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SOYPEPTIDE LUNASIN IN CYTOKINE IMMUNOTHERAPY FOR LYMPHOMA

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ABSTRACT

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Immunostimulatory cytokines can enhance anti-tumor immunity and are part of the therapeutic armamentarium for cancer treatment. We previously reported that chemotherapy-treated lymphoma patients acquire a deficiency of Signal Transducer and Activator of Transcription 4 (STAT4), which results in defective IFNγ production during clinical immunotherapy. With the goal of further improvement in cytokine-based immunotherapy, we examined the effects of a soybean peptide called lunasin that exhibits immunostimulatory effects on natural killer cells (NKCs). Peripheral blood mononucleated cells (PBMCs) from healthy donors and chemotherapy-treated lymphoma patients were stimulated with or without lunasin in the presence of IL-12 or IL-2. NK activation was evaluated, and its tumoricidal activity was assessed using in vitro and in vivo tumor models. Chromatin immunoprecipitation (ChIP) assay was performed to evaluate the histone modification of gene loci that are regulated by lunasin and cytokine. Adding lunasin to IL-12- or IL-2-cultuted NK cells demonstrated synergistic effects in the induction of IFNG and genes involved in cytotoxicity. The combination of lunasin and cytokines (IL-12 plus IL-2) was capable of restoring IFNγ production by NK cells from post-transplant lymphoma patients. In addition, NK cells stimulated with lunasin plus cytokines have higher tumoricidal activity than those stimulated with cytokines alone using in vitro tumor models. The underlying mechanism responsible for the effects of lunasin on NK cells is likely due to epigenetic modulation at target gene loci. Lunasin represents a different class of immune modulating agent that may augment the therapeutic responses mediated by cytokine-based immunotherapy.

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Natural Killer cells (NKCs) are an important part of the innate immune system particularly as they pertain to early defense against tumor malignancy. Understanding the circumstances in which NKCs are most active, and specifically what artificial or endogenously produced modulators affect their activity greatly benefits cancer research. The soypeptide lunasin has, in recent years, been described as a chemopreventative agent, and has been researched largely as a direct treatment for cancerous or potentially cancerous cells. In this study we explore lunasin as modulator of NKC activity. The purpose of this review is to provide a general background of NKCs, what is known about their mechanism of action, what stimulates them, their relationship to tumor cells, and how they can be used in immunotherapy. A special focus will also be placed on the background of the soypeptide lunasin which is explored as an immunomodulating agent of NKCs following the literature review.

1.2 NK Cell Biology

NKCs are large granular lymphocytes that represent about 15% of peripheral blood lymphocytes. When NKCs were first identified nearly 40 years ago it was observed that they did not require prior sensitization to kill target cells. They could spontaneously, or naturally, attack their targets and so were termed natural killer cells [1, 2]. It has since been established that NKCs are able to identify “missing self” due largely to the absence of major histocompatibility complex (MHC) I molecules[3]. MHC I is ubiquitously expressed on all nucleated cells in the mammalian body. One of its functions is to present antigens to CD8+ T cells, but another is to act as a ligand for inhibitory NKC receptors thus identifying targeted cells as originating from the same environment as NKCs, or “self” [4]. NKCs can be parsed into subsets identified as CD56bright and CD56dim which are superior cytokine producers and cytotoxic cells respectively [2, 5]. The breadth of known NKC receptors and their ligands is still being explored, but what is clear is that

NKC function is as complex and daunting a subject as any part of the adaptive immune system. NKCs are of particular interest to researchers pursuing immunotherapy. Their ability to survey the body and find cells that are “missing self” gives them several advantages over adaptive immunity. Adaptive immunity is dominated by B and T cells, which require gene rearrangement to create a highly specific receptor. When this highly specific receptor finds a corresponding antigen the B or T lymphocyte will duplicate itself via clonal expansion in order to develop enough cells for an adaptive immune response, a process that can take a minimum of several days to occur [6]. The most obvious advantage of the innate actions of NKCs over adaptive immunity is the speed at which NKC can react. The expedient responses of NKCs to foreign antigen allow them to halt, or at least slow, the progression of viral infections and tumorous mutations until an adaptive response can be mounted. Gaining control over these innate abilities could allow cancer researchers to prevent the recurrence of tumors after patients have undergone chemotherapy or even augment the immune system of individuals at risk for cancer

1.3 Recognition of Targets by the NKC: the “Missing self” Model

The “missing self” model proposes that NKCs primarily target cells with either reduced or absent MHC I expression (Figure 1). MHC I is ubiquitously expressed on all nucleated cells in the mammalian body; one of its functions is to present antigens to cytotoxic T lymphocytes (CTL), but another is to identify cells as “self” or originating from the same environment as NKCs [4]. Generally it has been noted that cells expressing MHC I molecules are better able to inhibit destruction by NKC [3] through the engagement of inhibitory receptors. Cancerous cells often have altered or reduced MHC I expression, which cannot trigger an inhibitory signal in NKC, and thus these cells are often more susceptible to NKC-mediated killing.

1.4 Licensing NKC to Engage Targets

Recent studies have suggested that NKCs must first become “licensed” before they actively defend against “missing self” cells [7, 8]. MHC I is the ligand for many inhibitory NKC receptors. NKCs developed from environments lacking MHC I, such as B2m -/- mice, cannot successfully target virally infected or tumorous cells derived from the same host [8-10]. This is interesting because early versions of the “missing self” hypothesis predicted that NKCs would attack any cell without MHC I proteins. That NKCs don’t become autoreactive in an environment

completely null of MHC I protein indicates that they must somehow need the molecule. They need to be “licensed” to survey for “missing self”. In support of this idea, one study showed that NKCs with Ly49A receptors (specific for H-2d haplotype) were inactive within B6 mice (H-2b haplotype), while Ly49C+ NKCs (specific for H-2b haplotype) were active [10]. Given that Ly49, and their human equivalent KIR, Killer immunoglobulin-Like receptors, are highly polymorphic it might be expected that many NKCs would not be in an environment with the appropriate MHC ligand and would become autoreactive. If, however, licensing is required for activation, NKCs that don’t have a ligand for their receptor would simply remain unlicensed and inert. Unlicensed cells represent an NKC population that is, under normal conditions, fully developed but otherwise unresponsive to activation. The differences between licensed and unlicensed cells seem to diminish under inflammatory conditions when the cytokines are present [10]. This supports a model in which licensed NKC avoid autoreactivity due to inhibitory ligands and unlicensed NKCs avoid autoreactivity due to being inert until infection occurs, while both are relevant to a healthy immune response.

1.5 Signaling Pathways Mediated by the Interaction of NKC Receptors and Their Ligands

The activity of NKC is now known to be regulated by the net signaling events triggered by activating and inhibitory receptors that recognize the ligands on target cells. When an inhibitory receptor bearing an ITIM (immunoreceptor tyrosine based inhibitory motif) is ligated the tyrosine of the ITIM is phosphorylated, often by a Src kinase, which provides a binding site for phosphatases with Src homology 2 (SH2) domains, like SHIP and SHP-1, which then can act in a number of inhibitory pathways [11, 12]. For an activating pathway to be initiated the activating receptor must be associated with an ITAM (immunoreceptor tyrosine based activation motif) [13]. Upon engagement of its ligand, a tyrosine residue on the ITAM becomes phosphorylated which in turn activates Syk or ZAP-70, allowing them to activate a number of signaling pathways [13, 14].

Both activating and inhibiting receptors are engaged whenever an NKC encounters a target cell. If enough activating receptors are ligated the inhibitory signal can be overcome and the cell will be targeted, if not then the inhibitory signal will prevail and the cell will be recognized as “self” (Figure 1). It’s important to note that a lack of inhibition doesn’t necessarily mean the NKC will engage a target. Erythrocytes for example lack nuclei and thus MHC I molecules, and neural

tissues have relatively low MHC I expression levels but neither is attacked by NKCs under normal circumstances [13]. Thus NKCs require an activating signal and not only a lack of inhibitory signals.

NKC receptors can be grouped into several broad categories including the Killer immunoglobulin-Like receptors (KIR), the C-type Lectin superfamily (CLR), natural cytotoxicity receptors (NCR) [32], Fc receptors and cytokine receptors.

1.5.1 Killer Immunoglobulin-Like Receptors (KIR)

KIR are polymorphic receptors, evolved from the Ig superfamily, that recognize HLA-A, B, and C proteins [4]. Individual NKCs inherit only some of the many potential KIRs, but at least one will usually be specific for a host MHC I molecule [15, 16]. An inhibitory and activating KIR can share the same specificity, but Inhibitory KIRs tend to have a greater avidity for their targets. This is why if both inhibitory and activating KIRs are ligated, the inhibitory signal will be stronger and the NKC will be pacified [17]. KIR's basic form and activating or inhibitory pathway is denoted in how they are named. KIR2DL, for instance, has 2 Ig like domains (2D) and an additional long cytoplasmic domain (L) which will contain at least one ITIM sequence. KIR2DS on the other hand has 2 Ig like domains (2D) and an additional short cytoplasmic domain (S) which means the receptor will need an accessory protein with an ITAM, like DAP12, in order to send activating signals (Figure 2) [4].

1.5.2 C-type Lectin Superfamily (CLR)

NKG2 (NK group 2) and CD94 are transmembrane proteins from the CLR which are often associated with one another and can either be activating or inhibitory. CD94 and NKG2A, for instance, are known to covalently bind to one another forming a heterodimeric receptor specific for the HLA-E haplotype, and because NKG2A has an ITIM motif it relays an inhibitory signal [12, 18, 19]. NKG2C, conversely, works through an ITAM via its association with adaptor protein DAP12 which serves to contain the ITAM and stabilizes the NKG2C-DAP12 complex [13]. Despite their opposite effects, NKG2A and NKG2C share a common ligand in HLA-E [13, 19]. This is noteworthy because NKG2A has a much higher binding affinity for HLA-E [20]. This means that if the two receptors were ligated at the same time the inhibitory signal would be stronger suggesting a possible evolutionary mechanism to prevent autoimmunity due to NKC activity.

NKG2D is unique in the NKG2 family. NKG2 A, C, and E all share a similar genetic sequence, are ligated by HLA-E , and form heterodimers with CD94 [21]. NKG2D, however, is genetically distinct, is ligated by stress induced non-classical MHC proteins like MICA and MICB, and does not form a dimer with CD94 [4, 21-23]. Additionally rather than using DAP12's ITAM, NKG2D-triggered cytotoxic activity is mediated via DAP10, which associates with NKG2D in a heterocomplex [24]. NKG2D is a potent activating receptor and can overcome signals from inhibitory KIRs [25, 26]. In addition to MICA/B, NKG2D can also be ligated by ULBP (UL binding proteins) [21, 26]. As a testament to the evolutionary age and importance of the NKG2D receptor several diseases seem to have evolved specific defenses against it. It has been suggested that UL 16, a human cytomegalovirus protein, can bind to ULBPs (UL Binding Proteins) 1-2 and MICB, effectively preventing or retarding NKG2D activation [27].

1.5.3 Natural Cytotoxicity Receptors (NCR)

Natural cytotoxicity receptors (NCR) are a group of receptors, belonging to the Ig superfamily, involved in many NKC activation events. The group is currently comprised of three receptors including NKp46, NKp30, and NKp44. NKp46 is thought to be important in the destruction of HLA deficient tumor cells. Studies have shown that NKCs expressing higher levels of NKp46 (NKp46bright) are better able to lyse HLA negative targets [28]. NKp46 cannot signal on its own but does associate with adaptor proteins, CD3ζ and FCεRIγ, which signal though ITAMs [29]. NKp44 is only present on activated NKCs, signals through a DAP12 mediated pathway, and is ligated by non-classical MHC molecules [30]. When NKp44 is blocked along with NKp46 it appears to have a greater inhibitory affect than if either one is blocked alone [30]. This suggests that the two could work in a synergistic fashion. NKp30 expression correlates with NKp46, in that both are present on all active and resting NKCs [31]. It has been noted that NKp30 and NKp46 have impaired expression in HIV patients possibly contributing to the increased occurrence of opportunistic tumors [32]. It has also been noted that in certain melanoma cell lines the masking of both NKp30 and NKp46 will decrease cytotoxic activity in freshly isolated NKC more than masking either alone [31], again suggesting that NCRs work in a synergistic fashion.

1.5.4 Fc Receptors

CD16, also known as FcγRIIIA, is the receptor responsible for antibody dependent cell cytotoxicity (ADCC) in NKCs. The receptor associates with either or both CD3ζ, and FcεRIγ, which are ITAM bearing adaptor proteins [4, 14]. When it comes into contact with cell with an IgG attached to its surface, CD16 is ligated by the Fc region of the IgG, which initiates the activation of the NKC and leads to the destruction of the target cell [33, 34].

1.5.5 Cytokine Receptor Signaling and the JAK/STAT Pathway

Cytokine regulation of NKCs is primarily mediated through JAK/STAT signaling pathways. When a receptor on the NKC encounters its cytokine there is a conformation change in the receptor which leads to the activation of JAK (janus kinase). The JAK will phosphorylate a tyrosine residue on the intracellular portion of the receptor creating a binding site for STAT (signal transducers and activators of transcription) [35]. STAT will bind to the receptor and be phosphorylated by JAK after which it will dimerize with another STAT and translocate to the nucleus where it activates transcription on target genes (Figure 3). In mammals the JAK family currently has four members including JAK1, JAK2, JAK3, and Tyk2 [36] and seven different STATs [35]. Different receptor families will utilize different JAK/STAT pathways (Figure 4). The IL-12R, for instance, is a member of the gp130 family and signals though a JAK2,Tyk2 / STAT4 pathway while *IFN*γRI is a member of the *IFN* family and signals via JAK1,JAK2/ STAT1 [35].

1.5.6 The Evasion of Immune Surveillance

NKCs have evolved into a unique role in immune system. When some viruses, for instance, enter a cell they are thought to down regulate MHC I expression [37]. It has been shown that adenovirus-2 encodes a protein, E19, which inhibits the transport of MHC I molecules to the cells surface [38]. By doing so the virus has effectively protects itself from attacks by CTL which rely on peptide presentation by MHC I molecules to clear pathogens. Tumor cells similarly tend to have decreased MHC I expression, as tumors which do express MHC will be targeted by T cells [37]. NKCs have receptors, like KIR and NKG2A, which exist specifically to survey for cells with altered MHC expression, making virally infected and tumor cells their prime targets. Viruses are constantly adapting to the human immune system and have, over time, evolved defenses against both T cells and NKCs. HIV (human immunodeficiency virus) is one of the most

successful. Much of the NKC's success in identifying target cells comes from its ability to recognize absent or altered HLA-C, via KIR receptors, and HLA-E proteins via NKG2D:CD94 receptors [4, 21]. HIV is reported to be able to down regulate HLA-A and HLA-B while HLA-C and HLA-E remain unaffected [39]. This allows HIV to effectively avoid both T and NKCs. Certain tumors have also been shown to have NKC evasion tactics. Tumors from human patients with stomach, colon, and rectal cancers were shown to produce high levels of soluble MICA which serves to provide alternative targets for NKG2D bearing immune cells [40]. These selected defenses against NKG2D and other NKC receptors are indicative of their utility and importance to the immune system.

1.6 Natural Killer Cell Subsets

Human NKCs are characterized as being CD 56+ and CD3- but beyond that they can be divided into subsets with specific functions and surface receptors. $CD56^{high}CD16^{low}$ cells represent approximately 10% of NKCs and produce cytokines at higher levels than $CD56^{low}$ $CD16^{high}$ cells in response to cytokines, such as IL-12, IL-15, and IL-18 [33, 41]. CD56dim CD16bright NKCs represent a majority of NKCs in the peripheral blood and produce fewer cytokines but are more cytotoxic than their CD56brightCD16dim counterparts [33].

1.7 Functions of NKCs

1.7.1 Immune Regulatory Function

NKCs regulate the immune responses by producing a number of cytokines and chemokines, including interferon gamma (*IFN*γ), tumor necrosis factor (TNFα), and granulocyte/macrophage colony stimulating factor (GM-CSF, CSF2) [42-44]. *IFN*γ is important to the immune system's response to a variety of tumors and virally infected cells [45-47]. *IFN*γ activates cytotoxic T lymphocytes (CTL) and macrophages in response to infection [48], retards tumor growth [49, 50], and, along with TNFα, stimulates the maturation of dendritic cells [51]. In addition, IFNγ enhances the immunogenicity of tumor cells by upregulating the MHC class I and class II expression which allows them to be recognized and eliminated by T cells more easily [52, 53]. Both spontaneously arising and chemically-induced tumors are more common in *IFN*γ receptor-

deficient and IFNγ-deficient mice compared to wild-type mice [54-57]. The Inhibition of IFNγ in vivo abrogates the ability of immunocompetent mice to reject transplanted syngeneic tumors [57].

Along with cytokines NKCs are known to produce chemokines including CCL3, CCL4, CCL5, CCL22, and CXCL8 when activated. It is thought that the production of these chemokines serves to attract T cells, B cells, and other NKCs to the vicinity of the active NKC [58]. One study showed that NKCs incubated with K562, chronic myelogenous leukemia, produced more CCL3, 4, and 5 as compared to NKCs alone, resulting in recruiting bystander NKCs [59].

1.7.2 Cytolytic Function

In addition to their immunoregulatory role through cytokine production, NKCs have the ability to spontaneously kill target cells without any prior immunization or stimulation. NKCs, in a process referred to as immunosurveillance, survey the body for foreign and mutated cells using an of array activating and inhibitory receptors. Upon recognition, NKCs are able to induce apoptosis in their targets. Apoptosis is a distinctive mode of programmed cell death necessary for the survival of living organisms. There are two main pathways of apoptosis referred to as intrinsic, in which an apoptotic suppressor within the cell itself is damaged due to external factors like radiation or toxins, and extrinsic, in which a receptor on the cell surface is ligated activating a “death domain”.

1.7.2.1 Fas and FasL Mediated Apoptosis

NKCs and CTLs (Cytotoxic T Lymphocytes) can utilize the extrinsic method or an additional method which requires the production of lytic granules containing granzymes and perforin. One of the better defined death domain pathways requires the Fas receptor. In this pathway a FasL on the immune cell will ligate a Fas receptor on the target cell. The ligation will trigger the binding of FADD, an adaptor protein, which will in turn associate with procaspase-8. A DISC, the death inducing signaling complex, will then form and cleave procaspase-8 leading to its activation, followed by the activation of caspase-3, a potent catalyst in the cleavage of key cellular proteins, ultimately leading to cell death [60-63] (Figure 5).

1.7.2.2 Release Lytic Granules

When an NKC or CTL encounters a target cell they can release granules, containing granzymes and perforin. Of the five known human granzymes, Granzyme B has the best understood pathway. It appears to induce apoptosis through a number of methods including direct activation of caspase-3, and the activation of caspase -10 which leads to the activation of caspase-3 [63] (Figure 6). There is also evidence showing that granzyme B can work though mitochondrial pathways involving the disruption of the mitochondrial membrane, leading to the release of pro-apoptotic proteins, and the release of cytochrome c, which activates caspase-9 leading to the activation of caspase-3 and apoptosis[63, 64] (Figure 6). Perforin is generally recognized as providing the mechanism for granzyme to enter the cell, though the exact method through which it does this remains a subject of debate [65]. Knockout experiments indicate that mutations in the perforin genes can be detrimental to the immune system, particularly in regards to tumor immunosurveillance. It has been shown that perforin deficient mice are unable to prevent the metastases of tumors [66]. In a similar study it was observed that perforin deficient mice develop larger tumors at a faster rate when challenged with a number of cell lines [67].

1.8 NKC-Based Cancer Immunotherapy

In a process referred to as immunosurveillance NKCs survey the body for foreign and mutated cells using an array of activating and inhibitory receptors. Harnessing this ability has been a long time goal for cancer researchers. A number of strategies have been used to manipulate NKC activity in an effort to enhance cancer immunotherapy. It is unlikely that any one method will become the new mainstay of cancer treatment. The future of immunotherapy will undoubtedly be a combination of different immunological and oncological practices.

1.8.1 Blocking Inhibitory Signals on NKC

One method involves blocking inhibitory receptors, so that NKCs will more readily attack cancer cells. It has been reported that the blocking of murine Ly49C, an NKC inhibitory receptor comparable to human KIR, with F(ab') monoclonal antibody fragments can decrease tumor size and increase the survival rate of mice [68].

1.8.2 Engagement of Activating Signals on NKC

Another study found that using rituximab, an anti CD20 mAb, to mark CD20+ lymphoma significantly enhances the ability of NKCs to identify and attack lymphoma tumors [69]. This is due to the NKC's CD16 receptor which can recognize the Fc portion of the antibody allowing the NKCs to attack the lymphoma cells in ADCC. The manipulation of NKC receptors is an exciting new area of research which could extend into epigenetic silencing of inhibitory receptor expression with siRNA, the overexpression of activating receptors, or the creation of chimeric receptors specific for tumor ligands [15, 70].

1.8.3 Adoptive NKCs Transfer and Bone Marrow Transplantation

Adoptive cell transfer is an approach to immunotherapy which requires the isolation and expansion of either autologous or allogenic immune cells followed by infusion back into the patient. The appeal of this method is that, in theory, immune cells specific to the cancer in question can be selected for ex vivo and expanded free from endogenous controls [71]. An extension of this idea is to use NKCs that don't respond to the same inhibitory ligands as the patients, which is the premise behind utilizing the graft versus leukemia (GvL) effect. For instance, a patient undergoing a bone marrow transplant needs new hematopoietic stem cells, which they will receive from a donor. Ideally the donor would have HLA antigens that are identical to the recipient's and the graft would avoid rejection and avoid attacking healthy host cells in what is called graft versus host disease (GvHD). NKCs have inhibitory KIR receptors with HLA ligands [15]. If an individual has an HLA-C haplotype, for instance, then they will likely have HLA-C specific KIR on their NKCs. If these cells are transferred to a patient with a different haplotype, whose own immune system would have been destroyed as a part of cancer treatment, the NKCs would attack any remaining leukemia cells because they do not have the HLA-C protein to ligate the inhibitory KIR receptor, this is the GvL effect [72]. Additionally the NKCs would attack any of the host's remaining dendritic cells, which could generate GvHD by presenting alloantigens to donor T cells [73, 74]. This thinking is supported by studies showing that AML (Acute Myeloid Leukemia) patients have higher remission rates when receiving KIR mismatched adoptive NKCs transfers [75].

1.9 NKC-Stimulating Agents

1.9.1 Cytokine Stimulation in Natural Killer Cells

Cytokine stimulation is an attractive immunotherapeutical option because it enhances the cytotoxicity of already established NKC populations rather than introducing new cells in an adoptive transfer and risking rejection. IL-2 has been clinically shown to enhance immune function in patients presenting with a variety of cancers [76, 77]. The ex vivo stimulation of NKCs with an IL-12, IL-18 combination in known to effectively increases NKC activity against MLL-3 leukemia cells, which express a wide range of inhibitory KIR ligands [78].

The cytotoxicity of NK cells can be activated by various cytokines and interferons which are often produced by monocytes or dendritic cells (DC). *IFN* α and β, enhance NKC cytotoxicity, and induce proliferation [46, 79, 80]. IL-2 and IL-12 can upregulate the expression of granzyme B by NK cells, resulting in increased cytolytic activity [81]. The combination of IL-12 and IL-18 is a potent inducer of IFNγ, particularly in CD56 bright cells, and combination of IL-15 and IL-18 has been noted as an inducer of GM-CSF in NKCs [82].

1.9.2 Natural Additive Stimulation in Natural Killer Cells

1.9.2.1 Mushrooms

Mushrooms, a food common to the palate of many cultures, have long suspected of having hidden health benefits. The white button mushroom, Agaricus bisporus, is commonly eaten in the U.S. and has considerable immunostimulatory effects. It has been shown, in mice, to increase IFNγ and TNFα production by NK and Th1 cells [83]. Similarly, a mushroom extract, Agaricus blazei Murill Kyowa, has been observed to increase NKC activity in gynecological cancer patients undergoing chemotherapy [84]. In another study D-Fraction, a β glucan extracted from the maitake mushroom Grifola frondosa, was shown to increase IFNγ and TNFα production by NKCs [85].

1.9.2.2 Other Food Products

In addition to mushrooms there are other NKC modulating additives. MGN-3 is a modified arabinoxylan derived from rice bran that is known to enhance murine NK activity [86]. Curcumin, a yellow pigment from the Indian herb Curcuma longa , has been identified as an

enhancer of NKC cytotoxic activity and is able to reverse some of the immunosuppressive effects of tumors on NKC [87, 88]. In a double blind study it was shown that terminally ill cancer patient receiving AGE (aged garlic extract) over the course of three months had a statistically significant higher number of NKCs [89].

1.10 Lunasin

1.10.1 Lunasin structure

Lunasin is a 43 amino acid peptide that was first isolated from the soybean and is now known to be present in wheat, barley, and other staple crops [90]. It was originally cloned as cDNA from a small-subunit peptide of the S2 albumin (Gms2S-1) [91]. Lunasin has been recognized to have an RGD (Arginine, Glycine, Aspartic acid) adhesion motif which is believed to facilitate the internalization of the peptide into cancerous cell lines, like C3H fibroblasts [92], although it has been noted that the RGD motif is not necessary for internalization in others, such as NIH3T3 fibroblasts [93]. Lunasin also contains a carboxyl end with a 9 amino acid long poly D tail, whose negative charge is thought to allow its binding to positively charged hypoacetlyated histones in cancerous cells [93].

1.10.2 Lunasin function

Lunasin has been researched largely as a chemotherapeutic agent. It been suggested that lunasin contributes to the cancer preventive benefits of soy. The consumption of which is often associated with the lower cancer rates prevalent in South East Asian cultures [93]. Early work showed that the transduction of lunasin cDNA into a number of cancer cell lines could interrupt mitosis leading to apoptosis; these included murine hepatoma, human breast cancer, and murine embryo fibroblasts C3H 10T1/2 [91]. Other studies have demonstrated that the lunasin peptide can be used to significantly reduce the transformation rate of normal nontumorgenic C3H cells treated with carcinogenic agents or tumor inducing viral transfections [94]. The same studies showed that the lunasin peptide could be applied topically to reduce murine tumor incidents due to carcinogens like DBMA [94]. More recent work has shown that lunasin inhibits the growth of HT-29 colon cancer cells via a decrease in anti-apoptotic Bcl-2 proteins and an increase in the pro-apoptotic Bax, as well as in caspase 3, an enzyme common to apoptotic

processes [95]. Lunasin's effect on histone acetylation is well documented. In one study a histone deacetylase inhibitor, sodium butyrate, was added to breast cancer cells with and without lunasin. The cells treated without lunasin had levels of acetylation several fold higher than the ones that had been treated with lunasin, the inference being that lunasin inhibited the acetylation of histones, thus preventing the activation of potentially oncogenic genes [94]. Around 4.5% of the total ingested lunasin can be detected in the human blood stream one hour after eating [96]. This suggests that lunasin is bioavailable and together with its observed antitumorgenic effect, indicates that lunasin may well contribute to the antitumor effect of soy. This antitumorgenic capacity of lunasin's, as well as its immediate presence in the food supply makes it a promising choice for both clinical treatment and the widespread epidemiological prevention of cancer.

1.11 Summary

Natural killer cells are integral to the immune systems response to cancer. Since their identification our understanding of how NKCs recognize "missing self" has increased dramatically [4]. The manipulation of NKCs, though cytokine stimulation or receptor alteration, opens many new avenues of cancer treatments. In order to take full advantage of our understanding of NKCs capability new immunomodulators and cytokine combinations must be developed. Lunasin is a peptide known to have chromatin binding and anti-carcinogenic properties [93]. The use of lunasin and new treatments with NKCs open novel and promising new areas of cancer treatment.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cytokine, Antibodies, and Lunasin Peptides

Recombinant human IL-2 was obtained from Prometheus Laboratories (San Diego, CA) and recombinant human IL-12 from PeproTech (Rocky Hill, NJ). Fluorochrome-conjugated monoclonal antibodies recognizing human CD3, CD4, CD8, CD14, CD56, and *IFN*γ were obtained from BD Biosciences (San Jose, CA). Ficoll-Paque^{TM} PLUS was purchased from GE Healthcare Bio-Sciences (Piscataway, NJ). Antibodies against acetyl-histone H3 (AcH3), histone H3 trimethyl Lys9 (H3K9me3), and non-immune rabbit serum were obtained from Millipore (Billerica, MA). The lunasin peptide with 43-amino acid was chemically synthesized to 97% purity by LifeTein (South Plainfield, NJ), and includes the following sequence: SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD. A truncated peptide (32-amino acids) lacking the RGD motif and the poly-D tail was synthesized by LifeTein, and includes the following sequence: SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQG. A negative control peptide with a scrambled sequence (RKMELQEGIHLKKGDQNTQSQSCQPKCIQVWH) was also synthesized. All the peptides were dissolved in sterilized water at stock concentration of 5 mM. FITC conjugated lunasin was purchased from LifeTein (South Plainfield, NJ).

2.2 Human Blood Samples and Primary Cell Cultures

Collection of blood samples was approved by the Institutional Review Board at Indiana University Medical Center and written informed consent was obtained from each study subject. Blood samples were obtained from patients with Hodgkin's or non-Hodgkin's lymphoma after treatment with high-dose chemotherapy and autologous Peripheral blood stem cell transplant (PBSCT). Healthy human blood samples were procured from the Indiana Blood Center (Indianapolis, IN). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque^{TM} PLUS, and aliquots of PBMCs were cryopreserved in liquid nitrogen. Human NKCs were isolated from normal control PBMCs using positive selection with CD56 magnetic beads

(Miltenyi Biotech, Auburn, CA). Human B-lymphoma cell line Raji cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, USA).

2.3 Evaluation of *IFN*γ Production

*IFN*γ production at the single cell level was evaluated using intracellular cytokine staining. Cells were treated with 3μM (golgi stop) monensin sodium salt (Sigma-Aldrich, Saint Louis, Missouri) after stimulation with specific cytokine for 24 hours. Cells were then incubated with flurochrome-conjugated antibodies against CD3 and CD56 to mark surface antigens, fixed in 4% paraformaldehyde, and washed with permeablization buffer (0.1% saponin) before being incubated with the fluorochrome-conjugate anti *IFN*γ antibody. Total PBMCs were gated for CD3- and CD56+ populations, which were then analyzed for *IFN*γ production. Flow cytometry was performed using a FACSCalibur APC 4 color flow cytometer (BD Biosciences, Mountain View, CA) and analyzed using BD CellQuest^{TM} Pro version 6.0. Secreted *IFN*γ protein collected from the supernatant was measured using standard ELISA practices, as previously described [97, 98].

2.4 Analysis of Gene Expression

Purified human NKCs were stimulated with medium only, lunasin at 20 μM, cytokine IL-12 at 10 ng/ml or IL-2 at 100 units/ml, and cytokine plus lunasin for 24 hrs. One day following stimulation, the cell pellets were subjected to analysis of gene expression using real time qPCR with Taqman Assay Primers for *IFNG*, *CSF2*, *GZMB*, *TGFB1*, *TGFBR2*, *CCL3*, and *CCL4*.

2.5 Analysis of STAT4 Activation by Western Blot

Purified human NKCs were stimulated for 3 hours and 22 hours. Western blot analysis was performed from total protein extracts of cultured NKCs to measure the activation of STAT4 using an anti-phospho-STAT4 (Y693) antibody (Cell Signaling Technology, Danvers, MA). The same blot was reprobed with an anti-STAT4 monoclonal antibody (BD Biosciences, San Jose, CA) for the total amount of STAT4. Ratios of phospho-STAT4 to total STAT4 (pSTAT4/Total STAT) is determined from the arbitrary units using densitometry (calculated with ImageJ, U. S. National Institutes of Health, Bethesda, Maryland). An anti-β-actin monoclonal (SC-47778) antibody (Santa Cruz Biotechnology,Santa Cruz, CA) was used for the loading control.

2.6 In Vitro Cytotoxicity Assays

Purified human NKCs stimulated as indicated for 1 day were washed and co-cultured with target cells (Raji) at the ratio of 10:1 for 4 hrs at 37C in a 5% CO_2 incubator. NK-mediated lysis was analyzed using the CytoTox 96 non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI).

2.7 Adoptive Transfer of Human NKCs in Xenograft Model In Vivo

The adoptive transfer of human natural killer cells into 2 months old NOD/SCID/gamma chain null (NSG) mice (The Jackson Laboratory, Bar Harbor, Maine) was performed by the In Vivo Therapeutics Core, Indiana University, Indianapolis. The mice were injected subcutaneously on day 1 with 0.5 x 10^6 Raji cells in 0.1 ml PBS mixed with 0.1 ml Matrigel (BD Biosciences, San Jose, CA). Human NK cells isolated from healthy control donors were stimulated as indicated for 1 day. On day 2, these pre-treated natural killer cells were injected at the tumor site (2.5 x 10^6/mouse). Tumor growth was monitored, and the volumes were measured using standard manual calipers.

2.8 Chromatin Immunoprecipitation (ChIP)

The ChIP experiment was performed using isolated human NKCs treated with medium only, IL-12 (10 ng/ml), IL-12 (10 ng/ml) + lunasin (20 μM) or lunasin alone (20 μM) for 24 hours following the established protocol [99, 100].

2.9 Confocal Staining

NKCs were treated with FITC conjugated lunasin (20μM) and IL-12 (10 ng/mL) overnight in a 5% CO_2 incubator at 37°C. NKCs were then dyed with DNA stain Hoechst 33342 (Thermo Scientific, Rcokford, IL) 0.5 mg/mL for 30 min in the same incubator prior to visualization at 400x. In a separate experiment NKCs treated under the same conditions were fixed in 4% paraformaldehyde and permabilized with ice cold 100% methanol. Histone H3 was identified using monoclonal antibodies (Cell Signaling, Danvers, MA) overnight at 4°C. Secondary antibodies, Alexa Fluor 555 (Cell Signaling, Danvers, MA), were incubated with the NKCs for 1-2 hours at room temperature. Cells were visualized with a Fluoview FV 1000 confocal laser scanning microscope (Olympus, Center Valley, Pennsylvannia).

2.10 Statistical Analysis

PASW Statistics (IBM-SPSS, Chicago, IL) was used to analyze the data. One-way ANOVA was employed to evaluate the difference of tumor volume among different groups followed by multiple rank comparisons with Tukey's HSD test. Statistical significance was set at $P \leq 0.05$.

CHAPTER 3: SOYPEPTIDE LUNASIN IN CYTOKINE IMMUNOTHERAPY FOR LYMPHOMA

3.1 Introduction

The use of cytokines in addition to traditional cancer treatments can have a profound impact on the treatment of the disease. Numerous cytokines have been used in clinical trials to enhance anti-tumor immunity [101]. *IFN*γ has been noted as particularly important to the immune system's response to a variety of tumors and virally infected cells [45-47]. Produced by T cells or NKCs, *IFN*γ activates macrophages [48], diminishes tumor growth [49], helps stimulate the maturation of dendritic cells [51], and is necessary in the early activation and differentiation of T helper cells [102]. Numerous cytokines are known to induce *IFN*γ production including IL-2, IL-12, IL-15 and IL-18 which have been used individually and in concert [33, 41, 103, 104]. The induction of endogenous *IFN*γ is considered, by many, to be superior to exogenous injections due to the wide distribution of *IFN*γ receptors and as compared to cytokine receptors, like the IL-12R, which is largely confined to NK and T cells [105].

IL-12, a potent inducer of *IFN*γ of in NKCs, signals through a STAT4 mediated pathway [101]. Earlier studies have established that patients treated heavily for cancer can acquire a STAT4 deficiency[106]. The soypeptide lunasin, which has previously been touted for its anti-carcinogenic effects, is explored here as a modulator of NKC activity and enhancer of *IFN*γ production. The goal of this study is to determine lunasin's ability to modulate *IFN*γ production as well as to asses if it can overcome deficiencies in the cells of immune compromised patients. We found that lunasin in combination with cytokines could modulate NKC activity and significantly enhances the *IFN*γ production capacity of NKCs. This observation indicates that lunasin could be a therapeutic capable improving current cancer immunotherapies.

3.2 Results

3.2.1 Lunasin Stimulates Human NKCs to Produce IFNγ

*IFN*γ is a pro-inflammatory cytokine associated with the immune response to tumors, viruses, and bacterial infections [45-48]. Its prevalence in the immune response makes it an appropriate candidate for gauging NKC activity. PBMCs were treated with IL-2 or IL-12, known inducers of *IFN*γ [101], with and without lunasin. Fluorochrome conjugated antibodies were used to identify several cell population markers as well as *IFN*γ. Flow cytometry indicated that lunasin did not affect *IFN*γ production in CD4+ or CD8+ T cell populations, known producers of *IFN*γ, (data not shown), but did enhance *IFN*γ production by NKCs when in combination with cytokines IL-2 or IL-12 (Figure 8A). This result was confirmed by measuring *IFN*γ levels in the supernatant of purified NKCs treated under the same conditions (Figure 8B). Using cells from the same culture qPCR was performed, and the mRNA levels of *IFN*G were higher in cells treated with both lunasin and cytokine as compared to cytokine alone (Figure 8C). That stimulation with both lunasin and cytokine, as opposed to lunasin alone, had such a prolific effect on *IFN*γ production, suggesting a synergistic mechanism of action.

3.2.2 Lunasin Regulates Gene Expression in NKCs

Along with *IFNG*, several other NKC genes appear to be affected by a lunasin-cytokine cocktail. qPCR results from samples obtained in Figure 8C show that adding lunasin to a treatment with either IL-12 or IL-2 significantly increases the expression of *GZMB* (granzyme B) and *CSF2* (Granulocyte-Macrophage Colony Stimulating Factor or GM-CSF), as compared to treatment with cytokine alone (Figure 9A). Stimulation with either IL-12 or IL-2 is known to downregulate *TGFB1* and *TGFBR2* expression in NKCs [107], and adding lunasin to cytokine-treated NKC cultures results in the further reduction of *TGFB1* and *TGFBR2* expression (Figure 9B). Interestingly lunasin was able to upregulate the expression of pro inflammatory chemokines CCL 3 and CCL 4 without the need for additional cytokine stimulation (Figure 9C).

3.2.3 Dose-Dependent Effects of Lunasin in Combination with Cytokines

Lunasin was titrated against different concentrations of IL-2 and IL-12 in ordered to determine the best dose response. It was found that in the presence of lunasin lower concentrations of

cytokine could induce *IFN*γ production at levels similar to that of a 10 fold higher concentration (Figure 10A). This observation suggests that the addition of lunasin to a therapeutic cytokine treatment could allow the concentration of cytokine to be decreased without abating its stimulatory capacity. In order to determine the dose response of lunasin in the presence of cytokine, lunasin was titrated in combination with IL-12 10 ng/mL. The EC_{50} was determined to be 5.64 $\pm$ 1.94 μM (n=4) (Figure 10B).

3.2.4 STAT4 Activation in Lunasin-Cultured NKCs

IL-12 induces *IFN*γ production via the STAT4 transcription factor in NKCs [97, 108, 109]. IL-2, though a less potent inducer than IL-12, can also activate the STAT4 pathway [110]. In order to determine if lunasin was influencing STAT4's activated form phosphorylated STAT 4 (pSTAT4) was assessed using western blots. Two time points were observed, 3 and 22 hours (Figure 11 A, B). IL-12 plus luansin stimulation elicited a higher amount of pSTAT4 than IL-12 alone at either time point. IL-2 showed a similar response though with much lower pSTAT4 levels than IL-12. The IL-12, IL-2, plus lunasin combination showed a similar amount of pSTAT4 compared to the two cytokines alone, at the earlier time point,, but had higher pSTAT4 levels at later time points. This result raises the possibility that lunasin's aptitude for increasing *IFN*γ production, in combination with cytokines, is related to increasing the initial amount of STAT4 activated and possibly prolonging pSTAT4 activity.

3.2.5 Effects of Lunasin Plus Cytokines on Rescuing *IFN*γ Production by NKCs from Lymphoma Patients Post-Transplant

STAT4 deficiency as a result of cancer treatment has been observed in post-transplant patient PBMCs [111]. Lunasin's ability to enhance, or perhaps prolong, the action of STAT4 means that it has the potential to nullify the deficiency created by cancer treatment. To test this hypothesis *IFN*γ production by the NKCs of healthy individuals was compared to *IFN*γ production by the NKCs of heavily treated post-transplant patients, deficient in STAT4. The results, as measured by flow cytometry, show that patient NKCs treated with both cytokines and lunasin produce a statistically equivalent (p=0.446) amount of *IFN*γ as compared to healthy cells treated with

cytokines alone (Figure 12). This finding implies that lunasin could serve to augment *IFN*γ production in patients receiving cytokine immunotherapy who have an acquired STAT4 deficiency.

3.2.6 Lunasin Augments the Cytotoxicity of Cytokine-Activated NKCs

To determine if the lunasin-cytokine combination that increases *IFN*γ production (Figure 8 A-C) also increases NKC cytotoxic activity, an in vitro cytotoxicity assay was utilized. Results show that NKCs treated with lunasin and cytokines together lyse Raji cells, a B lymphoma cell line usually resistant to NKCs, more effectively than NKCs treated with cytokines alone (Figure 13A). Cellular therapies employing NKCs have been clinically tested for several cancer types including acute myeloid leukemia, renal carcinoma, and malignant melanoma [15, 75]. In these therapies NKCs are stimulated and expanded *ex vivo* before being infused into the patients. *In vitro* cytotoxicity assay results indicate that the addition of lunasin to IL-2 or IL-12 cytokine treatments makes NKCs more capable of lysing tumor cells (Figure 13A) and better able to produce *IFN*γ (Figure 8 A-C). Such results suggest that lunasin-cytokine stimulation is a superior NKC activator for cellular therapies. In order to determine if the addition of lunasin to cytokine treatments can enhance NKC tumor fighting capacity *in vivo*, a xenographt model was utilized. Raji, B cell lymphoma, cells were injected into NSG (NOD/SCID/gc^{null}) mice. A day later the mice were infused with human NKCs stimulated with various combinations of lunasin and cytokines. The results show that mice treated with both lunasin and cytokine develop smaller tumors than mice treated with cytokine alone (Figure 13B).

3.2.7 Lunasin's RGD Sequence is Not Required for NKC Stimulation

Lunasin's RGD sequence has been identified as the sequence through which lunasin adheres to and is consequently able to enter some cancer cell lines [94]. NKCs also express integrins capable of binding the RGD motif [92], which allows for the possibility that lunasin enters NKCs in a similar fashion. To determine if this was the case, a mutant peptide (Figure 14A), lacking the RGD sequence and the aspartic acid tail, was evaluated for its ability to stimulate NKCs. The results show that the mutant peptide and wild type lunasin have equivalent stimulatory capacity (Figure 14B). Therefore it was determined that lunasin's effect on NKCs does not rely on its RGD

motif. In addition, the scrambled negative peptide did not induce detectable levels of *IFN*γ (Figure 14B), which ruled out the non-specific effects at the concentrations used (20 μM).

3.2.8 Mechanism of Synergistic Effects Mediated by Lunasin

Lunasin's apparent ability to affect NKCs without an RGD motif suggested that it may not be entering NKCs at all and perhaps binding to some unknown surface receptor [94, 112]. To determine if lunasin was entering the cell, NKCs were incubated with FITC conjugated lunasin and IL-12 10 ng/mL overnight and were stained with a Hoechst DNA stain 30 minutes prior to visualization. The results show that lunasin is present within both the cell and its nucleus (Figure 15A). Additionally NKCs, treated under the same conditions, were incubated with a histone antibody (Figure 15B). The results again indicate that lunasin is able to pass both the cellular and nuclear membranes.

The acetylation of histone H3, AcH3, is a well-recognized mark of an active gene locus [113]. It has been reported that lunasin can inhibit histone acetyltransferase (HAT) activity [112] as well as bind to deacetylated histones in cancer cell lines [94]. Lunasin's capacity to work synergistically with IL-2 and IL-12 to affect multiple genes (Figures 8C, 9), its presence in the NKC's nucleus (Figure 15), and its ability to alter the acetylation of histones in other cell types [91, 112], all indicate an epigenetic mechanism. To test this hypothesis a ChIP assay was performed to evaluate changes in AcH3 at the *IFNG* locus after stimulation with a cytokine-lunasin cocktail and cytokine alone (Figure 16). NKCs treated with a cytokine-lunasin mix showed higher levels of acetylation at the *IFNG* locus gene than cytokine alone. IL-12 suppresses *TGFB1* expression in NKCs, and stimulation with both IL-12 and lunasin further reduces *TGFB1* expression (Figure 9B upper panel). The acetylation of histone H3 at the TGFB1 locus follows this trend, with less acetylation at the TGFB1 locus in NKCs treated with both cytokine and lunasin. These results indicate that lunasin is able to modulate the AcH3 at target gene loci.

Histone mark H3K9me3, tri-methylated histone H3 at lysine 9, is commonly associated with transcriptional repression [114]. Using ChIP, H3K9me3 was examined at the *IFNG* and *TGFB1* loci but no alterations in methylation, regardless of stimulation of IL-12 or lunasin, were found (Figure 16B). This suggests that IL-12 does not alter H3K9me3 levels at these loci, and the addition of lunasin generates no additional effects.

3.3 Discussion

Lunasin is a 43 amino acid peptide present in wheat, barley, and soybean [90]. It has been touted largely for its cancer preventative properties [90, 112, 115], relatively little research, however, has been done on how lunasin affects immune cells. This study explores the use of lunasin as an immunomoduatory agent and specifically addresses how lunasin and selected cytokines alter NKC behavior. Lunasin was used in varying combination with IL-2 and IL-12. The combination of lunasin and selected cytokine, lunakine, was found to have significant immunomodulatory effects, which include increasing *IFN*γ production and the natural cytotoxicity of NKCs (Figures 8, 12). Lunakine accomplishes this immunomodulation through the upregulation of a number of genes needed for NKC activity (Figure 8C, 9) via what is most likely an epigenetic mechanism. Lunakine's ability to enhance NKC activity makes it a novel therapeutic and a promising new biologic for cytokine based therapies.

*IFN*γ is a pro inflammatory cytokine necessary for effective immunosurveillance [116]. It has been noted for its ability to inhibit angiogenesis [117], and has been linked to the development of CD4+ T helper type 1 (Th1) cells [118, 119]. The production of *IFN*γ and the lysis of target cells are two of the primary functions of NKCs. Additionally, NKCs produce GM-CSF, which has pro inflammatory and immune cell recruitment and development properties. NKC stimulation by lunakine induces enhanced production of GM-CSF and *IFN*γ, as well as the chemokines CCL3 and CCL4 (Figures 8, 9). All of which creates an unfavorable environment for tumor growth.

Lunasin was titrated against different concentrations of IL-2 and IL-12. It was found that in the presence of lunasin lower concentrations of cytokine could induce *IFN*γ production at levels similar to that of a 10 fold higher concentration (Figure 10A). In order to determine the dose response of lunasin in the presence of cytokine, lunasin was titrated in combination with IL-12 10 ng/mL. The EC_{50} was determined to be 5.64 ± 1.94 μM (n=4) (Figure 10B). This result suggests that the addition of lunasin to a therapeutic cytokine treatment could allow the concentration of cytokine to be decreased without abating its stimulatory capacity.

Previous studies have indicated that heavily treated bone marrow transplant and chemotherapy patients acquire a STAT4 deficiency, which leads to impaired *IFN*γ production [98, 106]. IL-12 induces *IFN*γ production via the STAT4 transcription factor in NKCs and T cells [97, 108, 109]. STAT4 is deficient but not null in these patients which is why the stimulation of PBMCs with IL-12 still leads to *IFN*γ production, though at lower levels than in healthy cells [120]. Flow

cytometry indicates that, when treated with lunakine, STAT4 deficient cells can be induced to produce *IFN*γ at similar levels to that of normal cells treated with cytokines alone (Figure 12). Given the lunakine's ability to enhance *IFN*γ production in STAT4 deficient PBMCs it is possible that lunakine could enhance the immune system of recovering cancer patients who have undergone chemotherapy or bone marrow transplants and acquired a STAT4 deficiency.

Along with cytokine production, the lysis of tumor cells is one of the main functions of NKCs. One way that NKCs accomplish this task is through the release of lytic granules containing, among other things, a serine protease called granzyme B [121]. Granzyme B is constitutively expressed in NKCs and can be up-regulated by multiple cytokines including IL-12 and IL-2 [81, 122]. Adding lunakine upregulates *GZMB* expression more than either IL-2 or IL-12 alone (Figure 9A, upper panel). Such upregulation is corroborated by cytotoxicity assays which demonstrate that NKCs stimulated with lunakine have more killing capacity than NKCs stimulated cytokine alone (Figure 13A). These results have implications on adoptive cellular therapies which require the *ex vivo* stimulation of cells. NKC based cellular therapy has been employed clinically for several cancer types including acute myeloid leukemia, renal carcinoma, and malignant melanoma [15, 75]. In order to determine if lunakine stimulation could be applied to this type of therapy a xenographt model, using NSG mice, was utilized. The mice were infused with a human B cell lymphoma, Raji, followed by human NKCs a day later. The results showed that mice injected with lunakine treated NKCs were best able to fight tumor growth (Figure 13B), supporting its potential use in cellular therapy.

Lunasin's mechanism of action is still uncertain, but there are numerous pieces of data suggesting that it is epigenetic in nature. Lunasin works synergistically with cytokines to alter the expression of several genes (Figure 8C, 9). The modulation of multiple genes suggests an underlying mechanism. IL-12 and IL-2 induce *IFN*γ production by NKCs, but show an enhanced production capacity when added together with lunasin (Figure 8 A-C). Lunasin by itself shows little ability to induce *IFN*γ production. GZMB and CSF-2 (GM-CSF) expression is upregulated in NKCs stimulated with IL-12 or IL-2, but is enhanced even more with the addition of lunasin (Figure 9A), while lunasin alone is comparatively ineffective. TGFB1 is downregulated by IL-12 [107], and once again lunasin seems to enhance this effect (Figure 9B upper panel). Lunasin's ability to work synergistically to enhance cytokine action but not induce strong results of its own indicates that it is somehow stabilizing or prolonging the effect of these cytokines. This type of

synergy is not always the case however. It should be noted that lunasin alone is able to upregulate CCL3 and CCL4 chemokines (Figure 9C), indicating that lunasin has many yet undefined properties.

IL-12 signals through a STAT4 pathway and is required for *IFN*γ production [97, 108, 109] and IL-2, though a less potent inducer than IL-12, can also activate the STAT4 pathway [110]. Because lunasin enhances the effect of IL-12 (Figure 8 A-C) it is possible that increased STAT4 activation is involved in the synergistic induction of *IFN*γ. Activated STAT4 (pSTAT4) was measured over two time points. Both the 3 and 22 hour time points showed an increase in pSTAT4 levels when treated with lunasin, for either IL-2 or IL-12 conditions (Figure 11 A, B). The IL-12 plus IL-2 condition had similar levels of pSTAT4 as compared to the two cytokines plus lunasin at the early time point but the condition with lunasin had higher pSTAT4 levels at the later time point. This result suggests that adding lunasin to cytokine stimulation can enhance over all STAT4 activation in cytokine treated cells, and that this enhancement can last well after the initial stimulation.

Numerous cell types, including NKCs and cancer cell lines, have integrins capable of binding to lunasin's RGD motif [92]. It has been noted in earlier studies that lunasin enters some cancer cell lines via this motif [94]. In order to determine if lunasin was entering NKCs in the same manner, a mutant peptide with no RGD sequence was produced (Figure 14A) and used to stimulate NKCs. The results showed no difference between the mutant and the wild type lunasin (Figure 14B), indicating that the RGD motif was not a required part of lunasin's mechanism. In order to determine if lunasin was entering the cell, NKCs were stimulated with FITC conjugated lunaisn and IL-12 (10 ng/mL) overnight and stained with a Hoechst DNA stain 30 minutes prior to visualization. The results indicate that lunasin can pass both the plasma and nuclear membrane of NKCs (Figures 15 A, B).

The acetylation of histone H3, AcH3, is a well-recognized mark of an active gene locus [113]. It has been reported that lunasin can bind to histone acetyltransferases (HATs) [112] as well as to deacetylated histones in cancer cell lines [94]. Lunasin's capacity to work synergistically with IL-2 and IL-12 to affect multiple genes (Figure 8C, 9), its localization to the NKCs nucleus (Figure 16), and its ability to alter the acetylation of histones in other cell types [91, 112], all indicate a likely epigenetic mechanism. To test this hypothesis, a ChIP assay was performed to evaluate changes in AcH3 at the *IFNG* locus after stimulation with lunakine and cytokine alone (Figure 16A). NKCs treated with lunakine show higher levels of acetylation at the *IFNG* locus than cytokine alone. IL-

12 suppresses *TGFB1* expression in NKCs, and stimulation with lunakine further reduces *TGFB1* expression (Figure 9B upper panel). The acetylation of histone H3 at the TGFB1 locus follows this trend, with less acetylation at the TGFB1 locus in NKCs treated with lunakine, as compared to cytokine alone. These results indicate that lunasin is able to modulate the AcH3 at target gene loci, created by initial cytokine exposure such as IL-12, thereby enhancing or prolonging the activation of target genes. To support this notion, we observed minimal amounts of the repressive H3K9me3 modification at either locus (Figure 10B) regardless of treatment with cytokine or lunakine, suggesting that H3K9me3 is not involved in IL-12-mediated gene regulation in NK cells, and adding lunasin has no effects on H3K9me3 to these loci.

Questions remain as to exactly how lunasin enters NKCs and precisely how it is augmenting the epigenetic state initiated by cytokine stimulation. These issues require further study. What is clear is that lunasin enhances the effect of cytokines on NKCs isolated from PBMCs. This finding indicates that lunasin could be used to increase the efficacy of cytokine based therapies without increasing the concentration of cytokines and risking toxic consequences.

3.4 Future Directions

There is still a great deal to be discovered about the effects of the soypeptide lunasin on immunological systems. In this paper it was proposed that lunakine prolongs the activating effects of STAT4 on *IFN*γ production. It was also noted, however, that lunasin alone was enough to induce the production of chemokines CCL3 and CCL4 (Figure 9C). The enhancement of chemokine production could generate significant immunoregulatory potential, and thus warrants further study. The enhancement of already existing immunotherapies is one of lunakine's most promising areas of use. In this study it was shown that lunasin can rescue *IFN*γ production in STAT4 deficient human PBMCs *In Vitro* (Figure 12). The next step is to utilize lunasin in mouse immunotherapy models, in which mice recovering after cancer treatment, or with a STAT4 deficiency, receive lunakine infusions. Lunasin would seem to have many applications as an immunomodulator which is relevant for individuals with compromised immune systems, and particularly those receiving cytokine therapy. The further study of its effects under various conditions with different cytokines may yet yield new and innovative treatments.

FIGURES

FIGURES

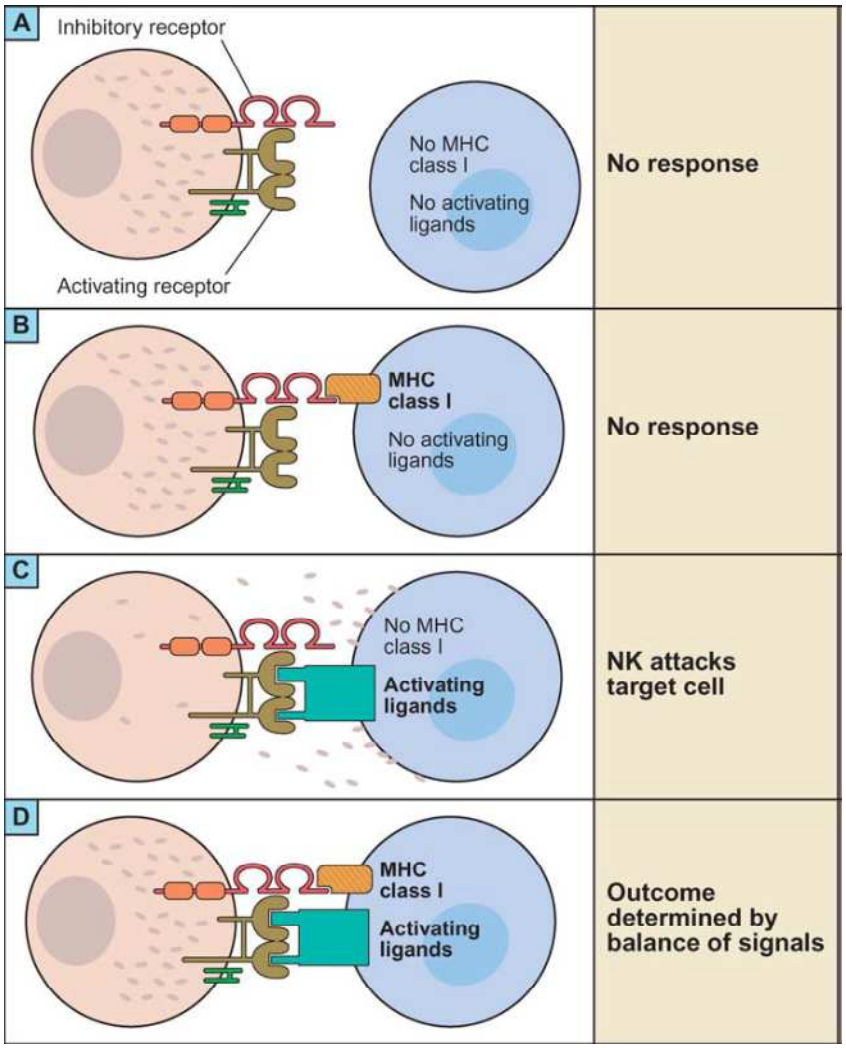


Figure 1. “Missing-self”. Graphic depiction of encounters between NK cells and potential targets and possible outcomes. In some circumstances, inhibitory receptors recognizing ligands other than MHC class I proteins may suppress NK cell responses. When interacting with target cells expressing ligands for both inhibitory and activating receptors, the outcome is determined by the summation of the strength of signals. The amount of activating and inhibitory receptors on the NK cells and the amount of ligands on the target cell, as well as the qualitative differences in the signals transduced, determine the extent of the NK cell response. Figure from Lanier [4].

Gene	Other Names	CD	Function	Signaling	Ligand
TYROBP	DAP12, KARAP		Activation	Syk, ZAP70	N/A
HCST	DAP10, KAP10		Activation	p85 PI3K	N/A
FCE1G	FcεRIγ		Activation	Syk, ZAP70	N/A
LILRB1	ILT2/LIR1	CD85j	Inhibition	ITIM	HLA-A,B,C,E,F,G, CMV UL18
KIR3DL3		CD158z	Inhibition	ITIM	?
KIR2DL3		CD158b2	Inhibition	ITIM	HLA-C S77/N80
KIR2DL2		CD158b1	Inhibition	ITIM	HLA-C S77/N80
KIR2DL1		CD158a	Inhibition	ITIM	HLA-C N77/K80
KIR2DL4		CD158d	Inhibition/ Activation?	FcεRIγ/ITIM?	HLA-G?
KIR3DL1		CD158e1	Inhibition	ITIM	HLA-Bw4
KIR3DS1		CD158e2	Activation	DAP12[a]	?
KIR2DL5A		CD158f	Inhibition	ITIM	?
KIR2DL5B			Inhibition	ITIM	?
KIR2DS3			Activation	DAP12	?
KIR2DS5		CD158g	Activation	DAP12	?
KIR2DS1		CD158h	Activation	DAP12	HLA-C, weakly
KIR2DS2		CD158j	Activation	DAP12	?
KIR2DS4		CD158i	Activation	DAP12	HLA-C, weakly
KIR3DL2		CD158k	Inhibition	ITIM	HLA-A?
KLRD1/ KLRC1		CD94/ NKG2A	Inhibition	ITIM	HLA-E
KLRD1/ KLRC2		CD94/ NKG2C	Activation	DAP12	HLA-E
KLRC3		NKG2E	?	?	?
KLRC4		NKG2F	?	?	?

[a]Based on sequence similarities, KIR2DS1, KIR2DS3, KIR2DS5, and KIR3DS1 probably associate with DAP12; however, this has formally been shown only with KIR2DS2 and KIR2DS4. The KIR2DL and KIR3DL all express ITIM and are assumed to transmit inhibitory signals. NKG2B is a splice variant of the *NKG2A* (*KLRC1*) gene and NKG2H is a splice variant of the *NKG2E* (*KLRC3*) gene.

Figure 2. Human NK receptors (and their signaling adapters) for MHC class I. Figure from Lanier [4].

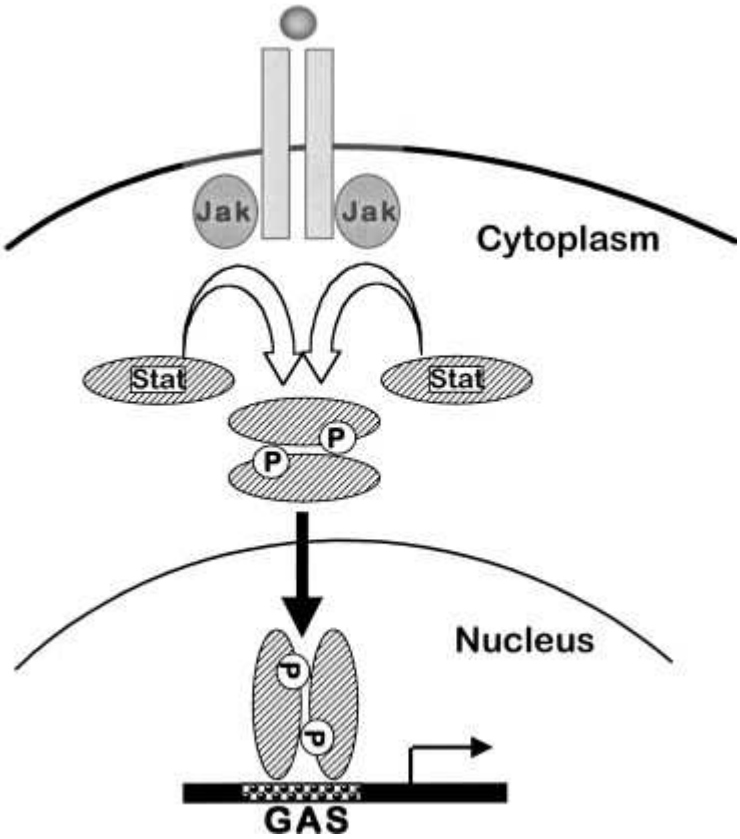


Figure 3. The JAK/STAT Signaling Pathway. When a cytokine ligates its receptor the cytokine receptor's associated JAKs become activated. The JAKs mediate the phosphorylation of tyrosine residues and recruited STATs. Activated (phosphorylated) STATs are released from the receptor, dimerize, and translocate to the nucleus where they bind to gamma associated sites (GAS) and initiate gene transcription. Figure from Kisseleva [35].

Ligands	Jak kinases	STATs
IFN family[a]		
IFN-αs[b]/β/ω/Limitin	Tyk2, Jak1	**Stat1, Stat2,** (Stat3, Stat4, Stat5)
IFN-γ	Jak1, Jak2	**Stat1**, (Stat5)
IL-10	Tyk2, Jak1	**Stat3**
IL-19	?	?
IL-20	?	**Stat3**
IL-22	?	**Stat3**, (Stat5)
gp130 family		
IL-6	Jak1, Jak2	**Stat3**, Stat1
IL-11	Jak1	**Stat3**, Stat1
OSM	Jak1, Jak2	**Stat3**, Stat1
LIF	Jak1, Jak2	**Stat3**, Stat1
CNTF	Jak1, Jak2	**Stat3**, Stat1
NNT-1/BSF-3	Jak1, Jak2	**Stat3**, Stat1
G-CSF	Jak1, Jak2	**Stat3**
CT-1	Jak1, Jak2	**Stat3**
Leptin	Jak2	**Stat4**
IL-12	Tyk2, Jak2	**Stat4**
IL-23	?	**Stat4**
γC family		
IL-2	Jak1, Jak3	**Stat5**, (Stat3)
IL-7	Jak1, Jak3	**Stat5**, (Stat3)
(TSLP)[c]	?	Stat5
IL-9	Jak1, Jak3	**Stat5**, Stat3
IL-15	Jak1, Jak3	**Stat5**, (Stat3)
IL-21	(Jak1), Jak3	Stat3**, Stat5, (Stat1)**
IL-4	Jak1, Jak3	**Stat6**
(IL-13)[c]	Jak1	**Stat6, (Stat3)**
IL-3 family		
IL-3	Jak2	**Stat5**
IL-5	Jak2	**Stat5**
GM-CSF	Jak2	**Stat5**
Single chain family		
EPO	Jak2	**Stat5**
GH	Jak2	**Stat5**, (Stat3)
PRL	Jak2	**Stat5**
TPO	Jak2	**Stat5**
Receptor tyrosine kinases		
EGF	(Jak1, Jak2)	Stat1, Stat3, Stat5
PDGF	(Jak1, Jak2)	Stat1, Stat3
CSF-1	(Tyk2, Jak1)	Stat1, Stat3, Stat5
HGF	?	Stat1, Stat3
G-protein coupled receptors		
AT1	Jak2	Stat1, Stat2

[a] mda7 and AK155 have not yet been functionally characterized.
[b] There are 12 IFNαs.
[c] Bind to related but γc independent receptors.

Figure 4. Receptor Families and JAK/STAT Pathways. Figure from Kisseleva [35].

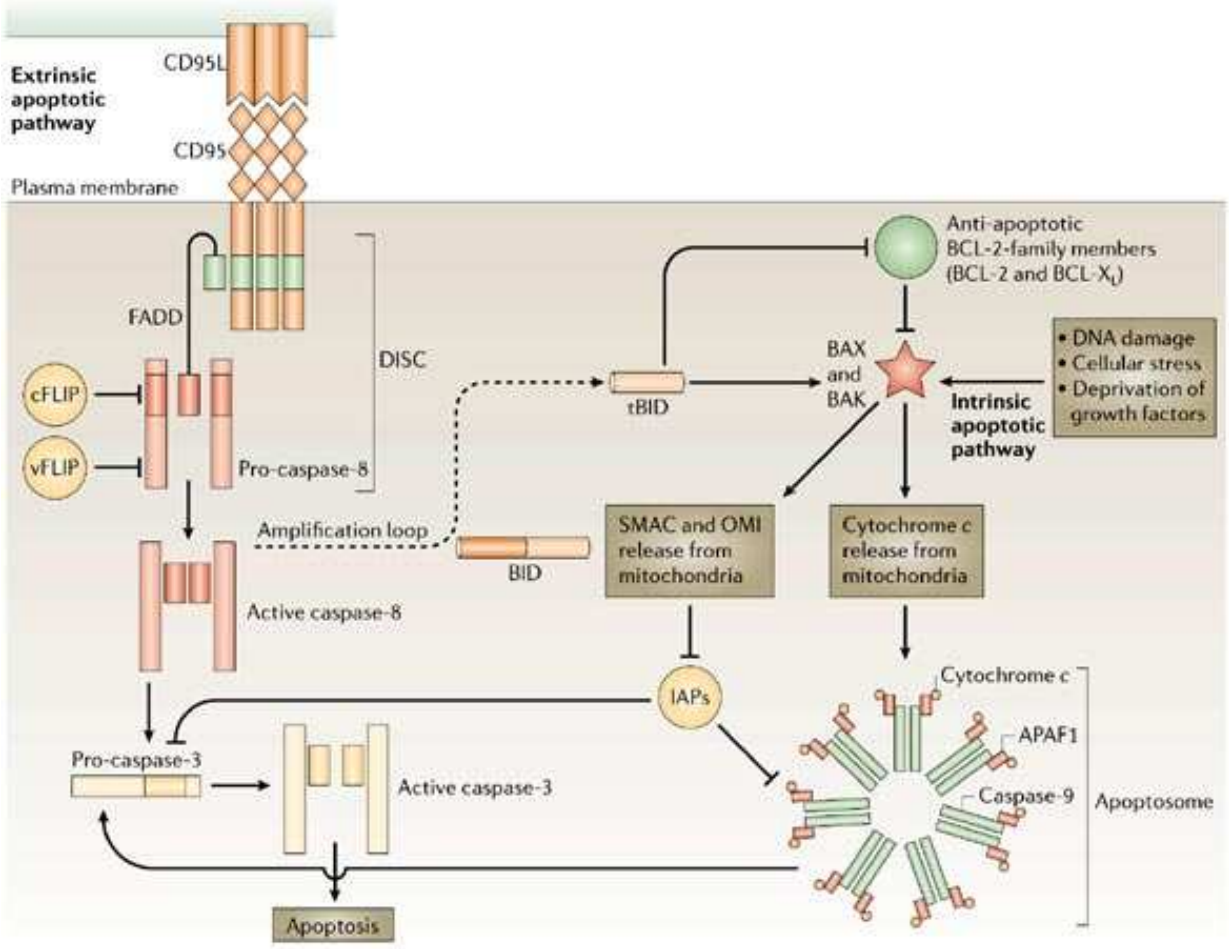


Figure 5. Death Receptor Apoptosis. Fas (CD95) is used as an example for the purpose of demonstating the extrinsic apoptotic pathway. The FasL (Fas ligand) on the immune cell will ligate a Fas receptor on the target cell. The ligation will trigger the binding of FADD, an adaptor protein, which will in turn associate with procaspase-8. A DISC, the death inducing signaling complex, will then form and cleave procaspase-8 leading to its activation, followed by the activation of caspase-3, a potent catalyst in the cleavage of cellular proteins, ultimately leading to cell death [60-63]. Figure from Siegel [123].

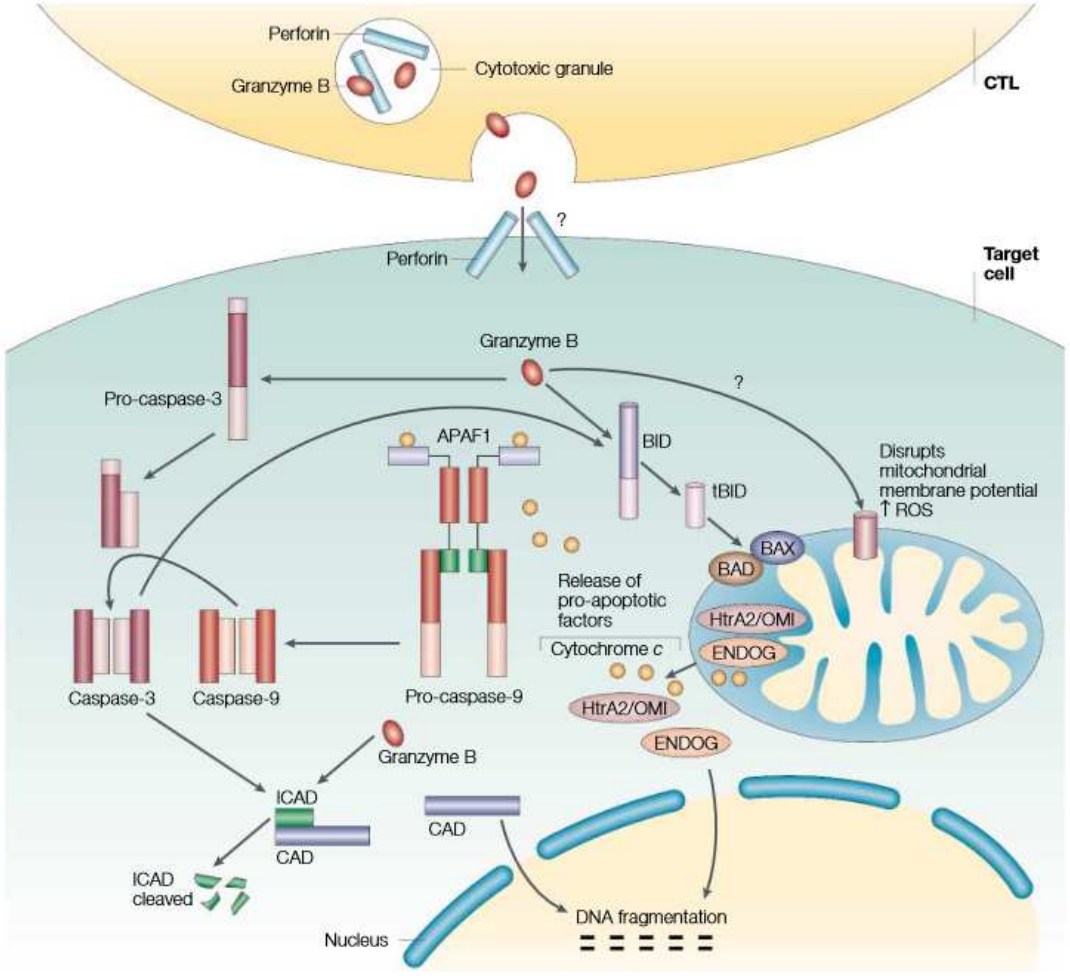


Figure 6. Granzyme B Apoptosis. Perforin is generally recognized as providing the mechanism for granzyme to enter the cell, though the exact method through which it does this remains a subject of debate [65]. Once in the cell's cytoplasm granzyme B can work though several pathways including the direct activation of caspase 3, which leads to apoptosis, and the disruption of the mitochondrial membrane which leads to the release of cytochrome c and the eventual activation of caspase 3. Figure from Lieberman [124].

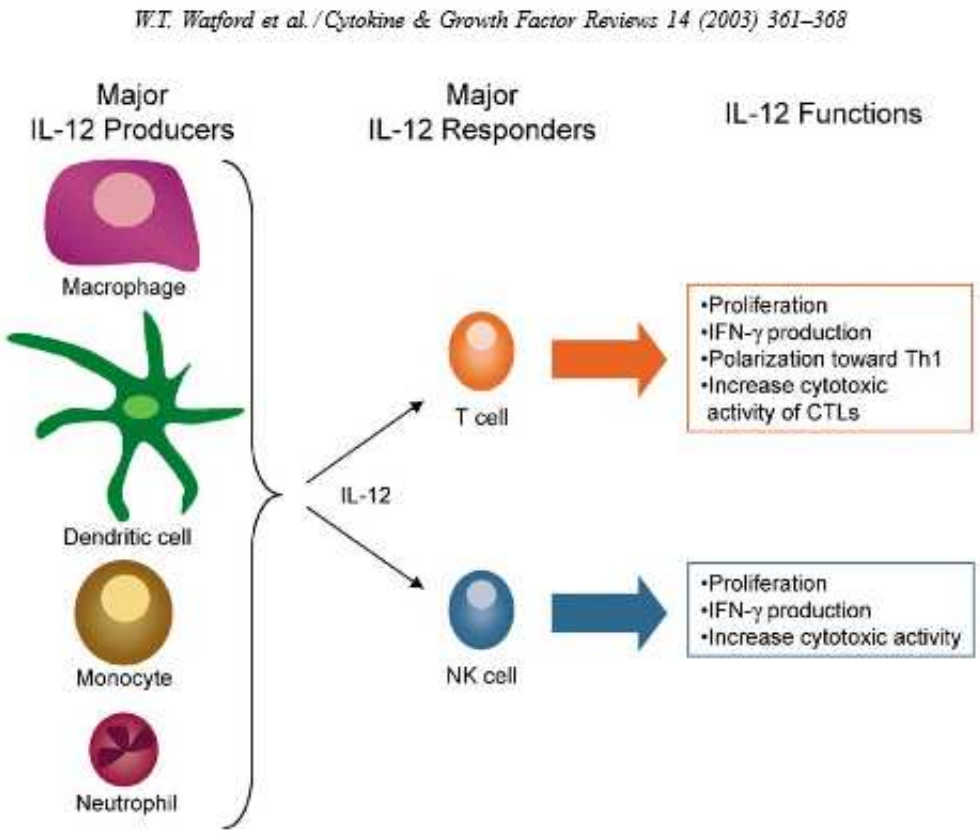


Figure 7. Cellular Sources and Responders of IL-12. Antigen-presenting cells and phagocytic cells, including monocytes and macrophages, dendritic cells, and neutrophils, are the primary producers of IL-12. The major actions of IL-12 are on T and NK cells. IL-12 induces proliferation, interferon production and causes increased cytotoxic activity in these cells. Additionally, IL-12 induces the differentiation of CD4+ T cells to the Th1 phenotype which is important in pro inflammatory responses. Figure from Watford [105].

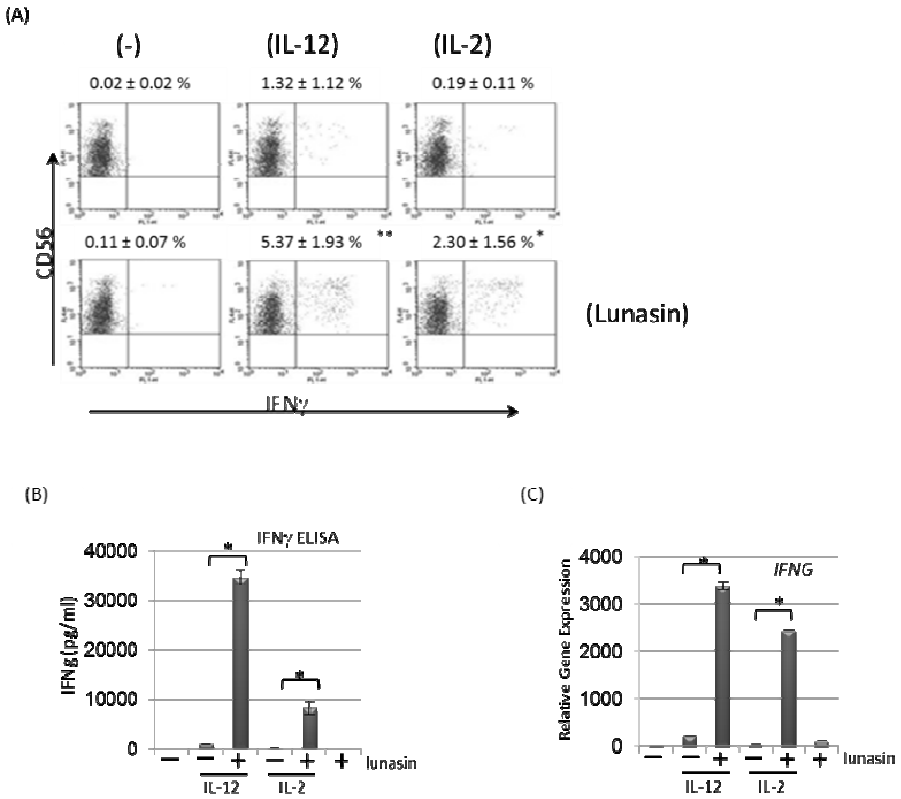


Figure 8. Effects of Lunasin on IFNγ Production by Human NKCs. Peripheral blood mononuclear cells (PBMCs) of normal controls were stimulated with medium only (-), lunasin at 20 µM (lu), cytokine IL-12 at 10 ng/ml or IL-2 at 100 units/ml, and cytokine plus lunasin for 24 hrs. The lunasin peptide was chemically synthesized by LifeTein (South Plainfield, NJ). The production of IFNγ at single cell levels was analyzed using intracellular cytokine staining (A). At the last 6 hrs of stimulation, golgistop (monensin) was added to block the secretion of IFNγ. Stimulated PBMCs were surface stained with FITC-conjugated CD3 and PE-conjugated CD56 monoclonal antibodies (BD), washed, fixed, and permeabilized. After washing, cells were incubated with APC-conjugated anti-IFNγ monoclonal antibody. Expression of IFNγ was evaluated using flow cytometry on 5000 events of gated CD3 negative and CD56 positive NKC populations. The % of NK populations producing IFNγ is labeled at the top of each dot plot in the parenthesis. An asterisks (*) indicates a p value < 0.05 for the same condition without luansin, (**) indicates a p value < 0.01, as calculated by IBM SPSS. n = 6 for all conditions. (B) The secretion of IFNγ by purified NKCs following stimulation was analyzed using ELISA. Freshly isolated human NKCs from PBMCs of normal controls using positive selection with CD56 magnetic beads (Miltenyi Biotec, Auburn, CA) were stimulated as in (A). Following 1 day of stimulation, cell-free supernatants were evaluated for IFNγ production. *P<0.05. (C) Analysis of IFNG gene expression. The cell pellets collected from (B) were resuspended in Trizol Reagents for total RNA extraction. The first-strand cDNA was synthesized followed by real time qPCR using Taqman Assay with primers for IFNγ (IFNG) in ABI 7300 (Applied Biosystems by Life Technologies, Carlsbad, CA). Data are presented as mean ± SD from duplicates. Results shown are representative over 5 different normal controls. *P<0.05.

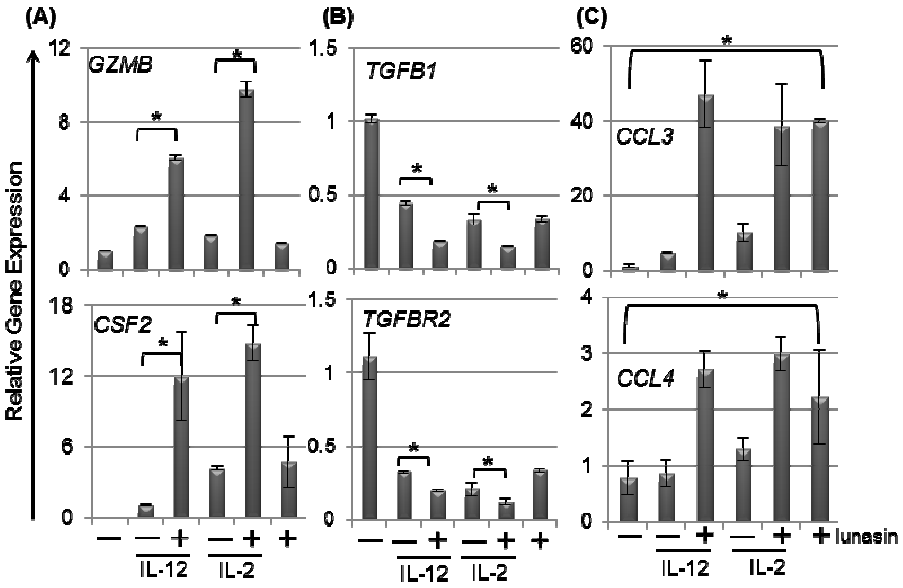
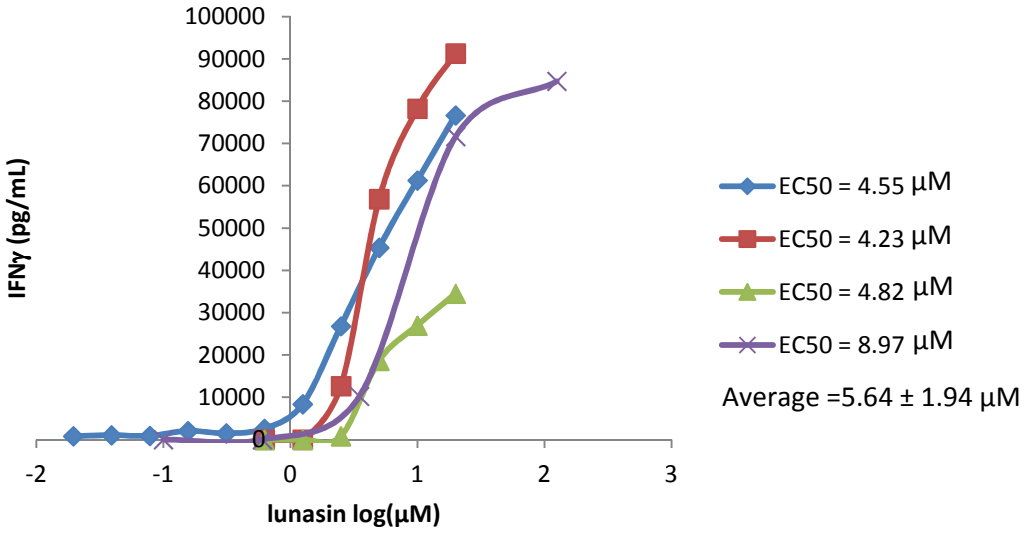


Figure 9. Effect of Lunasin on Gene Expression by Primary Human NKCs. Freshly isolated human NKCs from PBMCs of normal controls (described in Figure 8B) were stimulated with medium only (-), IL-12 (10ng/ml), IL-12 (10 ng/ml) + lunasin (20 μM), IL-2 (100 unit/ml), IL-2 (100 unit/ml) + lunasin (20 μM) or lunasin alone (20 μM). The lunasin peptide was chemically synthesized by LifeTein (South Plainfield, NJ). One day following the stimulation, the cell pellets were resuspended in Trizol Reagents for total RNA extraction. The first-strand cDNA was synthesized followed by real time qPCR using Taqman Assay with primers for granzyme B (*GZMB*), Granulocyte-Macrophage Colony Stimulating Factor (GM-CSF or *CSF2*), TGFβ (*TGFB1*), TGFβ receptor (*TGFBR2*), Chemokine (C-C motif) ligand 3 (*CCL3*), and CCL4 (*CCL4*) in ABI 7300 (Applied Biosystems by Life Technologies, Carlsbad, CA). Data are presented as mean ± SD from duplicates. Results shown are representative over 5 different normal controls. *P<0.05.

(A)



(B)

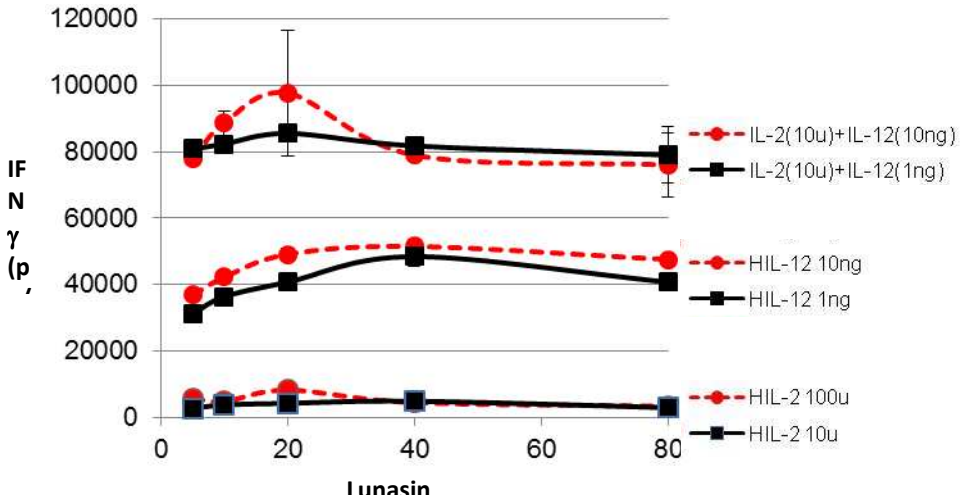


Figure 10. Dose-dependent Effects of Lunasin in Combination With Cytokines. (A) EC_{50} was calculated using the dose response curve function of OriginPro 8.1. NKCs were stimulated with IL-12 10 ng/mL and a half fold lunasin dilution series overnight. The production of IFNγ in the supernatants was determined using ELISA. (B) Human NKCs isolated as described in figure 8B were stimulated with single or both cytokines at low or high doses in the combination with different concentrations of lunasin. One day following stimulation, the production of IFNγ in the supernatants was determined using ELISA. Data are presented as mean ± SD from duplicates. Results shown are representative from over 3 different normal controls.

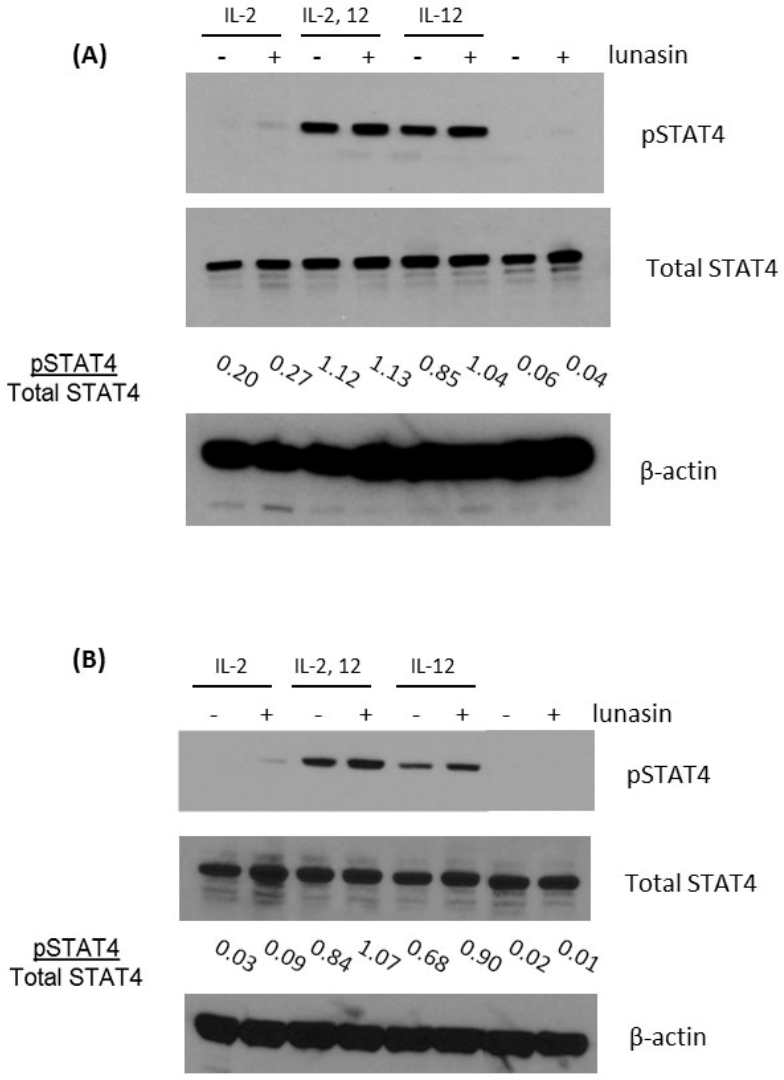


Figure 11. pSTAT4 at Different Time Points After Lunasin-cytokine Stimulation. Human NKCs were isolated as described in figure 8B and then stimulated with single or both cytokines at low or high doses in combination with lunasin. Activation of STAT4 was determined using western blot of total protein extracts from cultured NKCs. Ratios of phospho-STAT4 to total STAT4 (pSTAT4/Total STAT4) are indicated. (A) NKCs were stimulated for 3 hours. (B) NKCs were stimulated for 22 hours. Densitometry determined using ImageJ, U. S. National Institutes of Health, Bethesda, Maryland.

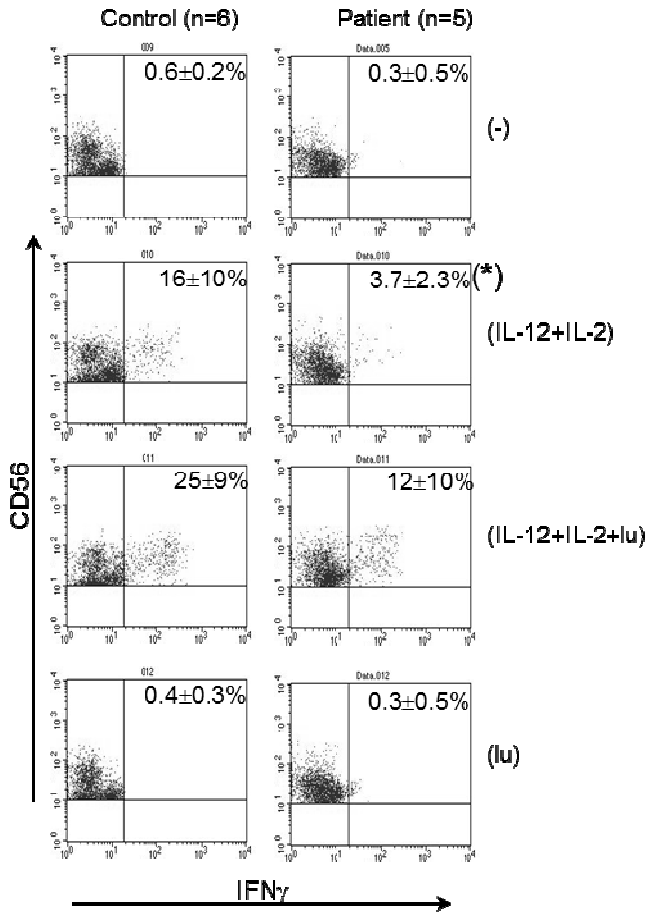


Figure 12. Lunasin Enhances Cytokine-induced IFNγ Production by PBMCs Obtained from Lymphoma Patients After Autologous Stem Cell Transplantation. PBMCs of normal controls and post-transplant lymphoma patients were stimulated with medium only (-), IL-12 (10ng/ml) and IL-2 (100units/ml), IL-12 and IL-2 plus lunasin (lu, 20μM), or lunasin alone (20μM) for 1 day. The production of IFNγ at single cell levels was analyzed using intracellular cytokine staining as described in figure 8A. The % of IFNγ-positive NK populations (CD3 negative and CD56 positive) are presented as mean ± SD from 6 normal controls and 5 patients, which is labeled at the upper right quadrant of the dot plot. *P<0.05, relative to normal controls stimulated with IL-12+IL-2.

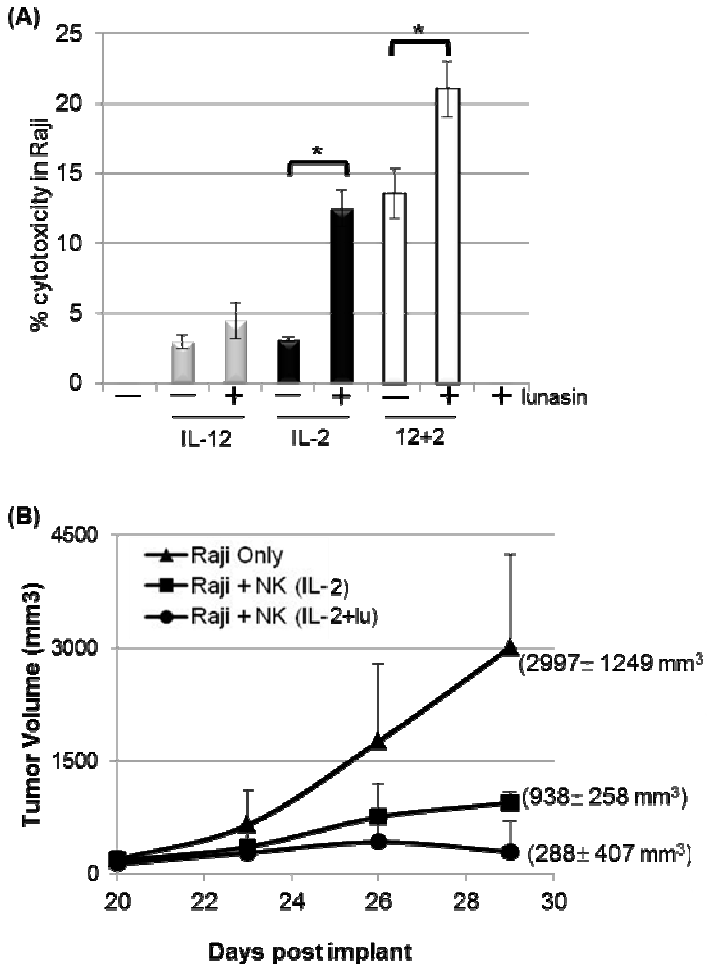


Figure 13. Effects of Lunasin on NKC-mediated Cytotoxicity Against Human B Cell Lymphoma Line. (A) NK-mediated cytotoxicity in vitro. Freshly isolated human NKCs (described in Figure 8B) were stimulated with medium only (-), IL-12 (1ng/ml), IL-12 (1ng/ml) + lunasin (20 µM), IL-2 (10 units/ml), IL-2 (10 units/ml) + lunasin (20 µM), IL-12 (1ng/ml) + IL-2 (10 units/ml), IL-12 (1ng/ml) + IL-2 (10 units/ml) + lunasin (20 µM) or lunasin (20 µM) alone for 1 day. The in vitro cytotoxicity assay was measured using lactate dehydrogenase (LDH)-releasing assay with the CytoTox 96 non-Radioactive Cytotoxicity Assay Kit (Promega, Madison, WI). The effectors were co-cultured with target cells at ratio of 10:1 for 4 hrs at 37C in a 5% CO_2 incubator. The % of cytotoxicity was calculated according to the manufacturer's instructions. Data are presented as mean ± SD from duplicates. Results shown are representative from 3 different normal controls. *P<0.05. (B) NK-mediated cytotoxicity in a human Raji lymphoma xenograft model. NOD/SCID/gc^{null} (NSG) mice at 2 months old were injected subcutaneously on day 1 with 0.5 x 10^6 Raji cells in 0.1 ml PBS mixed with 0.1 ml Matrigel (BD Biosciences, San Jose, CA). NKCs were isolated from the entire leucopack of the same donor and treated with IL-2 (10 units/ml) or IL-2 plus lunasin (lu, 20 uM) for 1 day. On day 2, these treated NKCs were washed and injected to the tumor site (2.5 x 10^6 NKCs/mouse). Tumor growth was monitored, and the volumes were measured using standard manual calipers. Tumor volume (mm^3) is presented as mean ± SD from 3 mice in the group of Raji (no NK), Raji with NK (IL-2), and from 2 mice in the group of Raji with NK (IL-2+lunasin), and values followed by the same letters in each group do not differ significantly (P>0.05) as determined by one-way ANOVA with post-hoc multiple ranking comparison with Tukey's HSD test. Results shown are representative from 2 different normal controls.

(A)

Wild type lunasin:	SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQGRGDDDDDDDDD
Mutant no RGD lunasin:	SKWQHQQDSCRKQLQGVNLTPCEKHIMEKIQG
NC (negative control)	RKMELQEGIHLKKGDQNTQSQSCQPKCIQVWH

(B)

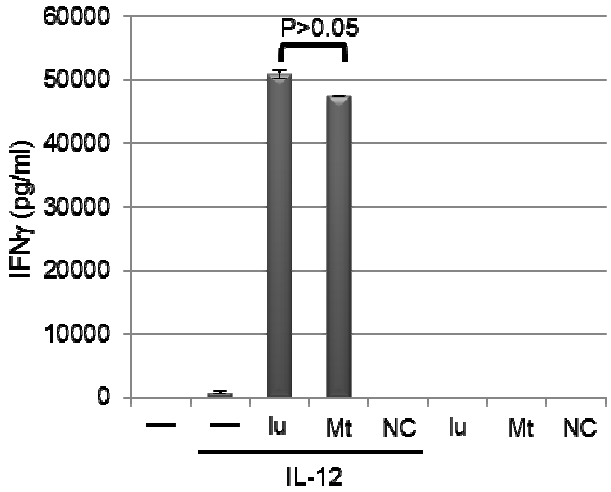


Figure 14. Mechanisms of Synergistic Effects Mediated by Lunasin. (A) The wild type lunasin amino acid sequence followed by a mutant no RGD sequence and a scrambled negative control. All were peptides prepared by LifeTein (South Plainfield, NJ). (B) The RGD motif and poly-D tails are not required for the synergistic effects of lunasin on *IFN*γ production by NK cells. Freshly isolated human NK cells (described in Figure 8B) were stimulated with medium only (-), IL-12 (10 ng/ml) without (-) or with the full-length lunasin (lu), mutant peptide (Mt) lacking the RGD motif and poly-D tail, or negative control (NC) peptide with scrambled amino acids as well as peptides only as indicated. The concentrations of the peptides used were 20 μM. One day following stimulation, the production of *IFN*γ in the supernatants was determined using ELISA. Results shown are representative from over 2 different normal controls.

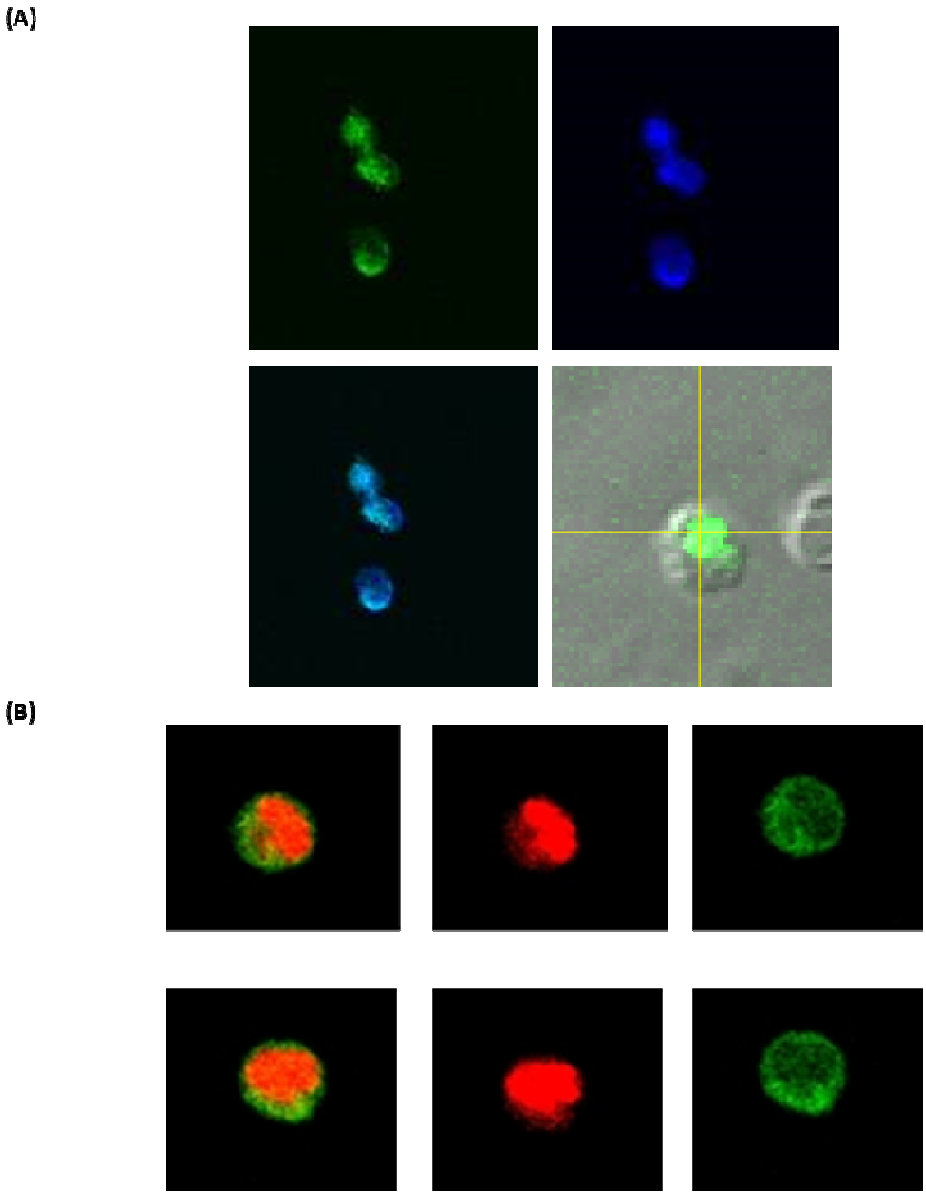


Figure 15. Lunasin Enters the Nucleus of NKCs. NKCs were treated with IL-12 (10 ng/mL) and FITC conjugated lunasin (20 µM)(green) overnight. (A) Hoechst 33342 DNA stain (0.5 mg/mL)(blue) was added 30 minutes prior to visualization at 400x. The two upper panels show independent color channels for FITC (green) and Hoechst 33342 (blue). The lower left panel shows the two channels together and demonstrates the co-localization of lunasin and NKC DNA. The lower right panel shows lunasin inside the nucleus of an NKC. (B) NKCs were fixed in 4% paraformaldehyde and permabilized with ice cold 100% methanol. Histone H3 (red) was stained using primary monoclonal antibodies from Cell Signaling (Danvers, MA) overnight at 4°C. Secondary antibodies (Alexa Fluor 555) were incubated with the NKCs for 1-2 hours at room temperature. Two different cells are shown. The two panels on the left show both channels. The two panels in the middle show only the H3 (red) channel and the two panels on the right show only the lunasin (green) channel.

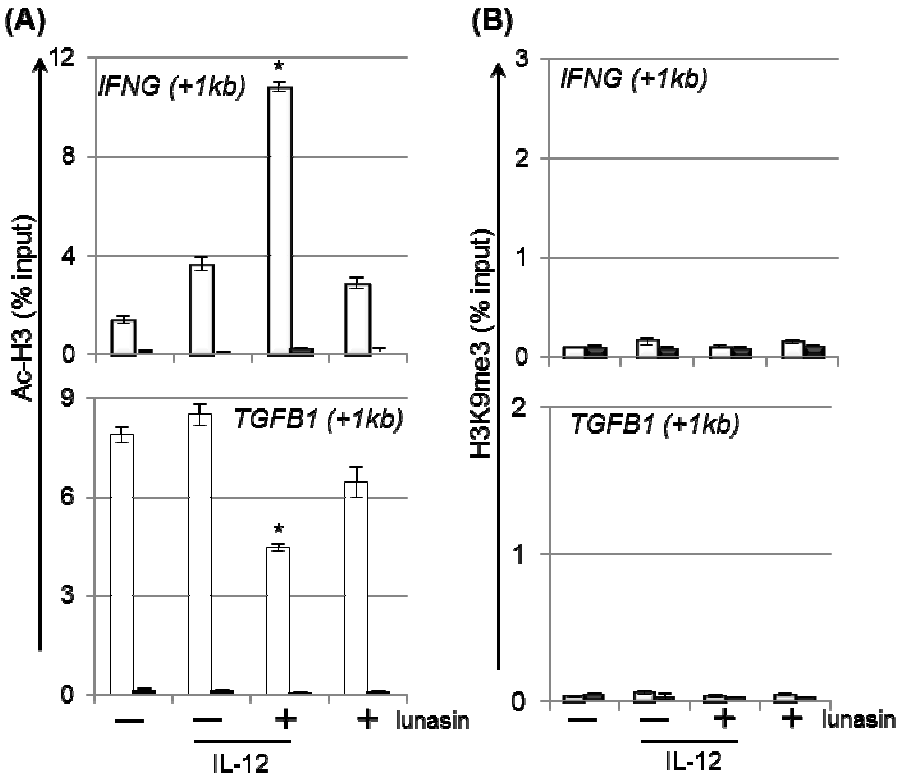


Figure 16. Chromatin Remodeling at the Loci of Target Genes. Freshly isolated human NKCs (described in Figure 8B) were stimulated with medium only (-), IL-12 (10 ng/ml), IL-12 (10 ng/ml) + lunasin (20 μM) or lunasin alone (20 μM). Following 1 day of stimulation, cells were subjected to the ChIP assay. Chromatin DNA fragments were immunoprecipitated with antibodies against acetyl-histone H3 (AcH3) (A) and histone H3 trimethyl Lys9 (H3K9me3) (B) along with non-immune rabbit serum (filled bars) (Millipore, Billerica, MA), individually. The relative degree of histone modification of *IFNG* and *TGFB1* loci was compared by qPCR using ChIP qPCR Primer Assay for human *IFNG* (+1kb) and *TGFB1* (+1kb), respectively (SABioscience Qiagen, Valencia, CA). For calculation of ChIP results, the amount of immunoprecipitated DNA is normalized to the input chromatin in each reaction as a percentage of input (% input). Data are shown as mean % of input ± SD from duplicates. Results are representative from 3 different controls. *P<0.05 relative to NK stimulated with IL-12 alone.

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REFERENCES

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APPENDIX

APPENDIX

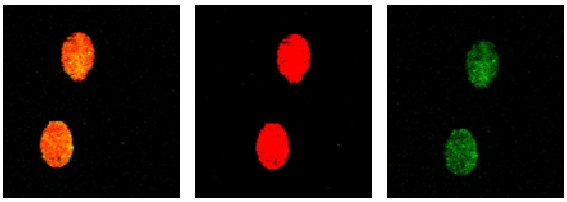

Figure A1. Lunasin in the Endoplasmic Reticulum. – NKCs were stimulated overnight with IL-12 (10 ng/mL) and FITC conjugated lunasin (green) from LifeTein (South Plainfield, NJ). NKCs were fixed in 4% paraformaldehyde and permabilized with ice cold 100% methanol. Calnexin (red), a protein associated with the endoplasmic reticulum, was stained using primary monoclonal antibodies from Cell Signaling (Danvers, MA) overnight at 4°C. Secondary antibodies (Alexa Fluor 555) were incubated with the NKCs for 1-2 hours at room temperature.

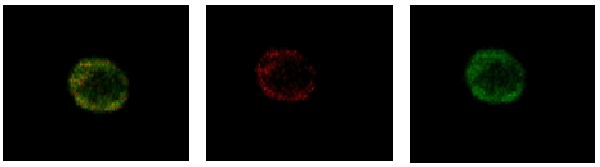

Figure A2. Lunasin in the Autophagosome. - NKCs were stimulated overnight with IL-12 (10 ng/mL) and FITC conjugated lunasin (green) from LifeTein (South Plainfield, NJ). NKCs were fixed in 4% paraformaldehyde and permabilized with ice cold 100% methanol. Autophagosome H3 (red) was stained using primary monoclonal antibodies from Cell Signaling (Danvers, MA) overnight at 4°C. Secondary antibodies (Alexa Fluor 555) were incubated with the NKCs for 1-2 hours at room temperature.