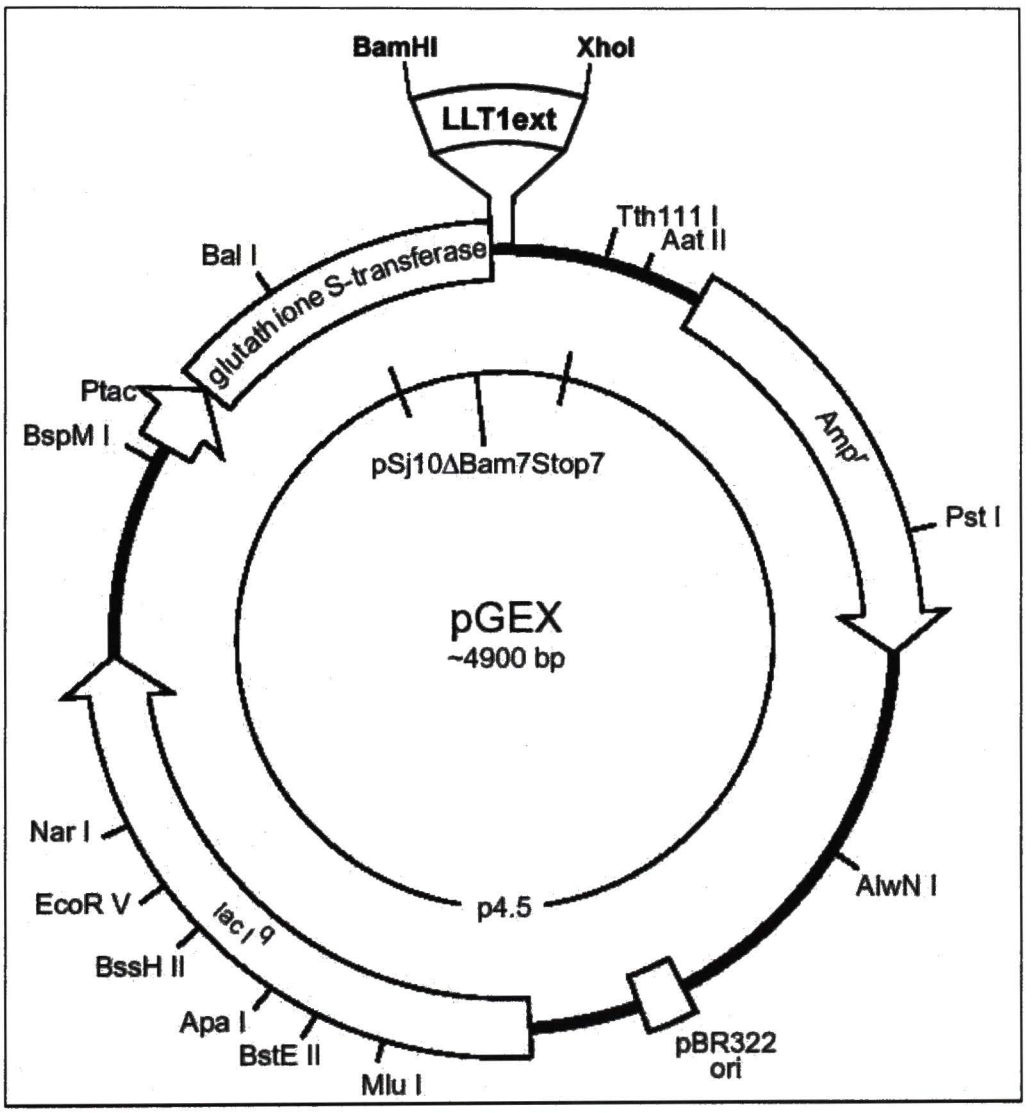

BamHI
XhoI
LLT1ext
Tth111 I
Aat II
Bal I
glutathione S-transferase
Ptac
BspM I
pSj10ΔBam7Stop7
Amp
Pst I
pGEX
~4900 bp
Nar I
EcoR V
lacI q
BssH II
Apa I
BstE II
Mlu I
p4.5
AlwN I
pBR322
ori

Figure 2.2 pGex-LLT1ext fusion protein. Schematic of pGex-LLT1ext fusion protein indicating three main regions, N-terminal glutathione-S-transferase, thrombin recognition site and LLT1 extracellular region

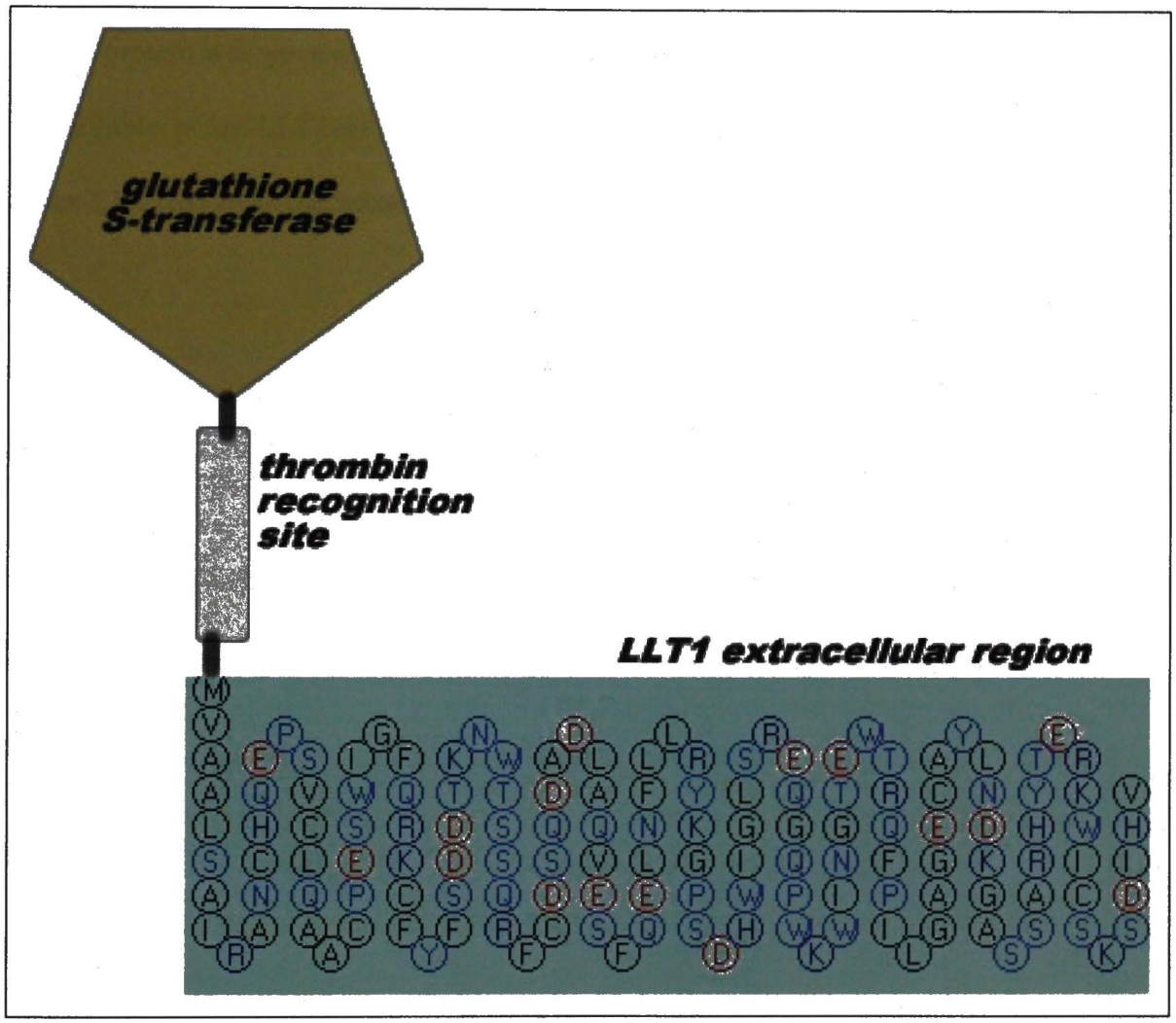

glutathione
S-transferase
thrombin
recognition
site
LLT1 extracellular region

Figure 2.3 pGex-LLT1ext SDS-PAGE. pGex-LLT1ext and empty pGex were expressed in BL21. Bacterial cells were lysed via sonication, and supernatants and bacterial pellet were analyzed separately. Empty pGex fusion protein (predominantly GST) was observed in both supernatant and bacterial pellet lanes indicating soluble and insoluble fusion protein was present. pGex-LLT1ext was observed only in the bacterial pellet lane. No soluble pGex-LLT1ext was detected in supernatant and no attempts to solubilize pGex-LLT1ext from the bacterial pellet were successful.

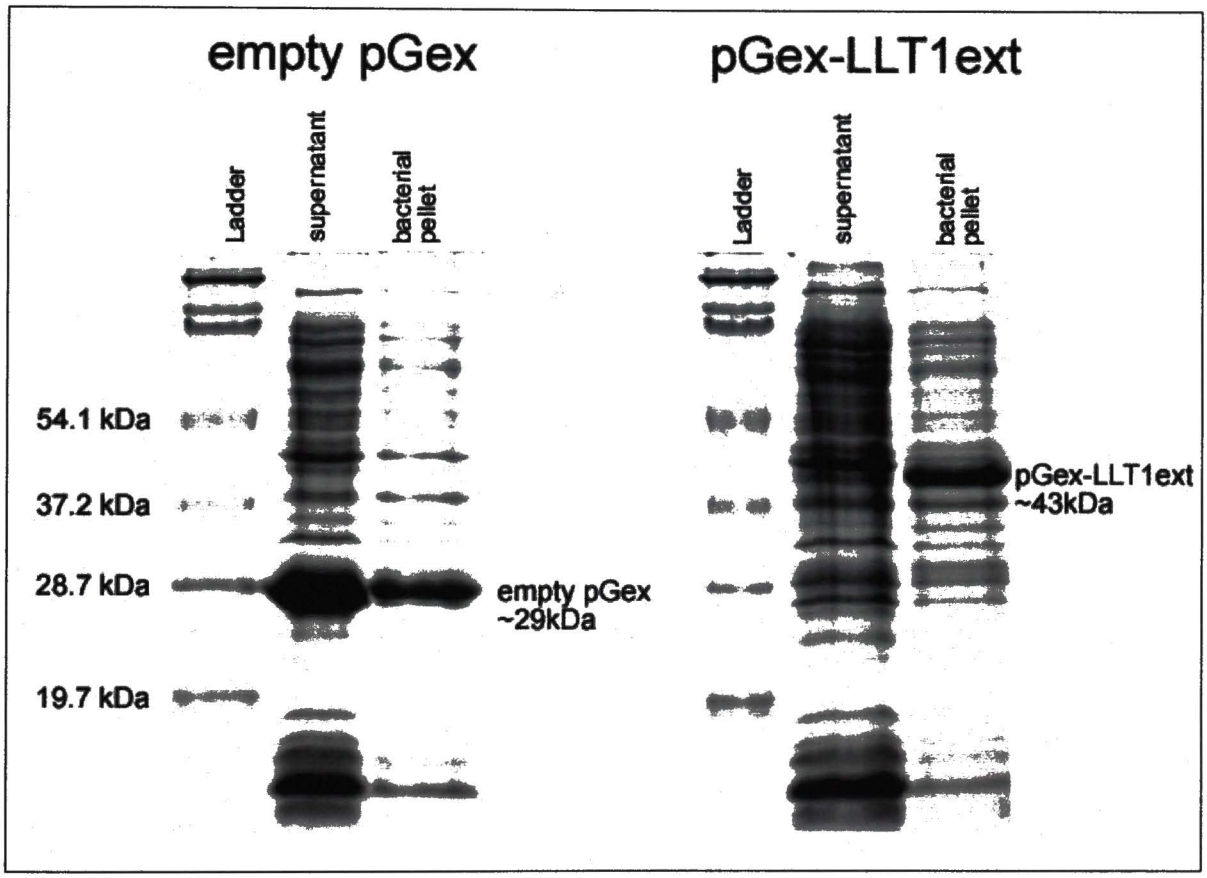
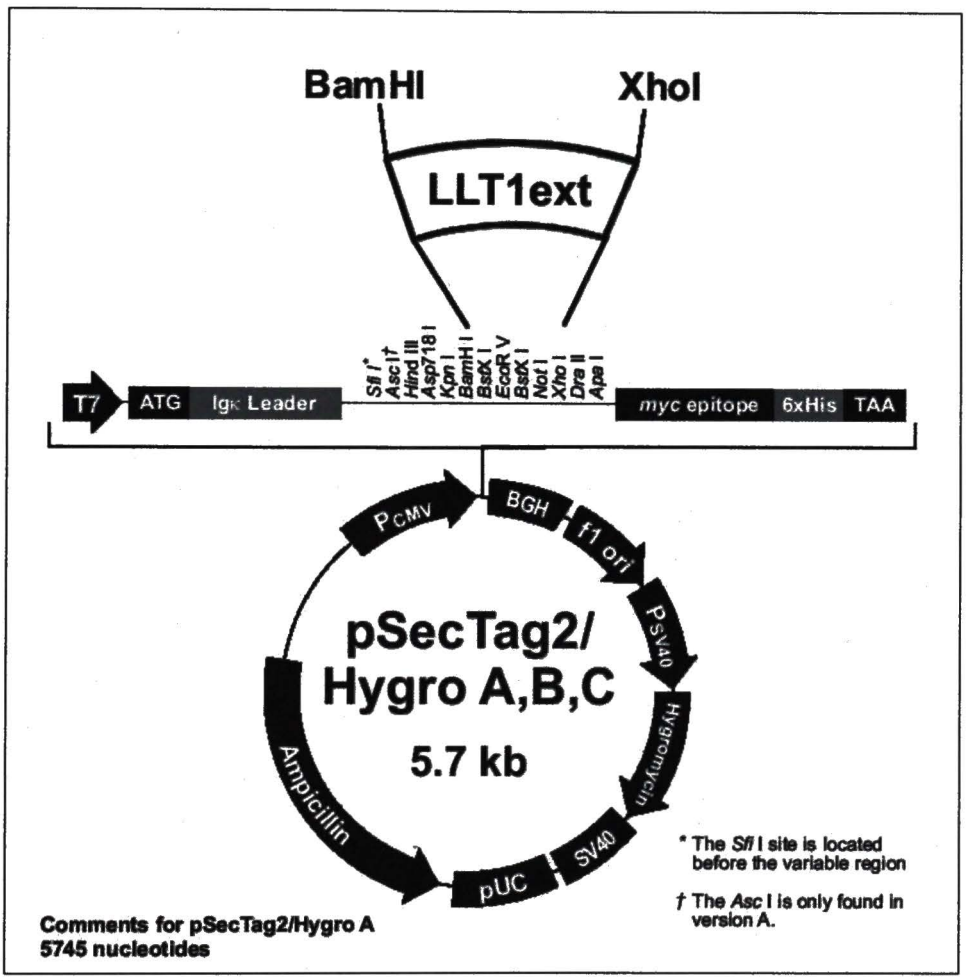

empty pGex
pGex-LLT1ext
Ladder
supernatant
bacterial pellet
Ladder
supernatant
bacterial pellet
54.1 kDa
37.2 kDa
28.7 kDa
19.7 kDa
empty pGex
~29kDa
pGex-LLT1ext
~43kDa

Figure 2.4 pSec-LLT1ext Vector Map. The extracellular region of LLT1 is approximately 450 base pairs long. It was ligated into the Invitrogen pSecTag2/Hygro B vector at the BamHI and XhoI restriction endonuclease cut sites.


BamHI
XhoI
LLT1ext
Sfi I*
Asc I†
Hind III
Asp718 I
Kpn I
BamH I
BstX I
EcoR V
BstX I
Not I
Xho I
Dra II
Ape I
T7
ATG
Igκ Leader
myc epitope
6xHis
TAA
PCMV
BGH
f1 ori
PSV40
Hygromycin
SV40
pUC
Ampicillin
pSecTag2/
Hygro A,B,C
5.7 kb
* The Sfi I site is located before the variable region
† The Asc I is only found in version A.
Comments for pSecTag2/Hygro A
5745 nucleotides

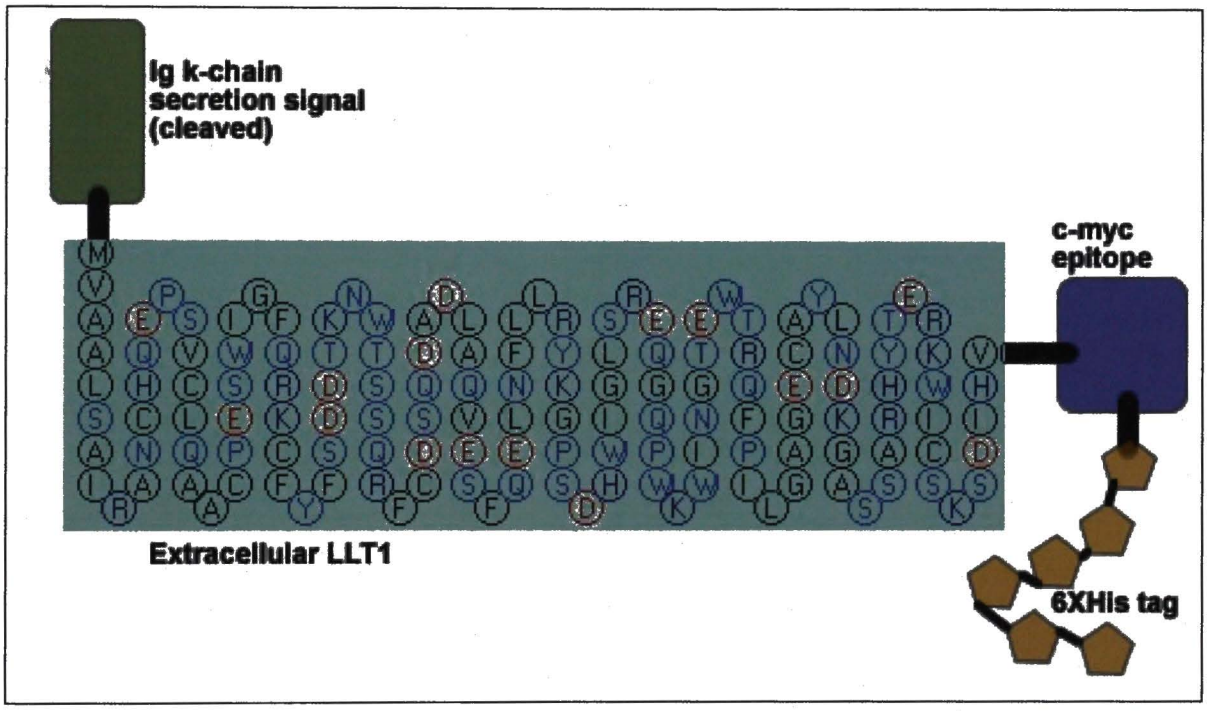

Ig k-chain
secretion signal
(cleaved)
c-myc
epitope
Extracellular LLT1
6XHis tag

Figure 2.6 pSec-LLT1ext Western Blot. pSec-LLT1ext expression vector was transiently transfected into B16 mouse melanoma cells. pSec-LLT1ext fusion protein was subsequently secreted into the supernatant which was concentrated and analyzed via western blot under native and reducing conditions. Blot was probed with mouse anti-c-myc-HRP antibody. pSec-LLT1ext fusion protein was observed under both native and reducing conditions. Recombinant c-myc protein served as a positive control.

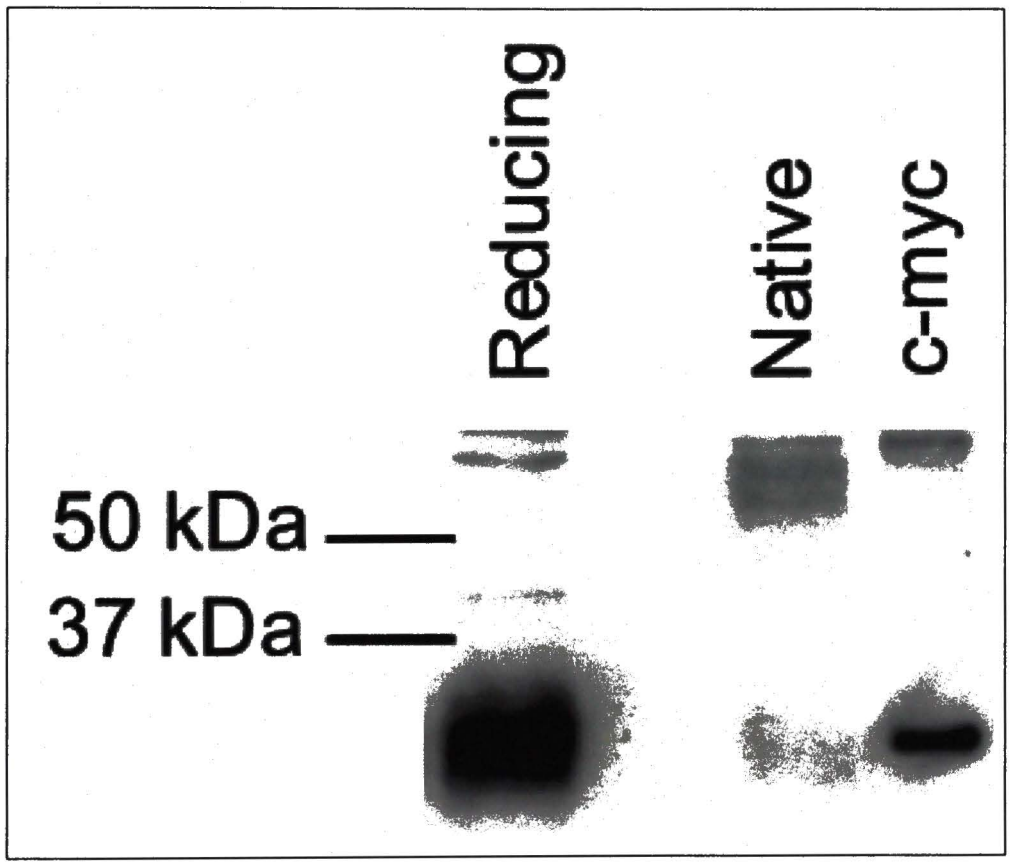

Reducing
Native
c-myc
50 kDa
37 kDa

Figure 2.7 Flow-cytometric analysis of peptide-derived LLT1 monoclonal antibody. After preliminary ELISA screening, approximately forty LLT1 hybridoma clones were selected for further screening by flow cytometry. Human PBMCs were isolated from peripheral blood and incubated with supernatant from the various hybridomas. A FITC-tagged goat-anti-mouse IgG polyclonal secondary antibody was used to detect any anti-LLT1 antibody that successfully stained the surface of PBMCs. C1.7 anti-2B4 antibody was used as a positive control and appears similar to what a positive LLT1 clone should look like. None of the anti-LLT1 hybridomas exhibited specificity for the LLT1 expressing PBMCs.

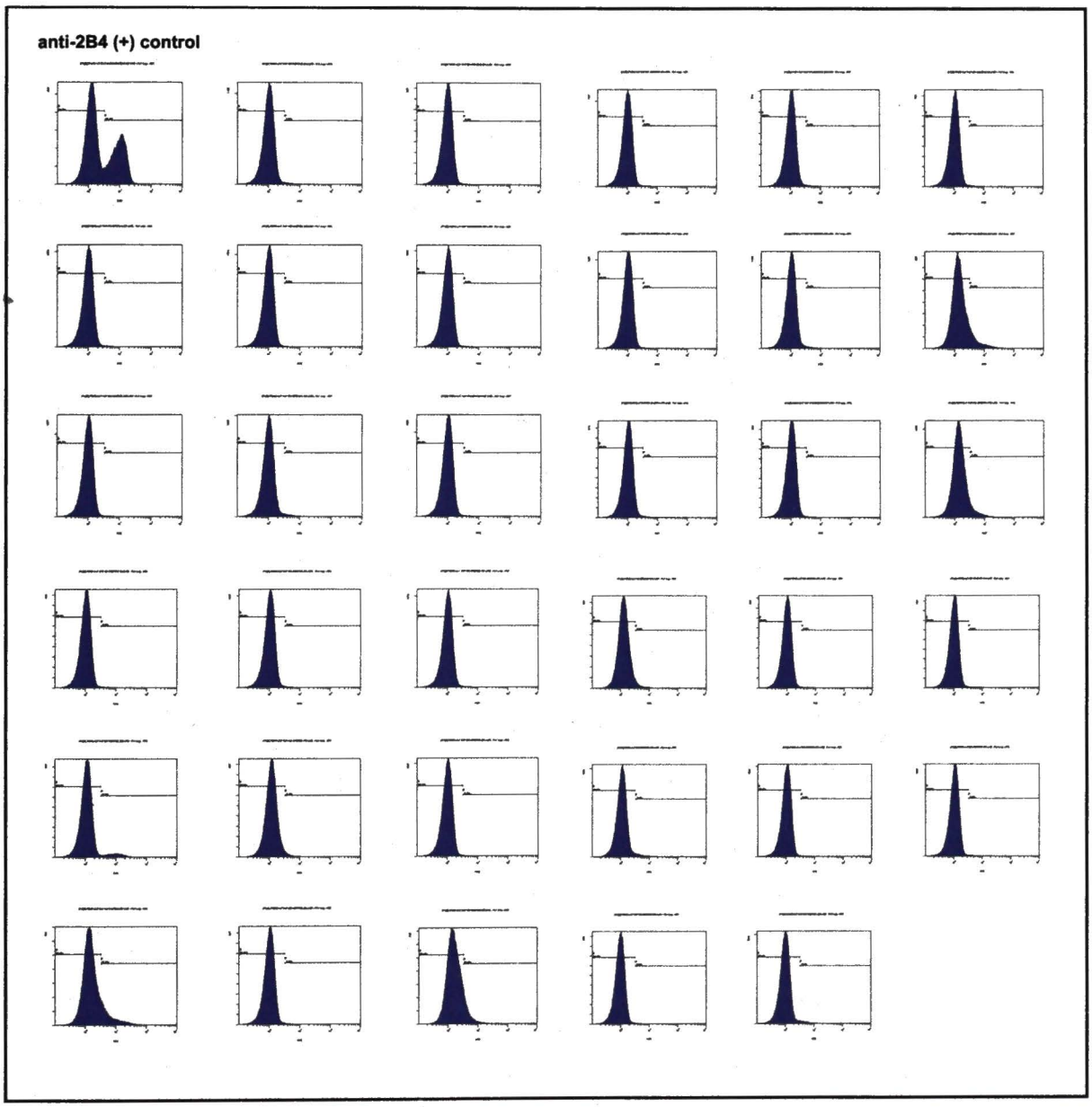
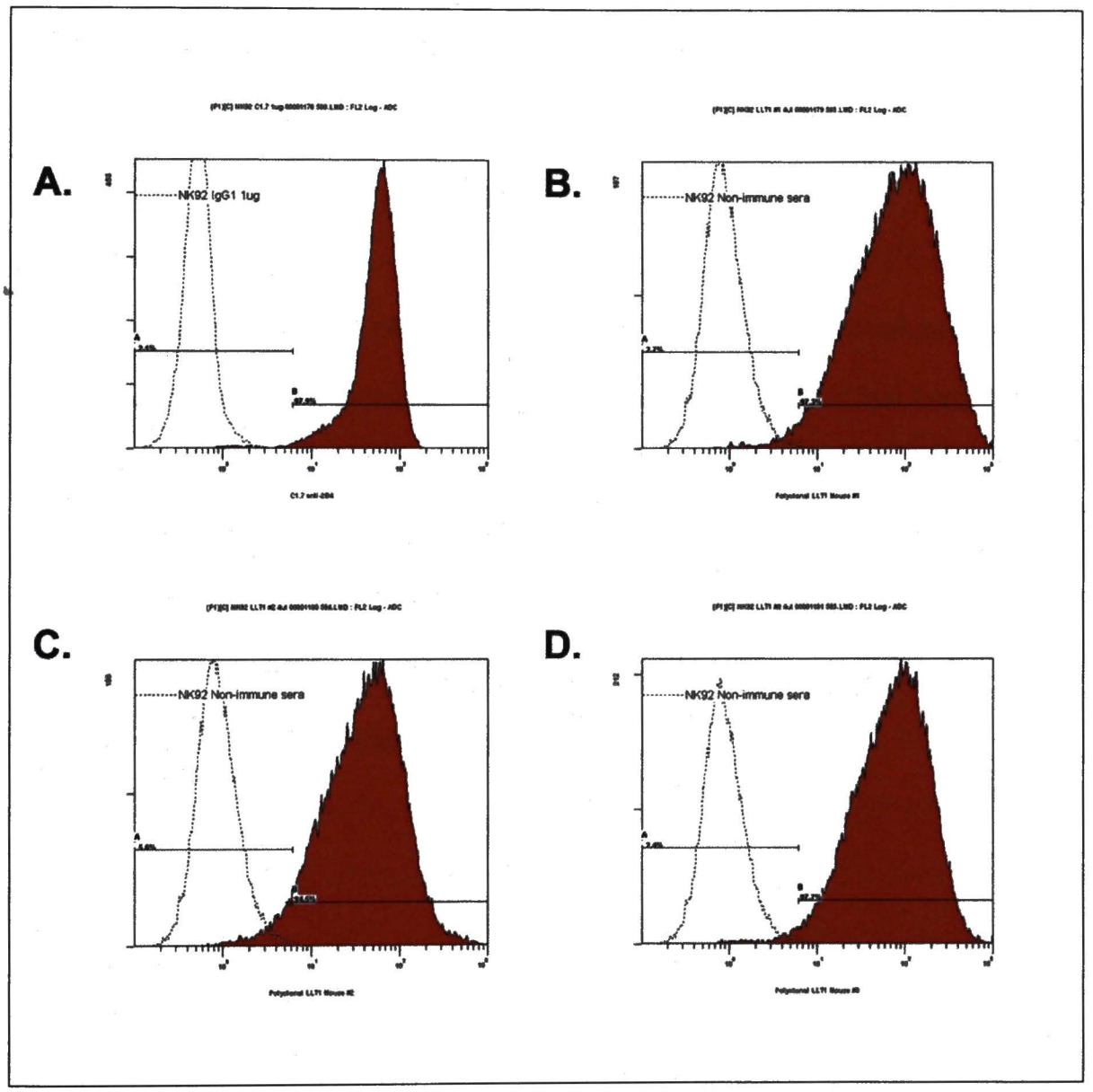

anti-2B4 (+) control

Figure 2.8. pSec-LLT1ext derived polyclonal mouse LLT1 antibody. Four (4) ul of mouse polyclonal sera from all three immunized mice were used to stain LLT1 expressing NK92 cells for flow cytometric analysis. Murine non-immunized sera served as an isotype control. Anti-h2B4 C1.7 monoclonal antibody served as a positive control. All three LLT1 immunized mice exhibited specificity for LLT1 on NK92. A. C1.7 stained cells. B. Mouse #1 anti-LLT1 sera. C. Mouse #2 anti-LLT1 sera. D. Mouse #3 anti-LLT1 sera.


A.
NK92 IgG1 1ug
B.
NK92 Non-immune sera
C.
NK92 Non-immune sera
D.
NK92 Non-immune sera

CHAPTER III

FUNCTIONAL ANALYSIS OF LLT1 ON HUMAN NK CELL LINE

SUMMARY

I evaluated the ability of the human NK cell line NK92 to function as a model of LLT1 function on activated human NK cells. To accomplish this, I first confirmed the expression of LLT1 on NK92 by flow cytometry. Secondly, I generated a CD161 expressing target cell line and used it to interact with LLT1 on the surface of NK92. This LLT1 activation is associated with IFN-G production, but not associated with altered cytotoxicity indicating that LLT1 is functional on NK92 in a manner similar to that observed on freshly isolated human NK cells.

INTRODUCTION

The human NK cell line NK92 served as an *in vitro* model for activated human NK cells. Before I could select this cell line, I needed to confirm that LLT1 is expressed on NK92. Prior to this, no group had published data indicating the presence or absence of LLT1 on the surface of NK92. Using flow cytometric analysis I was able to confirm that LLT1 is expressed on the surface of NK92. Additionally, before selecting NK92 as a functional model I needed to confirm that LLT1 on NK92 functioned in a fashion similar to that already reported on freshly isolated human NK cells and the YT cell line. To accomplish this I stably transfected K562 target cells with the LLT1 ligand CD161 and co-incubated these target cells with NK92. As predicted, surface expressed CD161 interacted with LLT1 and stimulated IFN-G secretion as our laboratory has previously observed with LLT1 stimulation by monoclonal antibody on human NK cells and YT cells. As predicted, CD161 interaction with LLT1 did not alter natural cytotoxicity as previous work with LLT1 antibody by our laboratory indicated LLT1 does not regulate NK cytotoxicity. By confirming that this NK92:K562-CD161 activation system was functional through LLT1 I was able to subsequently proceed and use this model to evaluate the signaling pathways of LLT1 associated with IFN-G production.

MATERIALS AND METHODS

Flow Cytometry:

To evaluate the expression of LLT1 on the cell surface, NK92 cells were analyzed by flow cytometry using a Beckman Coulter Cytomics FC 500 Flow Cytometer operated with CXP Analysis 2.1 software. Briefly, cells were harvested and counted by trypan blue exclusion assay. Fc receptors were blocked with 3 ug of human Fc fragment (Rockland Immunochemicals, Gilbertsville, PA) for every one million cells in PBS/1% BSA for 30 min on ice. Cells were washed with PBS/1% BSA and resuspended in wells on a 96-well, round bottom plate for staining with $1.0X10^6$ cells/well. To evaluate the presence of LLT1 on the cell surface, NK92 were resuspended in 50 ul PBS/1% BSA with 10 ug of 4C7 mouse anti-human LLT1 monoclonal antibody (Abnova, Taipei, Taiwan). Note that although this commercially available anti-LLT1 clone is capable of recognizing human LLT1 by western blot and flow cytometry, I have determined that it is not capable of inducing LLT1 associated IFN-G production. This is presumably due to the fact that 4C7 fails to recognize the region of LLT1 associated with CD161 interaction (141). After 40 min primary incubation on ice, cells were washed and resuspended with a PE conjugated goat anti-mouse IgG polyclonal secondary antibody. After 20 min incubation on ice, protected from light, the cells were washed, transferred to flow cytometry tubes and analyzed via flow cytometry. C1.7 anti-2B4 monoclonal antibody was used as a positive control reaction.

RT-PCR analysis of K562 CD161 Transcription:

Untransfected K562 cells were harvested and used to generate cDNA to analyze the lack of CD161 transcription. Cells were resuspended in 200 ul RNAStat60 (Ambion, Austin TX) mixed with chloroform and centrifuged to separate total RNA from cellular debris. Precipitated total RNA was used as RT-PCR template to generate cDNA using Qiagen Omniscript RT Kit (Qiagen, Valencia CA). CD161 PUB 75 FP 5' – CTT CCT CGG GAT GTC TGT CAG GGT TCA – 3' CD161 PUB 605 RP 5' ACA GTA CTC AGA ATA CAC AGA TGT CT – 3' Beta-actin primers were also used as a control. Beta-actin FP 5' – AAC GGC TCC GGC ATG TGC AA – 3' Beta-actin RP 5' – AGG ATC TTC ATG AGG TAG T – 3' PCR products were analyzed by electrophoresis on a 1% agarose gel with ethidium bromide and visualized by UV fluorescence. Beta-actin PCR product was approximately 500 base pairs. Correct CD161 product size was approximately 500 base pairs.

Generation of K562 and BW cell hCD161 and hLLT1 stable transfectants:

Mouse BW cells were stably transfected with pCI-neo mammalian expression vector containing cDNA encoding full length human CD161 and human LLT1. pCI-neo-LLT1 vector was previously generated by Dr. Stephen Mathew. pCI-neo-CD161 was generated as follows. Primers were designed to amplify full length CD161 cDNA previously cloned into pGEMT-easy vector from NKTRP cDNA library while inserting XhoI and XbaI restriction sites at 5' and 3' ends, respectively. NKRP1A-TA -32 XhoI FP

5' – GGC CGC GGG AAC TCG AGT CGG AAT TCG CCA CCA TGG – 3' NKRP1A-TA 704 XbaI RP 5' – CCG CGA ATT CAC TCT AGA TTC GGG ATC CTA TCA AG – 3' PCR product was cloned into pGEMT-easy vector and transformed into DH5alpha. Transformants were screened via blue/white colony method on LB plates with ampicillin/IPTG/X-gal. Several white colonies were selected and grown overnight in 5 ml LB broth with ampicillin. Plasmid DNA was isolated via mini-prep, restriction digested at XhoI and XbaI, run on 1.2% agarose gel and imaged via ethidium bromide fluorescence. Empty pCi-neo vector was prepared in the same fashion. Multiple clones exhibited a single DNA insert of ~700 base pairs, the correct size of full length CD161 amplified by PCR. Both CD161 and opened pCi-neo vector were gel extracted using Qiaquick Gel Extraction Kit (Qiagen, Valencia CA) and ligated together using T4 DNA ligase (Promega, Madison WI) (Figure 3.2). As described above, the ligation product was transformed into DH5alpha, screened on LB plates, restriction digested and analyzed via agarose gel and confirmed by sequencing. Sequence confirmed pCi-neo-CD161 was purified via CsCl maxi prep, linearized and stably transfected into mouse BW cells via electroporation using a BioRad Gene Pulser II at 300 volts, 950 microfaradays. pCi-neo-LLT1 previously generated by Dr. Stephen Mathew was also stably transfected into mouse BW cells using the same protocol. Transfected BW cells were plated on multiple 48-well tissue culture plates and grown in regular RPMI growth media containing 1000 ug/ml G418 (Mediatech, Herndon VA) for two weeks. After two weeks approximately twelve wells containing an individual cluster of viable cells were harvested and transferred to individual T25 flasks containing selective media with 400 ug/ml G418. To

confirm the presence of LLT1, whole cell RNA was isolated using RNAStat60 (Ambion, Austin TX) and analyzed via RT-PCR. The detection of PCR product of appropriate size was taken as confirmation that BW clones were stably transfected and expressing LLT1. CD161 stable transfectant surface expression was confirmed via flow cytometry using mouse anti-human CD161 (Clone DX12; BD Biosciences, San Diego, CA). BW-pCi-neo stable transfectant cells were prepared and analyzed in the same manner to serve as a control. K562 cells were stably transfected with pCi-neo-CD161 using the same protocols described above.

IFN-gamma release assay:

$2X10^5$ NK cells were co-incubated with $2X10^5$ BW or K562-CD161/-pCI-neo target cells in 1000 ul fresh alpha-MEM on a 24 well plate for 16 hours in tissue culture conditions. Cell-free supernatant was collected and IFN-gamma concentration was quantitated with a commercial ELISA kit per manufacturer's instructions (BD Biosciences, San Jose, CA). For a positive control $2X10^5$ NK cells were pre-incubated for 1 hour with 200 ng/ml C1.7 anti-2B4 antibody and subsequently incubated with untransfected K562 target cells for 16 hours. Alternatively, NK cells were incubated with 50 ng/ml PMA and 1 uM ionomycin for 5 hours as a positive control. Assays were conducted in triplicate with all proper standards and controls.

Chromium release killing assay:

K562-CD161/-pCI-neo target cells were labeled with chromium-51 by incubating one million cells with 2 MBq of $Na_2 51CrO_4$ (NEN Research Products, Boston, MA) for 90 min in standard tissue culture conditions. Labeled K562 target cells were incubated with an equal volume of NK92 cells under various conditions on a 96-well plate. After 4 hours incubation in standard tissue culture conditions the cells were pelleted at 250g for 5 min. 100 ul of supernatant was collected and radioactivity was measured. Percentage of specific lysis was calculated by the following equation: $(a\text{-}b/c\text{-}b) \times 100$, 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% Nonident P-40. In some reactions K562 target cells were pre-incubated with DX12 anti-CD161 monoclonal antibody to block CD161 interaction with LLT1 on NK92.

Statistical analysis:

Statistical analysis was conducted using one-way ANOVA with Tukey's post-hoc test using GraphPad Prism statistical software, 95% confidence interval. A P-value of 0.05 or less was considered significant.

RESULTS

NK92 LLT1 Surface Expression:

Flow cytometric analysis of NK92 with the 4C7 anti-LLT1 monoclonal antibody confirmed LLT1 is expressed on the surface of NK92 cells (Figure 3.3). Furthermore, as demonstrated in the previous chapter, polyclonal sera from three mice immunized with soluble pSec-LLT1ext were all capable of binding the surface of NK92 (Figure 2.8). Based upon these findings, I concluded that LLT1 is expressed on the surface of NK92 cells.

Generation of K562 and BW cell hCD161 stable transfectants:

To activate LLT1 in a manner that stimulates IFN-G production I generated a CD161 mammalian expression vector and stably transfected it into the CD161 negative K562 and BW cell lines. K562 was shown to be CD161 negative by our RT-PCR data (Figure 3.1). BW does not express human CD161 as it is a murine cell line. After stable transfection the stable expression of pCI-neo-CD161 was initially evaluated via resistance to G418. Surface expression of CD161 was subsequently confirmed on various BW and K562-CD161 clones via flow cytometric analysis using DX12 anti-CD161 monoclonal antibody (Figure 3.4 and Figure 3.5). Multiple clones of BW-CD161 and K562-CD161 were obtained, each exhibiting different levels of CD161 expression.

IFN-gamma release assay:

By co-incubating NK92 and K562-CD161 target cells in a one-to-one ratio CD161 was able to stimulate greater IFN-G production in NK92 compared to K562-pCI-neo transfectants and untransfected K562. I confirmed this IFN-G production was due to CD161 engagement of NK92 as blocking CD161 on K562 with DX12 anti-CD161 monoclonal antibody (142) was capable of abrogating this additional IFN-G production (Figure 3.7). Furthermore, K562-CD161 clones expressing a greater number of CD161 receptors were associated with more IFN-G production than clones expressing less CD161 (Figure 3.6). BW-CD161 targets were not capable of significant stimulation of IFN-G production when compared to BW-pCI-neo target cells (not shown). Based upon these results I subsequently proceeded to use the NK92:K562-CD161 co-incubation system to analyze LLT1 stimulation in NK cells.

Chromium release killing assay:

Under no conditions was an altered level of natural cytotoxicity observed between CD161 expressing or lacking target cells (Figure 3.8). At all ratios of effector to target cells approximately the same level of target cells lysis was observed. Blocking CD161 with DX12 anti-CD161 monoclonal antibody resulted in no significant change in observed cytotoxicity. These results are consistent with previously published data indicating that LLT1 activation does not alter NK cell cytotoxicity (117).

Figure 3.1 PCR analysis of K562 cDNA. Amplification of K562 cDNA with primers specific for human CD161 failed to produce specific bands of correct size, confirming K562 does not transcribe CD161. Beta-actin primers served as positive control and produced correct size ~500 base pair bands. Lane 1 is beta-actin primers, lane 2 is no primer (PCR negative control), lane 3 is CD161 primers.

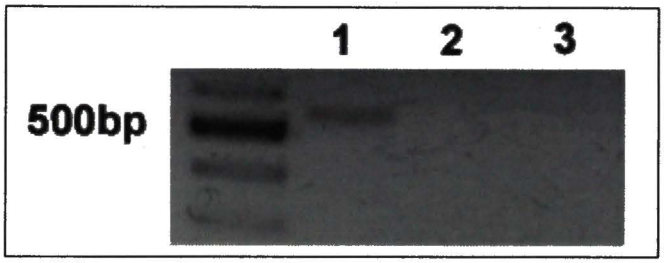

1
2
3
500bp

Figure 3.2 pCi-neo-CD161 Vector Map. The full length cDNA of human CD161 is 740 base pairs long. It was ligated into the Promega pCI-neo mammalian expression vector at the XhoI and XbaI restriction endonuclease cut sites.

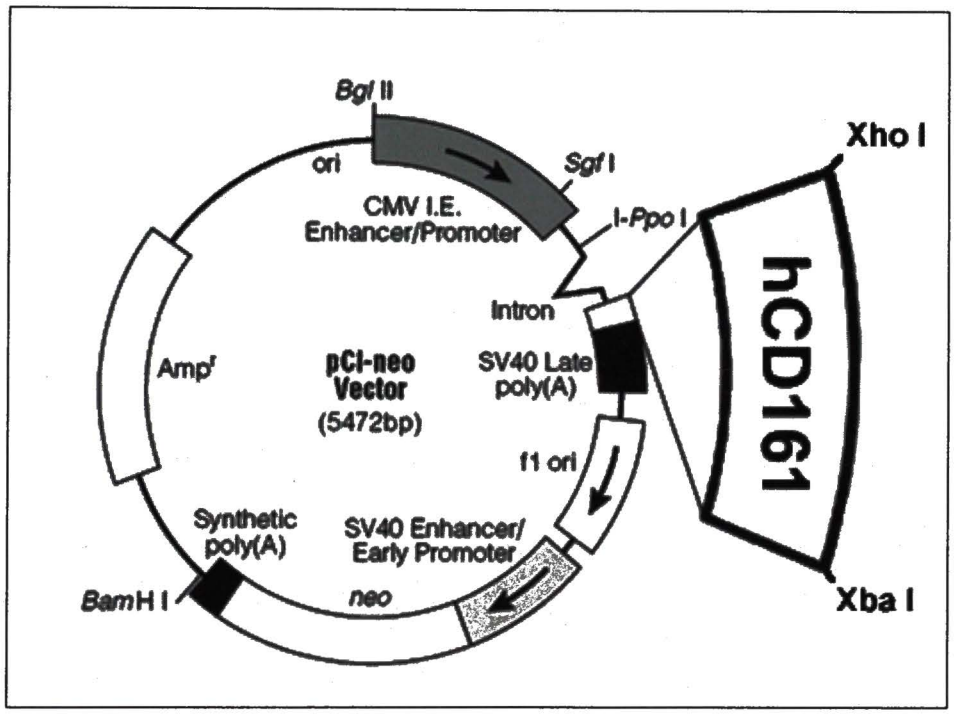

Bgl II
ori
Sgf I
CMV I.E.
Enhancer/Promoter
I-Ppo I
Xho I
hCD161
Intron
Amp^r
pCI-neo
Vector
(5472bp)
SV40 Late
poly(A)
f1 ori
Synthetic
poly(A)
SV40 Enhancer/
Early Promoter
BamH I
neo
Xba I

Figure 3.3 LLT1 expression on NK92. NK92 cells were analyzed via flow cytometry for surface expression of LLT1. 4C7 anti-LLT1 monoclonal antibody was used to detect LLT. A non-specific primary antibody of the same mouse isotype was used as a control. 4C7 anti-LLT1 exhibited a greater MFI than the control, indicating LLT1 is present on the cell surface.

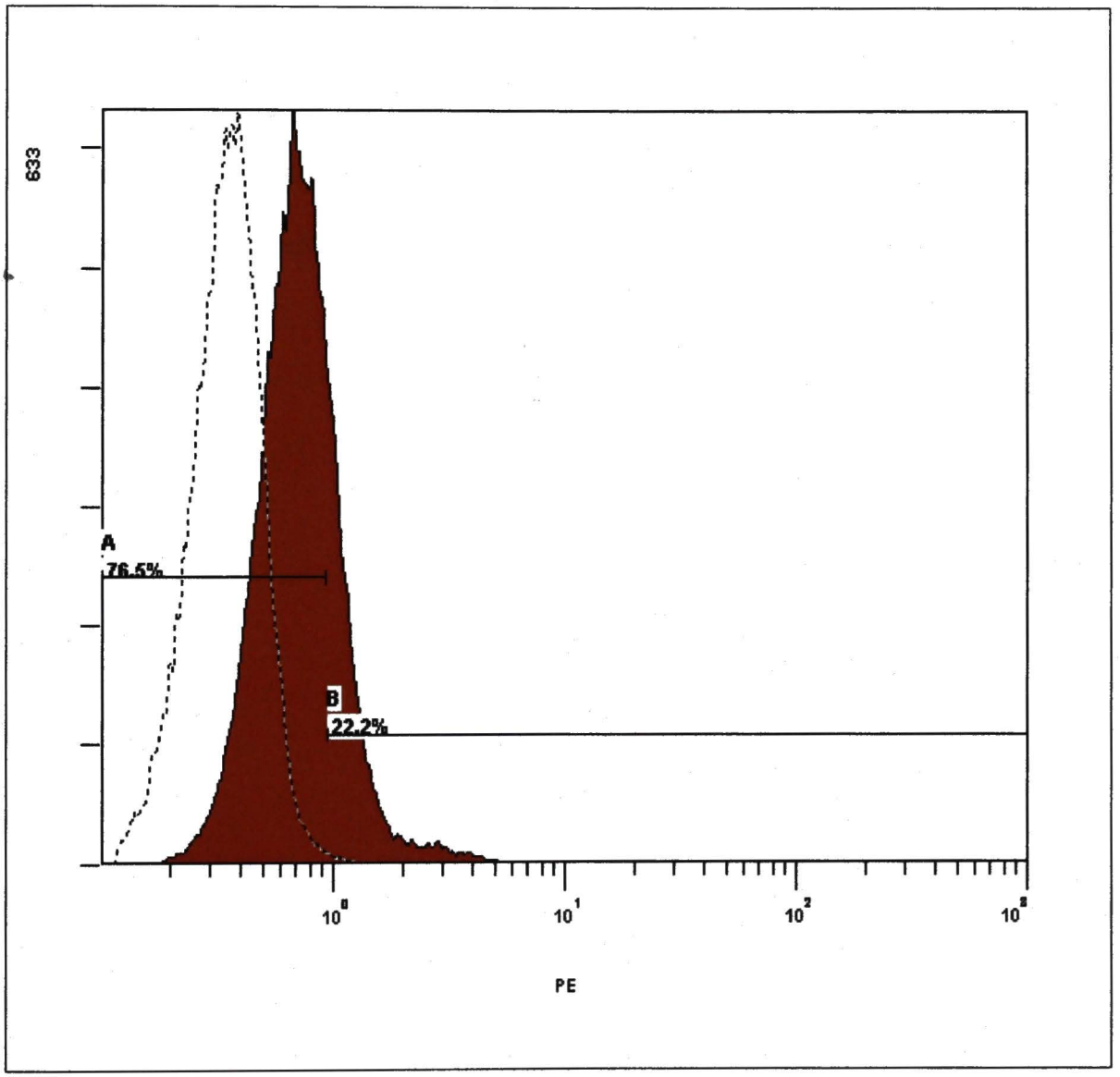

633
A
76.5%
B
22.2%
10^0
10^1
10^2
10^3
PE

Figure 3.4 BW-CD161 stable transfectants. G418 resistant clones of BW cells electroporated with pCI-neo-CD161 vector were analyzed by flow cytometry for human CD161 surface expression. DX12 anti-hCD161 monoclonal antibody was used to detect CD161. A non-specific primary antibody of the same mouse isotype was used as a control. DX12 antibody was associated with a greater MFI on multiple BW clones indicating CD161 is expressed on the surface of these cells. BW cells transfected with empty pCI-neo vector exhibited no MFI increase.

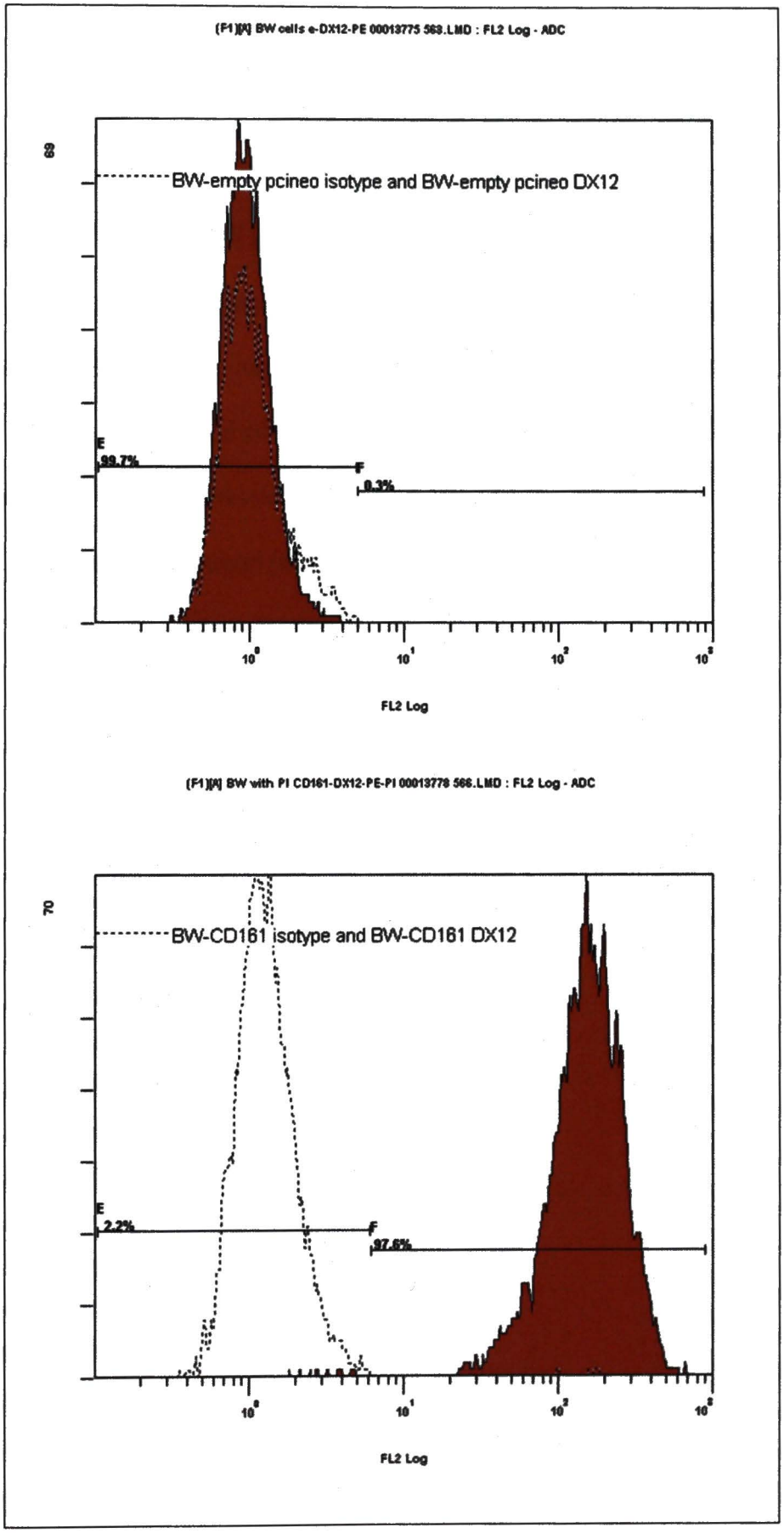

(F1)[A] BW cells e-DX12-PE 00013775 563.LMD : FL2 Log - ADC
BW-empty pcineo isotype and BW-empty pcineo DX12
E
99.7%
F
0.3%
FL2 Log
(F1)[A] BW with PI CD161-DX12-PE-PI 00013778 566.LMD : FL2 Log - ADC
BW-CD161 isotype and BW-CD161 DX12
E
2.2%
F
97.6%
FL2 Log

Figure 3.5 K562-CD161 stable transfectants. G418 resistant clones of K562 cells electroporated with pCI-neo-CD161 vector were analyzed by flow cytometry for human CD161 surface expression. DX12 anti-hCD161 monoclonal antibody was used to detect CD161. A non-specific primary antibody of the same mouse isotype was used as a control. DX12 antibody was associated with a greater MFI on multiple K562 clones indicating CD161 is expressed on the surface of these cells. Note that clone #12 exhibits a greater level of CD161 expression than clone #3. K562 transfected with empty pCI-neo vector exhibited no MFI increase (not shown).

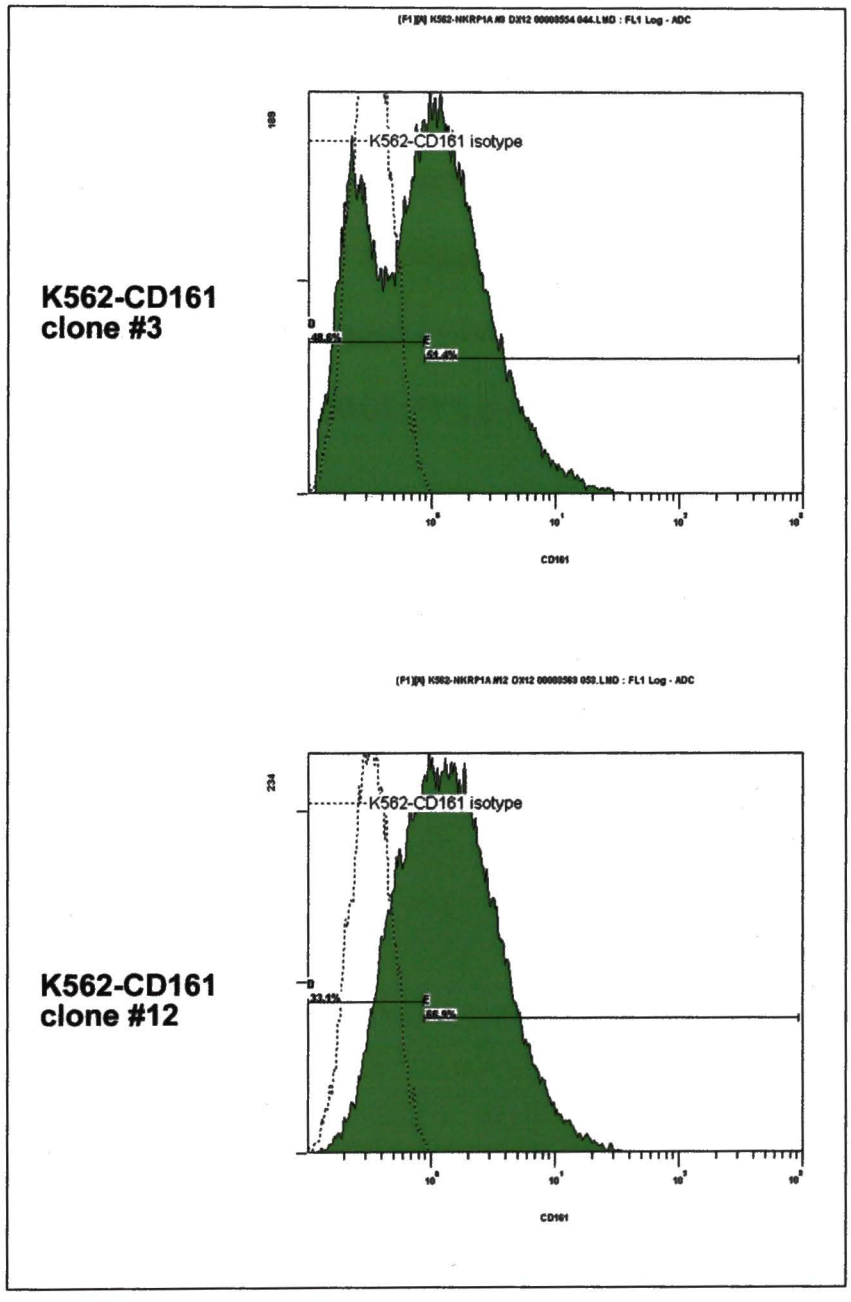

K562-CD161 clone #3
K562-CD161 isotype
CD161
K562-CD161 clone #12
K562-CD161 isotype
CD161

Figure 3.6 NK92:K562-CD161 co-incubation. NK92 were incubated with an equal number of K562-CD161/-pCI-neo target cells overnight to evaluate IFN-G production. Both K562-CD161 clones were associated with greater IFN-G production than K562-empty pCI-neo. Note that the clone previously shown to express a higher level of CD161, clone #12, was associated with a statistically greater level of IFN-G production than clone #3. Clone #12 was used for all subsequent K562-CD161 assays. Reactions were conducted in triplicate. Statistics analyzed by one-way ANOVA with Tukey's post-hoc test, 95% confidence interval. **, $p < 0.01$; ***, $p < 0.001$

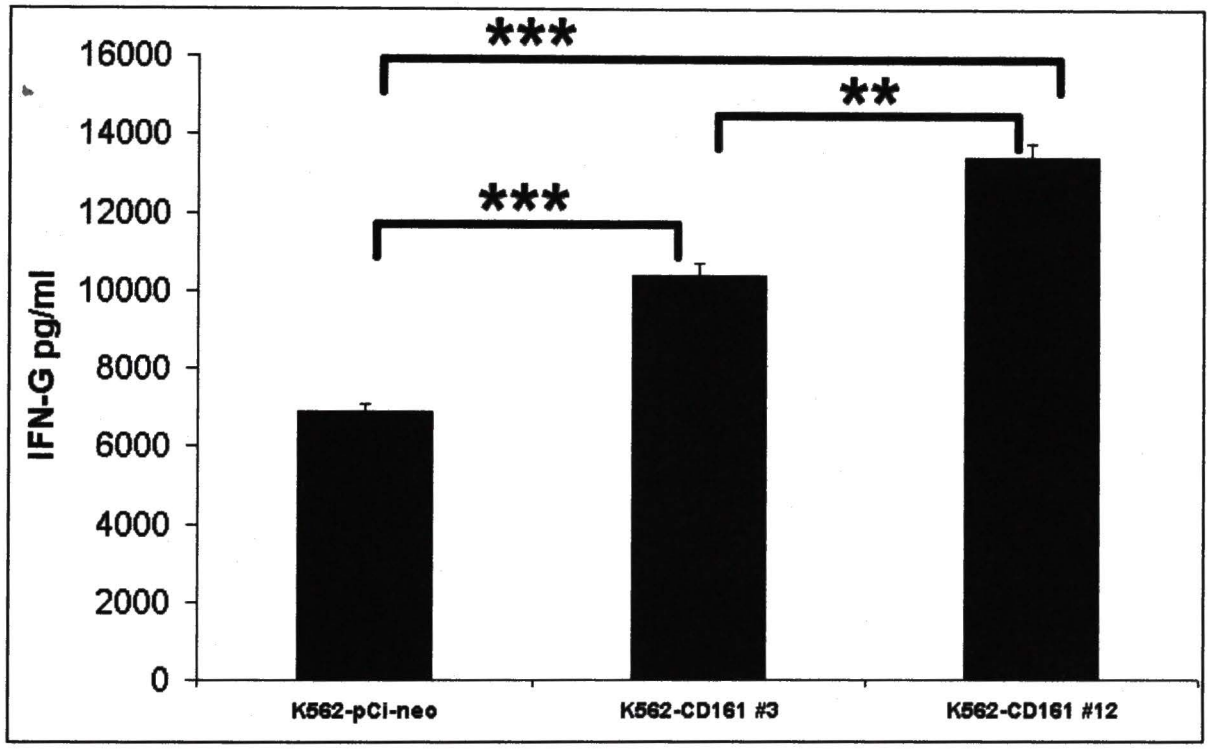

***
**
***
16000
14000
12000
10000
8000
6000
4000
2000
0
IFN-G pg/ml
K562-pCI-neo
K562-CD161 #3
K562-CD161 #12

Figure 3.7 NK92:K562-CD161 with DX12 blocking. To evaluate if the IFN-G production associated with K562-CD161 was due to the presence of CD161, NK92 were incubated with K562 target cells that had been pre-incubated with DX12 anti-CD161 monoclonal antibody. Pre-incubating K562-CD161 with DX12 resulted in a significant reduction of IFN-G production. This is presumably due to DX12 binding CD161 and preventing its physical interaction with its ligand on the surface of NK92. No statistically significant reduction in IFN-G production was associated with pre-incubating K562-pCI-neo cells with DX12, as was expected. Q-values are given to illustrate relative levels of significance between various reactions. Reactions were conducted in triplicate. Statistics analyzed by one-way ANOVA with Tukey's post-hoc test, 95% confidence interval. ***, $p < 0.001$

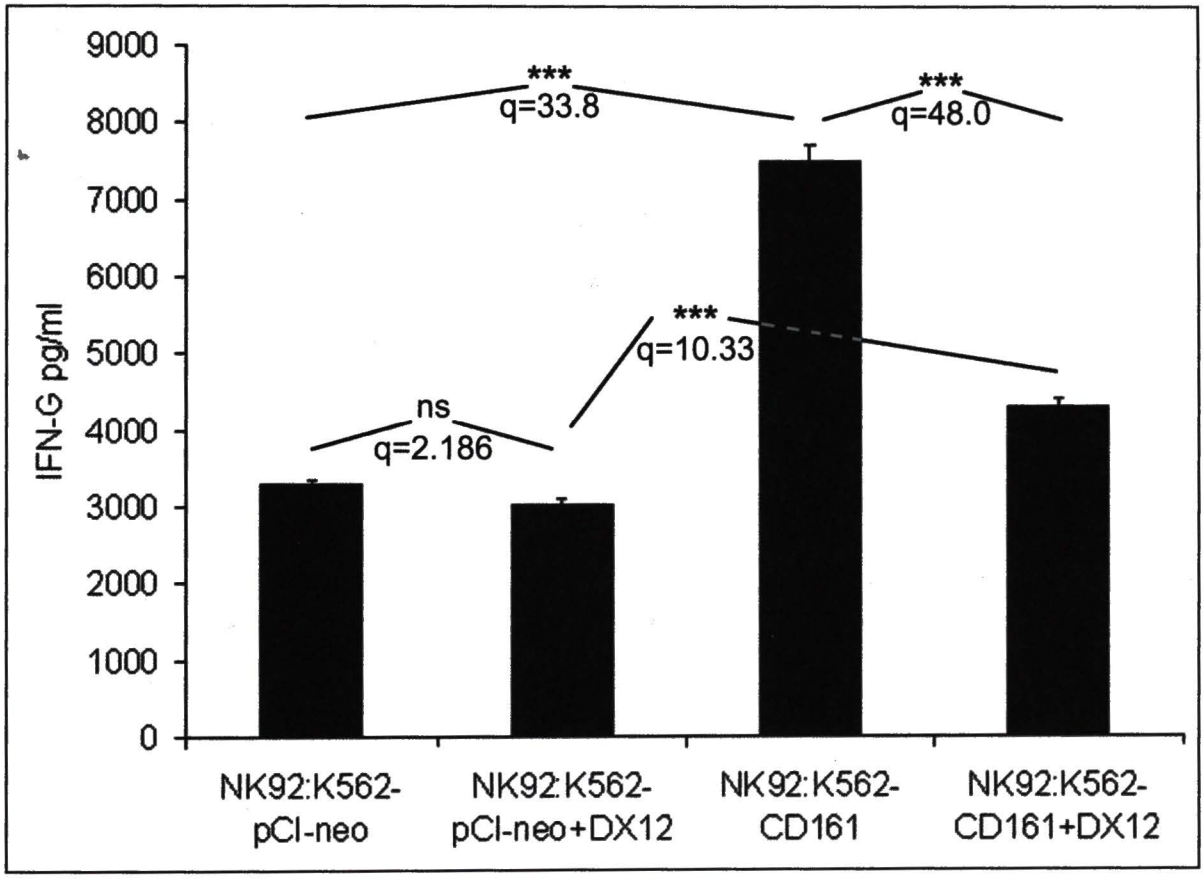
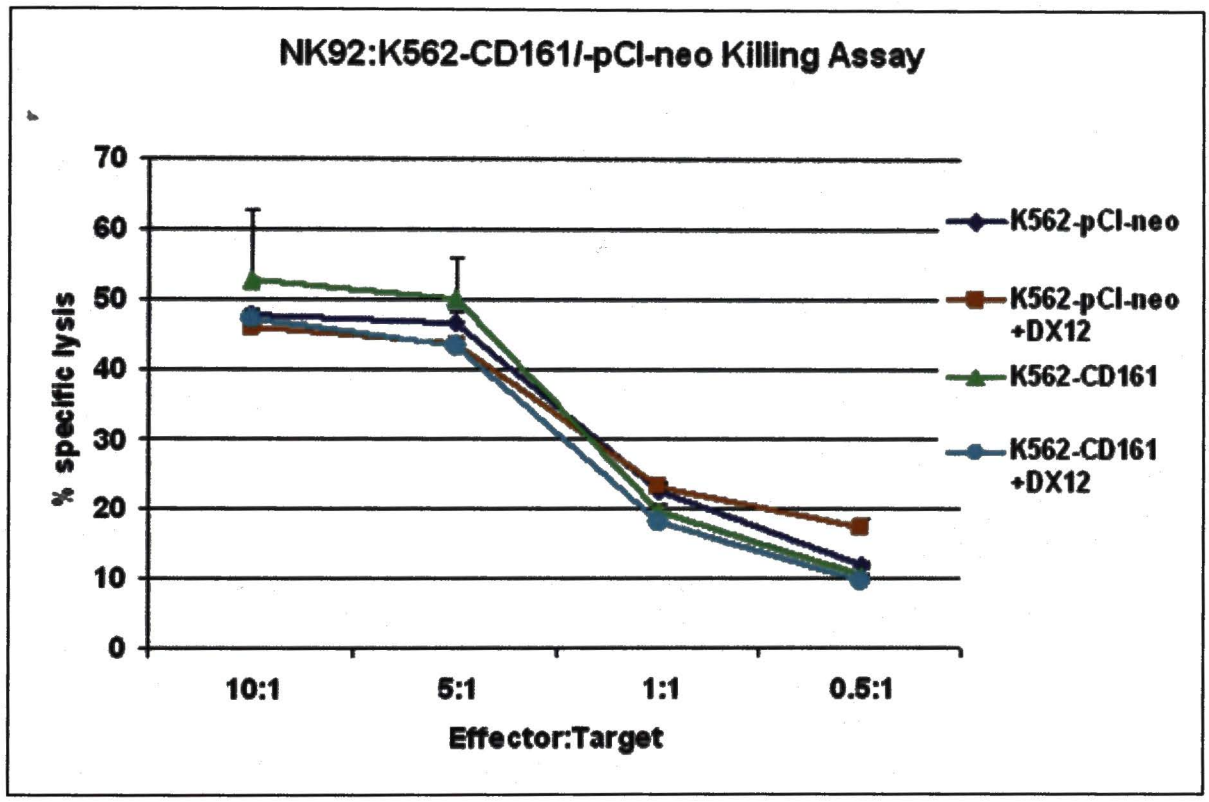

9000
8000
7000
6000
5000
4000
3000
2000
1000
0
IFN-G pg/ml
***
q=33.8
***
q=48.0
***
q=10.33
ns
q=2.186
NK92:K562-pCI-neo
NK92:K562-pCI-neo+DX12
NK92:K562-CD161
NK92:K562-CD161+DX12

Figure 3.8 LLT1 interaction with CD161 does not alter cytotoxicity. I performed cytotoxicity assays to determine whether interaction of LLT1 with CD161 plays any functional role in NK cell activation. NK92 cells were used as effectors against chromium labeled K562-CD161/-pCI-neo target cells. In some reactions, K562 target cells were blocked with DX12 anti-CD161 monoclonal antibody. K562-CD161 target cells were not associated with altered levels of killing compared to K562-pCI-neo targets and blocking CD161 was not associated with any altered levels of killing. These results suggest that LLT1 activation by CD161 does not regulate NK cell cytotoxicity. Reactions were conducted in triplicate.


NK92:K562-CD161/-pCI-neo Killing Assay
% specific lysis
70
60
50
40
30
20
10
0
10:1
5:1
1:1
0.5:1
Effector:Target
K562-pCI-neo
K562-pCI-neo +DX12
K562-CD161
K562-CD161 +DX12

CHAPTER IV

SIGNALING MECHANISMS OF LLT1 ON HUMAN NK CELL LINE

SUMMARY

I evaluated the signaling mechanisms of LLT1 using the NK92:K562-CD161 activation system already established. NK92 effectors and K562 targets were co-incubated in the presence and absence of various pharmacological inhibitors specific for various signaling pathways. IFN-G production was evaluated and a decrease in IFN-G production in the presence of a specific inhibitor was taken as evidence that the inhibited pathway is associated with LLT1 signaling. My data indicate that the ERK and p38 signaling pathways are associated with LLT1 activation. Additionally, time point analysis indicates LLT1 associated IFN-G secretion predominantly occurs after at least six hours of LLT1 stimulation. Furthermore, RT-PCR analysis indicates that LLT1 activation is not associated with an increase or decrease in IFN-G mRNA expression in NK cells.

INTRODUCTION

The signaling pathway mechanisms employed by LLT1 have been elucidated by a series of LLT1 activation reactions employing surface expressed CD161 on various target cells and the concomitant inhibition of individual signaling pathways using various pharmacological inhibitors. Surface expressed CD161 provides an *in vitro* mechanism for LLT1 activation that is identical to natural conditions of stimulation. The human myeloid leukemia cell line K562 has been stably transfected with a mammalian expression vector containing full-length human CD161 cDNA and I have confirmed its ability to stimulate IFN-G production in NK92 (Figure 3.6 and Figure 3.7). Using these same conditions I co-incubated K562-CD161 and NK92 cells in the presence or absence of various pharmacological inhibitors. These inhibitors specifically block known signaling pathways and by analyzing alterations in IFN-gamma production in the presence and absence of these inhibitors the pathways employed by LLT1 have been suggested. I have subsequently corroborated the results obtained by analyzing the phosphorylation status of the associated signaling proteins.

The production of IFN-G was also analyzed over various time points to evaluate when the bulk of IFN-G associated with LLT1 stimulation is secreted. Furthermore, the presence of IFN-G mRNA at various time points was analyzed to evaluate any possible alterations in transcription associated with LLT1 stimulation.

MATERIALS AND METHODS

Tissue culture:

Cell lines were handled in sterile, bio-safety level 2 conditions using a positive pressure laminar flow tissue culture hood. NK92 cells were maintained using alpha-MEM (Hyclone, Logan, UT) with 25% defined Fetal Bovine Serum (Hyclone, Hyclone, Logan, UT) and where appropriate 30 U/ml recombinant human IL-2 (Calbiochem, La Jolla, CA). All other cells were maintained using 4+RPMI 1640 (GibcoBRL, Grand Island, NY; with 10 mM MEM non-essential amino acids, 10 mM HEPES, 100 mM Sodium Pyruvate, 2 mM glutamine and penicillin/streptomycin) with 10% FetalPlex Animal Serum Complex (Gemini Bio-Products, Sacramento CA) at 37c, 5% CO2 in a water jacketed tissue culture CO2 incubator.

Pharmacological inhibitor incubations:

NK92 cells were pre-incubated with functional concentrations of various pharmacological inhibitors for an appropriate period of time prior to initiation of IFN-gamma release assay. Inhibitors, targeted pathways and concentrations are detailed in Table 4.1. A general overview of NK activating signaling pathways with pharmacological inhibitor targets is provided in Figure 4.1. Inhibitors were dissolved in DMSO. To control for this, NK92 cells not incubated with inhibitors were incubated with an equal amount of DMSO. To minimize the effects of DMSO, inhibitors were pre-mixed

into a volume of media to which cells were subsequently added and the volume of DMSO added never exceeded 0.5% of the total media volume.

IFN-gamma release assay:

$2X10^5$ NK cells were co-incubated with $2X10^5$ K562-CD161/-pCI-neo target cells in 1000 ul fresh alpha-MEM on a 24 well plate for 16 hours in tissue culture conditions. Cell-free supernatant was collected and IFN-gamma concentration was quantitated with a commercial ELISA kit per manufacturer's instructions (BD Biosciences, San Jose, CA). For a positive control $2X10^5$ NK cells were pre-incubated for 1 hour with 200 ng/ml C1.7 anti-2B4 antibody and subsequently incubated with untransfected K562 target cells for 16 hours. Alternatively, NK cells were incubated with 50 ng/ml PMA and 1uM ionomycin for 5 hours as a positive control. Assays were conducted in triplicate with all proper standards and controls.

RT-PCR analysis of IFN-G mRNA levels:

NK92 effector cells and K562 target cells from some IFN-G release assays were retained and used to generate cDNA to analyze IFN-G mRNA levels. Cells were resuspended in 200 ul RNAStat60 (Ambion, Austin TX) mixed with chloroform and centrifuged to separate total RNA from cellular debris. Precipitated total RNA was used as a template to generate cDNA using Qiagen Omniscript RT Kit (Qiagen, Valencia CA). cDNA was analyzed by PCR for IFN-G expression. GAPDH primers were also used as a control. hIFN-G 109 FP 5' - ATG AAA TAT ACA AGT TAT ATC TTG GCT TT - 3'

(143) hIFN-G 474 RP 5' - CGA ATA ATT AGT CAG CTT TTC GAA G - 3' (144) GAPDH FP 5' - ATG ACA TCA AGA AGG TGG TG – 3' GAPDH RP 5' - CAT ACC AGG AAA TGA GCT TG – 3' PCR products were analyzed by electrophoresis on a 1% agarose gel with ethidium bromide and visualized by UV fluorescence. IFN-G PCR product is approximately 370 base pairs. GAPDH PCR product is approximately 177 base pairs.

Statistical analysis:

Statistical analysis was conducted using one-way ANOVA with Tukey's post-hoc test using GraphPad Prism statistical software, 95% confidence interval. A P-value of 0.05 or less was considered significant.

Table 4.1 Inhibitors, targets, IC50s and working concentrations. IC50s are per manufacturer's data sheet. Working concentrations were selected based upon concentrations published as having been effective when used upon NK cells or PBMCs.

INHIBITOR	TARGET	IC50	CONCENTRATION	SOURCE
Actinomycin D	RNA polymerase		20 ug/ml	(82)
SB203580	p38	600 nM	10-50 uM	(82, 145)
PD98059	MAPK kinase 1 (MEK1)	2 uM	50-100 uM	(82, 145)
Ascomycin	calcineurin	10 nM	10 nM	(146)
PP2	Src-PTK	100 nM	10 uM	(147)
LY294002	PI3K	1.4 uM	25 uM	(148)
Bisindoylmaleimide I	PKC	10 nM	1 uM	(149)

RESULTS

IFN-G production with pharmacological inhibitors:

As expected, inhibition of all cellular transcription using actinomycin D completely abrogated detectable IFN-G production (Figure 4.2). This may be due to the inhibition of transcription of IFN-G, or of various other gene products required for IFN-G secretion or of both. Inhibition of Src-PTK with PP2 significantly reduced IFN-G production (Figure 4.2). This was expected as Src-PTK acts to phosphorylate ITAMs on the accessory proteins associated with NK activating receptors, one of which LLT1 is likely to associate with (125). Inhibition of PI3K using LY294002 failed to reduce IFN-G production, though a slightly significant increase in IFN-G production was observed in the presence and absence of LLT1 activation (Figure 4.3). Inhibition of the PKC pathway using bisindoylmaleimide I failed to significantly reduce IFN-G production compared to the same reaction incubated with DMSO alone (Figure 4.4). Based upon published data I would expect to observe a 50% reduction in IFN-G production with 1nM ascomycin, and maximal reduction with ascomycin concentrations of 5 nM and greater if calcineurin/NF-AT were associated with LLT1 stimulated IFN-G production (146). At 1nM ascomycin, no statistically significant reduction in IFN-G production was noted (Figure 4.4). However, at 10 nM ascomycin a slightly significant reduction in IFN-G production was observed. Based upon these results, I cannot entirely rule out the possibility that calcineurin/NF-AT is associated with LLT1 signaling. However, based upon the more

significant inhibition results associated with p38 and MEK/ERK I chose to focus on these pathways for further study. When I inhibited the p38 MAPK pathway using SB203580 IFN-G production was reduced to approximately the same level associated with DMSO alone (Figure 4.5). This was also observed when the MEK1 pathway was inhibited using PD98059 (Figure 4.6). Furthermore, when CD161 was blocked with DX12 antibody in combination with these inhibitors the detected amount of IFN-G fell below the level associated with DMSO alone (Figures 4.5 and 4.6). It was this fact that suggested that both the p38 and MEK/ERK pathways may be associated with CD161 induced IFN-G production. When p38 and MEK1 were simultaneously inhibited detectable IFN-G fell to almost zero (Figure 4.7).

IFN-G production over various time points:

IFN-G production was assayed at time points from 1 to 18 hours to determine when LLT1 stimulated IFN-G production becomes apparent. As Figure 4.8 clearly demonstrates the increase in IFN-G production associated with LLT1 activation becomes most apparent between 6 and 12 hours after co-incubation begins. This suggests that IFN-G secretion associated with LLT1 stimulation is not associated with release of pre-generated IFN-G but with *de novo* IFN-G production by NK cells.

PCR analysis of IFN-G mRNA levels:

NK92:K562 cells used for IFN-G timepoint analysis were retained and total mRNA was harvested. Levels of IFN-G mRNA were analyzed using RT-PCR. GAPDH

was amplified to confirm an equal amount of template was used from one reaction to the next. As shown in Figure 4.9, we were unable to detect any significant difference in IFN-G mRNA over the various time points in either the presence or absence of LLT1 stimulation by CD161. IFN-G mRNA detected came from NK92 as K562 does not produce IFN-G. This was confirmed by analyzing K562 cDNA alone for IFN-G mRNA (data not shown).

Figure 4.1 Overview of NK cell signaling pathways. An overview of possible signaling pathways associated with NK activating receptors such as LLT1 and their associated ITAM containing accessory proteins. Pharmacological inhibitors employed in this study are noted in red with their target proteins. Data adapted from MacFarlane et al. (125).

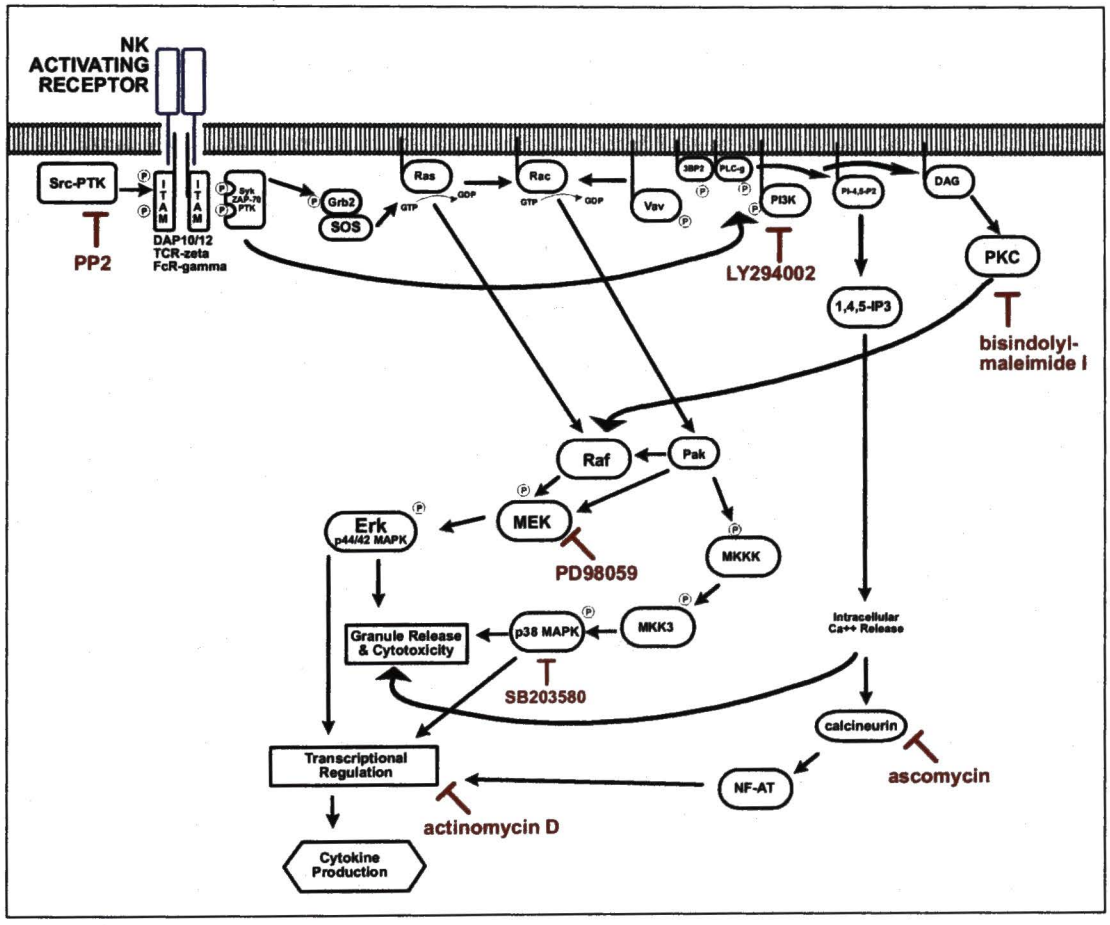

NK
ACTIVATING
RECEPTOR
Src-PTK
PP2
ITAM
ITAM
DAP10/12
TCR-zeta
FcR-gamma
Syk
ZAP-70
PTK
Grb2
SOS
Ras
GTP
GDP
Rac
GTP
GDP
Vav
3BP2
PLC-g
PI3K
LY294002
PI-4,5-P2
DAG
PKC
bisindolyl-
maleimide I
1,4,5-IP3
Raf
Pak
Erk
p44/42 MAPK
MEK
PD98059
MKKK
MKK3
p38 MAPK
SB203580
Granule Release
& Cytotoxicity
Intracellular
Ca++ Release
calcineurin
ascomycin
NF-AT
Transcriptional
Regulation
actinomycin D
Cytokine
Production

Figure 4.2 Inhibition of Src-PTK and RNA polymerase inhibits LLT1 associated IFN-G production. To evaluate signaling pathways employed by LLT1, NK92 was co-incubated with K562-CD161 target cells in presence of various pharmacological inhibitors and IFN-G production was measured. Inhibition of transcription with 20 ug/ml actinomycin D and Src-PTK with 10 uM PP2 was associated with complete abrogation of IFN-G production. These results indicate that LLT1 dependent IFN-G production requires Src-PTK and RNA polymerase. As a control, NK92 were co-incubated with K562 and anti-2B4 monoclonal antibody C1.7 or isotype control antibody and IFN-G production was measured. The level of IFN-G production associated with LLT1 activation was similar to that observed upon activation of 2B4 with a monoclonal antibody. Values normalized as percent change from control without inhibitor. Reactions were conducted in triplicate. Statistics analyzed by one-way ANOVA with Tukey's post-hoc test, 95% confidence interval. ***, $p < 0.001$

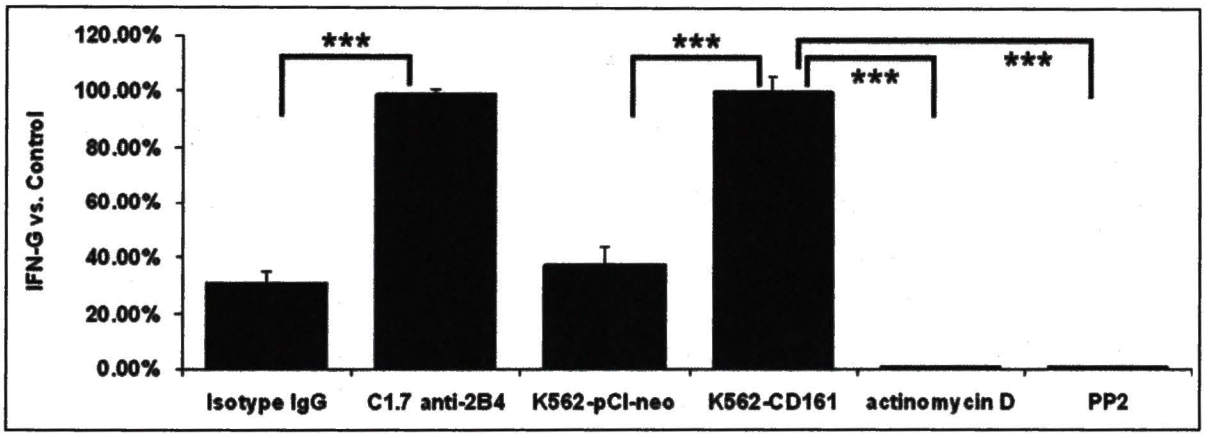

IFN-G vs. Control
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
***
***
***
***
Isotype IgG
C1.7 anti-2B4
K562-pCI-neo
K562-CD161
actinomycin D
PP2

Figure 4.3 Inhibition of PI3K with LY294002. To evaluate the role of the PI3K signaling pathways in LLT1 signaling, NK92 was co-incubated with K562 target cells in presence of 25 uM LY294002 and IFN-G production was measured. No significant decrease in IFN-G production was observed upon inhibition of PI3K with 25 uM LY294002, although a statistically significant increase in IFN-G production was observed (q=5.205). Inhibition of PI3K in the absence of LLT1 stimulation was also associated with a slight increase in IFN-G production, though this was not statistically significant (q=1.396). These results suggest that while PI3K may affect IFN-G production, it likely is not playing a major role in LLT1 stimulated IFN-G production. Q-values are given to illustrate relative levels of significance between various reactions. Reactions were conducted in triplicate. Statistics analyzed by one-way ANOVA with Tukey's post-hoc test, 95% confidence interval. *, $p < 0.05$; ***, $p < 0.001$

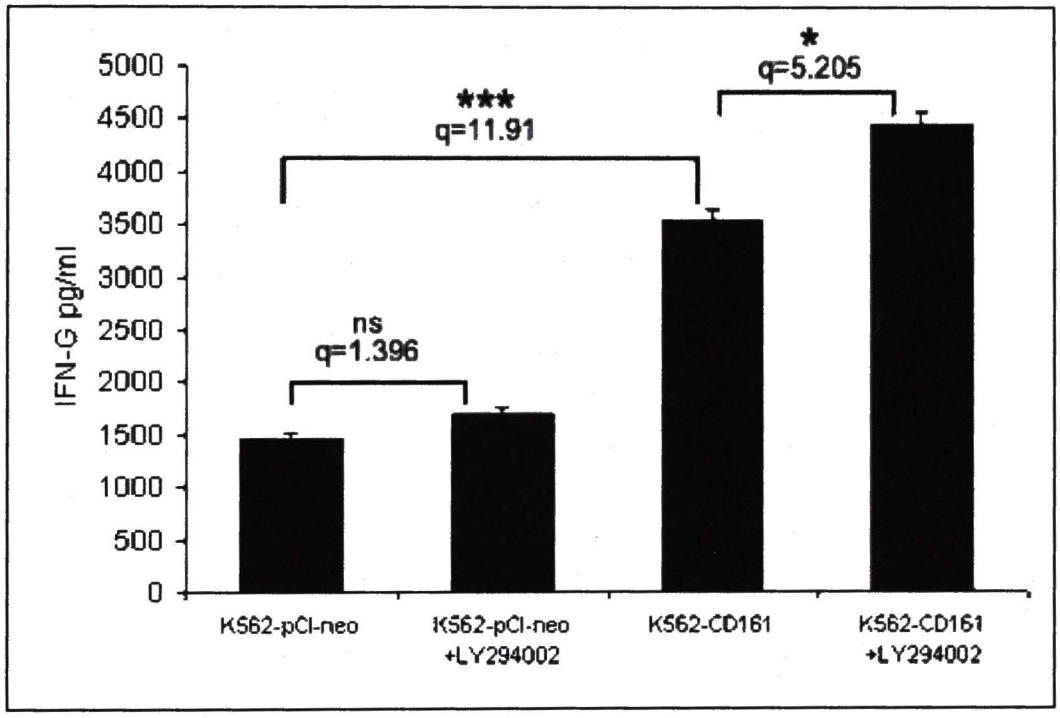

IFN-G pg/ml
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
***
q=11.91
*
q=5.205
ns
q=1.396
KS62-pCI-neo
KS62-pCI-neo +LY294002
KS62-CD161
KS62-CD161 +LY294002

Figure 4.4 Inhibition of PKC and calcineurin/NF-AT pathways. To evaluate the role of the PKC and calcineurin/NF-AT signaling pathways in LLT1 signaling, NK92 was co-incubated with K562 target cells in the presence of bisindoylmaleimide I (Figure A) or ascomycin (Figure B) and IFN-G production was measured. No significant decrease in IFN-G production was observed upon inhibition of PKC with 1 uM bisindoylmaleimide I, suggesting that PKC is not playing a significant role in LLT1 mediated IFN-G production. Calcineurin/NF-AT activity was inhibited using 1 nM and 10 nM ascomycin, concentrations as these have been reported as producing half-maximal and maximal levels of IFN-G production in PBMCs, respectively (146). 1 nM ascomycin was not associated with any significant decrease in LLT1 associated IFN-G production (q=4.955), whereas 10 nM ascomycin was associated with a statistically significant decrease in LLT1 associated IFN-G production (q=13.57). However, 10 nM ascomycin was also associated with a similar decrease in IFN-G production resulting from non-LLT1 stimulating K562-pCI-neo target cells (q=16.99). These results suggest that the calcineurin/NF-AT pathway plays a role in non-LLT1 associated IFN-G production related to NK92:K562-pCI-neo interaction. The current data does not enable us to rule out the possibility that the calcineurin/NF-AT pathway is associated with LLT1 dependent IFN-G production. Q-values are given to illustrate relative levels of significance between various reactions. Reactions were conducted in triplicate. Statistics analyzed by one-way ANOVA with Tukey's post-hoc test, 95% confidence interval.

***, $p < 0.001$

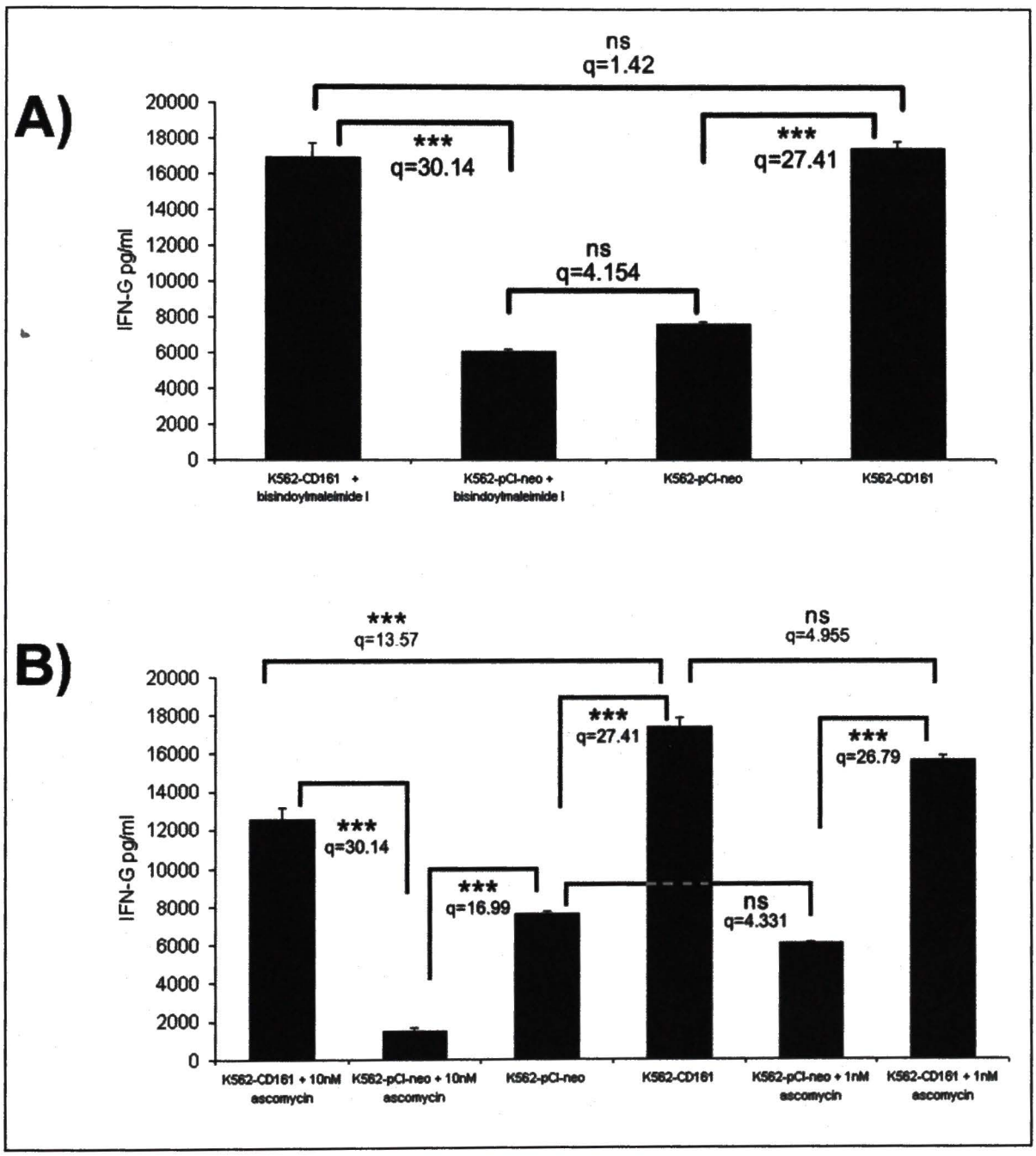

A)
ns
q=1.42
***
q=30.14
***
q=27.41
ns
q=4.154
IFN-G pg/ml
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
K562-CD161 + bisindoylmaleimide I
K562-pCI-neo + bisindoylmaleimide I
K562-pCI-neo
K562-CD161
B)
***
q=13.57
ns
q=4.955
***
q=27.41
***
q=26.79
***
q=30.14
***
q=16.99
ns
q=4.331
IFN-G pg/ml
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
K562-CD161 + 10nM ascomycin
K562-pCI-neo + 10nM ascomycin
K562-pCI-neo
K562-CD161
K562-pCI-neo + 1nM ascomycin
K562-CD161 + 1nM ascomycin

Figure 4.5 Inhibition of p38 with concomitant blocking of CD161. NK92 was co-incubated with K562-CD161 target cells in presence of 50 uM SB203580 and IFN-G production was measured. Inhibition of p38 MAPK with 50 uM SB203580 significantly reduced IFN-G production to levels comparable to that associated with lack of LLT1 activation (Figure A). K562-CD161 target cells were blocked with DX12 anti-CD161 antibody and subsequently incubated with NK92 effector cells that had been pre-incubated with SB203580 p38 inhibitor. This resulted in an even further reduction of IFN-G production than either method employed singly (Figure B). Together these data suggest that while p38 is associated with LLT1 stimulated IFN-G production its inhibition alone was not capable of fully eliminating IFN-G production and that additional pathways may be in use. Q-values are given to illustrate relative levels of significance between various reactions. Reactions were conducted in triplicate. Statistics analyzed by one-way ANOVA with Tukey's post-hoc test, 95% confidence interval.

***, $p < 0.001$

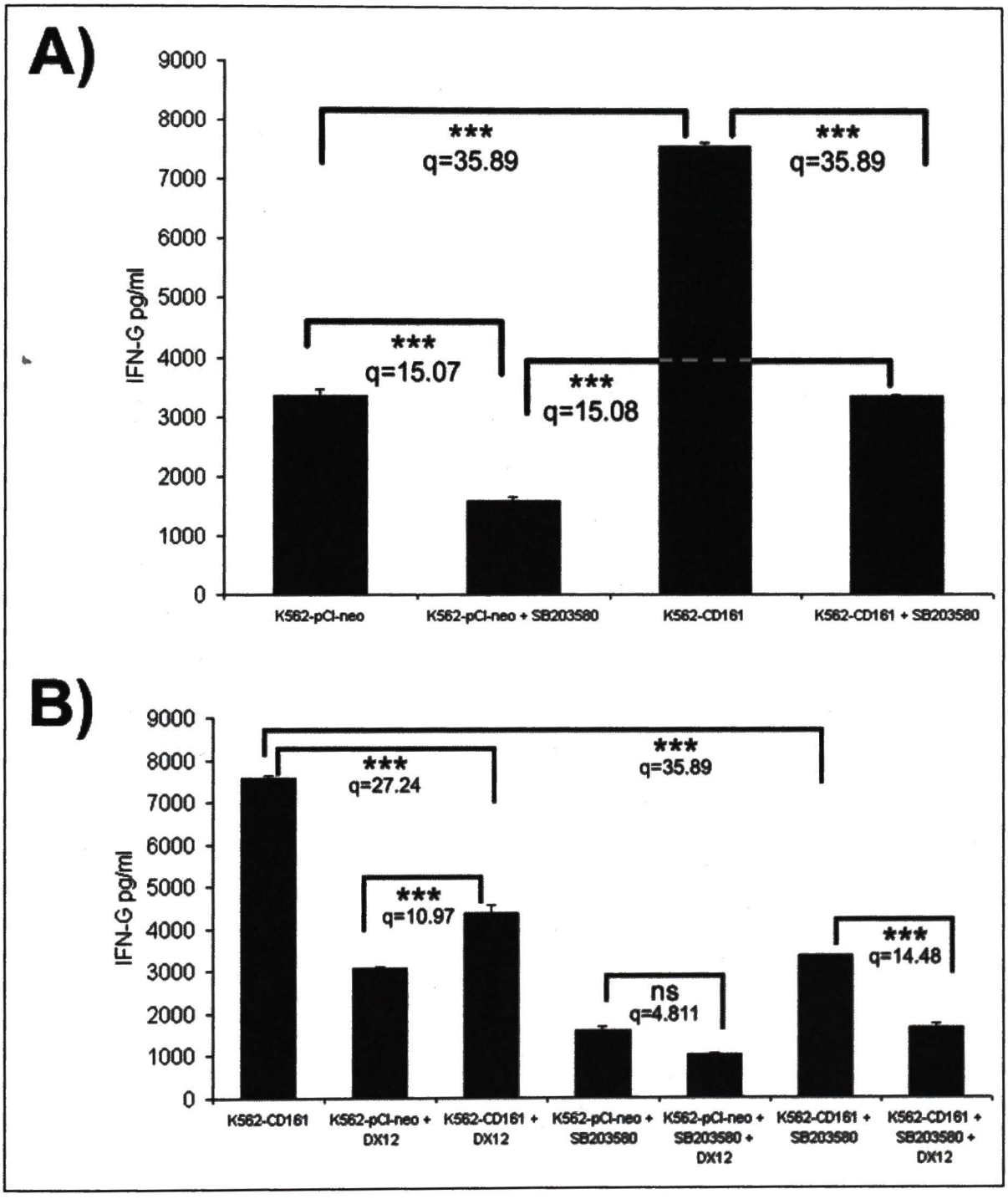

A)
IFN-G pg/ml
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
***
q=35.89
***
q=35.89
***
q=15.07
***
q=15.08
K562-pCI-neo
K562-pCI-neo + SB203580
K562-CD161
K562-CD161 + SB203580
B)
IFN-G pg/ml
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
***
q=27.24
***
q=35.89
***
q=10.97
ns
q=4.811
***
q=14.48
K562-CD161
K562-pCI-neo + DX12
K562-CD161 + DX12
K562-pCI-neo + SB203580
K562-pCI-neo + SB203580 + DX12
K562-CD161 + SB203580
K562-CD161 + SB203580 + DX12

Figure 4.6 Inhibition of MEK/ERK pathway with concomitant blocking of CD161. NK92 was co-incubated with K562-CD161 target cells in presence of 100 uM PD98059 and IFN-G production was measured. Inhibition of MEK/ERK pathway with 100 uM PD98059 significantly reduced IFN-G production to levels comparable to that associated with lack of LLT1 activation (Figure A). K562-CD161 target cells were blocked with DX12 anti-CD161 antibody and subsequently incubated with NK92 effector cells that had been pre-incubated with PD98059. This resulted in an even further reduction of IFN-G production than either method employed singly (Figure B). Together these data suggest that while the MEK/ERK pathway is associated with LLT1 stimulated IFN-G production its inhibition alone was not capable of fully eliminating IFN-G production and that additional pathways may be in use. Q-values are given to illustrate relative levels of significance between various reactions. Reactions were conducted in triplicate. Statistics analyzed by one-way ANOVA with Tukey's post-hoc test, 95% confidence interval.

***, $p < 0.001$

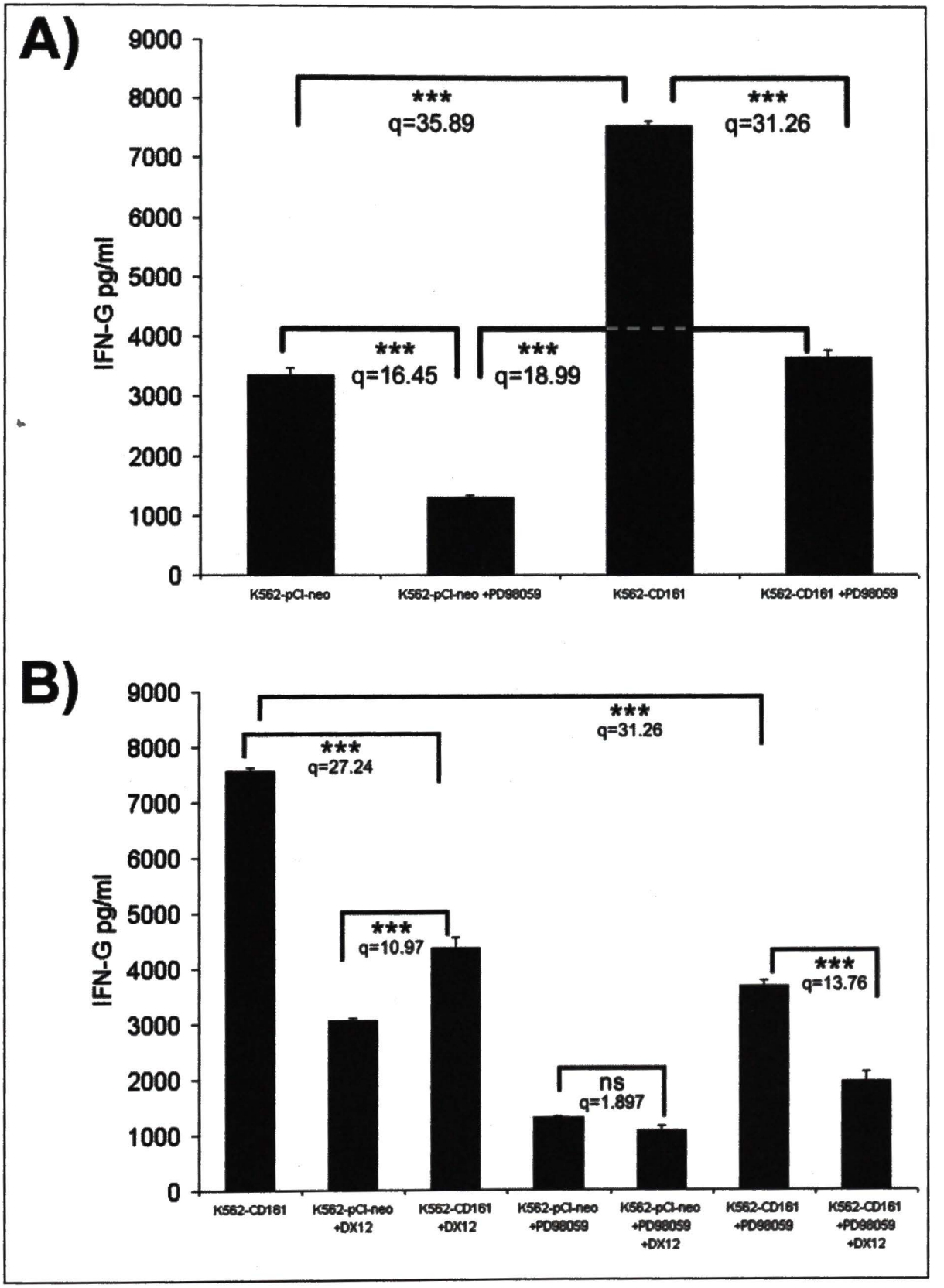

A)
IFN-G pg/ml
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
***
q=35.89
***
q=31.26
***
q=16.45
***
q=18.99
K562-pCI-neo
K562-pCI-neo +PD98059
K562-CD161
K562-CD161 +PD98059
B)
IFN-G pg/ml
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
***
q=31.26
***
q=27.24
***
q=10.97
***
q=13.76
ns
q=1.897
K562-CD161
K562-pCI-neo +DX12
K562-CD161 +DX12
K562-pCI-neo +PD98059
K562-pCI-neo +PD98059 +DX12
K562-CD161 +PD98059
K562-CD161 +PD98059 +DX12

Figure 4.7 Simultaneous Inhibition of p38 and MEK1 pathways. NK92 effector cells were pre-incubated with SB203580 and PD98059 inhibitors and then co-incubated with K562 target cells. This resulted in a statistically significant abrogation of IFN-G production suggesting that both the p38 and MEK1 signaling pathways play a role in IFN-G production associated with CD161 ligation of NK92. Reactions were conducted in triplicate. Q-values are given to illustrate relative levels of significance between various reactions. Reactions were conducted in triplicate. Statistics analyzed by one-way ANOVA with Tukey's post-hoc test, 95% confidence interval. *, $p < 0.05$; ***, $p < 0.001$

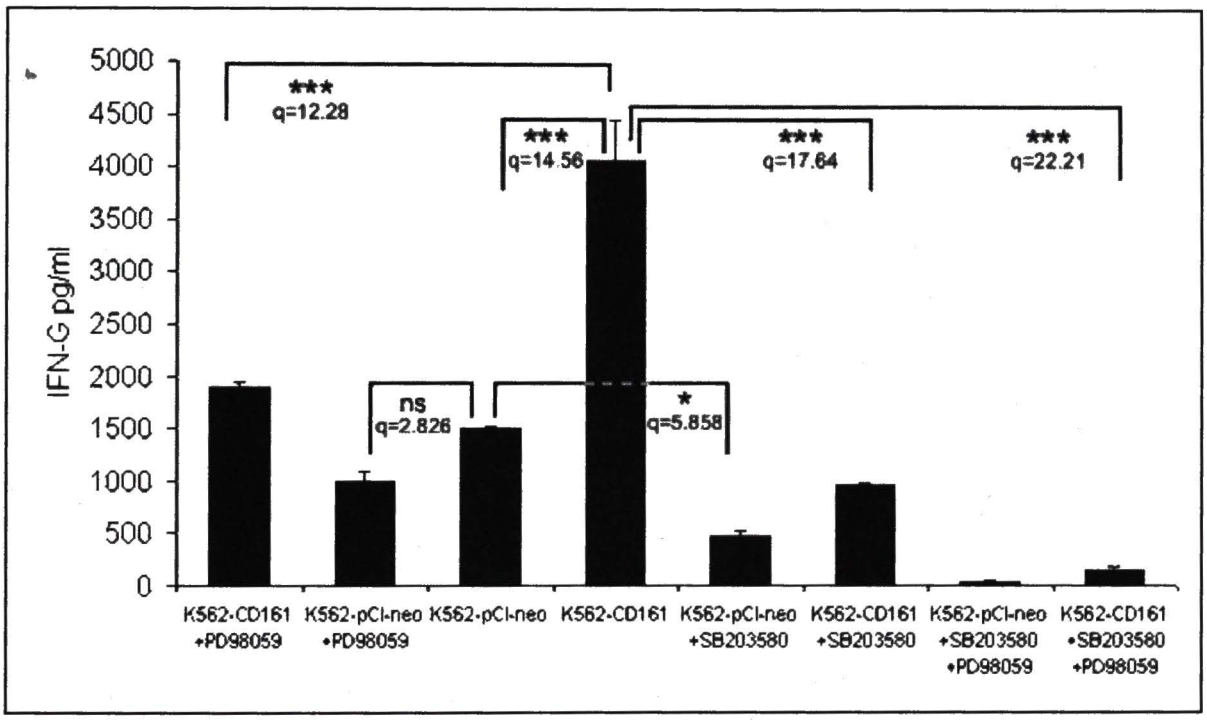

IFN-G pg/ml
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
***
q=12.28
***
q=14.56
***
q=17.64
***
q=22.21
ns
q=2.826
*
q=5.858
K562-CD161 +PD98059
K562-pCI-neo +PD98059
K562-pCI-neo
K562-CD161
K562-pCI-neo +SB203580
K562-CD161 +SB203580
K562-pCI-neo +SB203580 +PD98059
K562-CD161 +SB203580 +PD98059

Figure 4.8 IFN-G production over various time points. The standard NK92:K562-CD161/-pCI-neo IFN-G production assay was conducted with samples being harvested at 1, 2, 4, 6, 12 and 18 hours intervals. K562-CD161 samples are indicated by shaded bars, K562-pCI-neo control by open bars. The increase in IFN-G production associated with LLT1 ligation becomes significant between 4 and 6 hours after ligation, and remains significant through 18 hours post-ligation. Reactions conducted in triplicate. Statistics analyzed using one-way ANOVA with Tukey's multiple comparison test, 95% confidence interval. ***, $p < 0.001$

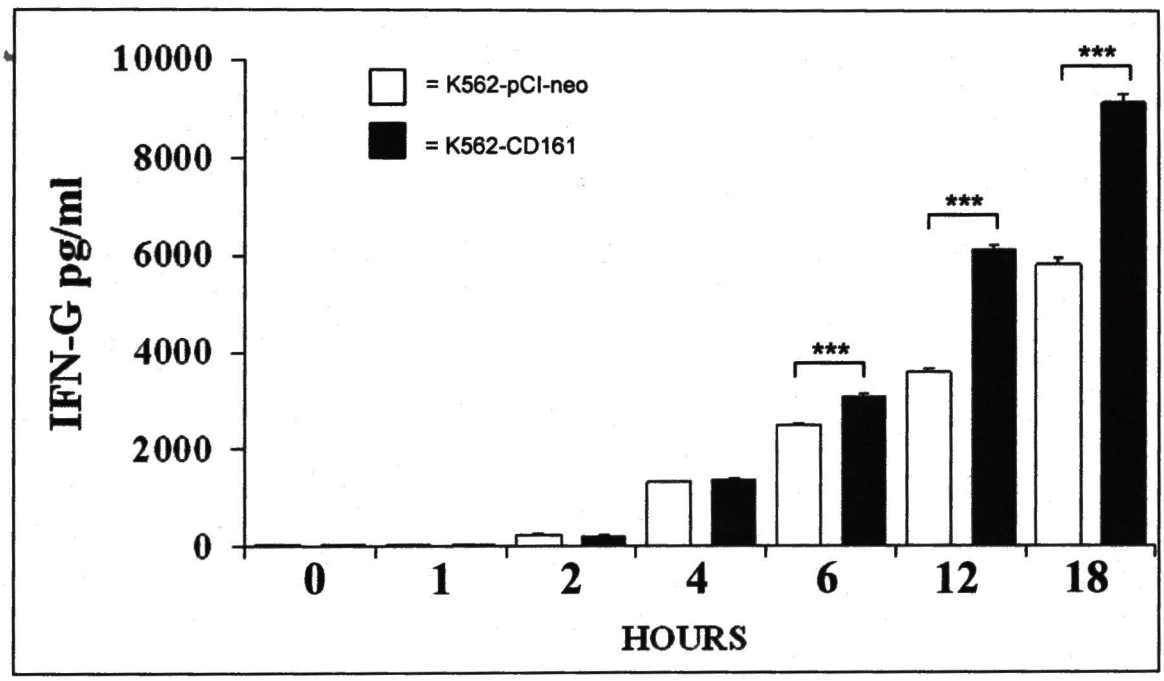
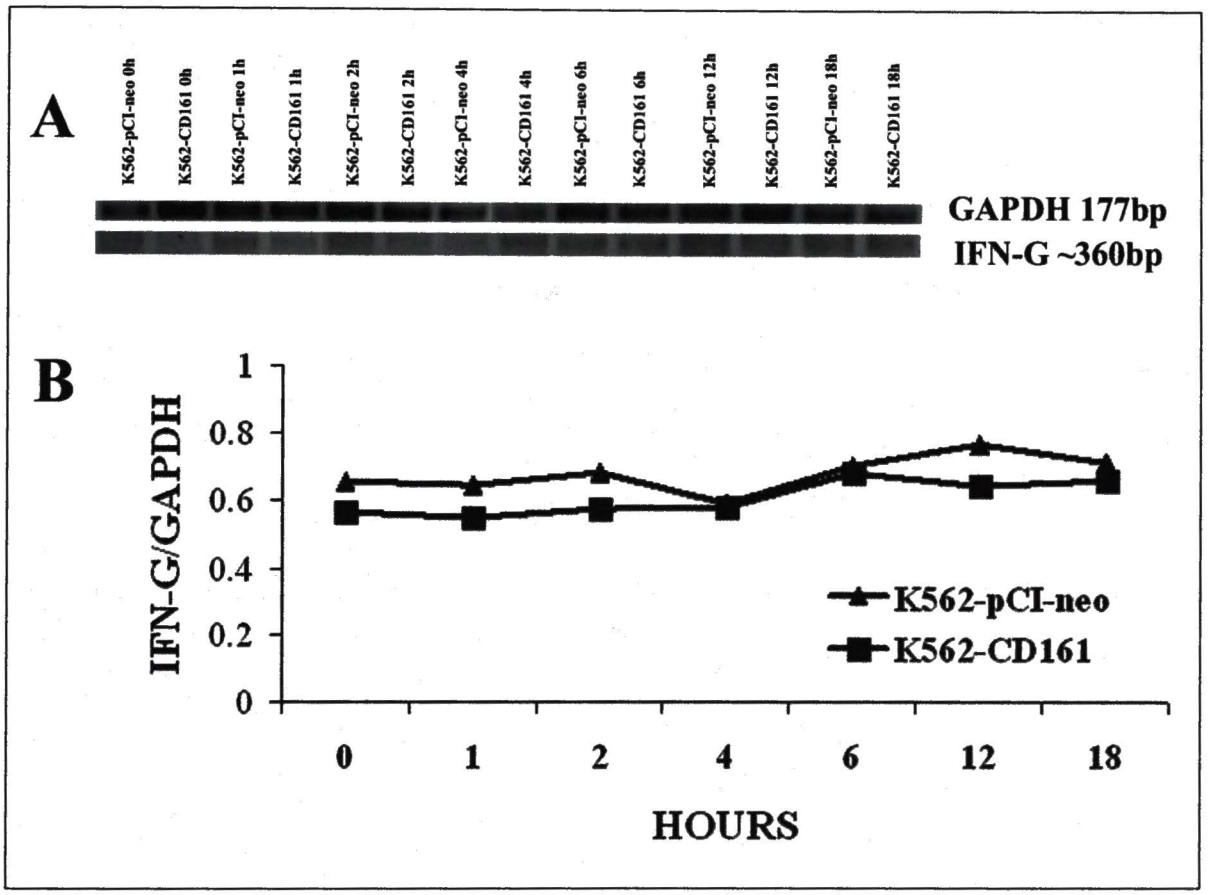

10000
8000
6000
4000
2000
0
IFN-G pg/ml
= K562-pCI-neo
= K562-CD161
***
***
***
0
1
2
4
6
12
18
HOURS

Figure 4.9 LLT1 activation is not associated with detectable alterations in IFN-G mRNA levels. NK92 was incubated overnight with K562-CD161 or K562-pCI-neo target cells. Cells were harvested at various time points and analyzed via RT-PCR. A, IFN-G mRNA expression was analyzed with GAPDH acting as a control. Visual analysis of PCR products indicates no difference in IFN-G mRNA levels at any time point regardless of LLT1 ligation. B, agarose gel underwent densitometry analysis to confirm visual results. The calibrated density of IFN-G mRNA divided by median calibrated density of GAPDH mRNA for NK92 incubated with K562-CD161 and K562-pCI-neo does not indicate any detectable increase or decrease in IFN-G mRNA expression associated with LLT1 ligation and IFN-G production. Image is representative of multiple experiments.


A
K562-pCI-neo 0h
K562-CD161 0h
K562-pCI-neo 1h
K562-CD161 1h
K562-pCI-neo 2h
K562-CD161 2h
K562-pCI-neo 4h
K562-CD161 4h
K562-pCI-neo 6h
K562-CD161 6h
K562-pCI-neo 12h
K562-CD161 12h
K562-pCI-neo 18h
K562-CD161 18h
GAPDH 177bp
IFN-G ~360bp
B
IFN-G/GAPDH
1
0.8
0.6
0.4
0.2
0
K562-pCI-neo
K562-CD161
0
1
2
4
6
12
18
HOURS

CHAPTER V

PHOSPHORYLATION OF SIGNALING MOLECULES INVOLVED IN LLT1 ACTIVATION OF NK CELLS

SUMMARY

Activation of signaling pathways through ERK and p38 requires phosphorylation of these molecules. Therefore, I evaluated the phosphorylation status of the ERK and p38 pathways to further confirm their role in LLT1 signaling. While LLT1 activation was clearly associated with an increase in ERK-phosphorylation, I was unable to detect a significant increase in p38-phosphorylation. This tends to confirm that the ERK pathway is employed for LLT1 signaling, while the role of the p38 pathway is unclear.

INTRODUCTION

My previous work with pharmacological inhibitors suggested that the p38 MAPK and MEK/ERK signaling pathways are associated with CD161 stimulated IFN-G production by NK92. To provide additional confirmation to this hypothesis I analyzed the phosphorylation status of p38 and ERK before and after LLT1 was activated on the surface of NK92. Both p38 and ERK become phosphorylated upon their activation by upstream signaling events (125). As I presently have no alternate method for engaging LLT1 in a manner that stimulates its function I was required to use the K562-CD161 co-incubation system to effect LLT1 stimulation. Analyzing intracellular protein phosphorylation requires the lysis of the cells under study. Using this LLT1 activation system, there is no option to separate the activating K562 target cells from NK92 and lyse NK92 in a timely fashion. Therefore, to prevent the detection of intracellular proteins from K562 when the NK92:K562 cell mixture lysate was analyzed by SDS-PAGE all K562 target cells were fixed with paraformaldehyde prior to co-incubation with NK92. Published data demonstrates that fixing cells in this manner prevents the detection of intracellular protein by SDS-PAGE and western blot (150-152). As this was a slight deviation from my previous IFN-G secretion assay that employed unfixed K562 target cells I repeated the standard NK92:K562 IFN-G production assay using paraformaldehyde fixed K562 targets to confirm that paraformaldehyde fixation does not abrogate the binding properties of surface expressed CD161.

MATERIALS AND METHODS

Paraformaldehyde fixing:

Following the protocol described by Djeu's Group, K562-CD161 and K562-pCI-neo target cells were resuspended in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) and incubated on ice for 30 min. They were subsequently washed four times with ice cold PBS before being resuspended in an appropriate volume of media for the NK92 co-incubation assay. This paraformaldehyde fixing prevents the detection of intracellular protein by SDS-PAGE and western blot (150-152).

Phosphorylation assay and cell lysis:

To stimulate phosphorylation of LLT1 downstream signals NK92 were co-incubated with an equal number of fixed K562 target cells for 5 to 30 min. Once the incubation was complete the cell mixture was quickly centrifuged and resuspended in Cell Signaling 1X Cell Lysis Buffer (Cell Signaling #9803) on ice for 5 min. Lysate was then centrifuged for 15 min at maximum speed at 4c to remove all cellular debris. Protein concentrations in supernatants were estimated via spectrophotometry using Bradford reagent to ensure equal loading on SDS-PAGE gels. All samples were run on SDS-PAGE under reducing conditions and transferred to PVDF membrane per standard protocols. To reduce background phosphorylation NK92 were incubated overnight in fresh media lacking IL-2 prior to incubation with fixed K562 targets.

Western blotting:

Cell lysates transferred to PVDF membranes were evaluated by western blot. Membranes were blocked using 3% BSA in TBST for one hour. Primary antibody was diluted in 3% BSA/TBST and incubated with membranes overnight at 4c with shaking. After washing three times with TBST membranes were probed with appropriate HRP linked secondary antibody for one hour in 3% BSA/TBST. After washing three times with TBST the membranes were then developed with Millipore Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica MA) and imaged using a UVP Bioimaging Systems EpiChemi3 Darkroom operating LabWorks Ver 4.6 (UVP, Inc Upland, CA). Antibodies used from Cell Signaling Technology (Danvers, MA) were rabbit anti-phospho-p38 MAP kinase (Cell Signaling #9211) at 1:3000 dilution, rabbit anti-total-ERK (Cell Signaling #9102) at 1:1000 dilution and HRP linked anti-rabbit IgG secondary antibody (Cell Signaling #7074) at 1:20000 dilution. Santa Cruz Biotechnology mouse anti-phospho-ERK (Santa Cruz Biotechnology #SC-7383) at 1:1000 dilution and HRP linked goat anti-mouse IgG secondary antibody (KPL, Gaithersburg MD) at 1:10000 dilution were also employed. Before membrane re-probing, to remove any remaining antibody PVDF membranes were stripped for 10-20 minutes using glycine stripping buffer (200 mM Glycine, 0.1% SDS, 1% Tween-20, pH 2.2) and re-subjected to the same western protocol using a different primary antibody. Antibodies specific for phosphorylated protein were always used prior to stripping as stripping may de-phosphorylate proteins. Abcam mouse anti-GAPDH (#AB9484; Abcam, Cambridge, MA) was used in a 1:20000 dilution to ensure an equal amount of protein was loaded in

each lane. To avoid possible background contamination, phosphate sources such as PBS and powdered milk were avoided.

IFN-gamma release assay:

$2X10^5$ NK cells were co-incubated with $2X10^5$ paraformaldehyde fixed K562-CD161/-pCI-neo target cells in 1000 ul fresh alpha-MEM on a 24 well plate for 16 hours in tissue culture conditions. Cell-free supernatant was collected and IFN-gamma concentration was quantitated with a commercial ELISA kit per manufacturer's instructions (BD Biosciences, San Jose, CA). Assays were conducted in triplicate with all proper standards and controls.

Statistical analysis:

Statistical analysis was conducted using Student's T-test for two samples with unequal variance, 95% confidence interval. A double sided P value of 0.05 or less was considered significant.

RESULTS

IFN-gamma release assay:

As paraformaldehyde fixing of cells was expected to result in some degree of membrane associated protein crosslinking, I analyzed the ability of K562-CD161 fixed target cells to stimulate IFN-G production in NK92 cells in the same manner as I have previously demonstrated unfixed K562-CD161 cells are capable. My results show that, although the absolute level of IFN-G produced was less than that associated with unfixed K562 target cells, paraformaldehyde fixed K562-CD161 target cells were capable of stimulating a greater level of IFN-G production than fixed K562-pCI-neo cells (Figure 5.1). Based upon these results, I am confident that phosphorylation data obtained using paraformaldehyde fixed K562 target cells may be accurately correlated to the previous IFN-G production data I have obtained using unfixed K562 target cells.

Phosphorylation assay:

My previous IFN-G production data suggested that the p38 and MEK/ERK signaling pathways were associated with CD161 stimulation of LLT1. Therefore, I hypothesized that upon incubation of NK92 with CD161 expressing target cells I would observe increased phosphorylation of both p38 and ERK proteins compared to NK92 incubated with CD161 lacking target cells. However, my western blot analysis was only capable of detecting an increase in phospho-ERK associated with K562-CD161 target

cells. Phospho-p38 was detected in both K562-CD161 and K562-pCI-neo reactions (Figure 5.2 and Figure 5.3). This does not entirely rule out the possibility that p38 is specifically associated with LLT1 downstream signaling. The method of LLT1 activation used herein requires CD161 expressed on the surface of K562 to activate LLT1. As phospho-p38 is detectable in NK92 incubated with K562 targets lacking CD161 it is possible that any p38 phosphorylation associated with LLT1 activation by CD161 is masked by p38 phosphorylation associated with the general engagement of K562 by NK92.

Figure 5.1 NK92:Fixed K562 IFN-G production assay. K562-CD161 target cells fixed with 4% paraformaldehyde were capable of stimulating greater IFN-G production than K562-pCI-neo target cells identically fixed. This was the same pattern previously observed using unfixed K562-CD161/-pCI-neo target cells. Both reactions conducted in triplicate, statistics analyzed by Student's T-test for two samples with unequal variance, 95% confidence interval. **, $p < 0.01$

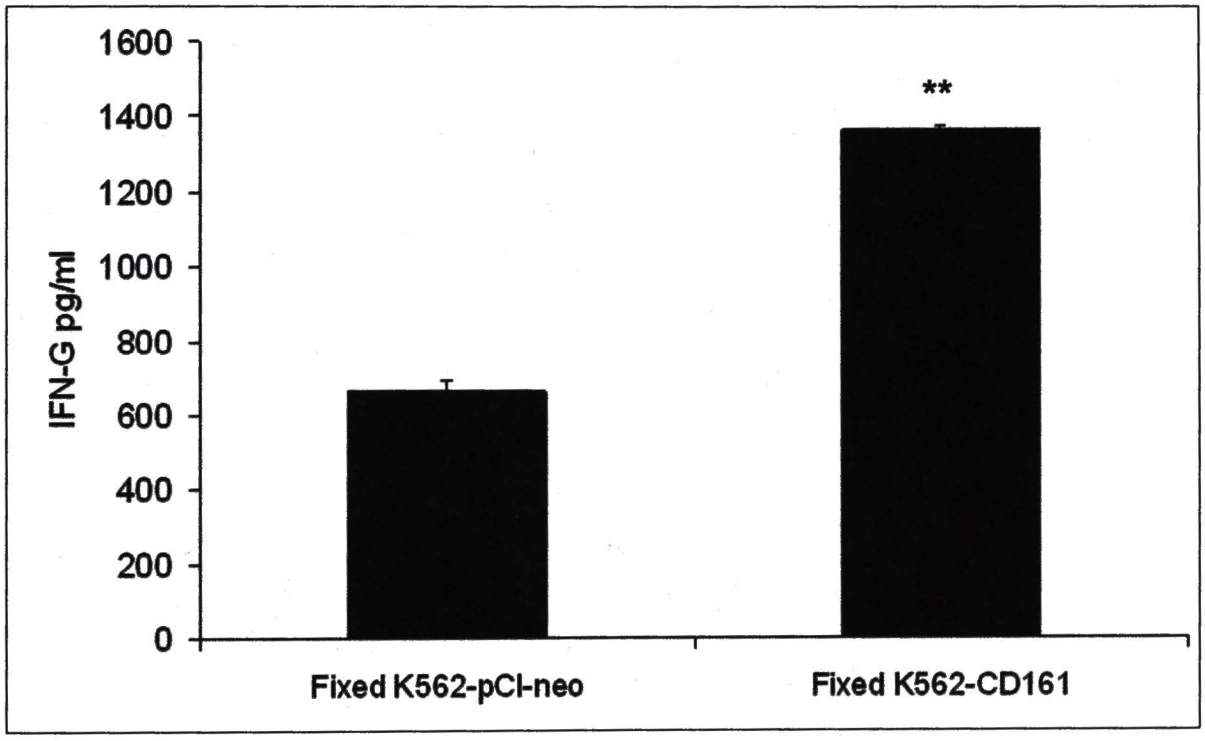

1600
1400
1200
1000
800
600
400
200
0
IFN-G pg/ml
**
Fixed K562-pCI-neo
Fixed K562-CD161

Figure 5.2 Phosphorylation of ERK pathway upon CD161 interaction with LLT1. An increase in phosphoryation of ERK was associated with the presence of CD161 on K562 target cells. Across all time points from 5 to 30 min additional phospho-ERK was observed in lanes of NK92 lysate incubated with K562-CD161 than in those incubated with K562-pCI-neo. No such association was observed with p38. Probing with GAPDH and total ERK antibodies confirmed equal loading of SDS-PAGE lanes. "K562-C" indicates K562-CD161, "K562-e" indicates K562-pCI-neo. Far right lane was NK92 incubated with PMA/ionomycin to serve as a positive control. Image is representative of multiple experiments.

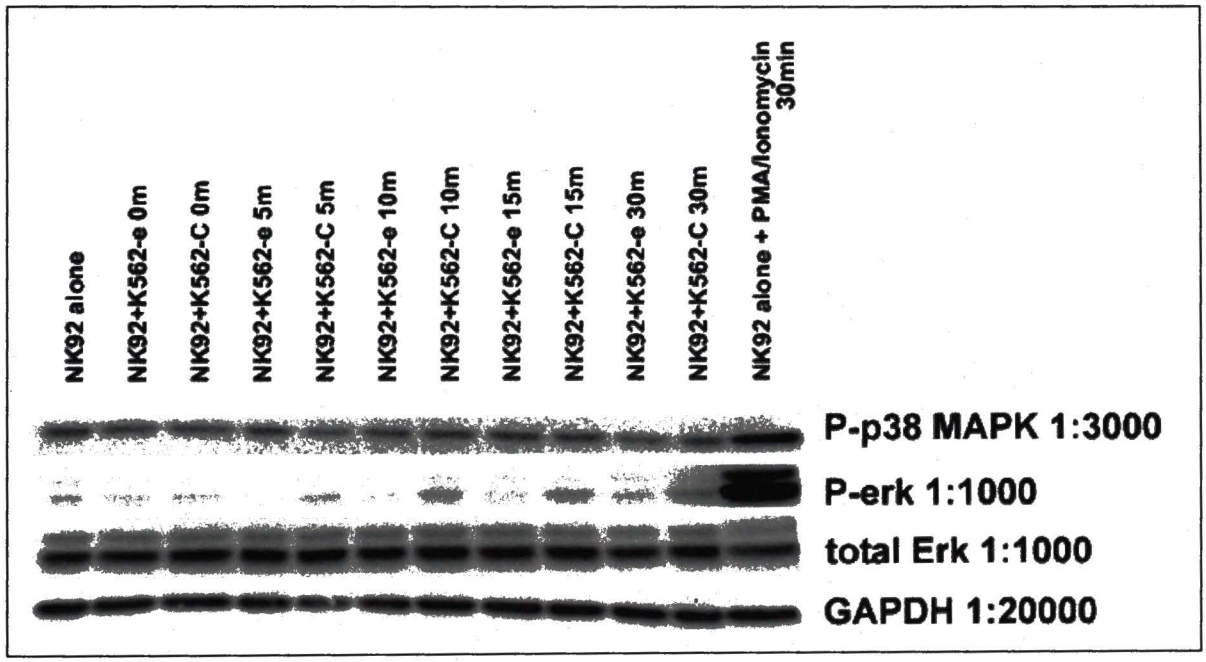
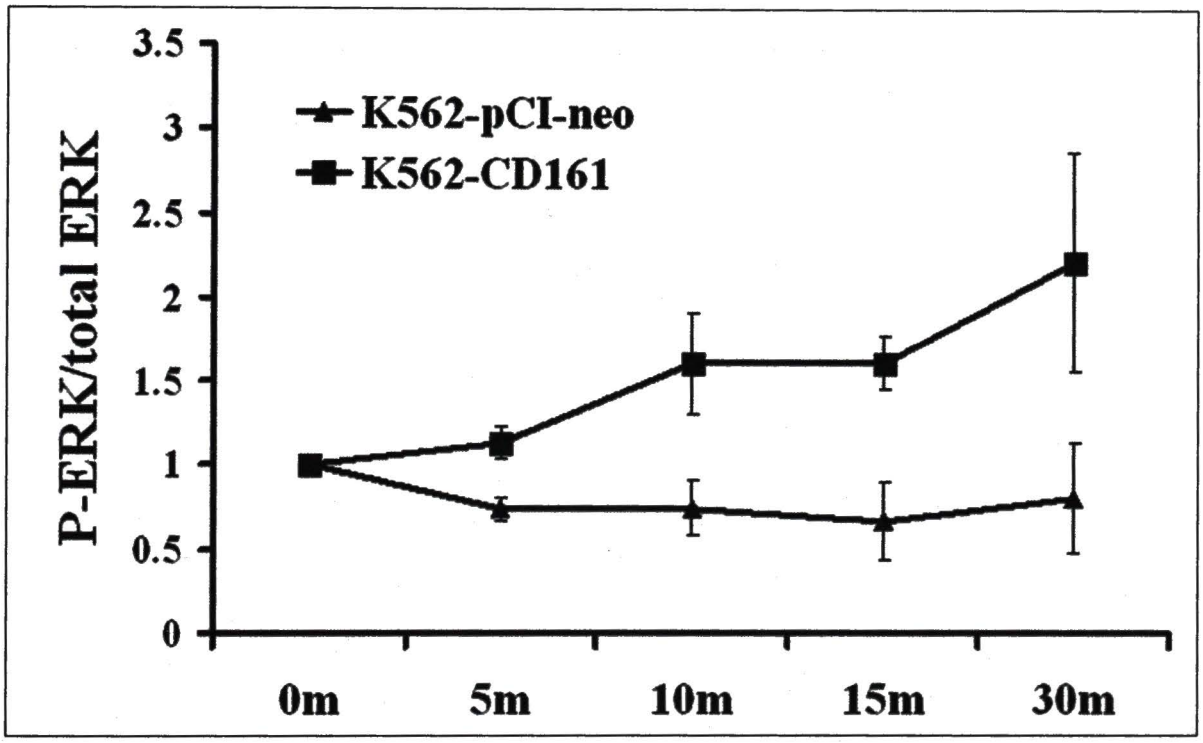

NK92 alone
NK92+K562-e 0m
NK92+K562-C 0m
NK92+K562-e 5m
NK92+K562-C 5m
NK92+K562-e 10m
NK92+K562-C 10m
NK92+K562-e 15m
NK92+K562-C 15m
NK92+K562-e 30m
NK92+K562-C 30m
NK92 alone + PMA/Ionomycin 30min
P-p38 MAPK 1:3000
P-erk 1:1000
total Erk 1:1000
GAPDH 1:20000

Figure 5.3. Densitometry analysis of P-ERK/total ERK Western Blot. The density of the western blot shown in Figure 5.2 was analyzed using the same UVP system used to capture the image. Densities of phospho-ERK for K562-CD161 and K562-pCI-neo at various time points were divided by the median density of their associated total ERK and normalized to their value at 0 minutes. K562-CD161 (represented by shaded squares) was associated with an increase in ERK phosphorylation compared to K562-pCI-neo (represented by shaded triangles) over 30 minutes. Image is representative of multiple experiments.


P-ERK/total ERK
3.5
3
2.5
2
1.5
1
0.5
0
K562-pCI-neo
K562-CD161
0m
5m
10m
15m
30m

CHAPTER VI

DISCUSSION

Previously, our lab has cloned the LLT1 receptor from a human NK cell cDNA library and shown that it is expressed by B cells, T cells, monocytes and NK cells. Additionally, our lab has demonstrated that LLT1 is a potent activator of IFN-G production on human NK cells (116, 117). In this study I have endeavored to develop additional methods to study the LLT1 receptor and employ these tools to identify the signaling pathways associated with LLT1. To study the mechanisms of LLT1 signaling I developed a novel model of LLT1 activation using NK92 and K562 cells stably transfected with the LLT1 natural ligand, CD161. I have demonstrated for the first time that LLT1 is expressed on the NK92 cell line, and that LLT1 is functional on NK92 in a manner identical to that observed on freshly isolated human NK cells and on the NK cell line YT. Using this LLT1:CD161 functional model I have demonstrated that LLT1 stimulated IFN-G production is associated with the ERK signaling pathway and possibly the p38 pathway as well. Furthermore, IFN-G secretion associated with LLT1 is detectable as little as six hours after ligation, and this IFN-G production is not associated with altered IFN-G mRNA levels.

For a thorough analysis of the expression and function of a surface receptor a monoclonal antibody is a highly desirable tool. I employed multiple strategies to generate

a monoclonal antibody against LLT1. Initial efforts to generate large quantities of LLT1 immunogen focused on harvesting pGex-LLT1ext fusion protein from BL21 bacterial cells transformed with my custom designed bacterial expression vector. While I was able to generate and detect pGex-LLT1ext fusion protein all my efforts to separate and purify this protein from the quantity of associated bacterial proteins were unsuccessful. This was likely due to the difficulties associated with solubilizing lectins (153, 154). An alternative source of immunogens initially was synthetic LLT1 peptides selected from theoretically well exposed regions of the extracellular region and mammalian expressed pSec-LLT1ext fusion protein for subsequent boostings. While these immunogens were capable of producing polyclonal sera against LLT1 none of the dozens of anti-LLT1 monoclonal hybridomas analyzed were specific for LLT1. As an alternative strategy I repeated the process of immunization using only mammalian expressed pSec-LLT1ext for all immunizations. Mammalian expressed LLT1 is theoretically the optimal immunogen as it should exhibit post-translational modifications such as glycosylation, phosphorylation and folding patterns similar to those observed naturally on LLT1, as opposed to bacterial expressed protein or synthetic peptide which are lacking these features. Preliminary analysis of polyclonal anti-LLT1 generated using mammalian pSec-LLT1ext indicates it is highly specific for the LLT1 receptor.

Rapid production of IFN-G is a critical role of NK cells responding to infection. Previously, our lab has identified the LLT1 receptor and demonstrated that its ligation with L9.7 anti-LLT1 monoclonal antibody stimulates IFN-G production by human NK cells (116, 117). LLT1 is a member of the C-type lectin like superfamily with its gene in

the NK gene complex on human chromosome 12 (116). NK cell activating receptors within this superfamily generally have short cytoplasmic domains that lack an ITAM or ITIM motif (127). LLT1 exhibits these properties. Additionally, the cytoplasmic domain of LLT1 lacks novel tyrosine motifs that are be associated with other signaling pathways such as those found on CD244 (2B4) and CD150 (155, 156). A basic residue present within the transmembrane domain of LLT1 suggests that LLT1 associates with an ITAM containing accessory protein such as DAP10, DAP12, TCR-zeta or FceRI-gamma (125, 126). As these accessory protein ITAMs are presumed to be phosphorylated by Src-PTK recruited to lipid rafts for that purpose (125), and my data indicated PP2 inhibition of Src-PTK abrogated LLT1 induced IFN-G production, it is likely that LLT1 associates with one of these four known activating accessory proteins and depends upon its ITAM to transmit regulatory signals into the cell.

Our present data consistently demonstrated that LLT1 activation on NK92 by its ligand, CD161, strongly stimulated IFN-G production. To our knowledge, this is the first study to report the expression and function of LLT1 on NK92 and confirms that CD161 interaction with LLT1 stimulated IFN-G production. My findings demonstrate that NK92 can serve as an effective model of LLT1 function on human NK cells. These results obtained using NK92 to model LLT1 function correlate with IFN-G production our lab previously observed when LLT1 on the YT cell line and freshly isolated NK cells was crosslinked with a functional monoclonal antibody, L9.7. However, LLT1 activation never been associated with an increase or decrease in natural cytotoxicity (117). These results illustrate the duality of NK activation pathways. Activating NK receptors are

known to exhibit multiple functions. KIR2DL4 ligation stimulates IFN-G production in resting NK cells and stimulates both IFN-G and cytotoxicity in activated cells (81). CD16 and 2B4 are capable of stimulating cytotoxicity in resting NK cells, but not IFN-G production (157). However, 2B4 is capable of stimulating cytotoxicity and IFN-G production in the activated NK cell line YT (158). Inhibition of either the p38 or ERK pathways abrogates 2B4 associated cytotoxicity, whereas only the p38 pathway is associated with 2B4 induced IFN-G production (82, 159). Furthermore, IFN-G production stimulated by KIR2DL4 is p38 dependent, whereas that stimulated by IL-2 is ERK dependent (81). These findings suggest there are different pathways associated with NK cell activation that overlap and exhibit varying degrees of multiplicity. With this in mind I endeavored to determine the signaling pathways associated with LLT1, a receptor heretofore associated exclusively with IFN-G production. My inhibition strategy indicates that successful LLT1 signaling requires Src-PTK, p38 and ERK pathways with the latter two possibly working in tandem. Inhibition of PKC, PI3K and calcineurin exhibited no affect upon LLT1 stimulated IFN-G production. While my phosphorylation assay confirmed the importance of the ERK pathway to LLT1 signaling, the lack of positive phosphorylation data associated with p38 does not completely rule out its importance to LLT1 function. One possibility is the current phosphorylation assay may not be sufficiently sensitive to detect an increase in p38 phosphorylation upon LLT1 stimulation. With these results in mind I have proposed a potential LLT1 signaling pathway depicted in Figure 6.1.

IFN-G plays an important role in the early response to intracellular infection and consequently IFN-G is the major cytokine produced by NK cells upon their detection of infected or cancerous cells (44). As NK cells do not store pre-synthesized IFN-G protein for rapid secretion, NK cells must constitutively express a quantity of IFN-G mRNA to facilitate rapid translation of IFN-G upon stimulation (41-43). NK cells are capable of secreting detectable levels of IFN-G within 5 hours of detecting the presence of infection (40). Time point analysis of LLT1 stimulated IFN-G production indicates statistically significant levels of IFN-G are detectable between 6 and 12 hours after LLT1 ligation. This suggests that LLT1 has a role in the synthesis of *de novo* IFN-G protein during infection. The known ligand of LLT1 is CD161, a receptor expressed on subsets of CD8+ T cells, CD4+ T cells and NK cells (142). I suggest that upon the arrival of NK cells at the site of infection, LLT1 is activated by CD161 expressed on cells already present, thereby signaling LLT1 to initiate IFN-G production. A recent study has shown an increased frequency of CD4+CD161+ T cells in cancer patients (160). Alternatively, a recent study indicates that overall CD161 PBL expression, and specifically CD161 expression on CD16+ NK cells is significantly less in patients with metastatic melanoma (161). Furthermore, CD56+CD161+ NK cells in HIV exposed-uninfected individuals exhibit significantly higher levels of CD161 expression than HIV infected and HIV unexposed individuals, though the frequency of CD161+ NK cells remains the same in all three groups (162), and significantly fewer CD161+ CD3+ T cells are detected in HIV positive patients than in healthy controls, regardless of HAART treatment (163). It is unclear if these alterations in CD161 expression are due to pathogenic alteration of the

immune system, the immune response to the pathogen or a combination of both. LLT1 stimulated IFN-G production likely serves as an additional mechanism by which the immune system can respond to infection under the appropriate conditions.

RT-PCR analysis of IFN-G mRNA over various time points was unable to detect any alteration of IFN-G mRNA levels in association with LLT1 activation. As LLT1 has been clearly demonstrated to stimulate IFN-G secretion, and IFN-G is not stored by cells but secreted immediately after synthesis (39), this evidence suggests LLT1 stimulates IFN-G production via some process of post-transcriptional or translational modification. There is precedence for such a model of immune cell cytokine production. CD28 is a stimulator of IL-2 production in T cells. CD28 mediates IL-2 production by activating the NF90 AU-binding protein, which binds an AU-rich element (ARE) in the 3' UTR of IL-2 mRNA, thereby stabilizing the mRNA allowing the rate of translation to increase (164). Human IFN-G is also known to be subject to post-transcriptional control. IL-12 + IL-2-induced NK92 IFN-G production is stimulated not by increased transcription but via post-transcriptional regulation of IFN-G mRNA trafficking, whereby IFN-G mRNA sequestered within the nucleus is transported to the cytoplasm where it then undergoes translation (165). Multiple AREs are found in the 3' UTR of IFN-G mRNA and they are associated with post-transcriptional regulation. The importance of these AREs is demonstrated by the fact that they have been highly conserved through evolution and are present in numerous species (166). Replacing these AREs with non-A + U-rich elements result in significantly higher levels of IFN-G expression, suggesting the presence of AREs are associated with IFN-G mRNA degradation (166). Conversely, splicing of the

IFN-G 3' UTR to a beta-globulin gene construct results in a significant decrease in the half-life of the associated mRNA compared to the native un-spliced beta-globulin mRNA when p38 MAPK is not stimulated. This instability is reversible upon p38 MAPK stimulation (167). 3' poly-A tails protect mRNA from degradation, and AREs appear to drive mRNA degradation by activating de-adenylation with mRNA degradation by subsequent 3'->5' exonuclease activity (168, 169). The precise mechanisms by which AREs mediate mRNA stability and degradation remain unclear but may be mediated by various mRNA binding proteins (169-171). ARE binding protein (ABP), such as HuR, are associated with mRNA stabilization, whereas other ABPs, such as tritetraprolin (TTP) and AU-binding factor 1 (AUF1), are associated with mRNA decay (169, 171). Stimulation of p38 MAPK and its downstream target MAPK-activated protein kinase 2 (MK2) reverses IFN-G ARE associated mRNA degradation and leads to increased protein expression (167). Work with knockout mice indicates MK2 plays a key role in post-transcriptional and translational regulation of multiple cytokines including TNF-a, IL-6, and IFN-G (172, 173), with MK2 double knockout mice exhibiting high susceptibility to intracellular bacterial infection, presumably due to impaired TNF-a and IFN-G production (174). MK2 presumably exerts its regulatory effect by activating mRNA binding proteins such as TTP and HuR rather than directly interacting with mRNA itself (175).

AREs are not exclusively associated with mRNA stabilization and decay as mechanisms of post-transcriptional control. TNF-a mRNA undergoes ARE dependent nuclear export and subsequent translation upon ERK stimulation (176). Furthermore, the

ABP TIA-1 is associated with inhibition of TNF-a translation (177), possibly driven by the same mechanism of de-adenylation associated with mRNA degradation (169). Although the exact mechanisms of 3' UTR ARE associated post-transcriptional regulation remain under investigation, multiple studies suggest they provide a potent method for tightly controlling the expression of various cytokines, including IFN-G, via multiple mechanisms of post-transcriptional and translational regulation (170, 178). The complex nature of these mechanisms are illustrated by a study demonstrating that TGF-b down-regulates IL-18 dependent IFN-G production by inducing mRNA degradation whereas it has no effect on IL-2 dependent IFN-G production (179). I hypothesize that LLT1 signaling regulates some form of IFN-G post-transcriptional regulation such as those described here, and future research should focus on identifying the specific mechanisms associated with this regulation. Potential mechanisms of LLT1 post-transcriptional control of IFN-G are depicted in Figure 6.2. However, the absence of detectable changes in mRNA levels after LLT1 ligation does not entirely rule out the possibility that LLT1 modulates IFN-G transcription. Actinomycin D dependent inhibition of RNA polymerase was associated with abrogation of IFN-G production (Figure 4.2). The RT-PCR analysis of IFN-G mRNA employed here can only suggest approximate mRNA levels. It may not necessarily be sensitive enough to detect some changes in IFN-G mRNA levels. Furthermore, RT-PCR does not directly evaluate the actual transcription of a given gene. Future investigations should analyze the actual transcription of IFN-G upon LLT1 ligation using techniques such as nuclear run-on and dot blot hybridization.

Our research has demonstrated a likely mechanism for LLT1 intracellular signaling pathways stimulating IFN-G production. Future research will focus on the precise mechanisms of LLT1 IFN-G post-transcriptional regulation, the precise identity of the LLT1 associated accessory protein, and the role of the p38 MAPK signaling pathway with LLT1 signaling. Our current method of LLT1 ligation, using CD161 expressing K562 target cells has worked well to this point for stimulating IFN-G production. However, K562-CD161 is a cumbersome method of LLT1 ligation better suited for overnight ligation protocols requiring K562 co-incubation. The process of K562 target cell fixation requires a significantly greater number of cells than are required for the actual NK92 co-incubation as a number of cells are lost during the process of fixation and subsequent washing. To analyze the nature of DNA and RNA binding proteins upon LLT1 activation, cell lysate must undergo ultrasonic pulsing to shear nucleic acids freeing any bound proteins. Such a harsh protocol will also be expected to lyse any cells remaining including paraformaldehyde fixed K562 targets, potentially contaminating the NK cell lysate with their DNA and RNA binding proteins. Additionally, K562-CD161 is not sufficient for LLT1 immunoprecipitation, only an anti-LLT1 antibody is suited for that protocol. Furthermore, analyzing IFN-G mRNA before and after LLT1 activation will require RNA isolation under various conditions from both the cytoplasm and the nucleus. To avoid contamination with K562 RNA, LLT1 must be ligated by an anti-LLT1 monoclonal antibody previously demonstrated to stimulate IFN-G secretion. We suggest that a functional anti-LLT1 monoclonal antibody must be

developed before any additional study of LLT1 signaling is attempted. Our laboratory is presently in the later stages of screening anti-LLT1 hybridomas.

Figure 6.1. Probable signaling mechanisms employed by LLT1. Our inhibitor and phosphorylation assays both indicate that the MEK/ERK signaling pathways are employed by LLT1 to stimulate IFN-G production. Inhibition of p38 MAPK abrogated IFN-G production, suggesting that it may be associated with LLT1 downstream signaling. However, lack of IFN-G abrogation by PI3K inhibition indicates that p38 MAPK would likely be stimulated through Ras activation of Rac rather than via PI3K/Vav activation of Rac. The absence of modified IFN-G mRNA levels upon LLT1 ligation indicates that post-transcriptional regulation is the final target of LLT1 signaling rather than transcription itself.

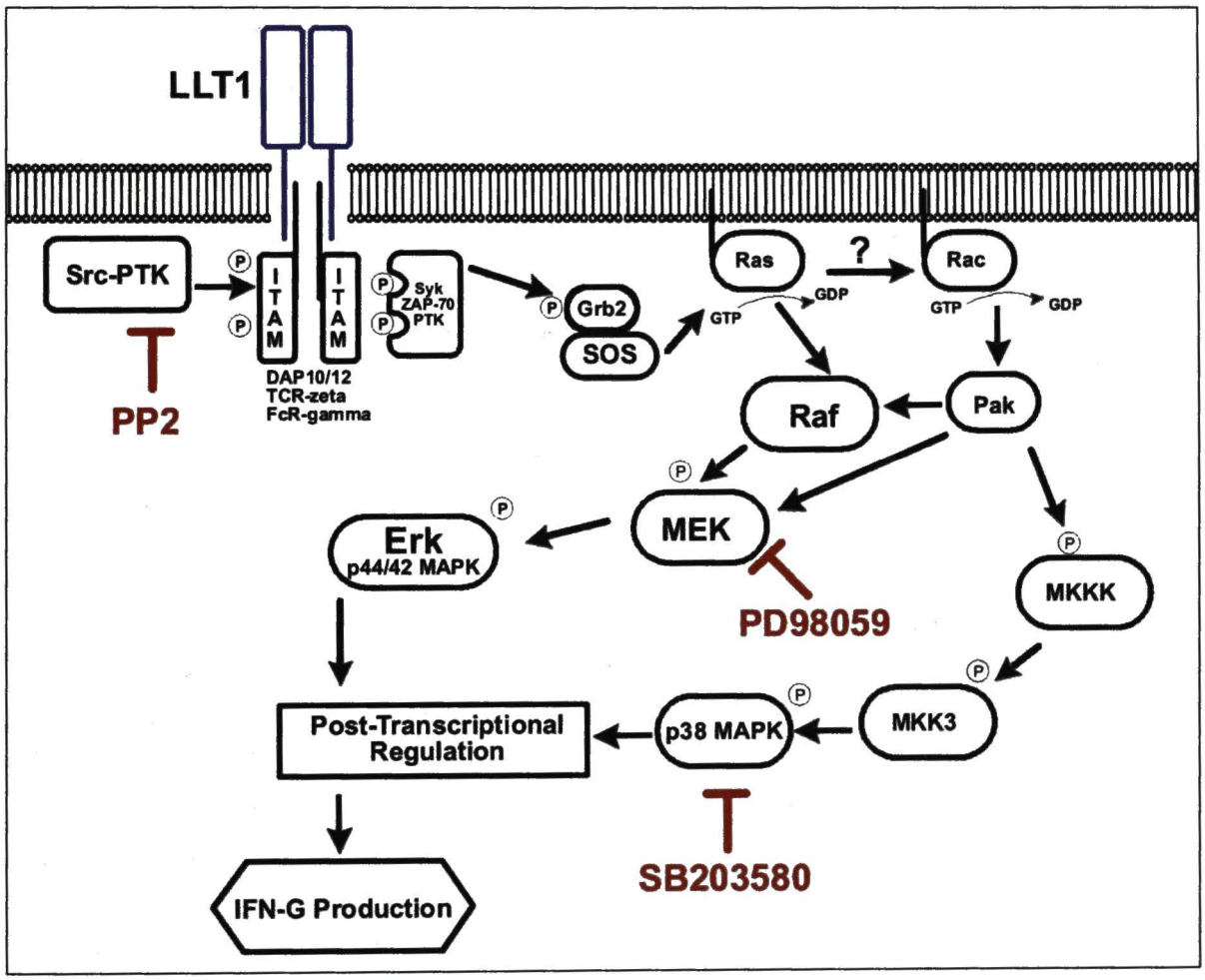
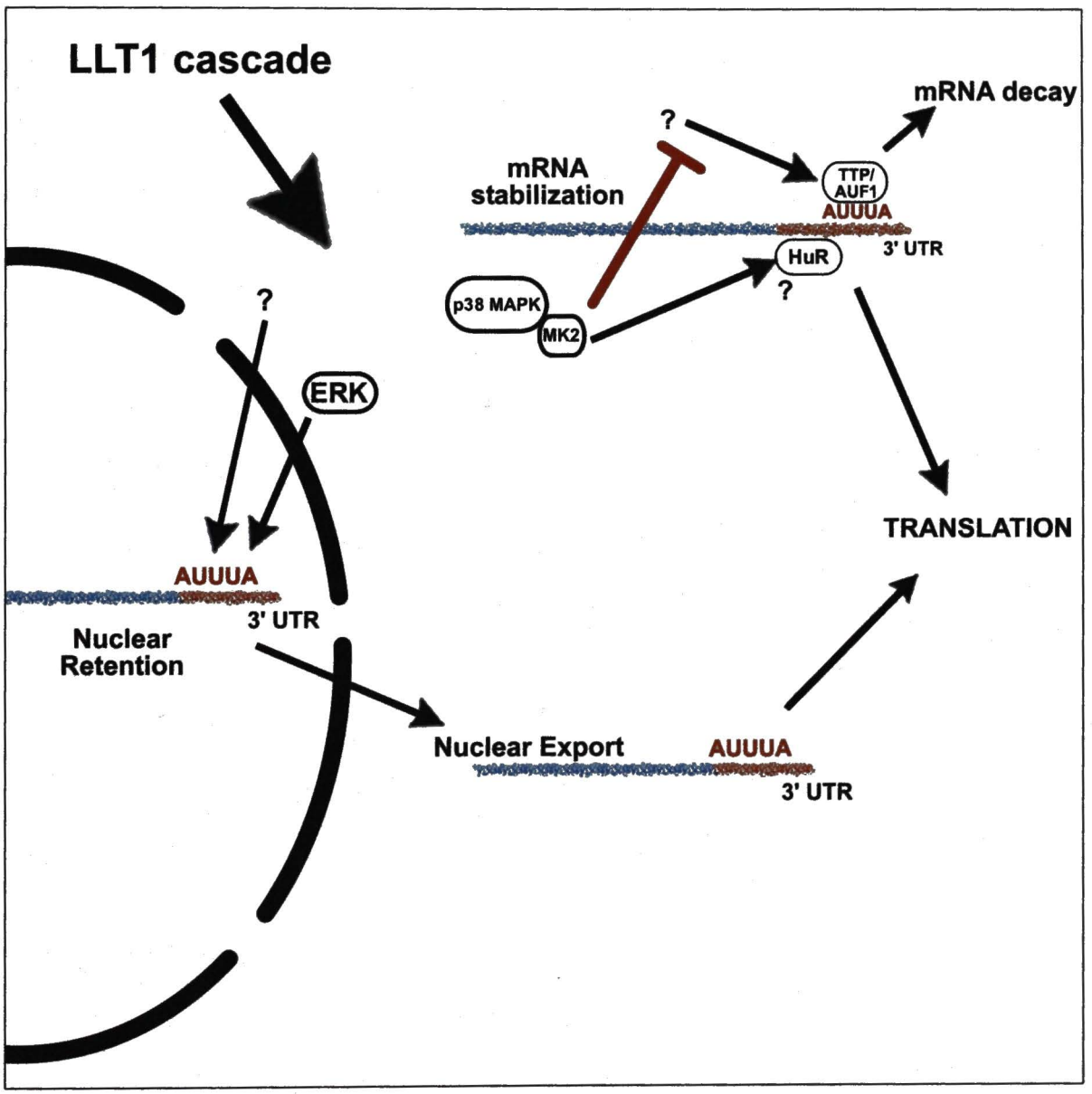

LLT1
Src-PTK
PP2
ITAM
ITAM
DAP10/12
TCR-zeta
FcR-gamma
Syk
ZAP-70
PTK
Grb2
SOS
Ras
?
Rac
GTP
GDP
GTP
GDP
Raf
Pak
MEK
PD98059
Erk
p44/42 MAPK
MKKK
MKK3
p38 MAPK
SB203580
Post-Transcriptional
Regulation
IFN-G Production

Figure 6.2. Possible mechanisms of IFN-G post-transcriptional regulation. Various mechanisms of cytokine post-transcriptional regulation have been suggested in recent years. A summary of mechanisms associated with IFN-G regulation is depicted below. As LLT1 activation is not associated with detectable changes in IFN- levels we suggest that LLT1 employs some mechanism of post-transcriptional regulation to stimulate IFN-G production. Future LLT1 research should evaluate these mechanisms in relation to LLT1 activation.


LLT1 cascade
mRNA decay
?
mRNA
stabilization
TTP/
AUF1
AUUUA
3' UTR
HuR
?
p38 MAPK
MK2
?
ERK
TRANSLATION
AUUUA
3' UTR
Nuclear
Retention
Nuclear Export
AUUUA
3' UTR

LIST OF ABBREVIATIONS

NK, natural killer

IFN, interferon

IFN-G, interferon-gamma

Ig, Immunoglobulin

LLT1, Lectin-like transcript 1

TCR, T-cell antigen receptor

MHC, major histocompatibility complex

APC, antigen presenting cell

ADCC, antibody dependent cell-mediated cytotoxicity

DC, dendritic cell

PBMC, peripheral blood mononuclear cell

KIR, killer-cell immunoglobulin-like receptor

ITIM, immunoreceptor tyrosine-based inhibitory motif

ITAM, immunoreceptor tyrosine activation motif

SHP, SH2-domain-containing protein tyrosine phosphatase

SHIP, SH2-domain containing inositol polyphosphate 5' phosphatase

NCR, natural cytotoxicity receptor

PI3K, phophatidylinositol 3-kinase

PTK, protein tyrosine kinases

PKC, protein kinase C

MAPK, mitogen-activated protein kinase

MEK, mitogen-activated protein kinase kinase

Grb2, growth factor receptor-bound protein 2

SOS, son of sevenless

TGF, transforming growth factor

TNF, tumor necrosis factor

ERK, extracellular signal-regulated kinase

XLP, X-linked lymphoproliferative disease

EBV, Epstein-Barr Virus

IL, interleukin

NF-AT, nuclear factor of activated T cells

ARE, adenine + uridine rich element

UTR, un-translated region

TTP, tritetraprolin

AUF1, AU-binding factor 1

MK2, MAPK-activated protein kinase 2

IPTG, isopropyl beta-D-1-thiogalactopyranoside

PE, phycoerythrin

FITC, fluorescein isothiocyanate

PMSF, phenylmethylsulphonyl fluoride

PBL, peripheral blood lymphocyte

BSA, bovine serum albumin

TBST, tris-buffered saline + Tween 20

HRP, horseradish peroxidase

ELISA, enzyme-linked immunosorbent assay

MEM, minimum-essential medium

ext, extracellular domain

HAART, highly active anti-retroviral therapy

BP, base pairs

GST, glutathione S-transferase

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