Induction of anti-tumor responses via adoptively transferred, cytokine-gene transduced A-NK cells

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Bachelor of Science, Geneva College, 1999

Submitted to the Graduate Faculty of

the School of Medicine in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

University of Pittsburgh

2008

UNIVERSITY OF PITTSBURGH

School of Medicine

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Induction of anti-tumor responses via adoptively transferred, cytokine-gene transduced A-NK cells

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University of Pittsburgh, 2008

Despite successes in animals, cytokine gene expression selectively in human tumors is difficult to achieve due to lack of efficient delivery methods. We previously demonstrated that adoptively transferred, IL-2 activated natural killer (A-NK) cells are very effective in trafficking to, infiltrating and, subsequently, reducing B16 lung tumors in tumor-bearing animals. We therefore speculated that the tumor-seeking A-NK cells could be used for the delivery of cytokines selectively to the tumor microenvironment. However, tumor infiltration by A-NK cells depends on high and often toxic doses of IL-2 to support the transferred A-NK cells' survival and anti-tumor functions. To address this problem, we hypothesized that A-NK cells transduced to express pro-NK cell cytokines would become less dependent on high and potentially toxic amounts of IL-2. Assessments of transduction efficiency *in vitro* demonstrated that adenoviral transduction consistently resulted in high (>60%) transduction rates and substantial expression of transgenes such as GFP, luciferase, and mIL-12 for at least 4 days. *In vivo* experiments illustrated that mIL-12 transduced A-NK cells localized and survived significantly better than mock transduced cells within lung metastases than in the surrounding normal lung tissue when supported with low, non-toxic doses of IL-2. The intratumoral survival of non-transduced "bystander" A-NK cells also increased when they were co-injected with IL-12 gene-transduced A-NK cells. The enhanced survival of exogenously delivered, IL-12 gene-transduced A-NK cells resulted in greater anti-tumor responsiveness. This led to a 7-10 day

increase in median survival time compared to tumor-bearing mice receiving mock-transduced A-NK cells. These data show that the presence of IL-12 around tumor-infiltrating A-NK cells enhances their anti-tumor activity while reducing their requirement for systemically administered IL-2. This A-NK cell delivered IL-12 has lead to an enhanced host anti-tumor reactivity, which appeared to be mediated through cytokine involvement, namely IFNγ, rather than T, B, and NK cellular activity. Thus, adoptive transfer of A-NK cells represents an efficient method for targeting products of genes to tumor sites and eliciting anti-tumor responses.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I need to acknowledge the very kind support of the Basse lab during my thesis work. Throughout the years, Qin Yang, Patricia Rice, Danielle Cary, Lubov Kublo, and most prominently, Lisa Bailey have helped me along the way. Each of these individuals has been instrumental in helping me reach this stage, from intellectual input to the all important laboratory activities. Next, I would like to express my gratitude to my thesis committee, whose vast expertise has guided and supported me. I would also like to mention and thank all those involved in the Office of Graduate Studies and the Department of Immunology, especially Dr. Olivera Finn and my inclusion on the NIH training grant for three years. Finally, my boss and mentor, Per Basse, has been absolutely incredible in every capacity in my experience as a graduate student. From his eternal optimism to his patience and understanding nature (to name only a few aspects), I am deeply indebted to him.

On a more personal note, there is the huge list of family and friends that have seen me through this major milestone to whom I owe a great deal of thanks. Most prominently are my parents, for their constant support and encouragement; my sister, for being my greatest cheerleader; and my wife, for walking down this road with me, experiencing it all right along with me. Finally, to the author and preserver of my life, Jesus Christ, I owe Him my all. The words of a song that have gotten me through some difficult times in my life, both professionally

and personally, and assure me for the future, say, "...*He has always been faithful. He will be again.*"

1.0 INTRODUCTION

1.1 OVERVIEW

Despite the years of research dedicated to eradicating cancer, it has remained one of the greatest killers in the United States. The difficulty lies in not being able to always translate what works in one person to others or all. In addition to fine-tuning protocols such that they require great specificity, such as with identifying and isolating tumor antigen specific T cells, it would be more advantageous to focus on broader, more general approaches that can generate long term individual specificities in their own capacity.

With continued research it is becoming clear that there are many issues that need to be addressed before widespread success can be expected. Tumors are apparently not considered dangerous by the immune system, as in the case of an infection. In many cases, tumors present antigenic material that is self-derived and therefore not foreign to the host. As a result, there is a certain level of tolerance toward the tumor by the immune system. It may be determined that "danger" signals are necessary to activate and maintain the immune system against this otherwise invisible threat. On the other hand, the tumor may not be recognized by the host due to the presence of immunosuppressive agents and regulatory cells within the tumor that could counteract any potential anti-tumor host response. Chemokines and anti-tumor cytokines may be essential in overcoming the tumor's evasive tactics by attracting immune cells to the tumor while

maintaining their proper function, respectively. The balance could be shifted, therefore, in favor of host anti-tumor immunity and tumor destruction.

Having illustrated what could improve anti-tumor immune reactivity, there needs to be a means to target and deliver these factors to tumors, including those in remote locations. There needs to be distinct specificity to limit the effects to the tumor and not surrounding normal tissues, that could lead to unwanted systemic burdens. IL-2 activated A-NK cells traffic to remote tumors and accumulate to high numbers. The potential of using these cells to act as delivering vehicles is far reaching. Non-protein based entities, such as viral or bacterial danger signals, can be shuttled into A-NK cells successfully with peptide transduction domains (PTDs), while proteins, including cytokines, chemokines, and prodrug activators, can be expressed following viral transductions. Furthermore, the combination of anti-tumor regulators is not restricted to single candidates, as A-NK cells can express multiple viral genes simultaneously. For the purposes of this dissertation, the focus will be on cytokine delivery to tumors by administration of adenovirally transduced A-NK cells. The cytokines chosen are due, primarily, to their pro-NK qualities and, secondarily, their anti-tumor and host stimulatory capacity.

1.2 NATURAL KILLER CELLS

Natural killer (NK) cells have been extensively studied for their ability to defend against pathogenic infections, to bridge innate immunity to adaptive responses, and mediate immunosurveillance of cancers. Comprising 5-15% of circulating lymphocytes, they are large granular lymphocytes of the innate immune system. Unlike T and B cells, NK cells generate inflammatory responses without prior sensitization toward specific antigens, but rather

coordinated stimulatory and inhibitory signals. Once activated by pro-inflammatory cytokines, NK cells exstravasate toward the site of infection to initiate an appropriate response. This can occur through the direct lysis of the target cells, occurring within hours to a day of infection, rather than requiring several days to prime and/or accumulate to sufficient numbers to mount an attack (as with B and T cells), or the release of cytokines and chemokines.

1.2.1 Natural killer cells in innate immunity

1.2.1.1 NK activation

The killing of target cells is a carefully balanced reaction in which normal cells are to be spared while virally-infected and tumor transformed cells are lysed. The surface phenotype of NK cells contains both activating and inhibitory receptors (Figure 1). Under normal conditions, NK cells interact with MHC-I expressed on other cells. This interaction overrides any killer stimulating signals received through activating receptor engagement. In mice, these inhibitory receptors belong to the Ly49 family while in humans they include a family of KIRs as well as CD94/NKG2A. An ITIM motif

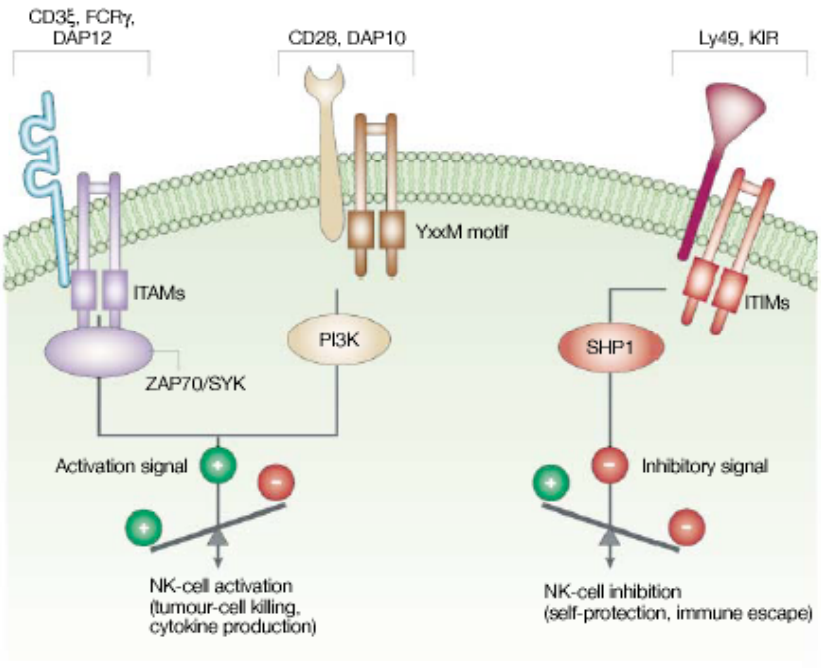

Figure 1. Control of NK-cell functions by the balance of activation/inhibitory signals. ***Smyth, MJ et al. Nat Rev Cancer. 2002. 2(11): 850-61.***

in the cytoplasmic tail of these receptors inhibits further signaling, and ultimately killing capacity.

When the expression of MHC-I is downregulated, in cases of viral infection, the engagement of activating receptors is not inhibited leading to the lysis of target cells, in accordance with the "missing self" hypothesis[1, 2]. These receptors include the human natural cytotoxicity receptors, NKp44, NKp46, and NKp30; mouse Ly49H and Ly49D; and NKG2D in both humans as well as mice[3]. In addition to cell-to-cell contact induced killing, NK cell-mediated lysis can be triggered through antibody dependent cellular cytotoxicity (ADCC). This involves the FcγRIII, CD16, on NK cells interacting with antibodies bound to infected or cancerous cells.

Apart from receptor/ligand communication, several cytokines/chemokines are integral in triggering NK cell responses. Type I interferons, IFNα/β, are produced early by virally infected cells and have been shown to increase the trafficking[4] and cytotoxicity[5] of NK cells. IFNα/β may also have a proliferative effect on NK cells, as does IL-2 (discussed further below) and IL-15[6]. IL-12 alone[7](discussed further below) and in synergy with IFNγ, IL-1α/β, IL-18 or TNFα[8, 9] activates NK cells, leading to the production of IFNγ. Furthermore, MIP-1α/β and RANTES are chemotactic for NK cells and may enhance their cytolytic function[10, 11].

1.2.1.2 Cellular responses

Upon activation, NK cells respond with (1) granule exocytosis; (2) death receptor mediated apoptosis; or (3) indirect killing through the release of cytokines or nitric oxide[12]. Control of viral infections, including MCMV infection, can occur through lysis of infected cells through the release of the pore-forming protein, perforin[13]. The expression of TRAIL[14] on the

surface of NK cells promotes apoptotic cell death pathway. Finally, NK cells influence immune responses with the release of cytokines, such as IFNγ, TNFα and GM-CSF. IFNγ, for instance, is a potent cytokine, generated predominantly by NK cells in response to cytokine stimulation[9, 15]. It aids in the protection against intracellular pathogenic infections[13, 16], which may involve hindering viral replication, inducing MHC molecule upregulation, and/or promoting T helper 1 (Th1) cell immunity. The production of nitric oxide from the IFNγ-induced stimulation of NK cells also contributes to innate immunity by activation of macrophages[17].

1.2.2 Natural killer cells help bridge innate and adaptive immunity

Natural killer cells are involved in shaping adaptive immunity through various mechanisms. After NK cells are recruited to a site of injury they become partially activated in response to cytokines such as type I interferons. The NK cells become cytolytic against the source of the threat, generating antigenic material for immature DCs (iDCs) to take up and process. DCs, in turn, produce pro-NK cytokines, such as IL-2[18] and IL-12, causing NK cells to become more cytolytic and secrete IFNγ, and chemokines that draw more NK cells to the site[19]. Activated NK cells may also begin to proliferate from the interaction with iDCs. The production of IFNγ and TNFα by activated NK cells, and cell-to-cell contact, further matures iDCs[20-22]. The "helper" function of NK cells requires two signals, where IL-2 or type I interferons must accompany target recognition[23]. If the ratio of NK cells to iDCs is high, iDCs are eliminated through interaction with NKp30 on NK cells[22]. However, in cases where the ratio of activated NK cells to iDCs is low, DCs are matured. As DCs mature their increased expression of MHC inhibits lysis by NK cells[22, 24]. Mature DCs (mDCs) then traffic to secondary lymphoid organs

where they initiate adaptive responses with T cell sensitization due to increased MHC/antigen (signal 1) and costimulatory (signal 2) expression by the mDCs.

NK cells can also directly affect the generation of cytotoxic T lymphocytes[25]. This cell contact dependent interaction, involving the expression of co-stimulatory molecules, such as CD86 and OX40L, on the surface of NK cells, as demonstrated by Zingoni et al., can also increase the proliferation and cytokine production of CD4+ T cells[26]. The expression of CCR7 on a subset of human NK cells[27] allows them to traffic to secondary lymphoid tissue where they can influence T cell function, or through DC cross-talk. IL-12-induced expression of IFNγ by NK cells skews T cells toward Th1 immunity[28].

1.2.3 Natural killer cells in cancer immunity

NK cells were discovered when it was found that there existed an inherent natural reactivity towards cancer[29-31] even when specific T cells were removed. Their ability to provide immunosurveillance against tumors was demonstrated with the clearance of tumor cells from the circulation of mice[32, 33] and rats[34]. Later, it was shown that NK cells eliminate MHC I deficient ("missing self") tumors[2]. NK cells have been investigated for their ability to protect against spontaneous tumor formation and have been loosely correlated with better anti-tumor prognosis when NK activity in patients is high[35]. The ability of NK cells to mount immune responses towards established, solid tumors in tissues has been more controversial. The presence of NK cells in various tissues[36], however, as well as enhanced anti-tumor function with cytokine activation[37] points to their potential role in controlling against established tumors. In addition, increasing evidence from NK-depleted mouse experiments has demonstrated, on a number of

occasions, that cytokine therapy is dependent upon NK cells for effective treatment of solid tumors[38-40].

In the same way NK cells control viral infections in innate immunity, they respond against tumors with perforin release, apoptotic pathways, and cytokine secretion. Perforin has been established to mediate tumor cell lysis[41, 42], involved in IL-12 activated killing of tumor cells by NK cells[43], and critical in the immunosurveillance against the formation of various tumors[44, 45]. The expression of FasL and TRAIL on the surface of NK cells promotes tumor cell apoptosis[46]. The apoptotic pathway is induced in many tumors upon engagement of TNF receptors on the tumor cells[47], to the point where NK cells have caused Fas expression by tumors[46]. TNFα has also been shown to mediate rejection of tumors by NK cells[48], while IFNγ production by cytokine stimulated NK cells enhances CTL activity through induction of MHC molecules on target cells and acts as an antiangiogenic agent[49-51].

1.3 INTERLEUKIN-2

Interleukin (IL)-2 acts on a number of cells of the immune system. It is a 15-kDa protein that interacts with the IL-2 receptor made up of a β, γ, and α subunit. The β and γ subunits make up the intermediate affinity receptor, while upregulation of the α subunit (CD25) creates a high affinity receptor. The γ subunit is a common subunit shared by many cytokine receptors including IL-7 and IL-15. Upon receptor engagement, STAT5, in conjunction with JAK1 and JAK3, is phosphorylated and transmigrates to the nucleus for increased survival and proliferation.

1.3.1 IL-2 effects in immunology

1.3.1.1 Pro-inflammatory roles

IL-2 was originally discovered for its ability to proliferate T cells[52]. The production of IL-2, induced primarily in CD4+ T cells, occurs in lymphoid tissue following the co-stimulatory signaling between CD28 on the T cells and B7 molecules (CD80 and CD86) on APCs[53], in conjunction with TCR engagement. Without co-stimulation, therefore, the T cells do not produce IL-2 sufficient to support expansion, even if they receive antigenic stimulation. Activated effector CD8+ and Th1 CD4+ T cells secrete IL-2 promoting their own survival and proliferation. IL-2 also aids in the proliferation of B cells[54, 55] and the activation of monocytes[56] and neutrophils[57]. The impact of IL-2 on NK cells has been shown to be important for their growth and cytotoxic function[58] and proliferation[59]. IL-2 also contributes to NK functionality. Cooper et al. have demonstrated a $CD56^{hi}$ subset of NK cells in humans that produces Th1 (IFNγ) and Th2 cytokines when stimulated by IL-2[60]. In addition to cytokine production, IL-2 has been shown to increase natural killer activity[61].

1.3.1.2 Anti-inflammatory roles

Inasmuch as IL-2 is necessary for proliferative responses in activated effector and help T cells, it is also critical in ensuring the cessation of such responses. One way this is accomplished is through CD4+CD25+ T regulatory cells. IL-2 signaling allows for the development, expansion and survival of CD4+CD25+ T cells[62]. In addition, several studies have demonstrated IL-2 to be essential for the functioning of T reg cells in establishing self-tolerance[63, 64], with the neutralization of IL-2 leading to autoimmunity from impaired T regulatory maintenance and function[65]. Furthermore, IL-2 affects the homeostatsis of CD8+ memory T cells by inhibiting

their proliferation and accumulation[66] through CD4+CD25+ T reg cells[67]. Another means of anti-inflammation is through activation-induced cell death (AICD)[68, 69], where the direct involvement of IL-2 has been shown from experiments using IL-2-/- mice[70]. Continued stimulation by IL-2, along with antigen restimulation, increases the expression of FasL on activated T cells while decreasing the expression of the inhibitor of apoptosis, FLIP, resulting in greater apoptotic T cell death[69]. IL-2 is integral, therefore, in maintaining peripheral tolerance toward self-antigens through the inhibition and/or removal of autoreactive T cells by CD4+CD25+ T regulatory cells and AICD.

1.3.2 IL-2 applications in cancer therapy

Since its discovery as a potent proliferative agent for T cells, IL-2 has been the most widely used cytokine adjuvant in cancer therapy. The treatment of renal cancer is difficult and has been unsuccessful with standard therapies, including chemotherapy. Pioneering work by Rosenberg et al. demonstrated, in a number of clinical trials involving patients with renal cell carcinoma, considerable efficacy using high-dose IL-2 with a response rate around 20-30%[71]. A lack of noticeable responses from the addition of lymphokine activated killer (LAK) cells[72]; application of low-dose IL-2, or coinjection of IFNα[73, 74] has further confirmed the superiority of high-dose IL-2 regimen. High-dose IL-2 has also been used as therapy of melanoma. Similar to renal cancer, initial studies demonstrated IL-2 to be effective in treatment of advanced melanoma when used alone[75].

Although results vary, the use of IL-2 lymphokine activated killer (LAK) cells[76, 77] has been increasingly studied, from enhanced proliferation and killing capacity through IL-2 signaling. More specifically, the identification and use of tumor specific T cells[78] and tumor

infiltrating lymphocytes (TIL)[79-81] has resulted in a considerable response rate. This response from TILs was actually twice that seen with IL-2 alone[82]. Immunotherapy involving IL-2 has also benefited from the application of activated natural killer (A-NK) cells (discussed further below)[37, 83]. Finally, IL-2 in conjunction with various cytokines, such as IL-12[84], IL-18 [85, 86], and IFNγ[87], established IL-2 as a model cytokine in tumor immunotherapy.

IL-2 does not come without its drawbacks. The toxicity generated from high dose IL-2 treatments has limited its application. This can manifest itself in "capillary leak syndrome" in which there is an increased movement of inflammatory proteins and activated lymphocytes towards the interstitial spaces[80, 88]. Although these issues have been reversible, there have been complications less manageable, especially regarding the heart. The toxicity of IL-2 is further compounded when combined with other cytokines, such as IL-12[89]. The benefits of joint cytokine therapy, especially involving IL-2, need to be carefully monitored to offset the concerns of toxicity.

1.4 INTERLEUKIN-12

Interleukin-(IL) 12 is a pleiotropic cytokine involved in many inflammatory responses. It was first identified for its ability to act as a natural killer stimulating factor (NKSF)[7], giving its name originally. It consists of two covalently linked subunits, a p40 and a p35 subunit, making up a 70kDa protein. Signaling through the β1 and β2 subunit, IL-12 receptor, expressed predominantly on NK and T cell, functions through STAT3 and STAT4[90].

1.4.1 IL-12 effects in immunology

1.4.1.1 Innate immunity

IL-12 is produced by APCs, neutrophils, and B cells following interactions with infectious particles or receptors on activated T cells (Figure 2)[91]. Acting as a chemotactic factor[92], IL-12 can draw NK cells to the site of infection and then lead to an increase in cytolytic activity and proliferation[93]. NK cell proliferation seems to occur when IL-2 amounts are low, possibly due to increased IL-2 receptor expression from IL-12 stimulation[94, 95]. IFNγ is produced in high levels by NK cells[96] following IL-12 stimulation. The IFNγ enhances MHC class I expression and antigen presentation on DCs[97] and T cell mediated immunity through T helper cell activation[98]. IL-12 also stimulates NK cells[94], as well as neutrophils, DCs, and B cells, to produce TNFα, IL-6, and IL-18, among others. IL-12 induced TNFα and IL-6 production has been shown to cause DCs to move to secondary lymphoid tissues where they interact with T helper cells[99]. A more direct effect of IL-12 on DCs is their maturation and enhanced antigen presentation[100], through MHC class II upregulation[101]. Each of these direct or indirect activities of IL-12 signaling on innate immunity, therefore, contributes to the initiation of adaptive immunity.

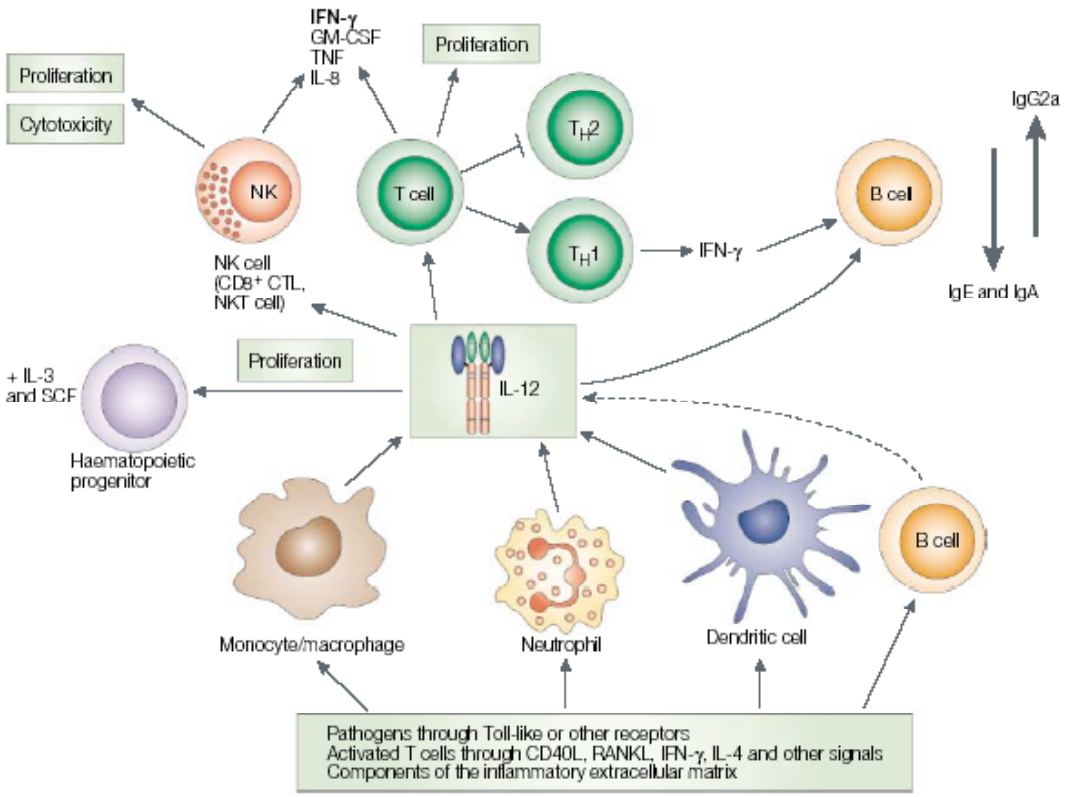

Figure 2. Summary of the biology of IL-12. ***Trinchieri, G. Nat Rev Immunol. 2003. 3:133-46.***

1.4.1.2 Adaptive immunity

IL-12 acts on naïve CD4+ T cells to differentiate them into Th1 cells[98, 102, 103]. It increases their proliferation along with generating a more activated phenotype with proinflammatory cytokine production[104]. Th1 cells express both subunits of the IL-12 receptor whereas Th2 cells lose the β2 subunit, making them unresponsive to IL-12. IL-12 enhances the presence of Th1 cells at the site of its production by maintaining the Th1 cells. This is accomplished with an increase in chemokine receptor (CCR1) and integrin expression, respectively. Following IL-12 stimulation of various cell types, the chemokine IP-10 is induced[105] which can mediate further Th1 cell homing through their selective expression of the

IP-10 receptor, CXCR3. As DCs interact with CD4+ T cells to prime efficient CD8+ cytotoxic T lymphocytes (CTL), their production of IL-12 is critical for this process. This is seen with increased differentiation of CTL, in addition to their cytolytic capabilities[105-108]. In fact, it has been demonstrated that IL-12, along with antigen, can directly expand and differentiate naïve CD8+ T cells[109] and may play a role in the generation of immunological memory[110]. Finally, IL-12 has also been shown to play a role in B cell differentiation[111] and in promoting Th1-associated antibodies like IgG2a directly[112], or via IFNγ[113], or increased production of antibodies in general[114].

1.4.2 IL-12 applications in cancer therapy

The anti-tumor capacity of IL-12 has been overwhelmingly studied. IL-12 has been shown to augment the *in vitro* anti-tumor functions of NK cells, against hematologic and solid malignancy[115], and TILs, toward melanoma and ovarian cancer[116]. In mouse studies, IL-12 promotes tumor regression, e.g. against melanoma, kidney and lung cancer when it is delivered systemically, alone[117-120] or in conjunction with peptide vaccination[121] and DC vaccinations[122]. Unlike IL-2, IL-12 has proven to be less toxic in treatment efficacy[117], but not completely without toxic effects[123]. Further studies have been conducted to establish local delivery of IL-12 to a tumor, most prominently with the gene transduction of tumor cells. This has included breast cancer[124], lung cancer[125], and melanoma[126, 127]; DCs[128, 129]; and fibroblasts[130, 131]. The anti-tumor effects of IL-12, through these various delivery methods, have been shown to be mediated through CD4+ T cells, CD8+ CTLs[119] and NK cells[43], leading to antigen specific immunity; memory recall upon rechallenges and prolonged survival of tumor-bearing mice[132]. As described above, IFNγ is a major down-stream product of IL-12 signaling and therefore has been shown to

contribute to IL-12 anti-tumor functions. When IFNγ is neutralized, IL-12 efficacy is abrogated[119], possibly through decreased immune activity[117] or inhibition of angiogenesis[133, 134].

Although less well supported, there have been a few clinical studies with the administration of IL-12. Melanoma patients receiving peptide vaccine and IL-12 demonstrated greater specific CD8+ T cell responsiveness, with regression of metastatic tumors, than if IL-12 is not given[135]. Other studies have seen the generation of tumor specific CTLs with protein IL-12[136] and varied clinical responses from IL-12 transduced tumor cell[137] and fibroblast[138] therapies.

1.5 TUMOR IMMUNOTHERAPY

In order to initiate effective responses against a tumor, there needs to be the involvement of a well-balanced immune system. When deficiencies exist within the host, intervention must be taken. This can be accomplished through immunostimulation and active or passive immunizations. With active immunization, the goal is to stimulate the endogenous immune system to generate specific immunity against the tumor. Passive immunization, however, involves the delivery of ex vivo-conditioned, activated cells capable of mounting anti-tumor responses without additional support from the host.

1.5.1 Active immunization

1.5.1.1 Cellular vaccines

DCs, presenting various tumor antigens, have been widely used to initiate host responsiveness toward tumors in mice[139-142] and, to a lesser extent, in clinical trials[143, 144]. As discussed above, the DCs can be gene modified to express cytokines. Their success, and alternatively their failure, has been shown to be dependent on the protocol used to mature the DCs, selecting of tumor antigens, and the delivery route and frequency of DC injection[145]. Maturation protocols have involved using a "cytokine cocktail" of TNFα, IL-1β, IL-6 and PGE2[146] or, more recently, a "megacytokine cocktail" of TNFα, IL-1β, PolyI:C, IFNα, and IFNγ[147]. This latter protocol allows for more potent CTL responses. Additionally, DCs can be matured in situ when antigen loaded immature DCs are injected into hosts pretreated with adjuvant[148]. Choosing the appropriate antigens to be loaded onto DCs to prevent autoimmunity presents more complexity. A further concern with this mode of tumor therapy involves very insufficient trafficking of DCs to lymph nodes once they have been injected[145] (most often subcutaneously)[149].

1.5.1.2 Non-cellular vaccines

The use of non-cellular vaccines has involved the application of tumor peptides. The identification of tumor-associated peptides has lead to their direct use in tumor therapy. Although anti-tumor results have been attained in mice[150], direct injection of single peptides with complete or incomplete Freund's adjuvant has been relatively ineffective, but not conclusive in ruling out all immunostimulatory signals. Ultimately, multiple peptides and danger signals could serve as better vaccine modalities[151]. Peptide vaccination with adjuvant and/or cytokine,

predominantly IL-2, IL-12[136] or GM-CSF[152], has generated tumor antigen specific CTL responses in patients with reactivity against melanoma, as well as cancers of the colon, ovary, and breast. The overall clinical responses, however, have been limited, even when DCs were first pulsed with peptides ex vivo[151].

Another technique for initiating host immunity has been the administration of immunostimulatory agents. IL-2 and IL-12, as discussed above, have been used in clinical trials. In similar fashion, other cytokines have been incorporated into pre-clinical and clinical trials[153]. IFNα, for example, has been systemically administered in the treatment of melanoma. Kirkwood et al. have shown an increased survival following tumor resection in melanoma patients receiving IFNα[154]. Systemic delivery of GM-CSF has also generated therapeutic results[155, 156]. Local presence of cytokines within the tumor has become a more appealing and effective approach in anti-tumor therapy. It has become evident that not only the more prominent candidate cytokines (IL-2, IL-12, and GM-CSF), but also Th2 cytokines, like IL-4 and IL-6, have proven effective in tumor therapy. This has been accomplished through intratumoral cytokine injection, intratumoral injection of cytokine-gene containing viruses, and ex vivo transduction of tumor cells[153]. Although each method can be effective, by and large, systemic cytokine strategies have not gained much ground, due to greater concerns from toxicity, while local presence relies on tumors being accessible for direct injection reducing their applicability for hard to reach tumors.

1.5.2 Passive immunization

1.5.2.1 T cell adoptive therapy

With the development of efficient methods for culturing and propagating tumor antigen specific T cells there has been increasing interest in using them in adoptive immunotherapy. CD8+ T cells stand as the prime candidate for cellular adoptive therapy in that they possess a distinct tumor specificity through their TCR with the potential of establishing long term memory, persisting for a long time within the host, and being genetically altered. Isolated from blood, tumors (TILs), or draining lymph nodes, tumor reactive T cells have been identified with specificity against overexpressed or mutated self-antigens that act as tumor-associated antigens (TAAs) including MUC-1, MAGE, and MART-1. Once identified, the T cells are expanded to sufficient numbers to overcome the tumor burden[157]. Basic ex vivo T cell stimulation protocols involve the use of anti-CD3 antibodies and IL-2 to induce clonal expansion[158, 159]. Additionally, irradiated peripheral blood mononuclear cells, or feeder cells, serve to present antigen. The cells are then reinfused back into the patient along with IL-2 coadminstration (as described above).

There are certain concerns attached to T cell adoptive therapy, however, that has lead to greater attention in their implementation. For one thing, advances in animal studies have not correlated as easily in clinical trials due to variability between antigens and greater self-tolerance in humans. The availability of vital cytokines *in vivo* can be competed out with the presence of other immune cells. The creation of this "cytokine sink" makes it difficult to amplify the anti-tumor responses when cytokines, such as IL-7 and IL-15, are consumed by endogenous, primarily NK[160], cells. Just as IL-2 is essential to CTL function, Treg cells depend on it heavily[63, 161], resulting in the increase of Treg numbers in cancer patients treated with IL-2[162]. Not only can they deplete cytokine resources, Treg cells are also potent inhibitors of CTL

reactivity directly[163, 164]. The differentiation stage of the T cell also affects their ability to respond appropriately[165], seen in mice[166] as well as humans[167]. Traditional CD3/IL-2 protocol for T cell transfer therapy may actually be detrimental to T cell function in that it may push them to a late effector stage ex vivo, making them proliferatively exhausted, prone to apoptosis and less responsive to cytokines than early stage effectors[168].

To circumvent some of these issues, researchers have investigated new ex vivo T cell protocols, genetic modification of the T cells, or host lymphodepletion. Reducing the time – to promote early effector cells[169] – and/or changing the cytokine cocktails, e.g. to include IL-15[170], in which T cells are expanded may enhance the efficacy of the CTLs. In addition to costimulatory molecules and cytokines, naïve tumor specific T cells, which normally express low affinity TCRs, have been transduced to express high affinity forms of these receptors[171, 172], making them more suitable for adoptive therapy. Finally, lymphodepletion removes cytokine competing cells from the host, especially Treg cells[168, 170]. It has been proposed that with the removal of host lymphocytes the body will try to maintain homeostasis by generating pro-lymphocytic cytokines. This cytokine storm can then benefit adoptively transferred cells in their survival and expansion.

1.5.2.2 A-NK cell therapy

It was found that lymphokine activated killer (LAK) cells could be isolated from bulk murine lymphoid preparations by their ability to adhere to plastic when cultured in the presence of IL-2[173]. They could then be further expanded with repeated passage of non-adherent cells onto new plastic. More importantly, as described above, LAK cells have been extensively used in cancer immunotherapy in pioneering work by Rosenberg et al. Their application, however, has been limited by their need for high, and ultimately toxic, doses of IL-2. It was later

determined that these IL-2 activated, plastic adherent cells (A-LAK), were predominantly comprised of activated natural killer (A-NK) cells, confirmed with negative staining for CD8, CD4, and CD3 and positive for the NK markers, asGM1 and NK1.1. A-NK cells once activated with IL-2 remain dependent upon it for their survival, proliferation and cytotoxic function[58, 61].

Adoptively transferred A-NK cells are capable of trafficking to established tumors. Within hours of intravenous injection into tumor-bearing mice A-NK cells localize within tumor nodules of different origins preferentially over normal lung tissue[174]. The A-NK cells accumulate in numbers over time. As evidenced *in vivo*, IL-2 plays a key role in this process. When no IL-2 is provided, A-NK cells cannot be found infiltrating lung tumors. A constant presence of IL-2, whether it be in the form of recombinant IL-2 given in 4-hour intervals or the more efficient, polyethylene glycol conjugated form (PEG-IL-2) with a longer half-life, provides for significant infiltration and accumulation of A-NK cells but does not depend on how the IL-2 is given[175]. The route of administration of IL-2 bears little significance compared to that of the A-NK cells which has proven to be crucial for selective trafficking to tumors, as systemic injection resulted in substantial numbers of A-NK cells in lung tumors but fewer cells being seen in extrapulmonary tumors, such as liver metastases. Direct injection into the portal vein, however, lead to a persistent presence of A-NK cells in the liver and liver metastases[176, 177].

Basse et al. further demonstrated the ability of A-NK cells to develop direct cell-to-cell contact with melanoma tumor cells in the lung[178]. As A-NK cells infiltrate tumors, this correlates well with anti-tumor reactivity, with numbers reaching levels greater than 1000/mm^2 of tumor tissue (Figure 3). Yang et al. demonstrated that tumors infiltrated by A-NK cells to such an extent can result in a 75-85% reduction in tumor burden compared to controls, in B16 and 3LL tumor models, as early as 120 hours after adoptive transfer. Generating these results

required 2 to 3 days of PEG-IL-2 support at 12-hour intervals[83]. The toxic nature of high dose IL-2, however, remains a major issue for the overall effectiveness of this therapy. If A-NK cells could be manipulated to express IL-2 and/or other cytokines many of the problems mentioned above could be addressed. This would provide an effective means to target specific cytokine therapy to remote tumors, supporting the A-NK cells and host immunity, without toxicity or compromising their efficacy or having to identify tumor antigens.

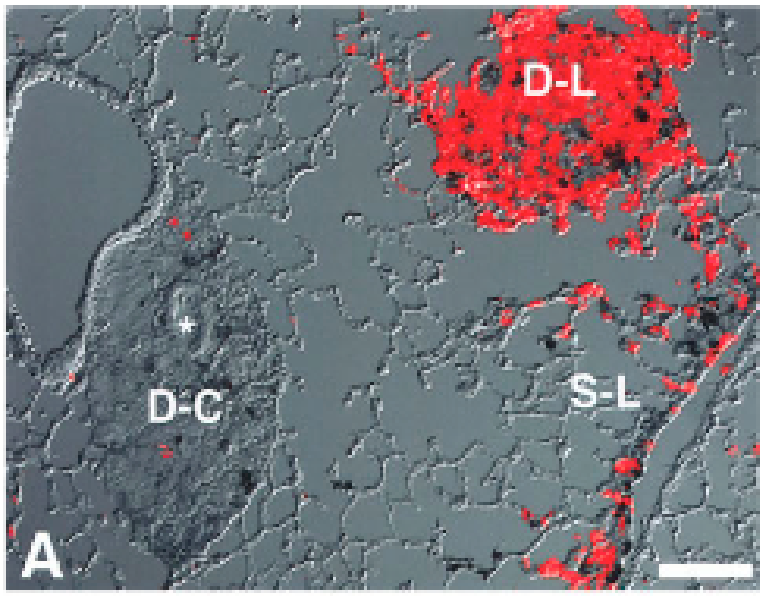

Figure 3. A-NK cell infiltration of B16 tumor metastases. Fluorescence/DIC image of lung tissue from animal treated with $20x10^6$ A-NK cells 24 hours earlier. The adoptively transferred A-NK cells are visualized by immunostaining with PE-conjugated anti-Thy1.1 antibody. Scale bar = 100μm.

SCOPE OF THIS THESIS

We set out to take advantage of the tumor-seeking nature of A-NK cells by cytokine-gene transducing them, such that they would maintain their own support within tumors, and, ultimately, create an environment that would promote further anti-tumor responsiveness. Reducing systemic cytokine toxicity, targeting tumors more effectively, and broad tumor specificity are critical issues that can be addressed using this technique. It should be noted that although we worked with transduced T-LAK cells (i.e., polyclonally activated CD8 cells) with noticeable success (Chapter 2), A-NK cells were the major focus of this thesis.

In chapter 2, we demonstrate successful production of various genes through adenoviral transduction. Production levels were high *in vitro* and correlated well *in vivo*. This was seen with greater survival of A-NK cells within B16 tumor nodules if they had been transduced to express IL-12 before adoptive transfer compared to no transduction. The increase in A-NK cell survival allowed for a prolongation in survival of tumor-bearing mice.

Next, we investigated further the functionality of IL-12-transduced A-NK cells. By transducing A-NK cells with IL-12 we have been able to reduce the amount of exogenous IL-2 administered without compromising their tumor trafficking and subsequent killing. Survival of mice (bearing lung tumors of different origin and age) was significantly greater in those receiving A-NK cells transduced with Ad-IL-12 cells than Ad-mock or mice receiving high dose IL-2 regimen. The latter is not surprising considering the toxic effects of continued IL-2 support.

Finally, we set out to investigate the mechanistic aspect of the A-NK_{12} cell therapy and the role of the host in the above studies. Through the use of NOD-SCID mice we found that increased survival of tumor-bearing mice receiving A-NK_{12} cells was likely not the result of host

T, B, or NK cell function. Despite the inability to significantly increase survival of tumor-bearing mice, the addition of TNFα to A-NK_{12} cell treatment was able to improve the presence of A-NK cells in tumors, while maintaining low exogenous IL-2 support. In other experiments, however, we discovered a role of the host through its production of IFNγ. When recipients of A-NK_{12} cells failed to produce IFNγ, they survived a significantly shorter amount of time.

Cancer Gene Therapy (2007) 14, 441–450
 0929-1903/07 $30.00
www.nature.com/cgt

ORIGINAL ARTICLE

Targeting of products of genes to tumor sites using adoptively transferred A-NK and T-LAK cells

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Despite successes in animals, cytokine gene expression selectively in human tumors is difficult to achieve owing to lack of efficient delivery methods. Since interleukin (IL)-2-activated natural killer (A-NK) and phytohemagglutinin and IL-2 activated killer T (T-LAK) cells, as previously demonstrated, localize and accumulate in murine lung tumor metastases following adoptive transfer, we transduced them to test their ability to deliver products of genes selectively to tumors. Assessments of transduction efficiency *in vitro* demonstrated that adenoviral transduction consistently resulted in high (>60%) transduction rates and substantial expression of transgenes such as GFP, Red2, luciferase, β-galactosidase and mIL-12 for at least 4 days. *In vivo* experiments illustrated that Ad-GFP transduced A-NK and Ad-Red2 (RFP) transduced T-LAK or mIL-12 transduced A-NK cells localized 10–50-fold more or survived significantly better than mock transduced cells, respectively, within lung metastases than in the surrounding normal lung tissue. Most importantly, mIL-12 transduced A-NK cells provided a significantly greater antitumor response than non-transduced A-NK cells. Thus, adoptive transfer of A-NK and T-LAK cells represents an efficient method for targeting products of genes to tumor sites. *Cancer Gene Therapy* (2007) **14,** 441–450. doi:10.1038/sj.cgt.7701019; published online 2 February 2007

Keywords: adenovirus; A-NK cell; T-LAK cell; tumor localization; B16 melanoma

2.0 TARGETING OF PRODUCTS OF GENES TO TUMOR SITES USING ADOPTIVELY TRANSFERRED A-NK AND T-LAK CELLS[1]

2.1 INTRODUCTION

Therapy of cancer involving cytokine genes is a new approach that has already shown promising results, especially in animal models. For example, ex vivo incorporation of a retroviral vector expressing IL-15 into colon carcinoma cells[179] or IL-18 cDNA into B16 cells[127] inhibited tumor growth *in vivo* following inoculation, and resulted in development of protective, systemic immunity against the tumors. In addition, IL-12 transduced tumor cells were not only capable of generating local but also systemic anti-tumor responses when intratumorally injected along with IL-15 protein[126]. IL-2 transduced colon carcinoma cells induced both specific and non-specific anti-tumor immune responses depending on the amount of IL-2 produced[180]. While these studies clearly demonstrate the very potent anti-tumor effects of intratumoral cytokine expression, difficulties targeting the cytokine gene expression selectively to tumors remain a major limitation of this therapeutic modality. In those few, select cases where the tumor is accessible, direct injection gene-therapy strategies have been widely explored and demonstrated a high degree of success. For example, when intratumorally injected as viral vectors containing the genes for IL-2, IL-18, or IFNγ retarded the growth of subcutaneous tumors and prolonged survival[40, 181, 182].

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[1] Chapter 2 has been published in Cancer Gene Therapy. (2007. 14:441-450.)

As an alternative to injection of viral vectors directly into tumors, several groups have injected non-transformed cells, such as fibroblasts, transduced with cytokine genes into established tumors. Bubenik et al. injected IL-2 gene transfected fibroblasts peritumorally into nude mice and found a growth inhibition of human carcinoma cell tumor xenografts[183]. Likewise, Duda et al. demonstrated an inhibition of colon carcinoma growth when tumor cells were co-injected s.c. with IL-12 transduced 3T3 fibroblasts[133]. Cytokine gene delivery directly to tumors has also been achieved by employing cationic liposomes. Ryuke et al. demonstrated a greater reduction in subcutaneous melanoma lesions when IFNβ plasmids, complexed to liposomes, were intratumorally injected as compared to recombinant IFNβ alone. The anti tumor effect appeared to be dependent on the presence of NK cells[39].

Although fibroblasts are easily transduced with a number of viral vectors, using transduced anti-tumor immune cells instead of fibroblasts provides an effector cell capable of responding directly to the cytokines they themselves deliver. In this scenario, anti-tumor responses may be generated by the expressed cytokines via both the delivery vehicle and the endogenous immune cells, enhancing the overall response. For example, Nishioka et al. have shown reduction in tumor burden following intratumoral injection of dendritic cells retrovirally transduced to express IL-12[128]. DCs, adenovirally transfected to express IL-12, have also been shown to reduce colon adenocarcinoma metastases in the liver and elicit systemic anti-tumor responses when injected intraportally[129].

In the majority of cases, though, tumors are disseminated and cannot all be reached by direct injection. Thus, in order to selectively target the tumor, antibody-cytokine fusion proteins[184] and modified adenoviruses[185] have been tested. Antibody-IL-12 together with antibody-TNFα fusion proteins were effective at eliminating small subcutaneous tumors but did

not completely protect against rechallenge[184]. Bauerschmitz et al. showed that modified adenovirus was more efficient than native virus at impeding growth of tumors[185]. Additionally, Sakurai et al. showed systemic, i.v. delivery of IFNβ or IFNγ lipoplexes to be effective prophylactically as well as therapeutically against colon carcinoma lung metastases[186]. However, it was found that empty lipoplexes performed nearly as well as IFNγ lipoplexes on many counts. Thus, despite the numerous methods developed for delivering cytokines or cytokine genes to tumors they still remain relatively ineffective in their selectivity.

We have shown that lymphokine activated killer (LAK) cells, namely IL-2 activated natural killer (A-NK) and IL-2 and PHA-activated T (T-LAK) cells, localize and accumulate within lung tumor metastases when administered intravenously[174, 187]. Based on their impressive ability to accumulate selectively at tumor sites (regardless of the antigen expression by the tumor), we hypothesize that A-NK or T-LAK cells transduced with cytokine genes may be used as vehicles to target cytokines to sites of tumors. This would, depending on the cytokine genes employed, result in a local production of innate and adaptive immune regulators, thereby limiting concerns as to their systemic availability, at the least, or toxicity, at the most.

Here we show that A-NK and T-LAK cells can be successfully transduced ex vivo with adenoviral vectors to express a variety of products of genes such as eGFP, RFP, luciferase, and, as an example of a pro-inflammatory cytokine, murine IL-12. Furthermore, adoptively transferred, adenovirally-transduced A-NK cells were found in 5-fold or higher density within B16 melanoma and 3LL tumors than in normal lung tissue after transfer into tumor-bearing mice. The A-NK cells expressed the gene product (eGFP) in the tumors for up to 72-96 hours post-injection. T-LAK cells, as well, were capable of expressing various genes selectively within tumor metastases. Most importantly, regarding cytokine delivery, mIL-12 transduced A-

NK cells demonstrated greater survival and anti-tumor effectiveness compared to mock transduced cells, at low doses of exogenous IL-2. On this background, we suggest that adenovirally transduced A-NK and T-LAK cells can be used for the selective delivery to tumors of an array of cytokines as well as other transgenic products displaying anti-neoplastic effects.

2.2 MATERIALS AND METHODS

2.2.1 Animals, tumor cell lines, and viral vectors

C57BL/6 and B6.PL-Thy1^{a}/CyJ mice were purchased from Jackson laboratories (Bar Harbor, ME). The animals were housed in a specific pathogen free facility and the University's IACUC authorized their use. F10 B16 melanoma cells and the Lewis lung, 3LL (ATCC), carcinoma cells, both syngeneic to C57BL/6 mice, were maintained in complete media (CM), which is RPMI1640 medium (Life Technologies, Gaithersburg, MD) supplemented with 10% heat inactivated FCS, 2mM glutamine, 0.8 g/L streptomycin, and 1.6 x 10^{5} U/L penicillin. Adherent tumor cells were harvested with 0.02% EDTA and washed in RPMI medium. The recombinant adenoviral vectors used in this study were E1/E3 deleted replication-defective type 5 adenoviruses[188] (obtained from the Preclinical Vector Core Facility, University of Pittsburgh). The cDNA encoding either Lac Z, murine IL-12, eGFP, or luciferase was inserted into the E1 region with gene expression driven by the early promoter of the human cytomegalovirus (CMV). High titer recombinant adenoviruses were generated as described previously[189], by Cre-Lox driven homologous recombination and permissive replication in CRE8 cells, a 293 cell line that expresses Cre recombinase. Viral titers were determined by optical density at 260 nm (OD_{260})

where 1 OD unit = 10^{12} viral particles[190]. Polylysine (pK7) and RGD-fiber-modified adenoviral Lac Z vectors were gifts from Dr. Imre Kovesdi (GenVec, Rockville, MD)[191].

2.2.2 A-NK cell preparations

A-NK cells were generated from congenic B6.PL- $Thy1^a$/CyJ spleens as previously described [83]. Briefly, spleens were asceptically obtained and a single cell suspension was generated. Red blood cells were lysed by incubating the splenocytes with red blood cell lysing buffer (Sigma-Aldrich, St. Louis, MO) at 1ml/spleen for 2 minutes with swirling every 30 seconds. Following filtration through a 70-µm filter, the remaining bulk splenocytes were plated in culture flasks, maintained at 37°C and 5% CO_2 in CM and 6,000 IU/ml human recombinant IL-2 (kindly provided by the Chiron Corporation, Emeryville, CA). Up to 72 hours after the start of culture, non-adherent cells were passaged to new flasks while the adherent cells were given fresh CM and IL-2 as needed, as described previously[173]. These plastic-adherent, A-NK cells were used on Day 5 or 6 of culture. Routinely, these A-NK cells were >95% Thy1.1+, >95% asGM1+, >90% NK1.1+, <2% CD8+, and <2% CD4+.

2.2.3 T-LAK cell preparations

Splenocytes were obtained as described above and transferred to T150 plastic flasks at 2 x 10^5 cells/ml of complete medium. The cells were activated with 600 IU/ml of rhIL-2 and 0.4 µg/ml of PHA-P (phytohemagglutinin-P; DIFCO, Detroit, MI, USA). After 2 days of incubation, clusters of T cells were transferred to 50 ml tubes and sediments were allowed to form for 5-7 min. before the supernatant was gently removed. The cell sediment was

resuspended at 1 x 10^5 cells/ml of fresh CM containing 600 IU IL-2/ml. Three days later, the cells were harvested, washed twice, and resuspended in RPMI-1640. Routinely, the T-LAK cells were found to be >95% Thy1, >95% asGM1+, >90% CD8 (Lyt-2)+, >90% CD3+, but <5% NK1.1+, and <2% CD4 (L3T4)+, in agreement with previous findings[187].

2.2.4 Viral transductions

A-NK or T-LAK cells were collected (A-NK cells with 0.02% EDTA), washed with CM, and resuspended in RPMI without serum at a concentration of 10 x 10^6 cells/ ml (or 40 x 10^6 cells/ml for *in vivo* studies). Virus was added at various multiplicities of infection (MOIs) and the cell suspensions were incubated at 37^{o}C for 90-120 minutes. During the incubation period the tubes were gently shaken every 15 minutes. The cell suspensions were then placed into culture flasks containing CM and 6,000 or 600 IU/ml IL-2 for A-NK or T-LAK, respectively. Cells were again incubated at 37^{o}C for the indicated number of hours for each assay. Before each assay and the *in vivo* tumor experiments, cells were harvested and washed 3 times in a large volume or in excess CM to remove unincorporated virus.

2.2.5 Flow cytometry

Adenovirally transduced A-NK cells were harvested, washed, and fixed in 0.25% paraformaldehyde. Samples were run on a Beckman Coulter Epic XL-MCL FACs machine, collecting 5000 events. Data obtained from histograms was compared to non-transduced control groups.

2.2.6 Luciferase, b-galactosidase, and cytokine production

The production of luciferase and β-galactosidase were assessed using substrates from Luciferase Assay System (Promega, Madison, WI) and Galacto-Light Plus (Tropix, Bedford, MA), respectively, according to the manufacturer's protocol. Samples were analyzed on an Autolumat LB-953 luminometer (Berthold Technologies, Bad Wildbad, Germany). Cytokine production was measured using sandwich ELISAs on supernatants for murine IL-12p70 and IFNγ (Endogen, Woburn, MA).

2.2.7 Adoptive transfer experiments

Lung tumor metastases were established by injecting B16 and 3LL tumor cells (4 x 10^5/0.4 ml of RPMI) intravenously into C57BL/6 mice pretreated 24 hours earlier with an i.p. injection of anti-asialo-GM1 antiserum (Wako PureChemicals, Wako, TX) at 25 μl in 0.5 ml PBS. Following 24 hours of *in vitro* culture, Ad-eGFP transduced A-NK cells were harvested from flasks and washed. Eight and twenty million cells/ 0.4 ml RPMI were injected i.v. into mice with day-10 3LL and B16 lung metastases, respectively. The transferred A-NK cells were supported by 0.5 ml i.p. injections containing 60,000 IU of IL-2 in complex with polyethylene glycol (PEG-IL-2, a kind gift from the Chiron Corporation, Emeryville, CA), starting at 0 hours and every 12 hours thereafter, up to 72 hours. For the RFP-T-LAK experiment, B16 cells were injected i.v. at 3.0 x 10^5/0.3ml RPMI. At day twelve of tumor growth, mice then received 22 x 10^6 Ad-Red2 (RFP) transduced T-LAK cells in 0.4 ml RPMI. Following the cellular transfer, mice received 30,000 IU/ml PEG-IL-2 injections every 12 hours up to 48 hours post injection

when all mice were sacrificed. For experiments employing mIL-12 transduced A-NK cells, B16 lung tumor metastases were established with 3.0 x 10^5 /0.4ml RPMI. Three days later, 5.0 x 10^6 Ad-IL-12 or mock transduced A-NK cells were injected i.v. in 0.4 ml RPMI. Injections of PEG-IL-2, at 60,000 IU/ml, were given two times within 24 hours of the injection of the A-NK cells.

2.2.8 Estimation of adoptively-transferred A-NK and T-LAK cell infiltration of lung metastases and survival analysis

Using the Thy1.1/1.2 congenic system the infiltration of the transferred A-NK cells was determined as described previously[83]. Briefly, one half of organ samples obtained from mice at various time points were fresh frozen at –70 ^{o}C. Eight micron thick sections of the organs were stained with PE-conjugated, anti-Thy1.1 Ab (Pharmingen) at a 1:200 dilution and compared to PE-conjugated IgG2b Ab controls. The other half of the organ samples were fixed in 1% paraformaldehyde for 3 hours then 30% sucrose overnight. These fixed tissues were then snap frozen, sectioned, and GFP+ A-NK, or RFP+ T-LAK, cells were identified by fluorescence microscopy. Fluorescent and DIC images of the tissue sections were obtained using a Nikon fluorescent microscope and analyzed for cell numbers using MetaMorph image analysis software[83]. Mice were code numbered and monitored for survivability by two observers with no knowledge of the code. When reaching a premorbid condition, as defined by our IACUC, mice were sacrificed and the lungs were removed, dried and weighed (the average dry weight of the lungs from the mock transduced A-NK and AdIL-12 transduced A-NK groups (Fig. 9) were 0.189g±0.01g versus 0.163g±0.03g, respectively, ensuring that the mice, at time of sacrifice, had reached the same stage of tumor development).

2.2.9 Statistical analysis

Two-tailed, unpaired student's t-tests were performed with a 95% confidence interval. For survivability curves, data were plotted according to the Kaplan-Meier method with statistical significance determined using the log-rank test.

2.3 RESULTS

2.3.1 A-NK and T-LAK cell expression of the marker protein eGFP following adenoviral transduction.

It was determined, first of all, to what extent the A-NK and T-LAK cells, capable of localizing to tumors, were transducible with adenoviral vectors. Since eGFP provides a quick assessment of successful transduction, an adenoviral (serotype 5) vector with the gene for eGFP, under the control of a CMV promoter, was chosen for this purpose. Within 24 hours of virus incubation, a considerable production of GFP was noticed by the A-NK and T-LAK cells compared to non-transduced cells (Figure 4, A and A). When high MOIs were used, the presence of GFP was evident for up to 168 hours, but expression peaked around the 48-hour time point, at which time the percentage of GFP positive A-NK cells reached levels of 70-80% (Figure 4, A and B, 80 and 160 MOIs). In general, higher transduction rates were achieved with A-NK cells than T-LAK cells (Fig. 4, A and B). The production of GFP by the A-NK and T-LAK cells was due to the original application of virus and not to viruses replicating in the A-NK or T-LAK cells or from transfer of GFP from transduced to non-transduced cells in that no GFP

positive cells were witnessed among non-transduced cells when these were co-cultured with cells transduced 24-48 hours earlier (data not shown).

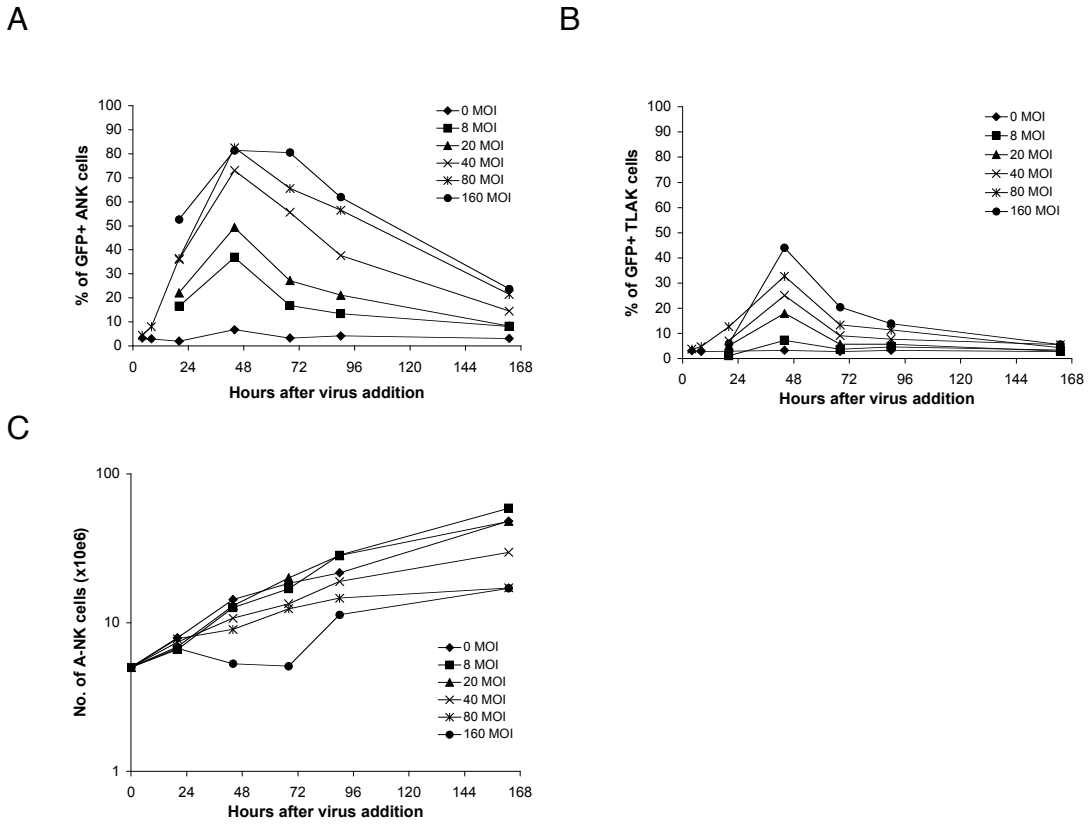


Figure 4. Ad-eGFP transduction of murine A-NK or T-LAK cells. $5x10^6$ A-NK (A) or T-LAK (B) cells were transduced with Ad-eGFP at the indicated MOI and tested for GFP expression by flow cytometry. Percentage GFP-positive cells were determined at five different times over the course of a week. (C) The cell numbers of transduced A-NK cells were calculated for extended growth of the $5x10^6$ cells originally plated.

A concern of viral transduction, especially at high MOIs, is that of toxicity to the transduced cells. The viability and proliferative capacity of A-NK and T-LAK cells transduced

at increasing MOIs was therefore examined. Trypan blue dye exclusion tests revealed a high viability of both A-NK cells and T-LAK cells at 24 hours at all MOIs tested, indicating a low immediate toxicity of the virus (data not shown). However, while analysis of cell growth one to five days after transduction demonstrated that all cells continued to grow, it was clear that the growth-rate of cells transduced at higher MOIs was somewhat suppressed - especially during the first days after transduction - compared to non-transduced control cells and cells transduced at lower MOIs (Fig. 4C).

2.3.2 Functional enzymes, luciferase and beta-galactosidase, are expressd by transduced A-NK cells.

It was of critical importance to demonstrate that gene delivery and expression in A-NK cells could result in production of proteins other than GFP. Adenoviral vectors with the genes for luciferase (Ad-luc) and beta-galactosidase (Ad-beta-gal), respectively, were therefore employed. After 48 hours of culture, lysates of Ad-luc transduced A-NK cells showed a marked increase, more than 100-fold increase in light activity seen after the addition of D-luciferin substrate as compared to non-transduced cells (Fig. 5A). Likewise, cells transduced with Ad-beta-gal reacted strongly when assayed with Galacto-Light Plus, a luminescent substrate system (Fig. 5B). Modified adenoviral vectors, such as Ad-RGD and –PK7 (Fig. 5B) enhance uptake

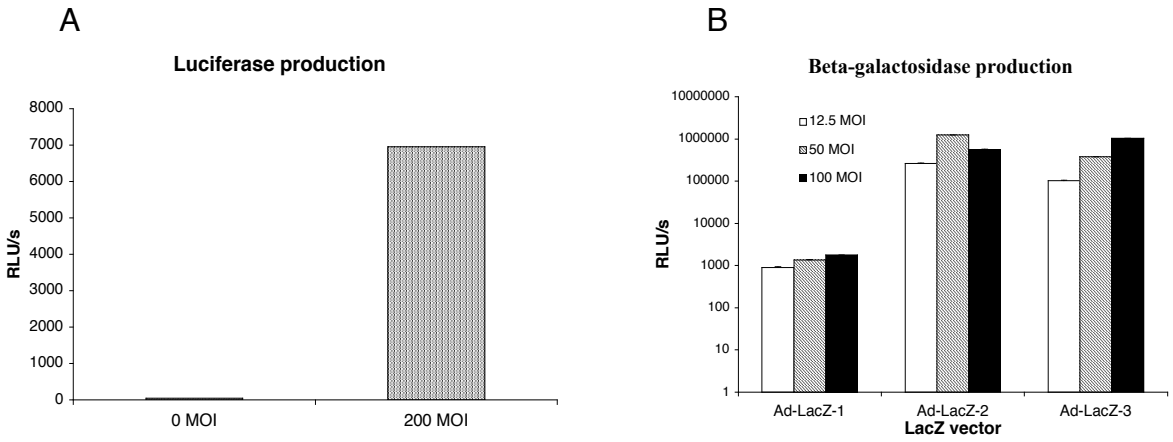


Figure 5. Ad-luc and Ad-LacZ transduction of murine A-NK cells. $2.5x10^6$ (A) or $1x10^6$ (B) A-NK cells were transduced with the indicated MOI of Ad-luc or Ad-lacZ vectors and tested for luciferase expression using the Luciferase Assay system or for β-galactosidase expression using the Galacto-Light Plus kit, respectively. Ad-LacZ-1, -2, and -3 correspond to wild type Ad-LacZ, -PK7-LacZ, and -RGD-LacZ, respectively. Samples were assessed for both genes after 48 hours of virus culture. Results are given as relative luminescent units/second (RLU/s) run in triplicate.

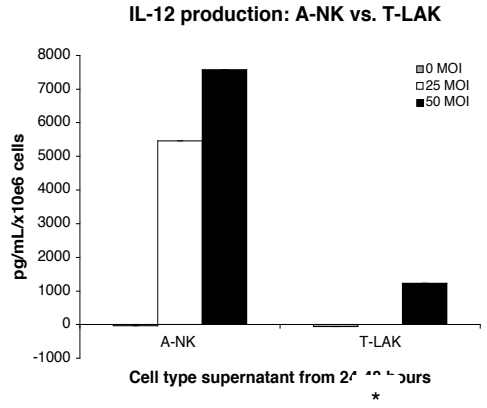
through unconventional receptors, the integrin and heparan sulfate receptors, respectively. Using these vectors, we achieved a greater transduction efficiency and gene expression of the A-NK cells compared to the unmodified Ad5 vector. Taken together, these data demonstrate that in addition to susceptibility to GFP gene-transduction, A-NK cells can be efficiently transduced to express genes coding for various enzymes.

2.3.3 Transduced A-NK and T-LAK cells can produce a potent regulator of adaptive immunity.

The next important step was to investigate whether A-NK and T-LAK cells could be transduced to over-express cytokines involved in anti-tumor immune responses. To test the

production of such an agent, an adenoviral vector expressing murine IL-12, a cytokine known for its ability to stimulate NK and T cells[192, 193] and to enhance CTL response and promote Th1 immunity[103, 194], was employed. As seen in Figure 6A, A-NK and T-LAK cells were successfully transduced with the Ad-mIL-12 vector to secrete high amounts of mIL-12. The production of mIL-12 by transduced A-NK cells followed similar kinetics as the GFP production, namely with a peak expression around the 48-hour time point (compare Fig. 4B with Fig. 6B). The functionality of the mIL-12 was confirmed by demonstration of an elevated IFNγ production from the transduced A-NK and T-LAK cells compared to non-transduced cells (Figure 6C). The IFNγ production by T-LAK cells transduced with the mIL-12 gene was not significantly different from that of A-NK cells despite their lower mIL-12 production.

A



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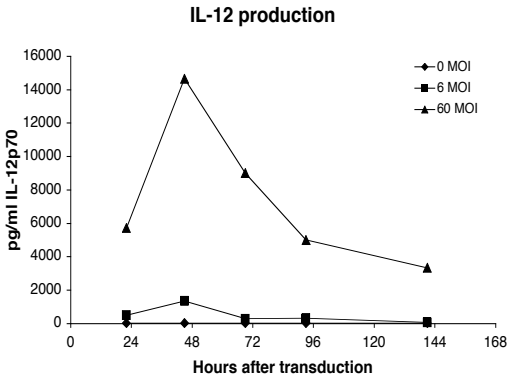

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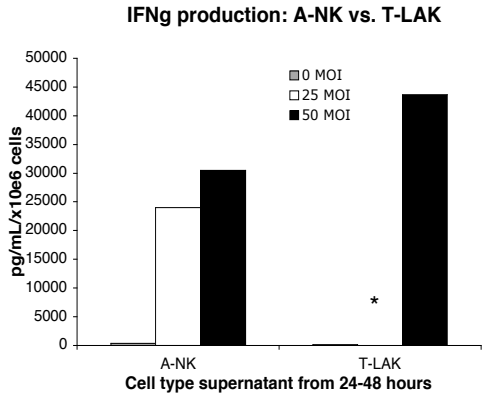

Figure 6. Primary IL-12 production and secondary IFNg production by Ad-mIL-12 transduced murine A-NK and T-LAK cells. $3x10^6$ A-NK or T-LAK cells were transduced with the indicated MOI of Ad-mIL12. Cells were washed after 24 hours culture and given fresh media. 24 hours later, supernatants were collected and analyzed for the presence IL-12 (A) or IFNγ (C). 25 MOI for T-LAK not tested (*). (B) $2x10^6$ ANK cells were transduced with the indicated MOI of Ad-mIL-12. Supernatants were harvested at various times and assayed using ELISA. Values are given in pg/ml for the total amount of mIL-12 present in the culture flask by multiplying the number of ml of supernatant by the ELISA readings for a 1/10 dilution of samples.

2.3.4 Selective tumor homing by adoptively transferred, A-NK and T-LAK cells transduced with various genes.

Having demonstrated the success of A-NK and T-LAK cell adenoviral transduction, we next studied the ability of the transduced cells to localize at tumor sites upon adoptive transfer and to express the transgene while in the tumor tissue. Mice were injected with 3LL or B16 tumor cells to induce pulmonary metastases and ten days later, 8 million Ad-eGFP transduced A-NK cells were injected i.v. Exogenous PEG-IL-2 injections were given every 12 hours to support the transferred cells as previously described[175]. At various times organs were harvested, fixed, cryosectioned and analyzed for the presence of A-NK cells and GFP. As early as 24 hours after injection, the density of the transferred A-NK cells was, as expected, significantly higher in

the tumor tissue compared to the normal lung tissue, and the A-NK cell density in the tumor tissue continued to increase for the next 3 days (400±138, 750±104, 810±290, and 1179±174 cells/mm^2 at 24, 48, 72, and 96 hours, respectively). The expression of GFP in the tumor tissue was also significantly higher in the tumor tissue than in the surrounding normal tissue from 24 hours and until 96 hours. Figure 7 shows the presence of GFP expressing A-NK cells at 72 hours in lung 3LL (Fig. 7A) and in B16 (Fig. 7B) tumors. As could be expected, though, based on the loss of GFP expression from the cells from 72 hours after transduction (Fig. 4), expression was maximal at 48 hours and declined thereafter (Fig. 7C) but remained significantly greater within the tumor tissue compared to normal tissue at all times evaluated, p-value <0.05. As seen in Figure 7D, T-LAK cells transduced to express RFP were found at a ratio of 10:1 in tumor tissue compared to normal lung tissue 48 hours after injection. Thus, although less transducable than A-NK cells, T-LAK cells may also serve to localize products of genes to tumor tissues.

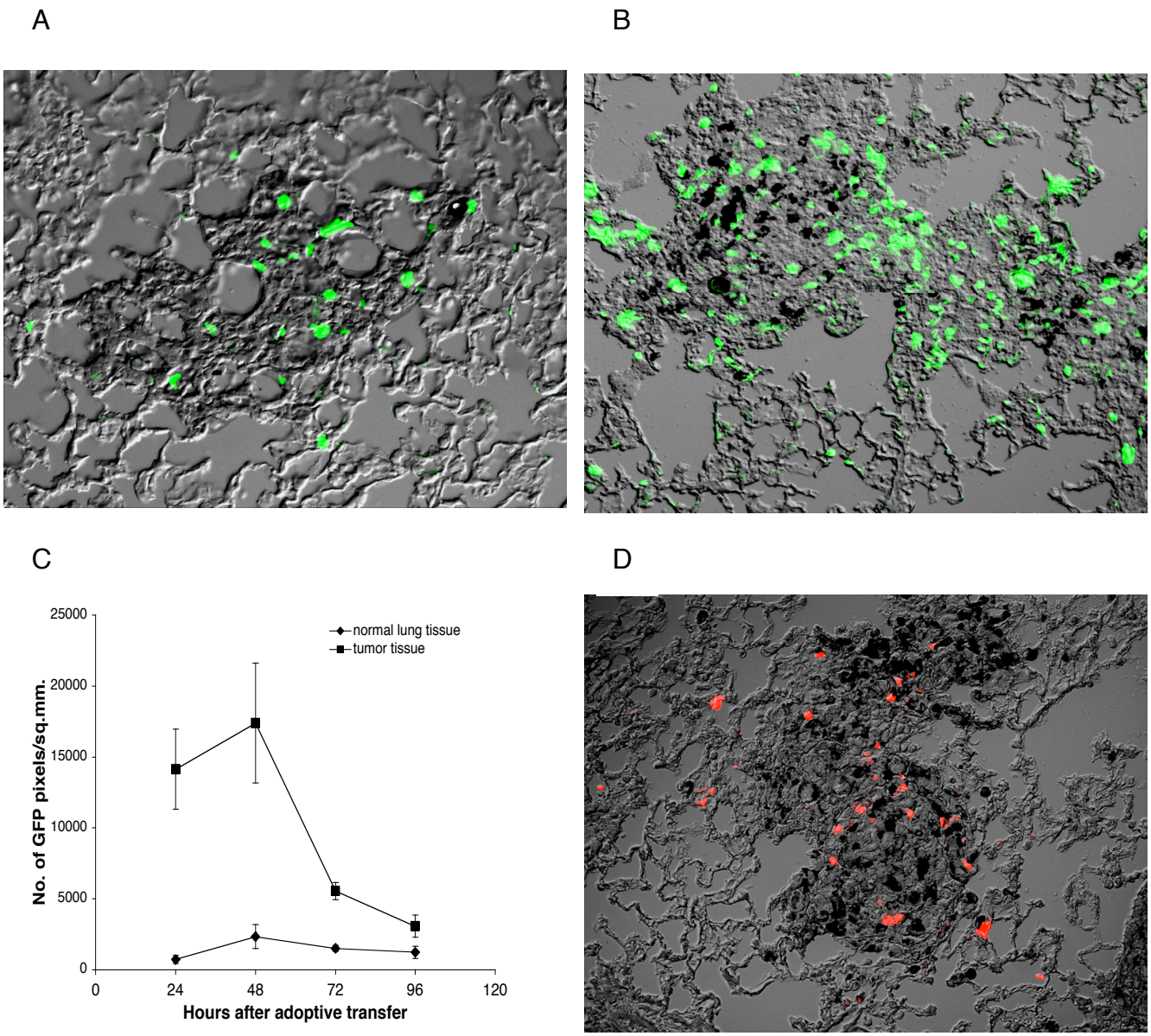

Figure 7. GFP or RFP expression within lung tumor metastases by adoptively transferred transduced cells. (A and B, respectively) 8 x 10^6 or 20 x 10^6 GFP-transduced A-NK cells were injected i.v. into 10-day 3LL or B16 (black pigmented) tumor-bearing mice. The 3LL tumor area (panel A) has been darkened for visualization. These panels illustrate lung tumor areas 72 hours post injection, infiltrated by A-NK cells capable of expressing GFP (green dots). Bars represent 100 microns. (C) Paraformaldehyde-fixed lung tumor sections were analyzed for the expression of GFP both within tumor tissue as well as normal lung tissue. Using Metamorph imaging software, the total number of pixels (corresponding to GFP expression) above background was measured. Data are expressed as number of pixels per square mm. normal lung and tumor tissue (average of 3 animals per time

point). P-values were significant for each time point analyzed, with values <0.005 for 24, 48, and 72 hours and <0.05 for 96 hours. (D) A representative image of a 12-day B16 lung tumor (black pigmented) at 48 hours after injection of 20 x 10^6 Red2-(RFP) transduced T-LAK cells is depicted. Bar represents 100 microns.

We also analyzed liver and spleen tissue sections for the presence of transduced cells, to rule out the possibility of non-specific targeting. We found GFP expression in the liver, for example, to be only 1.95% of that of lung tumors, with a similarly low density of Thy1.1 positive cells. The density of NK cells, as well as GFP expression, in other organs (such as kidneys and spleen) appeared to be substantially lower than the GFP expression in liver.

To illustrate the benefit of expressing a cytokine selectively within lung tumors, A-NK cells were transduced with Ad-mIL-12. B16 tumor-bearing mice received 5 million Ad-mIL-12 or mock transduced ANK cells along with two injections of full dose of IL-2 (60,000 IU/ml) only. Cells were quantitated in lung tumor metastases 72 hours after adoptive transfer. At this time we discovered a substantially greater number of exogenous A-NK cells within lung tumors when they were first ex vivo transduced to express mIL-12 (Fig. 8A) than if they had been mock transduced (Fig. 8B).

A B

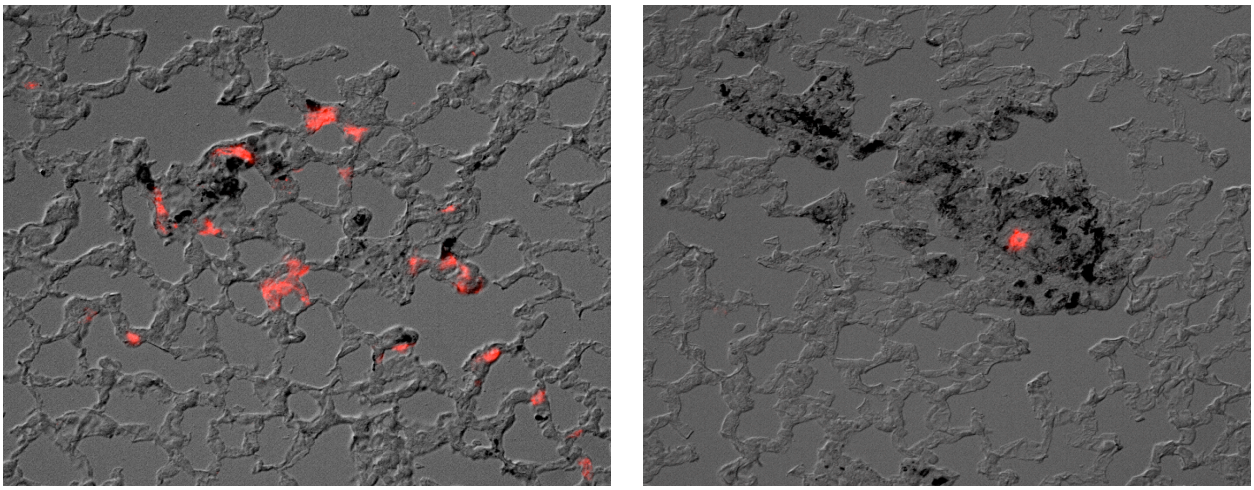

Figure 8. Selective mIL-12 expression in lung metastases enhances cell survival. (A and B, respectively) 5 x 10^6 mIL-12 or mock transduced A-NK cells were injected i.v. into 3-day B16 tumor-bearing mice, and supported with 2 injections of 60,000 IU/ml PEG IL-2. Fresh frozen lung tumor sections were analyzed for surviving adoptively transferred cells at 72 hours post injection. Representative images at this time point are given for mIL-12 transduced (A) or mock transduced (B) A-NK cells in B16 lung tumor-bearing animal. In both panels, the adoptively transferred A-NK cells are depicted with PE-conjugated anti-Thy1.1 Ab at a 1:100 dilution. Bars are equal to 50 microns.

We also found a significant anti-tumor response, as determined by mouse survivability, which accompanied the enhanced cellular survival of mIL-12 transduced cells. We found that mock transduced A-NK cells provided no benefit in mean survival of mice compared to that seen with the exogenous IL-2 only treatment group (Fig. 9). In contrast, mice receiving mIL-12 transduced A-NK cells survived significantly longer than Il-2 treated mice and mice treated with Mock transduced cells (Fig. 9, 26 days compared to 16 days for mock transduced mice, p value <0.005). We, therefore, conclude that A-NK cells can be used to deliver transgenic products, including pro-inflammatory cytokines such as IL-12, to sites of tumors to augment anti-tumor responses.

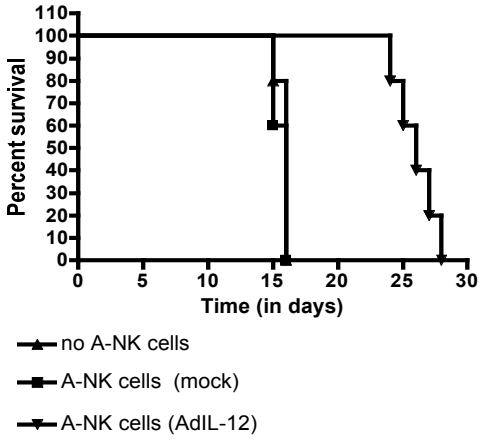

Figure 9. Selective mIL-12 expression in lung metastases provides for an overall greater survival in mice. Survival of mice (from Figure 8) was determined in a blinded fashion as described in Material and Methods and plotted using the Kaplan-Meier method. Log-rank test demonstrated a significant difference in survival time between mice receiving IL-2 only or mock transduced A-NK cells and mIL-12 transduced A-NK cells (p value<0.005, n=5).

2.4 DISCUSSION

Activation of macrophages involved in innate immunity, of dendritic cells in initiating adaptive immunity, and of T cells providing effector function and memory all depend on cytokine interactions. Numerous studies describe how administration of cytokines[43, 195] can help boost these immune reactions. For instance, Kodama et al. and Hashimoto et al. have demonstrated that the administration of recombinant IL-12 or IL-18 resulted in a noticeable anti-tumor effect in mice[43, 195]. Furthermore, the effects of IL-18 together with IL-2 were greater than either cytokine alone in reducing the number of lung metastases in mice[85]. However, caution must be taken in administering combinations of recombinant cytokines for therapeutic purposes, such as IL-2 and

IL-12[89] or IL-12 and IL-18[196], or even IL-18 alone[197], in that fatal toxicity may result. There is a fine balance, therefore, in ensuring that the amount of cytokine administered is sufficient to activate and sustain the immune system, but not so elevated that treatment-limiting toxicity is induced. In order to reduce toxicity, it seems logical to directly target the cytokine(s) to sites of established tumors. While the application of fibroblasts transduced with cytokine genes has delivered proof that selective presence of cytokine in tumors leads to tumor reduction with very little or no toxicity, this approach is limited by the fact that the cells must be injected directly into the tumors. Viral vectors or liposome complexes, when injected intravenously, may be mistargeted, degraded, or may never locate and infiltrate the actual tumor[198]. Furthermore, along with injection of recombinant cytokines, their systemic application may lead to development of toxicity. These, and other, shortcomings prompted us to investigate the use of lymphocytes capable of localizing at tumor sites as delivery vehicles of products of genes, including cytokines. We focused on A-NK and T-LAK cells as vehicles for tumor-selective targeting of transgenes, since we have previously demonstrated their ability to localize to remote, disseminated lung tumor metastases.

It was important to demonstrate, first of all, that the A-NK and T-LAK cells could in fact be successfully transduced with genes. Second, we wanted to determine if A-NK and T-LAK cells could express immunologically relevant genes, in particular those pertaining to cytokines. Finally, we needed to illustrate that the transduced cells maintain the capacity to traffic to tumors, without a loss in viability or ability to express the transgene.

For the gene transduction, we focused on adenoviral vectors since they were capable of infecting A-NK and T-LAK cells at high rates, could be generated to high titers, and could introduce genes of up to 8 kb into cells[199]. In fact, transduction of A-NK and T-LAK cells with

other types of vectors, e.g. retrovirus and lentivirus, resulted in very low transduction rates (0.1-~10%). Since A-NK and T-LAK cells divide for only a limited amount of time (2-4 weeks), cloning of a relatively low number of successfully retro- and lenti-virally transduced A-NK/T-LAK cells, therefore, was not feasible. We show here that a variety of genes, such as GFP, RFP, luciferase, as well as murine IL-12, could be successfully expressed by a high percentage of the A-NK and T-LAK cells. Judged by both GFP and mIL-12 expressions, the transduction efficiency was high and expression persisted, although reduced considerably with time, to at least a week after transduction. Thus, A-NK cells that were greater than 80% GFP-positive at 48 hours after Ad-GFP transduction, fell below 30% GFP-positive cells by day 7. This may be explained by the fact that adenoviruses are unable to integrate their genetic material into the host genome, and therefore incapable of replicating. Thus, the originally delivered viral genes became diluted with successive rounds of division of the rapidly proliferating A-NK and T-LAK cells. In addition, inactivation of the CMV promoter of the adenoviral vectors also contributes to the declining gene expression[200].

Somewhat surprisingly, T-LAK cells proved harder to transduce than A-NK cells. While up to 80% of the A-NK cells expressed eGFP at 24-48 hours after transduction, only about 30% of the T-LAK cells, transduced under the same conditions, were eGFP+ at this time. Whether this results from a lower expression of coxsackie and adenovirus receptor (CAR) on the T-LAK cells compared to A-NK cells is under investigation. To improve the transduction rate of T-LAK cells, it may be possible to employ fiber knob-modified or biotinylated adenoviral vectors[201] that can be taken up through various receptor interactions. A preliminary experiment in our laboratory indicates that DMRIE-C, a liposome-cholesterol reagent used to enhance DNA/RNA and virus transfection[202, 203], improved the T-LAK cell transduction rate. It is also possible that

other modified adenoviral vectors such as Ad-RGD and –PK7, which improved transduction of A-NK cells, will also be able to improve the transduction efficacy of T-LAK cells.

We also tested the ability of adenovirally transduced A-NK and T-LAK cells to express functional, anti-tumor cytokines. Both cell types were transduced with an Ad-mIL-12 vector. As early as 24 hours after transduction, both cell types produced significant amounts of mIL-12, with A-NK cells producing greater amounts. Again, the expression of this gene was evident for more than 72 hours. Also, the mIL-12, as well as mIL-18 (data not shown), gene transduction led to the induction of secondary cytokine/chemokine production. Levels of IFNγ, for example, were markedly elevated in supernatants from A-NK and T-LAK cells transduced with mIL-12 (and mIL-18, data not shown) genes as compared to non or mock transduced cells. In spite of the low mIL-12 levels generated by transduced T-LAK cells, the levels of IFNγ generated by the mIL-12 transduced T-LAK cells were equal to those generated by mIL-12 transduced A-NK cells, as confirmed from separate experiments. This is encouraging considering the significantly greater amount of mIL-12 produced by A-NK cells compared to T-LAK cells. Apparently, the mIL-12 produced by the T-LAK cells is fully capable of boosting downstream immune responsiveness as evidenced by the robust production of IFNγ.

Having demonstrated the efficiency of adenoviral vectors to transduce A-NK and T-LAK cells, we tested whether this procedure would translate to selective targeting of gene expression in our lung tumor model. Although previous reports have indicated A-NK and T-LAK cells possess the ability to home to and infiltrate lung metastases, we were uncertain of any deleterious effects adenoviral transduction may have on this. However, we did not find any indication of negative effects of gene transduction on tumor localization since Ad-eGFP and Ad-Red2 transduced A-NK and T-LAK cells, respectively, localized to tumors to the same degree as non-

transduced cells[83, 204]. Thus, for both A-NK cells and T-LAK cells, increasing cell densities within the metastases were found up to 96 hours. Accordingly, the density of GFP-positive cells also increased after injection, but, in contrast to the cell density, as measured by immunostaining, measurable GFP declined after 48 hours. We believe this is caused by both inactivation of the CMV promoter and dilution (due to cell proliferation) of the transgene, as discussed above. From these data, it is clear that adenoviral transduction of A-NK and T-LAK cells does not hinder their ability to traffic to and infiltrate lung tumors. In addition, the transduction process does not appear to be toxic to the cells, but results in successful expression of the transgenes by the tumor infiltrating A-NK and T-LAK cells.

The gradual loss of GFP or RFP signal due to dilution of the genetic material for each A-NK and T-LAK cell division clearly reduces the usefulness of these fluorescence products as markers for individual cells. However, as long as all A-NK and T-LAK progeny remain at the tumor site, one would expect that the overall gene expression would only become reduced due to inactivation of the viral genes. Thus, sufficient levels of pro-immune cytokine may be achieved by the combined effort of many cells each producing a modest amount of cytokine. Moreover, the A-NK/T-LAK cells infiltrating the lung tumors are confined to an environment with very little extracellular fluid. Therefore, even the production of a numerically modest number of cytokine molecules may result in cytokine-concentrations sufficient to stimulate both the exogenously delivered and endogenous immune effector cells. This cytokine benefit has been clearly demonstrated from our results illustrating a substantially improved effector cell survival and anti-tumor effect by mIL-12 gene transduced effector cells compared to mock transduced cells. It should be noted that the anti-tumor effect of the transduced cells was maintained even in the presence of only two initial injections of exogenous IL-2 support, a key factor in eliminating

systemic toxicity, which usually appears after two to three days of exogenous IL-2 treatment. Although the treatment of mice receiving mIL-12 transduced cells did not result in complete cure, these mice survived significantly longer compared to mock transduced cell recipient mice. With repeated adoptive transfer or multiple/different cytokines (as in ongoing experiments), it is imaginable that greater survival could be attained, ultimately leading to curative benefits.

All taken together, our data demonstrate the potential of A-NK and T-LAK cells to deliver anti-tumor products of genes, including cytokines, directly to the site of tumor growth. We envision that not only single cytokines, but also cytokine combinations, can be directed, at various times and intervals, to tumor tissues by transduced A-NK and T-LAK cells to induce strong and sustained anti-tumor immune responses. By not being dependent on injection of the genetic material directly into tumors, this technique can provide therapeutic benefit to multiple and remote tumors. In addition, achieving a selective, local production of cytokines should eliminate, or greatly reduce, the toxicity associated with systemic cytokine administration, since the cytokine effects will be concentrated right where they are needed, namely in the tumor tissue. This, in turn, should enhance the A-NK and T-LAK effectors' activity and survival, and, depending on the type of cytokines produced and host cells present in the tumor, should also boost various phases of the endogenous immune response.

HUMAN GENE THERAPY 18:701–711 (August 2007)

DOI: 10.1089/hum.2007.052

Cytokine Gene Therapy Using Adenovirally Transduced, Tumor-Seeking Activated Natural Killer Cells

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ABSTRACT

We previously demonstrated that adoptively transferred, interleukin (IL)-2-activated natural killer (A-NK) cells are effective in reducing B16 lung tumors in tumor-bearing animals. This effect depends on high and often toxic doses of IL-2 to support the survival and antitumor functions of the transferred A-NK cells. We hypothesized that A-NK cells transduced to express pro-NK cell cytokines would become less dependent on high and potentially toxic amounts of IL-2. Here, we demonstrate that A-NK cells adenovirally transduced to express mIL-12 survive well and function efficiently in mice bearing B16 lung tumors when supported with low, nontoxic doses of IL-2. The intratumoral survival of nontransduced "bystander" A-NK cells also increased when they were coinjected with IL-12 gene-transduced A-NK cells. The enhanced survival of exogenously delivered, IL-12 gene-transduced A-NK cells resulted in greater antitumor responsiveness. This led to a 7- to 10-day increase in median survival time compared with tumor-bearing mice receiving mock-transduced A-NK cells. These data show that the presence of IL-12 around tumor-infiltrating A-NK cells enhances their antitumor activity while reducing their requirement for systemically administered IL-2.

3.0 CYTOKINE GENE THERAPY USING ADENOVIRALLY TRANSDUCED, TUMOR-SEEKING A-NK CELLS[2]

3.1 INTRODUCTION

Although capable of eliminating circulating tumor cells from the blood-vascular system, it has long been thought that natural killer (NK) cells play a limited role in combating established tumors. However, we have shown that NK cells activated *ex vivo* with interleukin (IL)-2 (A-NK cells), and adoptively transferred back into tumor-bearing mice, not only localize selectively at tumor sites [174, 205], but also mediate a significant reduction of infiltrated host lung tumors within 4-5 days [83]. Activated NK cells do not, in contrast to cytotoxic T lymphocytes (CTLs), require recognition of specific antigen presented by MHC molecules to kill their targets, whether these are virally infected, malignantly transformed, or otherwise aberrant cells. In fact, the NK cell preferentially kills cells that fail to express normal levels of MHC [2, 44]. Thus, inclusion of activated NK cells with CTL-based immunotherapies may be crucial to avoid tumor escape via immunoselection of tumor cells expressing low levels of, or no, MHC.

However, the *in vivo* survival, tumor localization, and, consequently, the anti-tumor effect of the A-NK cells are strongly dependent on a continuous support of relatively high doses of exogenous IL-2. Even injections of IL-2 every 4 hours for 3 days is not sufficient to sustain

[2] Chapter 3 has been published in Human Gene Therapy. (2007. 18:701-711.)

optimal tumor-localization [175] and anti-tumor activity of the A-NK cells. This is probably related to the very short half-life of IL-2 (~5 min), because impressive tumor-localization and substantial anti-tumor activity are seen when the adoptively transferred A-NK cells are supported by injections of the long-lived (t1/2 ~ 4 hours) polyethylene glycol-conjugated IL-2 (PEG-IL-2) [83]. However, while the use of PEG-IL-2 secures the anti-tumor function of the A-NK cells, it often results in toxicity in the form of weight-loss and pulmonary edema/vascular leakage syndrome. It is therefore clear that before adoptive A-NK cell therapy can be clinically applicable, it will be necessary to circumvent the demand of A-NK cells for exogenous IL-2 in order to reduce toxicity.

Other cytokines, such as IL-12 and IL-18, which strongly augment NK cell proliferation and effector functions [7, 192, 206, 207], may substitute for IL-2. However, systemic injection of combinations of these cytokines with IL-2 induces even more severe side effects than IL-2 alone. For example, IL-2 in combination with IL-12 [89, 208], and combinations of other cytokines, such as IL-12 and IL-18 [196], used as anti-tumor reagents, resulted in severe toxicity to the point of death of treatment mice. To circumvent these toxic side effects others have looked into intratumoral injection of transduced cells, localizing the cytokine activity [128, 129]. For example, Niskioka et al. and Satoh et al. found the delivery of IL-12 by transduced dendritic cells resulted in systemic immunity leading to tumor regression. For more remote tumors that cannot be injected directly, treatments need to be tumor-specific, such as with cytokine-antibody fusions [184]. Fusion protein technology is dependent upon appropriate antigens being well presented within the tumor for adequate cytokine localization, which is not always the case.

Here, we set out to demonstrate that A-NK cells are capable of gene-delivery specifically to sites of tumors and this would lead to a reduced need for exogenous IL-2 as well as greater

anti-tumor efficacy of the A-NK cells. We base this on our recent observation that adenovirally transduced A-NK cells expressing green fluorescent protein (eGFP) accumulate selectively within tumor nodules and produce eGFP at the tumor site for several days (Goding *et al.*, 2007). A-NK cells transduced with the gene for IL-12, a cytokine that stimulates a variety of NK cell proliferative and cytotoxic functions, survived better *in vivo*, as do nontransduced coinjected A-NK cells, in low amounts of exogenous IL-2. Additionally, the IL-12 gene transduction of the A-NK cells lead to a significant increase in survival of tumor-bearing mice, when compared to mice receiving mock-transduced A-NK cells. These data illustrate that it is possible to reduce the tumor-infiltrating A-NK cells' need for IL-2 support - and thereby the risk of systemic toxicity - while maintaining anti-tumor effectiveness by transducing them with cytokine genes such as IL-12 before the adoptive transfer.

3.2 MATERIALS AND METHODS[3]

3.2.1 A-NK cell preparation by negative selection

For tracking purposes, A-NK cells were generated from congenic B6.P1-Thy-1aCy spleens as previously described [83]. Briefly, spleens were aseptically obtained, and a single cell suspension was generated. Red blood cells were lysed by incubating the splenocytes with red blood cell lysing buffer (Sigma-Aldrich, St. Louis, MO) at 1ml/spleen for 2 minutes with

[3] General materials and methods pertaining to this chapter but not mentioned in this section can be found in Chapter 2, Materials and Methods.

swirling every 30 seconds. Following filtration through a 70-μm filter, the remaining bulk splenocytes were plated in culture flasks, maintained at 37°C and 5% CO_2 in CM and 6,000 IU/ml human recombinant IL-2 (kindly provided by the Chiron Corporation, Emeryville, CA). Following 48 hours of culture, bulk splenocytes were depleted of CD8+ and CD3+ cells, using anti-CD8 hybridoma supernatant (Lyt2, ATCC, Manassas, VA), at 2 ml/100x10^6 cells, and purified anti-CD3 Ab (clone 17A2, BD Pharmingen, Franklin Lakes, NJ), at 25 μl/100x10^6 cells, respectively. Antibody-coated cells were incubated with magnetic beads conjugated to sheep anti-rat IgG Ab (Dynal Biotech ASA, Oslo, Norway), and A-NK cells were negatively selected according to the manufacturer's protocol. Up to 72 hours after depletion, non-adherent cells were passaged to new flasks while the adherent, purified A-NK cells were given fresh CM and IL-2. These plastic-adherent, A-NK cells were used on Day 5 or 6 of culture. Routinely, these A-NK cells were >95% Thy1.1+, >95% asGM1+, >90% NK1.1+, <2% CD8+, <3% CD3+, and <2% CD4+.

3.2.2 Proliferation assay and cytokine production

Non-transduced A-NK cells were plated into 96-well plates at 15,000 cells/200μl/well, following 3 washes to remove residual IL-2. rhIL-2 (Chiron Corporation, Emeryville, CA) was added in 10-fold dilutions and recombinant murine IL-12 (Endogen, Woburn, MA) was added at the indicated concentrations. After 48 hours incubation, 10μl of Cell Titer Reagent (Promega Corporation, Madison, WI) was added to each well. At this time a standard curve of known cell numbers was generated and run in parallel with the treated cells. The plates were incubated another 3 hours at 37°C and then analyzed on a plate reader at 490nm. For IL-12 production in presence of titrated IL-2, 1x10^6 Ad-IL-12 transduced A-NK cells were cultured in 0, 6, 60, 600,

6000 IU/ml rhIL-2 for 72 hours. Supernatant was collected and IL-12 production was measured using sandwich ELISAs on supernatants for murine IL-12p70 (Endogen, Woburn, MA).

3.2.3 Adoptive transfer and survival experiments

Lung tumor metastases were established by injecting B16 or MCA205 tumor cells ($3x10^5$/0.4 ml of RPMI) intravenously into C57BL/6 mice pretreated 24 hours earlier with an ip injection of anti-asialo-GM1 antiserum (Wako PureChemicals, Wako, TX) at 25 µl in 0.5 ml PBS to remove endogenous NK cells. Following 24 hours of *in vitro* culture, Ad-IL-12, mock, or non-transduced A-NK cells were harvested from flasks and washed. Five million cells/ 0.4 ml RPMI were injected iv into mice with day 3, 7 or 10 B16 lung metastases. The transferred A-NK cells were supported by 0.5 ml ip injections containing 60,000 IU of IL-2 in complex with polyethylene glycol (PEG-IL-2, a kind gift from the Chiron Corporation, Emeryville, CA), at 0 and 12 hours and then either 0, 2220, 6600, or 60,000 IU (for the titration experiment) or 4,500 IU (for iv versus ip experiments) every 12 hours thereafter, up to 72 hours. For the survival experiments (involving B16 and MCA205 tumors) mice received only two injections of 60,000 IU Peg-IL-2 on the day of adoptive transfer. Mice were code numbered and monitored for survivability by two observers with no knowledge of the code. When reaching a premorbid condition, as defined by our IACUC, mice were euthanized and the lungs were removed, dried and weighed (the average dry weight of the lungs from Ad-mock and Ad-IL-12 transduced A-NK groups (Fig. 9) were 0.189g±0.01g versus 0.163g±0.03g, respectively, ensuring that the mice, upon sacrifice, had reached the same stage of tumor development).

3.2.4 Estimation of adoptively transferred A-NK cell infiltration of lung metastases and tumor burden

Using the Thy1.1/1.2 congenic system the infiltration of the transferred A-NK cells was determined as described previously[83]. Briefly, one half of organ samples obtained from mice at various time points were fresh frozen at –70 ^{o}C. Eight-micron thick sections of the organs were stained with anti-Thy1.1-PE Ab (Pharmingen) at a 1:200 dilution and compared to PE-conjugated IgG_{2b} Ab controls. Sections from fresh-frozen lungs with B16 tumors were stained with a 1:1 mixture of polyclonal rabbit anti-Tyrp1[209, 210] and Pmel17[211, 212] antibodies (kindly provided by Dr. Vincent Hearing, NIH, USA) and subsequently with PE-conjugated anti-rabbit antibody (#2771, Molecular Probes Inc., Eugene, Oregon, USA) to reveal B16 tumor cells. Fluorescent and DIC images of the tissue sections were obtained using a Nikon fluorescent microscope and analyzed for cell numbers using MetaMorph image analysis software[83].

3.3 RESULTS

3.3.1 IL-12 enhances survival of A-NK cells supported by low doses of IL-2.

A-NK cell survival depends on continuous stimulation by high doses of IL-2 both *in vitro* (6,000 IU/ml) and *in vivo*[175]. Regarding *in vivo* settings, it is of great interest to find ways to maintain NK cell survival while avoiding the continuous use of systemic, toxic, high-dose IL-2 support. Because IL-12 acts as an activating signal for NK cells[192, 193], in addition to its effects as a helper T cell type 1 (Th1) cytokine [103, 194], we tested whether A-NK cells adenovirally

transduced to express IL-12 would require less IL-2 for their survival. We first set out to determine if A-NK cells transduced with an Ad-IL-12 vector, as recently described [213], could survive *in vivo* without any exogenous IL-2 support. We found that by 72 hours post injection, none of the IL-12 gene-transduced A-NK ($A\text{-}NK_{12}$) cells could be located within any of the tissues analyzed, i.e. lung, lung tumors, liver, and spleen (data not shown). This indicated that IL-12 alone is not a survival factor for murine NK cells. We then set up an *in vitro* assay in which recombinant IL-12 and IL-2 were titrated to determine how much IL-2 was needed to augment the effects of the transgenic IL-12 on NK cell survival. As seen in Figure 10A, when IL-2 is not provided the A-NK cells do not survive in any of the tested doses of IL-12, confirming that this cytokine alone is not sufficient to maintain A-NK cell survival. However, once IL-2 is given, even at 6 IU/ml, IL-2 and IL-12 have a synergistic effect on cell survival. The survival benefit was even more pronounced at the 600-IU/ml IL-2 level (Fig. 10A). Increasing IL-2 to 6,000 IU/ml (the normal A-NK cell culture dose) did not enhance A-NK cell survival above that seen with 600 IU/ml, but their survival was still greater than that of A-NK cells cultured without IL-12 at these IL-2 levels (Fig. 10A). Thus, IL-2 is necessary as a survival factor, but the IL-2 concentration can be greatly reduced (10-100 fold) in the presence of IL-12.

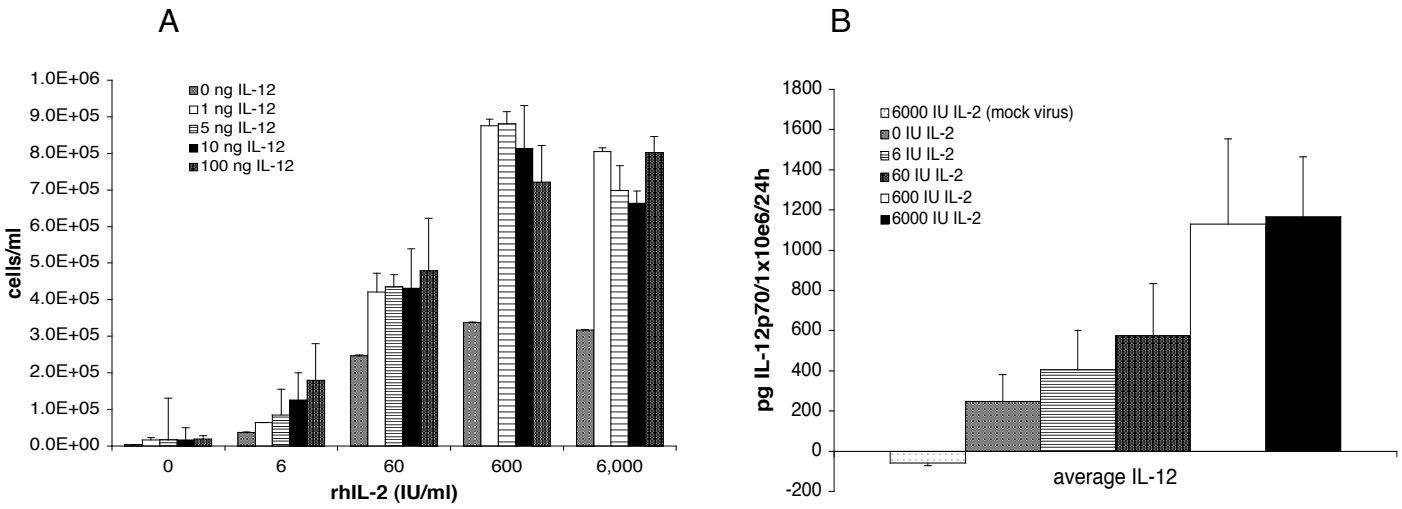

Figure 10. IL-12 survival benefits on A-NK cells and IL-12 production by transduced A-NK cells depend on IL-2. (A) A-NK cells were plated at $1.5x10^4$ cells per 200μl CM per well of a 96-well plate. To the wells was added rhIL-2 and/or rmIL-12 (ng/ml) in the concentrations indicated. Following a 48-hour incubation, 10μl of Cell Titer substrate was added to each well. Plates were then read after a three-hour incubation at 490nm. Data are presented as number of cells/ml run in triplicate, with error bars representing SD. (B) $5x10^6$ A-NK cells were transduced with Ad-IL-12 at 32 MOI in RPMI. $1x10^6$ transduced cells were then cultured in CM in 0, 6, 60, 600, or 6,000 IU/ml IL-2. Mock transduced cells cultured in 0 or 6,000 IU/ml IL-2 served as controls. Supernatants were collected after 72 hours of culture and mIL-12 was measured using ELISA, according to manufacturer's protocol. Values are represented as the average pg/ml IL-12p70 from two separate assays, with SD error bars.

Having demonstrated an IL-2/-12 synergy, we next tested the ability of A-NK_{12} cells to produce IL-12 when maintained at various concentrations of IL-2. A-NK_{12} cells receiving no IL-2 produced only 247 pg of IL-12 per $1x10^6$ cells per 24 hours (Figure 10B). However, values reached 1,130 pg/$1x10^6$ cells/24 hours with the addition of 600 IU/ml of IL-2, but did not increase further even after addition of 6,000 IU/ml IL-2. These data illustrate, as could be expected, the need for a source of IL-2 to be present for A-NK cells to express the IL-12 transgene in considerable amounts. They also illustrate that a substantial amount of IL-12 is produced by A-NK_{12} cells even when supported by low doses of IL-2.

3.3.2 IL-12 production results in an increase in CD25 expression by transduced A-NK cells.

IL-12 is known to induce an increase in expression of CD25 on T cells [214] and NK cells [95]. Along with the beta and gamma subunits, CD25, the alpha subunit, makes up the high affinity complex of the IL-2 receptor. Thus, an increase in CD25 expression induced by IL-12 could explain the reduced IL-2 dependency of the A-NK_{12} cells. Indeed, 44.6% of the A-NK_{12} cells were positive for the expression of CD25, compared with 3.3%+ for nontransduced A-NK (A-NK_{non}) cells (Figure 11).

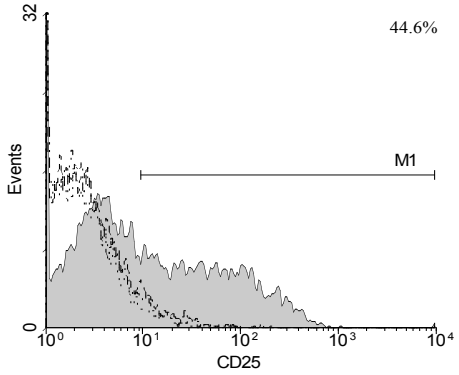

Figure 11. CD25 expression on A-NK cells increases following Ad-IL-12 transduction. $5x10^6$ A-NK cells were transduced with Ad-IL-12 at 25 MOI. A-NK_{12} (filled curve) and A-NK_{non} (open curve) cells were stained with anti-CD25-FITC Ab after 48 hours of culture. Cells were analyzed by flow cytometry, collecting 5000 events and compared with isotype control.

3.3.3 IL-12 produced *in vivo* by transduced A-NK cells enhances their survival in B16 lung metastases.

Having demonstrated the capacity of A-NK_{12} cells to survive *in vitro* in low concentrations of IL-2, we wanted to confirm this finding *in vivo*. In the B16 lung tumor model, four separate experiments demonstrated that when IL-2 was administered at low doses, adoptively transferred A-NK_{12} cells survived to a much greater extent than A-$NK_{non/mock}$ cells. In a representative experiment, in which we titrated the exogenous PEG-IL-2, a dose response was clearly evident, with the A-NK_{12} cells persisting in the tumor tissue (72 hours after adoptive transfer) in higher numbers than A-NK_{mock} cells at all of the ranges of PEG-IL-2 below 60,000 IU tested, (Fig. 12A).

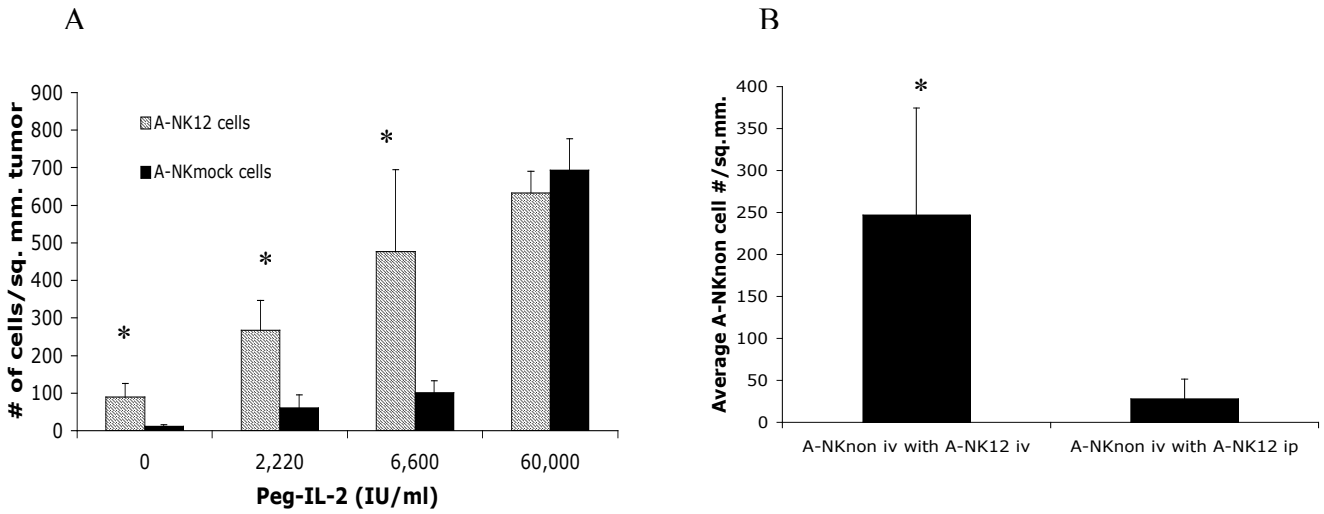

Figure 12. IL-12 expressed by transduced A-NK cells provides a survival benefit to transduced cells, at low doses of Peg-IL-2, as well as non transduced cells when expressed locally. (A) Mice bearing day 10 B16 lung tumors received $4x10^6$ A-$NK_{12/mock}$ and $5x10^6$ A-NK_{non} cells iv with two injections of 60,000 IU Peg-IL-2, followed by 4 injections at 0, 2,220, 6,600, or 60,000 IU Peg-IL-2. (B) Mice bearing day 10 B16 lung tumors

received either $5x10^6$ A-NK_{12} and $5x10^6$ A-NK_{non} iv along with $5x10^6$ A-NK_{non} ip or $10x10^6$ A-NK_{non} iv along with $5x10^6$ A-NK_{12} ip. At twelve-hour intervals, the mice then received two injections of Peg-IL-2 at 60,000 IU, followed by 4 injections at 4,500 IU. At 72 hours post adoptive transfer, mice were sacrificed and tissues harvested (n=3). A-NK cell densities in tumors were measured as described in Materials and Methods, and represented as number of cells (A-NK_{12}/$_{mock}$ (A) and A-NK_{non} (B)) per square mm lung tumor tissue. Values were found to be statistically significant with a p value<0.05, *, and p value=0.0056, **, with error bars representing SD.

As expected, when full dose of PEG-IL-2 was given (60,000 IU/ml every 12 hours in the first 3 days post adoptive transfer), the exogenous A-NK cells were found in equal numbers in lung tumors whether they were expressing IL-12 or not. The A-NK_{12} cells preferentially localized to the lung metastases rather than normal lung tissue. This provided support that adenoviral transduction did not diminish the transduced cells' ability to traffic to and localize within lung tumors. At a PEG-IL-2 dose of 6,600 IU/12h, the A-NK_{12} cells were present at 75% maximal (i.e., the value seen with 60,000 IU/12h PEG-IL-2) compared with only 15% for A-NK_{mock} cells (Table 1).

Table 1. Effect of interleukin-12 on survival of both transduced and nontransduced activated natural killer cells *in vivo*.

		Exogenous PEG–IL-2 support[a]			
		0 IU	*2220 IU*	*6600 IU*	*60,000 IU*
IL-12 transduced[b]	Thy1.1	90.41[c] (35.19)[d]	267.48 (79.95)	477.41 (217.71)	632.54 (57.41)
	% maximal	14.3	42.3	75.5	100
Nontransduced[e]	CD45.1	62.85[c] (16.90)[d]	154.07 (91.99)	310.22 (150.92)	693.00 (55.32)
	% maximal	9.1	22.2	44.8	100
Mock transduced[b]	Thy1.1	12.30[c] (4.32)[d]	61.64 (34.60)	101.75 (31.06)	692.86 (83.72)
	% maximal	1.8	8.9	14.7	100
Nontransduced[e]	CD45.1	11.98[c] (2.46)[d]	33.81 (28.38)	93.20 (24.76)	692.33 (136.11)
	% maximal	1.7	4.9	13.5	100

[a]Mice received two injections of 60,000 IU of PEG–IL-2 after adoptive transfer of transduced A-NK cells. Mice then received 0, 2,220, 6,600, or 60,000 IU of PEG–IL-2 every 12 hr for 2 days.
[b]Mock- or IL-12-transduced Thy1.1 A-NK cells (4×10^6) were administered to Thy1.2 hosts bearing day-7 B16 lung tumors.
[c]The exogenously delivered A-NK cells were quantitated within lung tumors and are expressed as number of cells per square millimeter of tumor tissue ($n = 3$).
[d]Standard deviation for $n = 3$ mice per group.
[e]Nontransduced CD45.1 A-NK cells (5×10^6) were coadministered along with mock- or IL-12-transduced A-NK cells.

The numbers were equally as dramatic for the lowest (2,200 IU) and no PEG-IL-2 doses with A-NK cell densities in the tumor tissue being 4- and 8-fold better, respectively, for the A-NK12 cells compared with A-NKmock cells (Table 1 and Figure 12A). Thus, it appears that A-NK12 cells are able to survive and localize to tumor tissue when supported by as little as one-tenth the dose of exogenous IL-2 needed for A-NKmock cells ($p<0.05$, for all three PEG-IL-2 doses).

3.3.4 IL-12 produced *in vivo* by A-NK12 cells enhances the survival of nontransduced, coadoptively transferred A-NK cells in B16 lung metastases.

We wanted to determine whether the IL-12 produced within tumors could also maintain the survival of nontransduced, coadministered, A-NK cells. Using CD45 and Thy1 congeneic systems, we could distinguish the nontransduced from the transduced effectors. When supported by low doses of PEG-IL-2 (2,220 IU and 6,600 IU respectively), 4.5- to 3-fold more A-NK_{non} cells survived within lung tumors when coinjected with A-NK_{12} cells than A-NK_{mock} cells (Table 1). The IL-12 produced by transduced A-NK cells is, therefore, able to maintain survival of not only the transduced cells themselves, but of neighboring A-NK_{non} cells as well.

3.3.5 Tumor-selective versus tumor-distant IL-12 delivery.

To evaluate the importance of the IL-12 being produced at the tumor site (as opposed to a site distant from the tumor) on the survival of nontransduced A-NK cells in lung tumors, we injected 5 million A-NK_{non} cells by the iv route plus 5 million A-NK_{12} cells by either the iv or ip route. We have previously shown that A-NK cells injected ip do not leave the ip cavity and therefore do not localize into the lungs or lung tumors [83]. Even though ip injection of A-NK_{12} cells resulted in the survival of a few more A-NK_{non} cells (iv injected) in lung tumors compared with the control group (which did not receive any A-NK_{12} cells), 8.8-fold more iv-injected A-NK_{non} cells survived in lung tumors when the A-NK_{12} cells were injected by the iv route than by the ip route (Fig. 12B, p value=0.0056). Thus, in maintaining A-NK cell survival in the tumor tissues, delivery of the IL-12 cytokine to the tumor microenvironment is superior to a tumor-distant delivery.

3.3.6 Intratumoral IL-12 expression by transduced A-NK cells enhances the reduction of B16 tumor metastases.

Next, we wanted to determine how successful the local expression of IL-12 by iv injected A-NK_{12} cells would be in supporting NK cell-mediated responses against established B16 lung tumor metastases. We began by looking at tumor tissue from mice receiving 6,600 IU/ml PEG-IL-2 and either A-NK_{12} or A-NK_{mock} cells iv. Having IL-12 expressed within lung tumors provided a superior benefit in regards to anti-tumor effect as well as A-NK cell survival (Fig. 13A-C). To investigate anti-tumor responses from IL-12 produced distally rather than intratumorally, some mice received A-NK_{12} cells by the ip route. The presence of A-NK_{12} cells in the ip cavity caused some reduction in tumor burden compared with control animals, but this reduction was not significant (Fig. 13C). By comparing the efficacy of A-NK_{12} cells injected iv with that of cells injected ip, it was evident that targeting the A-NK_{12} cells to the lung tumors, by iv injection, resulted in a larger tumor reduction than ip injection (p value=0.023).

3.3.7 Co-injection of nontransduced A-NK cells enhances the anti-tumor efficacy.

We next wanted to determine if the treatment efficacy of A-NK_{12} cells could be increased by simultaneous administration of A-NK_{non} cells (that could benefit from the IL-12 produced by A-NK_{12} cells in the tumor microenvironment). This seemed to be the case since the 64% tumor reduction seen following iv injection of A-NK_{12} plus A-NK_{non} cells was significantly greater than the 48% reduction following iv injection of A-NK_{12} alone (p=0.011, Fig. 13D). It could be argued that the reason we did not observe a comparable tumor reduction in mice receiving A-

NK_{12} cells ip to that of mice receiving them iv, as in Fig. 13C, was due to a lack of A-NK effector cells present to respond within the lungs.

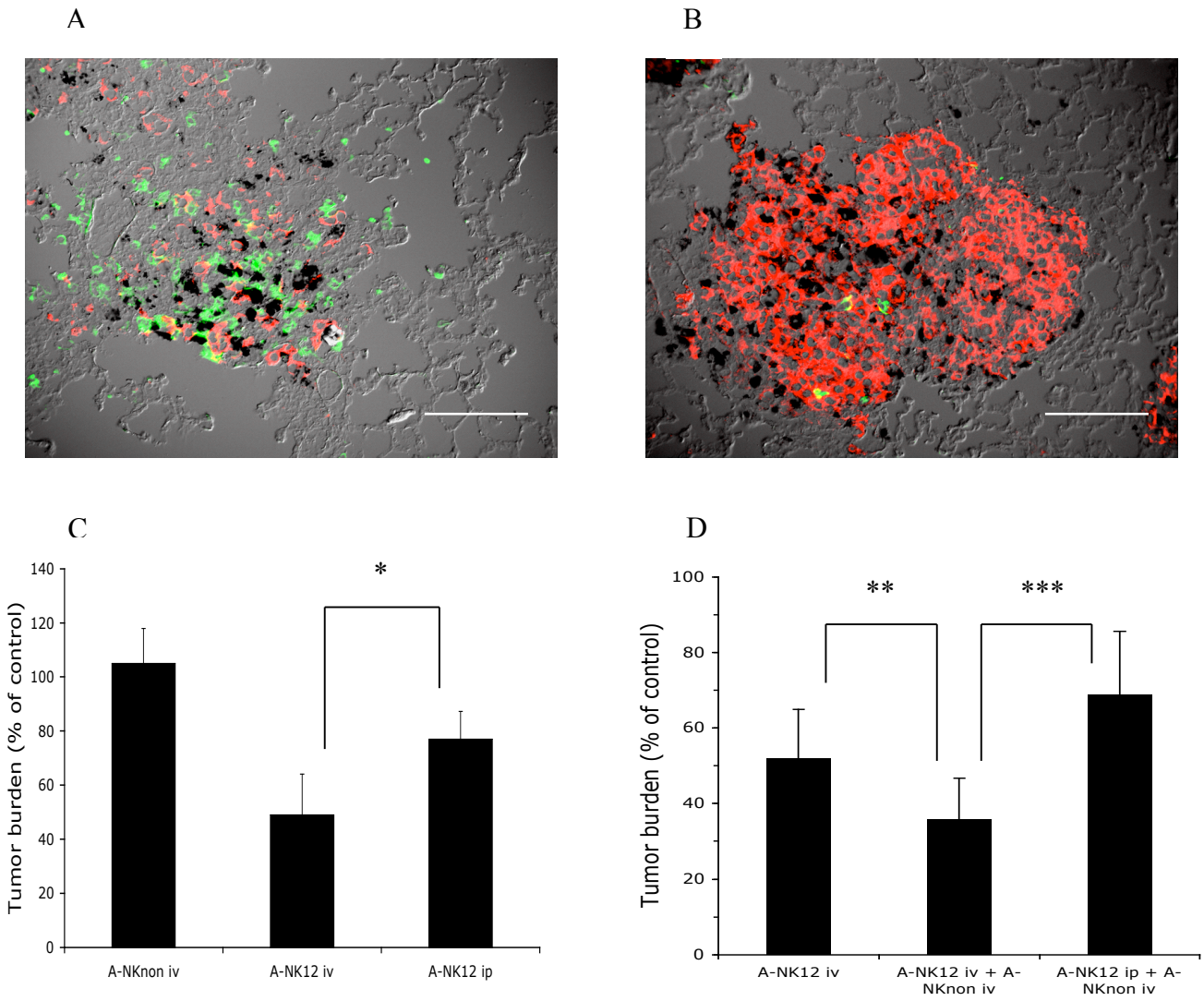

Figure 13. Presence of IL-12 within B16 lung metastases leads to greater tumor reduction. A representative image of a lung tumor from mice receiving A-NK_{12} (A) or A-NK_{mock} (B) cells together with 6,600 IU/ml Peg-IL-2, following 2 injections of 60,000 IU Peg-IL-2 co-administered along with A-NK cells, illustrates B16 staining (in red) contrasted with the presence of adoptively transferred A-NK cells (in green). Images were obtained using 20x magnification. Bars represent 100 microns. Note the abundant volume of A-NK cells compared to tumors cells when IL-12 is expressed within the tumor. (C and D) Mice bearing B16 lung metastases received

$5x10^6$ A-NK_{non} or A-NK_{12} cells (iv or ip) only (C), or in conjunction with $5x10^6$ A-NK_{non} cells (iv) (D). 60,000 IU Peg-IL-2 was administered to all mice at t=0 and 12 hours post adoptive transfer, and thereafter 4 injections of 4,500 IU Peg IL-2 were given 12 hours apart. Data are represented as percent reduction in tumor tissue at 120 hours post adoptive transfer compared to mice treated with IL-2 alone (n=4 (C), n=9 (D)) (*, p value=0.023, ** p value=0.011, and ***, p value=0.0001). Error bars represent SD.

However, as seen in Figure 13D, mice receiving an ip injection of A-NK_{12} cells plus iv injection of A-NK_{non} cells (to ensure the presence of A-NK cells in the lung tumors) demonstrated some reduction (31%) in tumor burden compared with control animals (receiving IL-2 only), but this was significantly less than the 64% tumor reduction achieved by iv injection of both A-NK_{12} plus A-NK_{non} cells (ensuring the presence of both A-NK_{non} cells and A-NK_{12} cells in the lung tumors) (p=0.0001). Thus, A-NK_{12} cells are able to significantly boost the anti-lung tumor activity of A-NK_{non} cells but only if the A-NK_{12} cells are also located in or near the lung tumors.

3.3.8 Prolonged survival following treatment with IL-12 transduced A-NK cells compared with mock transduced A-NK cells.

The results described above indicate that A-NK_{12} cells survive better *in vivo* and combat the tumors they infiltrate better than A-NK_{mock} cells. To evaluate if the observed tumor reduction was sufficient to increase survival of tumor-bearing animals, A-NK_{12} and A-NK_{mock} cells were adoptively transferred into mice with established lung tumors of B16 or MCA-205 origin. In an early (day-3) B16 lung tumor model, a 10-day increase in survival time was found

following iv injection of A-NK_{12} cells compared with A-NK_{mock} cells or treatment with PEG-IL-2 alone (Figure 14A, p value=0.0031). Thus, although A-NK_{mock} cells lose their therapeutic efficacy when supported by only two injections of 60,000 IU of PEG-IL-2, this nontoxic regimen of IL-2 support is sufficient to support the anti-tumor effect of A-NK_{12}. In fact, the therapeutic effect of A-NK_{12} cells is still maintained, and significant, in mice receiving even less IL-2 (two PEG-IL-2 injections of only 4,500 IU [Fig 14A, A-NK_{12} low IL-2, p value=0.0016]).

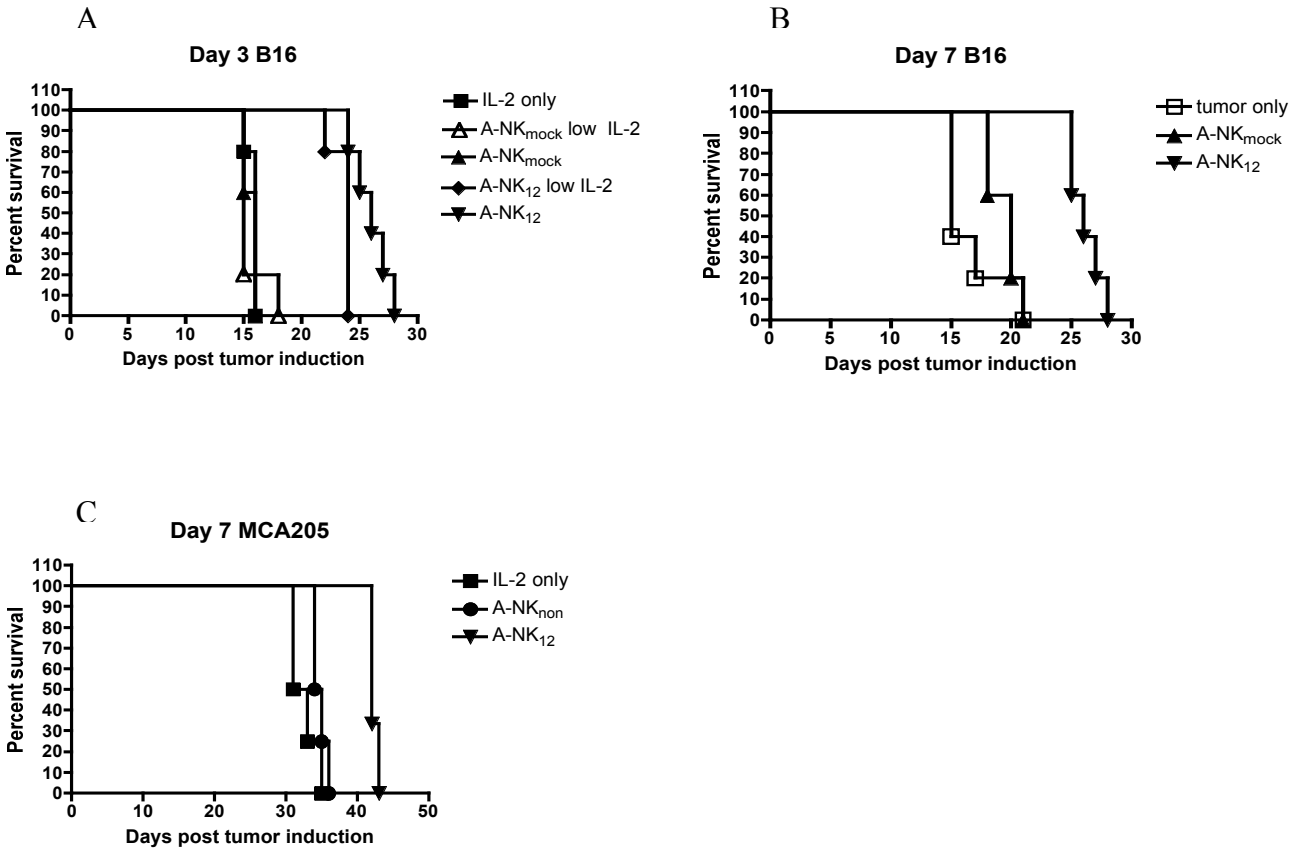

Figure 14. IL-12 prolongs the survival of mice when expressed locally by A-NK12 cells. Survivability of mice (n=5) was monitored, as described in Materials and Methods, after the mice received $5x10^6$ A-NK_{non}, A-NK_{mock} or A-NK_{12} cells. The survivability of mice bearing day 3 B16 (A), day 7 B16 (B), or day 7 MCA205 (C) lung metastases was plotted using the Kaplan-Meier method for the number of days post tumor induction. Statistical significance was calculated, using log-rank test, for mice receiving A-NK_{12} compared to A-NK_{mock} cells, with p

values=0.0031 (A), 0.0019 (B), and 0.0158 (C). Δ = A-NK_{mock} low IL-2: A-NK_{mock} plus 2 injections of 4,500 IU (low) Peg-IL-2; ▲=A-NK_{mock}: A-NK_{mock} plus 2 injections of 60,000 IU (high) Peg-IL-2; ■=IL-2 only: 2 injections of high Peg-IL-2 only; ◆=A-NK_{12} low IL-2: A-NK_{12} plus low Peg-IL-2; ▼=A-NK_{12}: A-NK_{12} plus high Peg-IL-2; □=tumor only: tumor cells only; ●=A-NK_{non}: A-NK_{non} plus high Peg-IL-2. Some data from panel (A) have previously appeared as a separate figure in a *Cancer Gene Therapy* article (Goding *et al*., 2007).

To determine the efficacy of A-NK_{12} cells in a more aggressive advanced tumor setting, mice bearing 7-day-old B16 lung tumor metastases were treated with A-NK_{12} cells. These mice survived a median 26 days compared with 20 days for mice receiving A-NK_{mock} cells (Figure 14B, p value=0.0019). Treatment with A-NK_{12} cells as compared with A-NK_{non} cells also prolonged the survival of mice bearing day-7 MCA-205 lung tumors, by 7.5 days (Figure 14C, p value=0.0158). This clearly illustrates the enhanced anti-tumor efficacy of A-NK_{12} cells compared with A-$NK_{non/mock}$ cells and demonstrates the advantage of using adenovirally transduced A-NK cells to deliver and express cytokines specifically within lung tumors.

3.4 DISCUSSION

We suggest that adoptive transfer of A-NK cells, adenovirally transduced with cytokine genes, can be used to ensure the presence of cytokines in the tumor tissue. Our data show *ex vivo*-transduced A-NK cells are clearly capable of expressing a transgene in the tumor microenvironment following adoptive transfer. Depending on which cytokine gene the A-NK

cells express, this strategy may help support the adoptively transferred NK cells themselves, endogenous NK cells or cells involved in adaptive immunity, such as dendritic cells and T cells. We found that IL-12 gene transduction of A-NK cells significantly reduced their demand for exogenous IL-2 as a survival factor both *in vitro* and *in vivo*, reducing concerns of IL-2-related toxicity. In addition, greater survival of nearby, nontransduced, A-NK cells was observed *in vivo*. Most importantly, at low doses of exogenous IL-2 support, selective IL-12 gene therapy to lung tumors using IL-12 gene-transduced A-NK cells resulted in significantly greater anti-tumor responses, as compared with nontransduced A-NK cells.

IL-2 is involved in a number of ways in generating and perpetuating anti-tumor responses. NK and T cells require it for activation, proliferation, and survival [61]. IL-2 is a key component in triggering other anti-tumor cytokines cascades, such as interferon (IFN)-γ [215] and tumor necrosis factor (TNF)-α [216]. We have also found that IL-2 is needed to ensure trafficking to and localization within established tumors by adoptively transferred A-NK cells [175]. The amount of IL-2 that can be given to support the A-NK cells, however, is greatly limited by the adverse effects IL-2 has when applied at high doses over an extended period [88, 217]. To solve this problem, we investigated whether provision of the A-NK cells, via adenoviral gene transduction, to produce IL-12 would enable them to survive and function *in vivo* without the support by high and toxic doses of IL-2.

When recombinant murine IL-12 was added to A-NK cell cultures, we found, as expected, enhanced A-NK cell proliferation at all IL-2 levels tested. However, the A-NK cells died rapidly if IL-2 was absent, indicating that although IL-12 works synergistically with IL-2 in terms of survival and proliferation, IL-12 alone is not a survival factor for the A-NK cells. Accordingly, A-NK_{12} cells also survived much better *in vitro* at low levels of IL-2 compared

with A-NK_{mock} cells, but when no exogenous IL-2 was given most of the A-NK_{12} cells died within 24 hours. It was not surprising, therefore, that the ability of the transduced A-NK cells to produce IL-12 depended on the presence of IL-2. However, maximal IL-12-production (>1.1 ng/24 hours/1x10^6 cells) was achieved at low IL-2 levels (600 IU/ml IL-2). It is very likely that this increased sensitivity to IL-2 induced by the IL-12 gene transduction is caused by the IL-12-driven upregulation of CD25.

Adoptive transfer experiments revealed that IL-12 gene transduction of the A-NK cells prior to injection enhanced their survival *in vivo* as well. When supported by high (120,000 IU/day) doses of IL-2, the densities of A-NK_{mock} and A-NK_{12} cells in lung tumors were the same. However, lowering the IL-2 support by 10-fold resulted in an almost complete loss of the A-NK_{mock} cells, whereas A-NK_{12} cells were found at 75% of maximal density in the lung tumors. Furthermore, A-NK_{non} cells survived significantly better in lung tumors when coinjected with A-NK_{12} cells than with A-NK_{mock} cells, due to the benefit of IL-12 produced by A-NK_{12} cells at the tumor site.

The enhanced survival of the A-NK_{12} cells compared with A-$NK_{mock/non}$ cells translated into a therapeutic benefit, in that a significantly greater reduction in growth of infiltrated tumors was seen following treatment with A-NK_{12} cells versus A-$NK_{mock/non}$ cells. Furthermore, the therapeutic effect of the combined treatment with A-NK_{12} and A-NK_{non} cells was superior to either treatment alone, indicating that not only survival, but also the anti-tumor effector mechanisms of A-NK_{non} cells is enhanced by IL-12 (or IL-12-induced IFNγ) produced by nearby A-NK_{12} cells.

To evaluate whether the observed effect on A-NK cells survival and anti-tumor potential was dependent on the presence of IL-12 produced locally, i.e., in the tumor microenvironment,

or at a site distal from the lung tumors, A-NK_{12} cells were, in some experiments, injected by the ip route. Even though ip injected A-NK cells do not leave the ip cavity and therefore do not home to tumor sites in the lungs [83], the IL-12 (and/or IFNγ) produced by these cells could reach systemic level high enough to stimulate intratumoral A-NK cells. Whereas IL-12 was undetectable in serum from any of the treated animals, IFNγ levels were increased in all animals receiving A-NK_{12} cells, compared with mice receiving IL-2 only, despite the route of their administration. IFNγ was measured in the range of 1000-1500 pg/ml in the serum of mice receiving A-NK_{12} cells whereas approximately 250 pg/ml was demonstrated in serum of mice receiving IL-2 only treatment. This provides evidence that, despite direct proof for IL-12 production by A-NK_{12} cells *in vivo*, unlike with the production of GFP within tumors previously, IL-12 is being produced by the A-NK_{12} cells in the tumors. Although iv injected A-NK_{non} cells survived slightly better and displayed slightly enhanced anti-tumor activity when coadministered with A-NK_{12} cells ip compared with animals not receiving A-NK_{12} cells, both parameters were significantly better in animals receiving the A-NK_{12} or A-NK_{12} plus A-NK_{non} cells iv, illustrating even further the effects of IL-12 production in A-NK_{12} recipient mice. Thus, tumor-localization of A-NK_{12} cells seems to be a prerequisite for achieving the full IL-12 effects generated by these cells.

We have recently demonstrated that the morphology ("loose" versus "compact") of experimental mouse lung tumors predicts their permissiveness to A-NK and T cell infiltration [218]. In the experiments described above, measurements of A-NK cell tumor homing and anti-tumor effect were performed on lung tumors of the infiltration-permissive, "loose" phenotype, usually comprising 65-75% of all B16 lung metastases. However, despite the fact that most metastases of the infiltration-resistant, "compact" phenotype became only poorly infiltrated by the injected

A-NK cells, the overall efficacy of the A-NK_{12} cell therapy was highly significant in both day-3 and -7 tumor models as judged by survival time of treated animals (resulting in 10- and 7-day increases, respectively). Although such prolongation in survival, especially in the aggressive B16 model, is impressive given the fact that only one treatment with about five million effector cells was given, it is clear that the anti-tumor effect is only transient and that the remnants of the tumors attacked by the A-NK cells continues to grow exponentially. Apparently, no efficient adaptive immune responses against the B16 or MCA-205 tumors were induced, despite the massive destruction of tumor tissue by the A-NK cells and the intratumoral presence of the Th1 cytokines, IL-12 and IFNγ. Experiments are in progress to elucidate whether any adaptive responses are generated at all after A-NK_{12} treatment or whether the low MHC-I expression, especially by the B16 tumors, allows the evasion from MHC-restricted CTLs. If no signs of adaptive immunity are detected, it is possible that such immunity can be induced by cotransducing the A-NK_{12} cells with genes encoding for additional immunostimulatory factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), CD40, and so on.

In conclusion, we have demonstrated that A-NK cells can be effectively used for cytokine gene therapy, due to their ease of transduction with adenoviral vectors expressing transgenes encoding immunostimulatory cytokines such as IL-12. The success of this novel mode of gene therapy is evident in greater survival of both A-NK_{12} and bystander A-NK_{non} cells, allowing for efficient A-NK cell-based cancer treatment that is not dependent on continuous support by high and toxic doses of exogenous IL-2. Although the A-NK_{12} treatment leads to significant tumor reduction and survival prolongation, it does not result in long-lasting inhibition of tumor growth. Nevertheless, these experiments demonstrate the potential of employing cytokine gene-transduced A-NK cells in cancer immunotherapy and we predict that, because A-NK cells can be

transduced to express several genes simultaneously [213], we will eventually be able to manipulate the intratumoral cytokine milieu such that long-lasting adaptive anti-tumor responses can be generated and maintained without systemic injection of cytokines and therefore without the induction of limiting side effects and toxicity.

4.0 ENHANCING A-NK$_{12}$ EFFICACY WITH TNFα

4.1 INTRODUCTION

Treatment with IL-12 gene-transduced A-NK cells has been effective in promoting significant benefits with respect to increased animal survival and decreased cytokine toxicity, but the mice eventually succumbed to the tumor burden. Since the A-NK cells are relatively short-lived cells, it is very likely that as A-NK cell numbers diminish in tumors, the overall anti-tumor response was subsequently diminished. Regardless of the tumor-destruction mediated by the treatment and the presence of pro-inflammatory cytokines IL-2, IL-12, and IFNγ, the survival benefit was limited to 6-10 days and therefore did not indicate that cellular adaptive anti-tumor immune response were being induced. To compensate for A-NK-IL-12's lack of long-term host anti-tumor induction, we decided to investigate the addition of another immune-promoting cytokine, TNFα, to the IL-12 transduction. This seems to be an appropriate choice in that TNFα functions to increase the permeability of vasculature to allow immune cells and humoral immune factors to enter an inflamed tissue. Thus, TNFα could enhance the movement of cells, including endogenous and transferred NK cells into the tumor[48, 219]. TNFα may stimulate and mature intratumoral DCs[220], and this, along with the presence of antigens released from tumor cells killed by the A-NK$_{12}$ cells and the presence of the proinflammatory cytokines IL-12 and IFNγ within tumors, may then lead to the induction of a Th1-polarized anti-tumor immune response.

TNFα could also benefit the A-NK_{12} cells themselves in that TNFα has been shown to increase the proliferation of NK cells[221]. Finally, when combined with IL-2, TNFα may increase lymphokine activated killer activity[222]. Thus, an increased efficacy of the A-NK cell treatment and possibly induction of adaptive anti-tumor responses could be expected when TNFα gene-transduction is added to A-NK_{12} cell treatment.

We show here that in spite of considerable production of TNFα and an inhibitory effect of A-$NK_{12/TNF\alpha}$ cell supernatants on B16 growth *in vitro*, as well as a remarkable persistence *in vivo* of A-NK cells transduced with both IL-12 and TNF, co-transducing A-NK_{12} cells with TNFα did not result in a robust and significant survival benefit compared to IL-12 transduction alone.

4.2 MATERIALS AND METHODS[4]

4.2.1 Animals and viral vectors

C57BL/6, B6.PL-Thy1^a/CyJ, and NOD.CB17-Prkdc/J mice were purchased from Jackson laboratories (Bar Harbor, ME). The animals were housed in a specific pathogen free facility and the University's IACUC authorized their use. The recombinant murine TNFα adenoviral vector used in the proceeding studies were E1/E3 deleted replication-defective type 5 adenoviruses[18] (obtained from the Preclinical Vector Core Facility, University of Pittsburgh).

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[4] General materials and methods pertaining to this chapter but not mentioned in this section can be found in Chapter 2 or 3, Materials and Methods.

4.2.2 TNFα production and B16 growth inhibition

A-NK cells were transduced with Ad-mTNFα at 5, 25 or 50 MOI, Ad-IL-12 at 20 MOI or together at 5 MOI TNFα/20 MOI IL-12 (for cytokine analysis) or 25 MOI TNFα/20 MOI IL-12 (for B16 growth inhibition). A-NK cells transduced with 50 MOI Ad-TNFα were cultured over the course of 96 hours. Supernatant was collected every 24 hours. For A-NK cells transduced with TNFα and IL-12, supernatants were obtained 72 hours after initial viral incubation. Supernatants were then tested for TNFα production using multi-cytokine Luminex assay (Luminex core facility, University of Pittsburgh) or added to B16 cell cultures diluted in complete media. After a 48-hour incubation, 10μl of Cell Titer Reagent (Promega Corporation, Madison, WI) was added to each well. The plates were incubated another 3 hours at 37°C and then analyzed on a plate reader at 490nm.

4.2.3 Adoptive transfer and survival experiments

Lung tumor metastases were established by injecting B16 tumor cells ($3x10^5$/0.4 ml of RPMI) intravenously into NOD.CB17-Prkdc/J and/or C57BL/6 mice pretreated 24 hours earlier with an ip injection of anti-asialo-GM1 antiserum (Wako PureChemicals, Wako, TX) at 20 μl in 0.5 ml PBS to remove endogenous NK cells. Following 24 hours of *in vitro* culture, Ad-IL-12, Ad-TNFα, Ad-TNFα/IL-12, or mock-transduced A-NK cells were harvested from flasks and washed 3 times. Five million cells/ 0.4 ml RPMI were injected iv into mice with day-7 B16 lung metastases. The transferred A-NK cells were supported with 0.5 ml ip injections containing

60,000 IU of IL-2 in complex with polyethylene glycol (PEG-IL-2, a kind gift from the Chiron Corporation, Emeryville, CA), at 0 and 12 hours.

4.3 RESULTS

4.3.1 A-NK_{12} cell therapy is not dependent on endogenous T, B or NK cells.

Before evaluating the ability of TNFα to further improve the A-NK_{12} cell-based therapy, we wanted to determine if the survival increases we have seen previously with A-NK_{12} cell therapy were due primarily to the A-NK cells only or if the host has contributed to the anti-tumor therapy via T, B or NK cells. We therefore conducted an experiment involving a comparison of A-NK_{12} cells transferred into wild-type (WT) mice versus NOD-SCID mice. NOD-SCID mice are deficient for T and B cells and NK cell activity. Before assessing the survival of these two types of mice following A-NK_{12} therapy, we needed to ensure that A-NK_{12} cells are not impeded in any way in trafficking to lung tumors in the NOD-SCID mice, as this could skew results. As expected, A-NK_{12} cells were found in tumors at the same level in both WT and NOD-SCID mice (data not shown). As seen in Figure 15, WT animals receiving A-NK_{12} survived 8 days longer than WT animals receiving A-NK_{mock} cells (p value=0.0012). Interestingly, NOD-SCID mice survived an equal amount of time following A-NK_{12} treatment (Figure 15), illustrating a lack of involvement of host anti-tumor activity mediated by T, B, and NK cell.

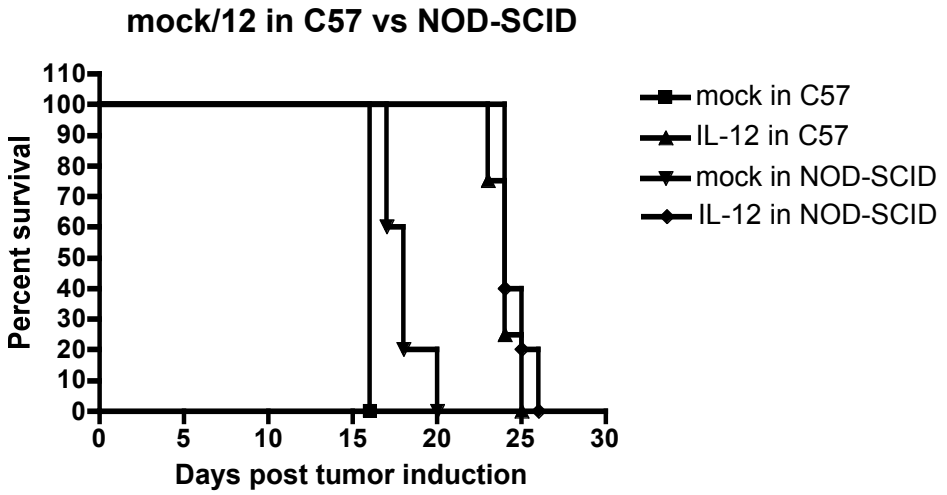

Figure 15. C57BL/6 mice and NOD-SCID mice bearing B16 lung tumors survive significantly longer following A-NK$_{12}$ cell therapy than A-NK$_{mock}$ therapy. 5 x 10^6 mIL-12 or mock transduced A-NK cells were injected i.v. into 7-day B16 tumor-bearing WT and NOD-SCID mice, and supported with 2 injections of 60,000 IU/ml PEG IL-2. Survival of mice was determined in a blinded fashion as described in Material and Methods and plotted using the Kaplan-Meier method. Log-rank test demonstrated a significant difference in survival time between mice receiving mock transduced A-NK cells and mIL-12 transduced A-NK cells (p value<0.005, n=5, except for IL-12 in C57 mice where n=4).

4.3.2 TNFα-transduced A-NK cells can produce TNFα and the production is increased following IL-12 co-transduction.

Although IL-12 has been successful in enhancing A-NK cell therapy without generating toxicity, this does not seem to be sufficient to boost long-term adaptive host immunity, according to the survival readout. An early initiator of inflammation, TNFα, activates DCs and promotes

CTL generation[223, 224]. In addition to its other beneficial effects listed above, TNFα is therefore an attractive choice to add to the A-NK-12 therapy to further promote adaptive immune responses. We began by demonstrating the ability of A-NK cells to produce TNFα following adenoviral transduction. Figure 16A demonstrates that TNFα can be detected in supernatant in the range of ng/ml from A-NK cells transduced at 50 MOI with Ad-TNFα. Production peaks around 72 hours post transduction at 1750 pg/ml and can be measured out to at least 96 hours. Furthermore, Figure 16B shows a 25-fold increase in TNFα production with 5 MOI virus compared with no virus. When the A-NK cells were cotransduced with IL-12, the level of TNFα increased 2-fold from that seen with TNFα transduction alone (Fig. 16B, 109pg/ml vs. 216pm/ml for TNFα and TNFα/IL-12, respectively).

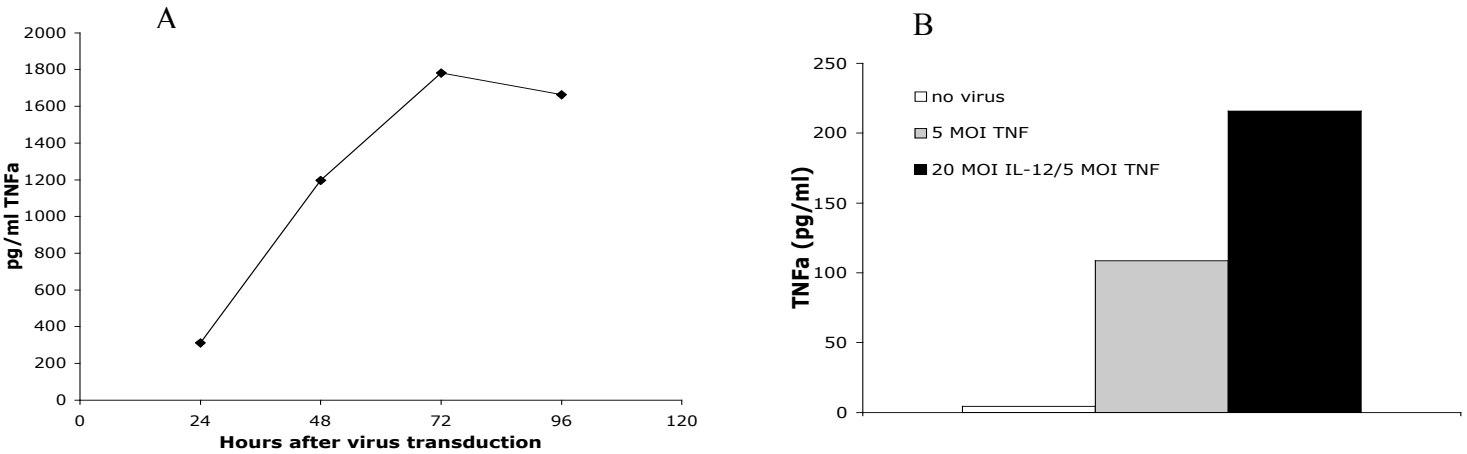

Figure 16. TNFα is produced by transduced A-NK cells. (A) $5x10^5$ A-NK cells were transduced with Ad-TNFα at 50MOI or (B) $5x10^5$ A-NK cells were transduced with Ad-TNFα or Ad-TNFα/IL-12 at 5 and 5/20 MOI, respectively, in RPMI. Transduced cells were then cultured in CM with 6,000 IU/ml IL-2. Non-transduced cells served as control. Supernatants were collected at the times indicated (A) or after 72 hours of culture (B) and TNFα was measured in a luminex assay.

4.3.3 Anti-B16 activity of supernatants from A-$NK_{12/TNF\alpha}$ cells.

We wanted to determine the combined effect of TNFα and IL-12 in tumor cell killing mediated by supernatants from transduced A-NK cells. When supernatants from TNFα gene-transduced A-NK cells were added to B16 cell cultures, fewer B16 cells survived compared with no virus and mock virus controls (Fig. 17). There was a slight decrease in the number of B16 cells seen with TNFα over IL-12 alone but this was only obvious at the 1:2 dilution. The effect on B16 cell growth was more pronounced when TNFα and IL-12 are combined, as seen at the 1:8 dilution of supernatant (Fig. 17, p value<0.001). These data illustrate that supernatants from A-NK cells transduced with TNFα, in addition to IL-12, inhibit the growth of B16 cells. We also investigated the ability of A-NK cells transduced with TNFα and IL-12 to directly kill B16 cells in a 4-hour chromium release assay. Unlike with B16 growth inhibition, A-NK cytotoxicity was not significantly enhanced by co-transducing A-NK_{12} cells with TNFα.

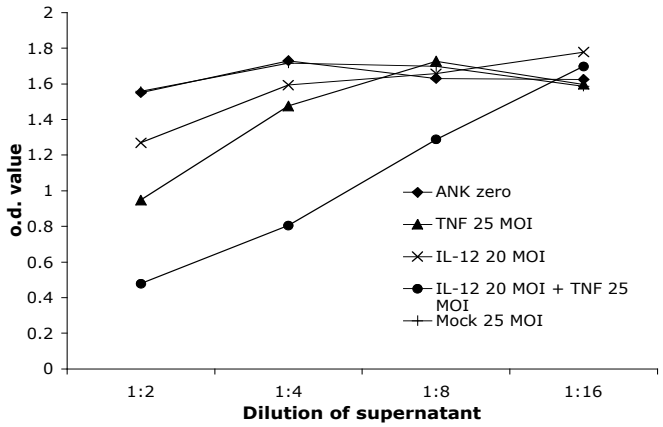

Figure 17. TNFα adds to the IL-12 induced growth inhibition of B16 cells *in vitro*. $15x10^3$ B16 cells were set up in 96-well culture plates for 24 hours. Supernatants from cultures of A-NK cells transduced with IL-12, TNFα, or IL-12 and TNFα, at the MOI indicated, were added at varying dilutions to the B16 cell cultures. B16 cells were then cultured for another 48 hours. Cell titer assay substrate was added to the wells and following a 3-hour incubation period the plates were read on a plate reader at 490nm. Values are given in average o.d. value from wells run in triplicate.

4.3.4 Transduction with TNFα and IL-12 enhances *in vivo* survival of adoptively transferred A-NK cells.

Having demonstrated that TNFα can be over-expressed by transduced A-NK cells, enhanced with IL-12 co-transduction, and an improved B16 cell growth inhibition by A-$NK_{12/TNF\alpha}$ cell-secreted factors, we set out to determine if this could translate into improved anti-tumor responses in an *in vivo* setting. We decided to investigate the early effects of TNFα in terms of the dynamics of the lung and tumor microenvironment infiltrate. We began by looking at the survival of adoptively transferred A-NK cells in B16 lung tumor-bearing animals. Mice were given 5 million A-NK_{12}, A-$NK_{TNF\alpha}$, or A-$NK_{12/TNF\alpha}$ cells iv and two ip injections of PEG-IL-2 (60,000 IU/ml), one given at the time of the injection of A-NK cells, the next given 12 hours later. At 96 hours after adoptive transfer we found, as expected based on previous experiments, relatively few A-NK cells in the lung tumors when transduced with IL-12 or mock virus (approximately 53.4 A-NK_{12} cells/mm^2 tumor tissue in the lungs, and only 10.1 A-NK_{mock} cells/mm^2 (Fig. 18A and B)). This low A-NK cell density at a late time point is not surprising considering the fact that IL-12 on its own is not a survival factor for A-NK cells and that

exogenous IL-2 support of the NK cells was administered only the first day (120,000 IU PEG-IL-2 given within 12 hours of the A-NK cell injection). We

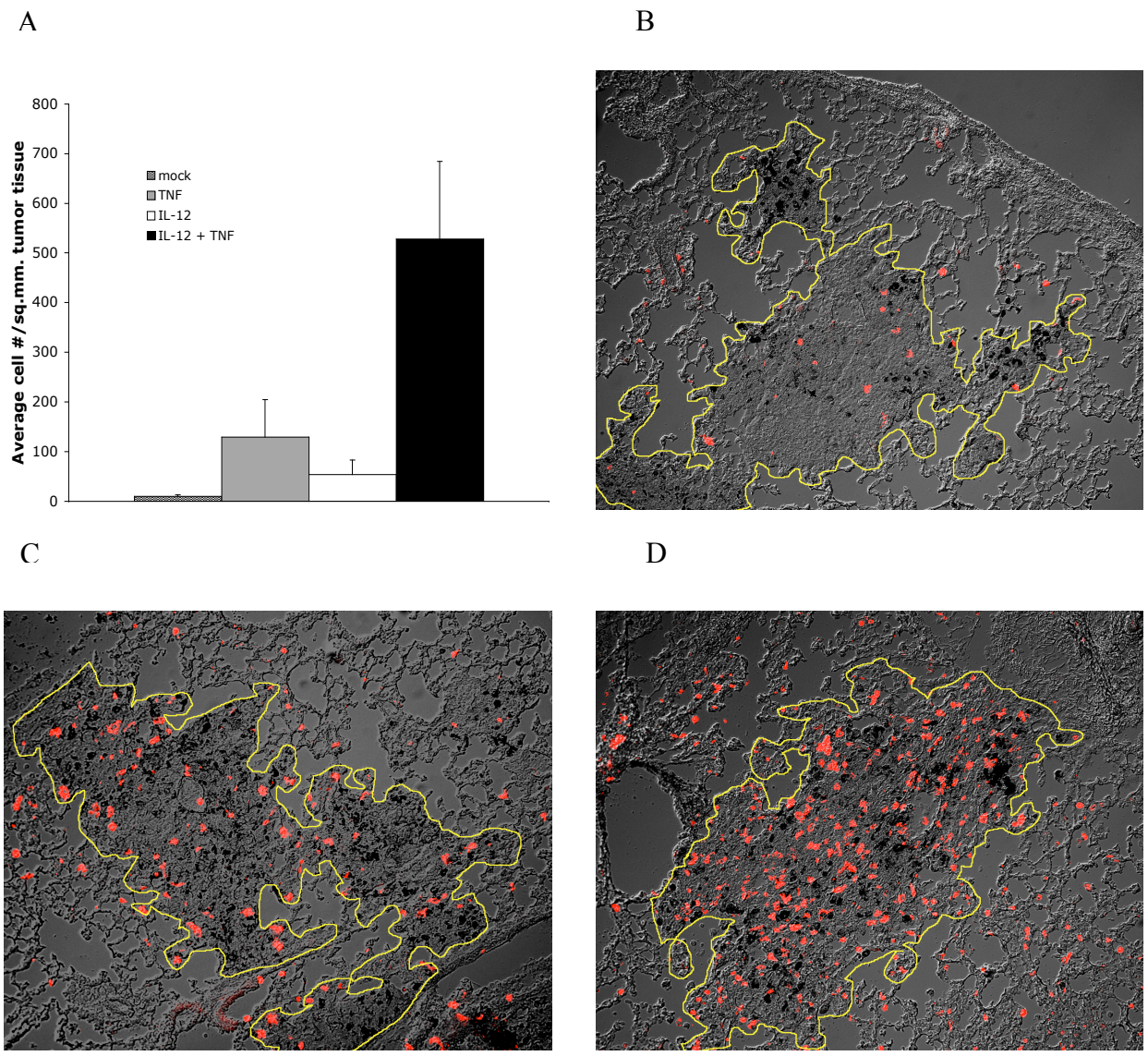

Figure 18. Presence of TNFα within B16 lung metastases leads to greater presence of A-NK cells. (A) Mice bearing day-7 B16 lung metastases received $5x10^6$ A-NK_{mock}, A-NK_{12}, A-$NK_{TNF\alpha}$, or A-$NK_{12/TNF\alpha}$ cells iv. 60,000 IU PEG-IL-2 was administered ip to all mice at t=0 and 12 hours post adoptive transfer. Data are represented as average number of A-NK cells per square mm tumor tissue at 96 hours post adoptive transfer compared (n=5) (*, p value=0.0021). Error bars represent SD. (B-D) Representative images of a lung tumors from

mice receiving A-NK_{12} (B), A-$NK_{TNF\alpha}$ (C), or A-$NK_{12/TNF\alpha}$ illustrating the presence of adoptively transferred A-NK cells (in red). The B16 tumor areas have been outlined in yellow for identification purposes. Images were obtained using 10x magnification. Note the high density of A-NK cells when IL-12 and TNFα are expressed within the tumor compared with IL-12 alone (B and D).

were, however, surprised to find an abundance of A-NK cells persisting in lung tumors, when they had been transduced with TNFα. There was a 2.4-fold increase in the number of cells if they expressed TNFα as opposed to IL-12 (Fig. 18A and C), although not statistically significant. However, when the A-NK cells were cotransduced with IL-12 and TNFα, the cell numbers reached levels of 528/mm^2 tumor tissue (Fig. 18A and D, p value=0.0021), i.e. a 9.9-fold increase compared to A-NK_{12} cells. We also tested for differences in the presence of endogenous cells, such as CD4+ and CD11c+ cells, but each treatment resulted in relatively equal numbers.

4.3.5 TNFα, when combined with IL-12, does not result in significant survival of tumor-bearing mice.

We next wanted to know if the TNFα/IL-12 induced increases in the amount of A-NK cells present within lung tumors, along with the production of TNFα, IL-12 and possible down-stream (TNFα)-induced pro-inflammatory cytokines and chemokines, could translate into a significant enhancement of survivability in tumor-bearing mice. A-$NK_{12/TNF\alpha}$ cells were injected into B16 tumor-bearing animals. In a 7-day B16 model, A-$NK_{12/TNF\alpha}$ cell treated mice survived 8 days longer than A-NK_{mock} cell treated mice (Fig. 19, p value=0.012).

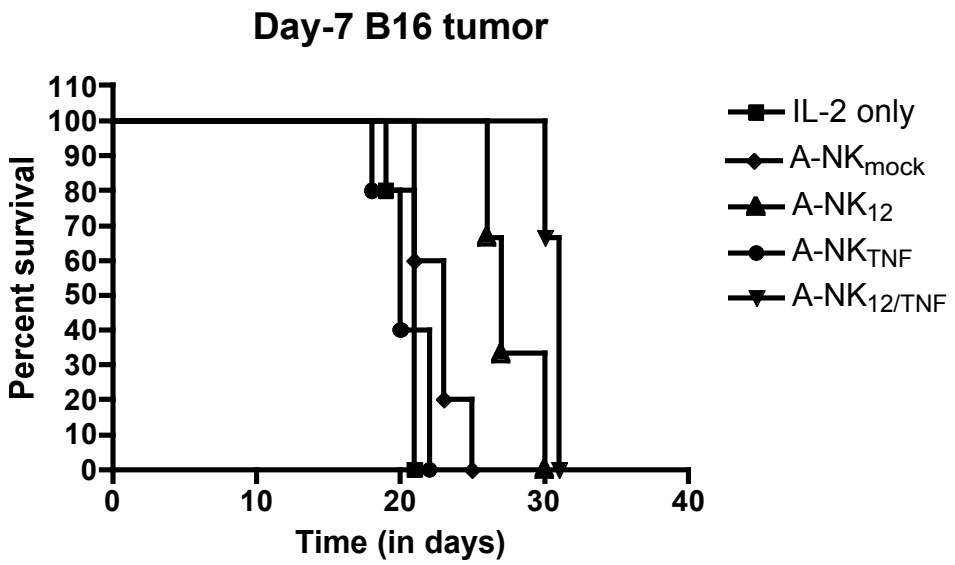

Figure 19. Co-transducing A-NK$_{12}$ cells with TNFα provides an increase in survival, albeit non-significant, in mice bearing B16 lung tumors. B16 tumor-bearing mice received 5x10^6 A-NK$_{12}$ cells. Survivability was monitored, as described in Materials and Methods, and plotted using the Kaplan-Meier method for the number of days post tumor induction. Statistical significance was calculated, using log-rank test, for mice receiving A-NK$_{12}$ compared with A-NK$_{12/TNF\alpha}$ cells in the B16 background, p value=0.063 (n=5 for all groups except A-NK$_{12}$ and A-NK$_{12/TNF\alpha}$ where it was n=3).

Compared with A-NK$_{12}$ cell treated mice, however, there was only a 4-day difference in survival (p value=0.063). This indicates a lack of improved therapy of A-NK$_{12/TNF\alpha}$ treatment, over A-NK$_{12}$ treatment alone, contrary to the significant A-NK cell survival benefit by combining TNFα and IL-12, as described above.

Considering that induction of adaptive immunity could take place without being "visible" by survival as endpoint, we investigated if the initiation of T cell responses was in fact being induced by A-NK$_{12/TNF\alpha}$ treatment. For these studies, we used the OT-I/MO5 model. MO5/OT-I offers the B16 tumor cell line that stably expresses ovalbumin and the ovalbumin transgenic,

MHC-I restricted (OT-I) CD8+ T cell, which recognizes the OVA-derived peptide, SIINFEKL. However, two out of three attempts incorporating this model with A-NK_{12} or A-$NK_{12/TNF\alpha}$ cell therapy resulted in no signs of OT-I cell proliferation in draining lymph nodes. From these results, TNFα, in the capacity it has been employed, was not able to significantly enhance the therapeutic efficacy of A-NK_{12} cells, neither in terms of improved survival of the treated animals, nor at the level of T cell activation.

4.4 DISCUSSION

Here we have shown that A-NK cells can be transduced with multiple genes, in this case IL-12 and TNFα. The expression of these genes resulted in a high production of both cytokines and greater TNFα with TNFα/IL-12 co-transduction than if the cells were transduced with the TNFα gene alone. It was hypothesized that transduction with this additional cytokine gene would improve the efficacy of the A-NK cell treatment and possibly promote adaptive immunity unlike the apparent inability of IL-12 alone to accomplish this. We found, however, that the co-transduction of A-NK cells with IL-12 and TNFα did not promote T cell activation and gave only a modest, non-significant 4-day increase in survival.

At the 96-hour time-point, when one day of PEG-IL-2 support has been insufficient in maintaining A-NK cell survival, even with IL-12 gene transduction, the presence of a high number of A-$NK_{12/TNF\alpha}$ cells in lung metastases was a remarkable finding. This increased A-NK cell survival trend was also observed with the transduction of the A-NK cells with TNFα alone, although not significantly greater compared to IL-12 alone. When we further investigated how

this increased presence of cells would translate in survivability, we found a 4-day increase in overall survival when mice received A-$NK_{12/TNF\alpha}$ cells compared to A-NK_{12} cells, but these data turned out to be non-significant. Of course, with a larger number of mice per group, the mouse survival difference could turn out to be significant. We believe that two conclusions can be drawn from these results. First, the addition of TNFα gene transduction does not improve the A-NK cell treatment to the extent that can be measured with survivability readouts. Second, the number of A-NK cells present within the lung tumors does not correlate strictly with the therapeutic outcome, i.e. the survival of recipients. The abundance of A-NK cells when co-transduced with IL-12 and TNFα would suggest a greater effector population with the capacity to kill tumor targets. Having 9.9-fold more A-NK cells present in the lungs with IL-12/TNFα gene transduction compared to IL-12 alone, we imagined, therefore, a more prolonged survival than the short, 4-day increase we observed. It was a little surprising that while supernatants from IL-12/TNFα transduced A-NK cells were able to reduce the growth of B16, the cytotoxicity of the A-NK cells, as measured in a 51-Cr release assay, was not improved by the TNFα co-transduction. This result may not be so surprising, however, since the 51-Cr release assay was only run for 4 hours, and this may not have been sufficient TNFα expressed to mediate a substantial difference in tumor cell killing compared to A-NK_{12} cells. The growth inhibition study involved supernatant containing TNFα accumulated over a 24-hour period.

It could be that TNFα maintains the presence of the A-NK cells in lung tumors without significantly enhancing their direct anti-tumor functions. The increased persistence of A-$NK_{12/TNF\alpha}$ cells beyond that seen with IL-12 transduction alone is somewhat puzzling in light of the established apoptotic role of TNFα, including NK cell apoptosis[225]. TNFα may stimulate the production of PGE_2 by the immune system, which can then lead to the suppression of LAK cell

activity, in mice[226] and humans[227]. These and other negative effects of TNFα, most prominently its major role in inducing toxicity as seen during sepsis, should be considered for the effectiveness of TNFα. Although the mechanisms involved in the double transduced A-NK cells persisting to such a high degree without mediating greater therapy have not been elucidated, in any case this phenomenon provides a great deal of promise for future use of transduced A-NK cells.

5.0 IMPORTANCE OF IFNγ FOR THE ANTI-TUMOR EFFECT OF A-NK_{12} TREATMENT

5.1 INTRODUCTION

The fact that mice eventually succumb to tumor burden after A-NK_{12} cell treatment, as disparaging as this may be, does not mean that the host does not or cannot contribute to the anti-tumor effect, in a non-T, B, or NK cell manner. While A-NK_{12} cells do not lead to induction of long-term adaptive immunity they do provide greater survival of mice either via their cytolytic function or by releasing and inducing the release of various cytokines, or both. For example, IFNγ is produced in high amounts by A-NK_{12} cells and we believe its production is also induced in the host by the IL-12 from A-NK_{12} cells. IFNγ has been extensively shown to be a key component of NK cell functions. Apart from granule exocytosis of perforin and granzymes, NK cells kill target cells through the indirect action of IFNγ[12]. If adaptive immune reactivity had been induced, the IFNγ could also help the CTLs via MHC upregulation which would improve CTL recognition of the tumor cells[49]. Also IFNγ is known to act as a strong inducer of macrophage activity[228]. Based on our finding that the A-NK_{12} cell therapy is fully effective in NOD-SCID mice (Chapter 4), we believe that host-mediated anti-tumor effects, if important at all, could be mediated via IL-12 and/or IFNγ activated macrophages rather than T, B, or NK cells.

Using IFNγ KO mice, we determined the importance of IFNγ for the anti-tumor activity of the A-NK_{12} cell treatment and identified which source, the A-NK cells and/or the host, is important in supplying the IFNγ by comparing the survival of IFNγ KO and WT mice receiving A-NK_{12} cells derived from IFNγ KO and WT mice.

5.2 MATERIALS AND METHODS[5]

5.2.1 Animals

C57BL/6 and B6.129S7-Ifng/J mice were purchased from Jackson laboratories (Bar Harbor, ME). The animals were housed in a specific pathogen free facility and the University's IACUC authorized their use.

[5] General materials and methods pertaining to this chapter but not mentioned in this section can be found in Chapter 2 or 3, Materials and Methods.

5.3 RESULTS

5.3.1 A-NK$_{12}$ cells generated from IFNγ KO mice produce high amounts of IL-12 and home to established B16 lung tumors.

With the goal of determining the impact of IFNγ in A-NK$_{12}$ cell therapy, we needed to determine first if A-NK cells derived from IFNγ KO mice would be capable of functioning like A-NK cells derived from WT mice, i.e. could they be transduced to express IL-12 and still traffic to tumors if they are missing the ability to produce IFNγ. We found that A-NK cells can be successfully generated from IFNγ KO mice and possess a similar phenotype as WT A-NK cells. Following Ad-IL-12 transduction, a high level of IL-12 was produced by IFNγ KO A-NK cells, roughly 2100 pg, comparable to that seen with A-NK$_{12}$ cells derived from normal mice (Figure 20).

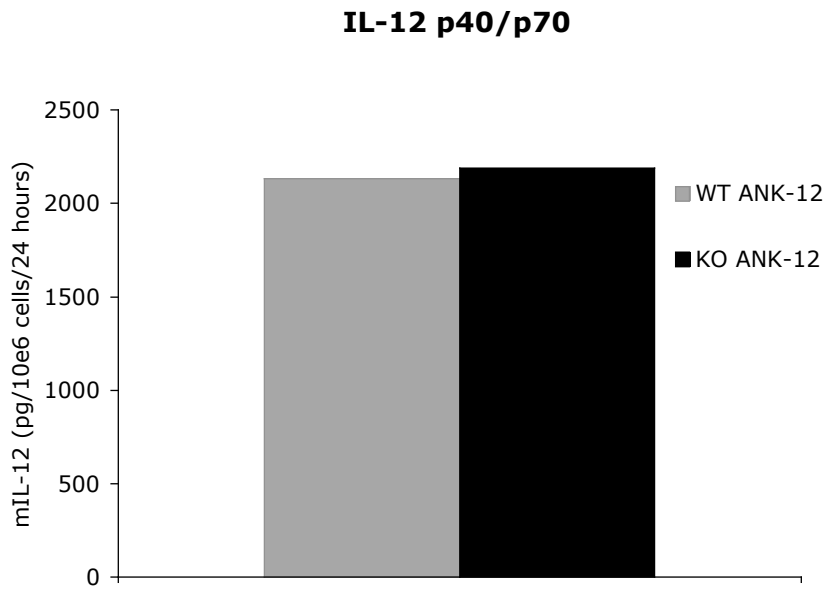

Figure 20. IL-12 production is comparable in transduced A-NK cells from C57BL/6 and IFNγ KO mice. A-NK cells generated from WT and IFNγ KO donor mice were transduced at 20 MOI of Ad-IL-12. $3x10^6$ transduced cells were cultured for 72 hours after the cells were washed 3x in an excess volume of RPMI. Supernatants were then collected and tested for IL-12 production with a Luminex assay. Values are calculated for $1x10^6$ cells per 24 hours.

We also confirmed that IFN γ KO A-NK_{12} cells, as expected, do not produce IFN γ, unlike WT A-NK_{12} cells (data not shown), which produce approximately 25ng/million cells/24 hours. Finally, we measured a similar level of infiltration by IFNγ KO A-NK_{12} cells, compared to WT A-NK_{12} cells, into B16 lung tumor metastases, 48 hours after adoptive transfer (Figure 21).

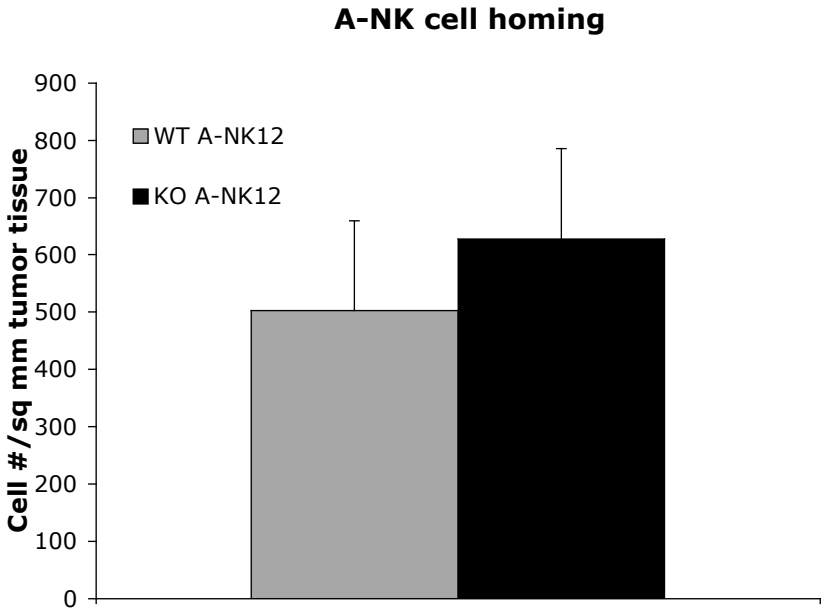

Figure 21. A-NK_{12} cells from C57BL/6 and IFNγ KO mice traffic to and localize within lung metastases. $3.5x10^6$ A-NK_{12} cells were intravenously injected into WT recipient mice and supported with 2 ip injections of PEG-IL-2 at 0 and 12 hours after adoptive transfer. 48 hours after cell injection mice were euthanized, lungs removed, and cells enumerated as previously described.

5.3.2 IFNγ supplied by the host is a critical factor for the anti-tumor effect of A-NK$_{12}$ treatment.

We wanted to determine if the high presence of IFNγ we have observed in the serum of A-NK$_{12}$ cell treated mice (generated by the A-NK$_{12}$ cells, the host, or both) is important for anti-tumor survival benefits and from which source, i.e. A-NK cell-derived or host-derived. We conducted two separate experiments in which 4.5x10^6 A-NK$_{12}$ cells, generated from wild-type C57BL/6 and IFNγ KO mice, were adoptively transferred into wild-type mice or IFNγ KO mice. These mice received 120,000 IU PEG-IL-2 (60,000 IU at time of transfer and again 12 hours later) and were monitored for survival. As we have seen on a consistent basis, there was a significant 6-day increase in the survival of WT mice receiving normal A-NK$_{12}$ cells compared to normal A-NK$_{mock}$ cells (Figure 22, p value=0.0031).

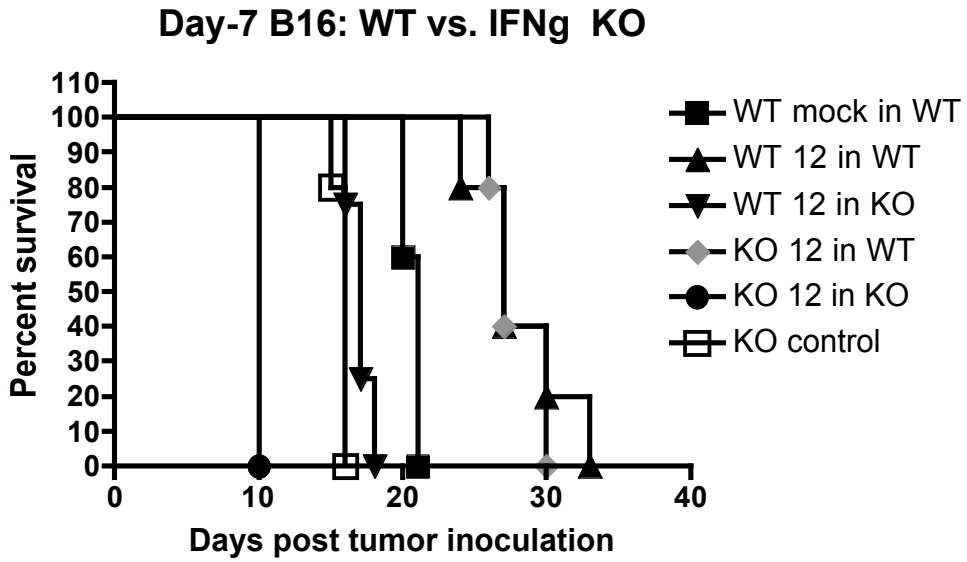

Figure 22. IFNγ produced by the host has a greater impact on the survival of tumor-bearing mice than IFNγ produced by the IL-12 transduced A-NK cells. 4.5x10^6 WT A-NK$_{mock}$ or WT or IFNγ KO A-NK$_{12}$ cells were injected into WT or IFNγ KO recipient mice bearing day-7 B16 lung tumors, as indicated in the legend.

Survivability was monitored, as described in Materials and Methods, and plotted using the Kaplan-Meier method for the number of days post tumor induction. Statistical significance was calculated, using log-rank test (n=5 for all groups except WT A-NK_{12} into KO mice, where it was n=4). These results represent 1 of 2 identical experiments that produced similar results without significantly different survivals in the same groups of the two experiments. Median survivals in days for experiment 1 (presented above)/ experiment 2 are 21-21 for WT mock in WT, 27-29 for WT 12 in WT, 17-17 for WT 12 in KO, 27-29 for KO 12 in WT, and 10-10 for KO 12 in KO. Median survival of IFNγ KO control mice was 16 days and only included in experiment 1.

In both experiments, there was no difference in survival of WT tumor-bearing mice when A-NK_{12} cells were generated from WT or IFNγ KO mice, illustrating a minimal requirement for A-NK cell-derived IFNγ production. However, when WT A-NK_{12} cells were administered to IFNγ KO tumor-bearing recipients, the mice survived a median 17 days, a 10-day decrease from WT A-NK_{12} in WT mice (p value=0.0029), and, more surprisingly, a 4-day decrease from mock control mice (p value=0.0029). These data represent a clear role for IFNγ produced by the host. We included in one experiment a group of IFNγ KO mice to serve as a control for B16 tumor development in these mice. The difference in survival between these mice and IFNγ KO mice receiving WT A-NK_{12} cell treatment was only a day. Their survival seems to point to a faster growth rate of B16 tumors in IFNγ KO mice, as we have seen a 4-day increase in survival of B16 lung tumor-bearing WT mice that had received A-NK_{mock} cells. In previous experiments, mice receiving these cells do not survive significantly longer than WT mice not receiving treatment. Finally, in the situation where no IFNγ could be made, namely A-NK_{12} cells from IFNγ KO mice injected into IFNγ KO recipients, all mice surprisingly died within 3 days of the adoptive transfer. Upon further investigation, it was apparent that mice died due to what appeared to be

cytokine-induced toxicity (including vascular leakage) rather than advanced growth of tumor (i.e. both the tumor burden of the lungs and extrapulmonary sites at this time point were low or undetectable macroscopically). Interestingly, in both experiments one IFNγ KO mouse receiving WT A-NK_{12} cells died several days (approximately 5 days) before the remainder of the mice in that group. This illustrates a fine balance in which the presence of IL-12 and IFNγ leads to therapeutic effect, however, in this case where IL-12 is present while IFNγ is absent, severe toxicity is induced.

Thus, it is clear that IFNγ produced by the host rather than by the A-NK cells is critical for the anti-tumor effect of A-NK_{12} treatment.

5.4 DISCUSSION

Our work with IL-12 gene transduced A-NK cells has demonstrated a profound effect of IL-12 on the production of IFNγ by both the A-NK cells and the host. By employing IFNγ KO mice, we have demonstrated that IFNγ is crucial for the anti-tumor effect mediated by A-NK_{12} cells and it became clear that host-derived IFNγ is much more important than the contribution of the A-NK_{12} cell-derived IFNγ. Surprisingly, when both sources were knocked-out for IFNγ production, all of the mice died within 72 hours of the transfer of A-NK_{12} cells. Thus, the removal of IFNγ from the system actually produced a toxic, lethal environment as witnessed by fluid build-up in the lungs.

A-NK_{12} cells generated from WT mice provided a significant survival benefit in tumor-bearing WT recipient mice, as seen before, compared to A-NK_{mock}. Since WT tumor-bearing mice that had received A-NK_{12} cells from IFNγ KO mice survived as long as those receiving A-

NK_{12} cells from WT mice, we can conclude that the contribution of IFNγ from A-NK cells is not important. In addition, when WT A-NK_{12} cells are transferred into IFNγ KO recipient mice the therapeutic benefit is abrogated, again illustrating a major role of the host in producing IFNγ. The A-NK_{12} cells, with their limited presence *in vivo* following transfer, can probably produce only a limited amount of IFNγ. This is apparently sufficient to protect from toxicity but not benefit therapy. However, to mediate anti-tumor therapy, i.e. increase animal survival, the A-NK cell delivered IL-12 is needed to stimulate the host to produce IFNγ. From the NOD-SCID experiment and these IFNγ KO experiments, we found that host T and NK cells cannot account for the IFNγ contribution in anti-tumor therapy. The abundance of CD11c+ cells in the tumors could provide a good source of IFNγ. It has been shown that macrophages[229] and neutrophils[230] can, in fact, produce IFNγ in response to IL-12 stimulation. Since host T and NK cells are not important for the increase in animal survival A-NK_{12} cells/IFNγ generates, we believe that the IFNγ, consequently, is acting predominantly back on the host macrophages and/or exogenously delivered A-NK cells, in mediating anti-tumor therapy. Thus, despite evidence for T, B, or NK cells' lack of direct activity in survival benefits, as seen in NOD-SCID mice, we have established that the host is indeed mobilized through our A-NK_{12} therapy, at least through a clear and instrumental role of IFNγ.

When IFNγ KO mice received A-NK_{12} cells generated from IFNγ KO mice, all animals surprisingly died within 48-72 hours. It is apparent from two separate experiments that as long as some IFNγ is present in the system (and the tumor microenvironment, presumably), produced by the host or A-NK cells or both; mice can be rescued from the toxicity induced when IFNγ is completely absent. When the donor A-NK_{12} cells are generated from WT mice, no toxicity is seen; neither in WT nor IFNγ KO recipients, and the A-NK_{12} cell treatment significantly extends

the life of WT mice by a week. Interestingly, though, one IFNγ KO mouse per experiment receiving WT A-NK_{12} cells died several days (approximately 5 days) before the remaining mice in that group. While these animals may have died from other reasons, their death may indicate that the IFNγ produced from the WT A-NK_{12} cells is in most, but not all, cases sufficient to inhibit treatment-induced toxicity in the IFNγ KO recipients.

We have seen cytokine induced toxicity in other experiments and could easily attribute the toxicity seen in the IFNγ KO recipients receiving A-NK_{12} cells from IFNγ KO mice to the presence of excess cytokine (especially IL-2). This is the first time, however, that the removal of a predominantly stimulatory cytokine resulted in exacerbated toxicity. It could be that sensitivity of the host to IL-2 is increased when IFNγ is absent while IL-12 is present. Carson et al. also found severe toxicity after systemic treatment along with exogenous IL-2 plus IL-12 treatment, but they could not provide an explanation for the exact course of this toxicity[89]. Another possibility involves indoleamine 2,3 dioxygenase (IDO), which has been shown to act suppressively in inhibiting T cell proliferation[231, 232]. IDO is induced in DCs[233, 234] and macrophages[232] by IFNγ. Thus, in situations of total IFNγ deficiency, immune cells could proliferate and/or function uncontrolled. We did not find any significant difference in the number of immune cells in tumors from the double KO group compared to other groups, but we cannot exclude that when IFNγ is missing, and therefore IDO missing as well, there may be a more severe reaction by the infiltrating immune cells (possibly through TNFα production).

It is important to mention that the tumor burden, in terms of tumor number and density rather than in size of individual tumors, in both experiments was relatively high. Thus, any adverse effect of tumor-infiltrating immune cells probably affect the whole lung, leading to fatal toxicity. We speculate that if fewer, but not necessarily smaller tumors are present, more normal

lung tissue would be left unaffected by the cytokines produced in the tumors and toxicity may, in that situation, be less severe. However, while this may hinder overt toxicity, it still does not explain why only the double KO mice developed fatal toxicity.

6.0 DISCUSSION

Immunotherapy of cancers has involved the use of T cells, B cells/antibodies, dendritic cells, and, to a lesser extent, NK cells. T cells offer an effector cell that can be directly targeted towards a specific tumor antigen. Unfortunately, this same feature may limit their broad application. Tumors may down regulate MHC expression, making them less susceptible to T cell killing, or there may be tumor variants that express antigens not recognized by the adoptively transferred T cells or lost TAA expression all together. Antibody treatment has similar difficulties, along with maintaining a suitable systemic level and ensuring delivery to the center of tumors. DC-based immunotherapy can present tumor antigens that have been pulsed onto the cells as well as providing co-stimulation for T cell activation. The use of cytokine therapy benefits a systemic response but can often, therefore, also lead to toxicity. Cytokine gene-transduced A-NK cell therapy, however, provides an efficient effector cell which is not antigen specific and therefore can eliminate T cell resistant tumor cells, and combines with it – due to the tumor-seeking properties of the A-NK cells - an effective means to localize anti-tumor cytokine expression specifically within the tumor.

6.1 IL-12

A goal of this dissertation project was to enhance, or at least maintain, the function of A-NK cell anti-tumor therapy while reducing the amount of exogenously delivered IL-2 (a necessary, but potentially toxic, component for A-NK cell trafficking, infiltration, and proliferation in tumors), via cytokine gene transduction. Transduction of A-NK cells with IL-12 has provided a superior benefit to standard A-NK cell therapy. By itself, as we have shown, it has resulted in significant survival benefit of A-NK cells in mice receiving far less PEG-IL-2 support than what is needed to support non-transduced WT A-NK cells. The A-NK_{12} cells also provide greater overall survival of tumor-bearing mice, with as little as one PEG-IL-2 injection, compared to both mice receiving mock transduced A-NK cells and mice receiving non-transduced A-NK cells supported with high dose IL-2. The increase in survival of mice bearing these rapidly growing tumors reached 10 days in an early, day-3, tumor model and 7 days in a more aggressive day-7 tumor model. This could translate into months or years of increased survival in cancer patients (whose tumors usually grow much slower than murine tumors) without the adverse effects of prolonged (or continuous) exogenous IL-2 administration. Thus, the primary goal of this project has been attained through transduction of A-NK cells with IL-12, mediating greater anti-tumor responses and requiring significantly less exogenous IL-2 support. Another goal, namely to deliver cytokines to the tumor microenvironment to stimulate host anti-tumor responses, has also been established.

6.1.1 IL-12 works through IFNγ

IL-12 acting on A-NK cells, and the host as well, could be through a number of functions. First, IL-12 may be increasing the A-NK cell lytic function. Although we did not see this clearly in cytotoxicity assays with A-NK_{12} cells, we cannot exclude the possibility that this is occurring in the more vibrant tumor microenvironment. Likewise, the IL-12 may be stimulating endogenous immune cells to be more efficient killers. Second, IL-12 may simply be maintaining A-NK cell survival in low amounts of IL-2 through the upregulation of CD25. It would be imagined that a longer presence of efficient killers would produce a greater therapeutic outcome. Finally, as established in Chapter 5, IL-12 is important for its induction of IFNγ. IL-12, formerly known as natural killer cell stimulatory factor, has demonstrated a remarkable downstream effect in the production of IFNγ. We have shown host IFNγ to be integral, whereas IFNγ produced by the A-NK cells themselves does not seem to play as significant a role for the efficacy of the A-NK cell treatment, but surprisingly very important for preventing treatment-induced toxicity. We have found that the host is critical for the production of IFNγ. IFNγ may be important, as described above, for its antiangiogenic function. With IFNγ removed from the system, tumors in the lungs could develop new vasculature, supporting their growth. It has been established, however, that in the B16 lung tumor model, the B16 tumors are not heavily dependent on neovascularization[235]. The contribution of IFNγ, therefore, is not likely due to its antiangiogenic capacity. Further studies using IFNγR KO mice will be needed in order to determine which component(s) is/are responding to the IFNγ, leading to tumor growth inhibition.

It could be argued that if IFNγ is so important for treatment effect when the host provides it, how could there be an equally efficient therapeutic effect in NOD-SCID mice receiving A-

NK_{12} cells when the primary producers of IFNγ, namely T and NK cells, are absent. Puddu et al. has demonstrated an ability of macrophages to produce IFNγ following IL-12 stimulation[229], while Munder et al. have shown similar results with IL-12 and IL-18 stimulation[236]. In addition, it has been shown that human neutrophils can be induced to produce IFNγ upon IL-12 stimulation[230]. Thus, these findings clearly establish the potential for other cells present in NOD-SCID mice to act as a viable source of IFNγ. We have attempted to measure the production of IFNγ in NOD-SCID mice, as well as WT mice, that have received A-NK_{12} cells. Unfortunately, no IFNγ could be found in serum obtained 72 hours after adoptive transfer, at least not at the level of detection (calculated to be ~300pg/ml). This may be explained by the fact that tumors were not induced in the mice used in this experiment. It appears that the A-NK cells will not persist and reach high enough numbers without tumors to accumulate in. The IL-12 expressed by A-NK_{12} cells will, therefore, not reach levels to stimulate macrophages to produce detectable IFNγ in the serum.

6.1.2 IL-12 does not induce host cellular immunity

We have shown the host to be important to the anti-tumor responses generated with A-NK_{12} cells through its production of IFNγ. This demonstrates that we, via the cytokine-transduced A-NK cells, are able to "communicate" with the host immune system to generate anti-tumor reactivity. We wondered, however, why we could not observe the induction of long-term adaptive cellular immunity despite the presence of pro-inflammatory cytokines and A-NK cells in the tumor microenvironment. If cytokine gene transduced A-NK cells are capable of activating specific T cell responses there may be a number of reasons why this, in our

experiments, did not manifest itself in terms of survival benefits. An obvious reason, considering survivability readouts, could have to do with the time that is needed for the efferent arm of adaptive responses to be mobilized. It would take 4 or 5 days from the time that antigen is taken up, presented to antigen specific T cells, T cell expansion, and T cell traffic back to the site of insult. Considering this time period, the growth kinetics of the tumor may exceed the ability of a well-armed immune response to overcome it. By the time effector cells arrive at the tumor, the challenge may be insurmountable. However, even in an experiment involving a day-3 lung tumor model and another involving a lower B16 cell inoculum, giving more time for T cells to expand to critical numbers than in the more advanced day-7 tumor model, mice did not generate long-term adaptive immunity. This indicates that there could be more inherent problems with the efferent arm of the immune response, i.e. antigen specific T cells may proliferate but not be fully armed or may have been rendered anergic, have difficulty in trafficking back to the antigenic source (tumor), or be impeded by regulatory or suppressive factors.

A second possibility involves a more fundamental problem with the afferent arm, such that the efferent arm would not be properly activated. This could be due to an inability of immature DCs to traffic to lung metastases, become matured in response to appropriate stimuli, or traffic back to secondary lymphoid tissue for T cell priming. We do not feel there is an inadequacy of immature DC trafficking into the tumors, as we have found relatively high densities of CD11c+ cells within tumors, regardless of whether the animals had received any treatment or not. Although we are not certain that these CD11c+ cell are in fact DCs and not macrophages, given their high expression of CD11c, we do believe that DCs are present to some degree. Most of the CD11c+ cells are filled with phagocytosed melanin, clearly demonstrating

their ability to take up tumor cell debris. In spite of what we perceive as a suitable DC maturation environment, i.e. an abundance of cytokines such as IL-2 (injected) and IL-12 (from the transduced A-NK cells), liberation of tumor antigen(s) due to tumor cell killing by the A-NK cells, and the possibility for cross-talk between NK and DC, there is less evidence to support the idea that DCs, if they are present, are actually matured appropriately. Thus, an inability of matured DCs to traffic to lymph nodes remains a possibility for a defect in the afferent arm. This is quite conceivable considering that the lymph nodes draining the lungs are more involved with airborne antigens, while in B16 metastases that are established "deeper" in the lung parenchyma where there may be fewer antigen presenting cells available, unlike at the air/lung interface. It is possible that the intratumoral CD11c+ cells are efficient in acting as antigen presenting cells. Even if they are genuine DCs, there may be insufficient lymph vessels in the tumors to secure a functional path for the DCs by which they can reach the draining LNs. A lack of proliferation of antigen specific T cells in the draining lymph nodes following A-NK cell treatment could point to any of these deficiencies in the afferent arm.

By looking in the draining lymph nodes of the lungs (i.e. the mediastinal lymph nodes), we could get a better idea if there are defects in the afferent or efferent arm of the immune response, or, in other words, if early events in effector cell induction/priming (i.e. T cell proliferation) in fact is taking place following A-NK cell treatments. We supplied the system with both a T cell that recognizes a specific antigen and a tumor that will express it with the OT-I/MO5 model. By doing so, we are starting with a greater T cell pool that can become activated, providing a greater window of opportunity to observe T cell responses (in a shorter amount of time). The MO5/OT-I offers the B16 tumor cell line that stably expresses ovalbumin and the ovalbumin transgenic, MHC-I restricted (OT-I) CD8+ T cell, which recognizes the OVA-derived

peptide, SIINFEKL. We decided to investigate the ability of A-NK_{12} to promote T cell proliferation, and how the addition of TNFα gene-transduction of the A-NK cells might affect this process. We also tested A-NK_{12} cells transduced with CD40L. Interacting with CD40 on DCs, CD40L can act to mature DCs[237, 238]. Considering a lack of long-term response from A-NK_{12} and A-$NK_{12/TNF\alpha}$, we hypothesized that CD40L stimulation of antigen presenting cells, e.g. DCs, would initiate T cell responses that IL-12 or IL-12/TNFα may not mediate. Disappointingly, we did not observe any signs of OT-I proliferation in the draining LNs, indicating a lack of proper stimulation of the afferent arm of the adaptive immune response following cytokine-gene transduced A-NK cell treatment. It could be argued that CD8+ T cells need CD4+ T cell help to proliferate efficiently. We included OT-II (ovalbumin-specific CD4+ T cells) to a couple of the experiments without noticeable effects on OT-I or OT-II proliferation. In case DCs traffic from the lungs parenchyma more readily through the bloodstream rather than the lung lymphatics we investigated the possibility of systemic activation by looking for OT-I cell proliferation in the spleen. We found no increase in OT-I cell proliferation at this site in any experiments.

Since we did not observe any changes in OT-I cell numbers in mediastinal lymph nodes or spleens of mice receiving A-NK_{12} or A-$NK_{12/TNF\alpha}$ treatment, we set up an experiment in which transduced A-NK and MO5 tumor cells were mixed and injected subcutaneously to initiate responses in inguinial lymph nodes. This is a more widely used model that, along with the incorporation of adjuvant and ovalbumin protein as a positive control, should provide a means to observe T cell proliferation if this technique is successful. We found that only the positive control group, consisting of Freund's adjuvant and ovalbumin protein, was successful in promoting OT-I cell proliferation in the inguinial lymph nodes. Adjuvant alone and each of the

transduced cell/MO5 treatments (including IL-12/TNFα and IL-12/CD40L) could not initiate T cell proliferation. This again indicates that the A-NK cell mediated killing of tumor cells, even in an environment containing IL-12, in conjunction with TNFα or CD40L, is not sufficient to stimulate T cell proliferation. CD40L may be a good candidate for activating DCs but it may be more effective when present in the lymph nodes rather than in the lung tumors (where transduced A-NK cells are expressing it). Therefore, there is a need to identify alternative cytokines and chemokines and amounts of them that, when delivered by A-NK cells, will stimulate the afferent arm of adaptive immunity and the proliferation of antigen specific T cells.

6.1.3 Concerns with IL-12 application

Interestingly, it has been shown that IL-12 may decrease the early CTL activity in mice when given as adjuvant. It plays a more extensive role following this period of suppression[239]. Ribas et al. demonstrated a detrimental effect of chronic therapy involving IL-12 administration in which anti-tumor reactivity was suppressed[240]. On the other hand, Kilinc et al. demonstrated reductions of FoxP3+ Treg cells, along with increases in CD8+ effector T cells, in tumors with the intratumoral injection of IL-12, illustrating the usefulness of IL-12[241]. In addition, A-NK_{12} cells produce significant amounts of IL-10 which clearly could suppress T cell responses. The conflicting immunostimulatory and immunosuppressive results obtained with IL-12 point to great impact of the model, timing, dosage, and delivery method. Clearly, IL-12 does not, in our system, contribute much to the host's T, B, or NK cell effector responsiveness, as these effector cells did not seem to be important when we investigated them in WT vs. NOD-SCID tumor-bearing mice. If IL-12 is acting to suppress host T cells this may explain, in part, why we cannot see long-term immunity being generated in WT mice either. However, even with possible

suppression of host T cells occurring, the A-NK cells can still respond to the IL-12 being produced, along with downstream IFNγ produced by the host. In our model, A-NK_{12} cells reach the tumors where they produce IL-12, and destroy tumor cells without the need of a high and constant supply of IL-2, due to the presence of the IL-12 (leading to CD25 upregulation). As the A-NK cells die off, the IL-12 levels will also drop and may soon reach levels unable to maintain any host response that may be developing. Thus, before the CTL responses are fully developed, the presence of IL-12 within the tumors/draining LNs may have diminished to suboptimal levels. The argument would then be to continue or repeat the injections of A-NK_{12} cells to overcome this lag period. While we, in one experiment where two A-NK_{12} injections were given 4-days apart, did not see any increase in therapeutic efficacy or signs of a further developed adaptive host immune response, it is still very likely that with the right timing, serial injections of transduced A-NK cells may support the initiation of adaptive anti-tumor responses.

In addition to the potential lag period, we have, as mentioned above, found a dramatic production of IL-10 by IL-12 transduced A-NK cells. Others have demonstrated this with human NK cells following IL-2 and/or IL-12 stimulation[242]. It is possible that a lack of host anti-tumor immunity could be explained by the A-NK cells' ability to produce IL-10. Most widely known for its effects in Th2 immunity, IL-10 could be a key target in dampening potential IL-12-mediated host immunity, possibly by preventing the accumulation of DCs in tumors[243]. It may also prevent T cell stimulation by impairing antigen cross presentation by DCs[244]. Along with TGFβ, IL-10 is used by Tregs to act immunosuppressively[245]. In contrast, IL-10 has been shown, to act as an NK cell stimulator[246, 247]. IL-10 has been used extensively, with success, in animal tumor models[248-251] and, in at least one instance, found to be associated with better prognosis in human tumor, a leukemia, B-CLL[252]. More prominently, the expression of IL-12

and IL-10 together, by transduced tumor cells, synergizes to mediate anti-tumor immunity against subcutaneous and lung tumors[253]. Additional experiments (e.g. KO mice or cytokine depletions) are needed, therefore, to determine the role of IL-10 as a stimulator or suppressor in this model.

6.2 IL-2

6.2.1 IL-2 transduction to support A-NK cells

In spite of a number of attempts with different IL-2 gene containing adenoviral vectors, we have not been completely successful in transducing A-NK cells to become totally independent of exogenous IL-2. We do not believe, however, that IL-2 gene transduction is completely unsuccessful altogether. Instead, it is more likely a matter of insufficient amounts being produced. According to our calculations, the amount of IL-2 required for maintaining A-NK cells *in vitro* would be in the range of 30-300ng/x10^6 cells/ml which is 50 to 500-fold more than what we have obtained with IL-2 transduction thus far. The objective then became a matter of how a more suitable, diminished presence of IL-2, either exogenously delivered or transgene expressed, could suffice in maintaining A-NK cell survival and functionality. One possibility that has provided favorable results combines IL-2 transduction with IL-12 transduction. In an experiment in which A-NK cells were co-transduced to express both IL-12 and IL-2, mice receiving A-$NK_{12/2}$ cells were capable of preserving the ~7-day increase in survival (median 28 days compared to 21.5 for A-NK_{mock}, p value=0.0005). Remarkably, this has been accomplished without any support from exogenous PEG-IL-2. These results were consistent with treatment

with A-NK_{12} cells plus one injection of PEG-IL-2 given at time of the A-NK cell injection. (30 days with A-NK_{12} and 28 days with A-$NK_{12/2}$, p value=0.3916). Thus, when coupled with the high production of IL-12, the modest IL-2 production by IL-2/12 transduced A-NK cells apparently was capable of maintaining viability and function of the A-NK cells and thereby completely eliminating the need for exogenous IL-2 support.

6.2.2 Concerns with IL-2 application

IL-2 needs to be carefully considered in its application. Most important, if too much IL-2 is produced, toxicity may occur. As stated, however, with the low amounts of IL-2 produced by our transduced A-NK cells, this is not likely to be a concern. Nevertheless, as intratumoral IL-2 allows for the survival and proliferation of effector T cells, it produces similar effects in T regulatory cells as well. How much of a factor Tregs play in preventing long-term immunity toward the tumor needs further investigation in this model system (i.e. via Treg depletions). Nagai et al. demonstrated greater therapeutic benefit of IL-12 when it was combined with CD4+CD25+ T cell depletions[254]. We have tested for the presence of FoxP3+ T regulatory cells in the B16 lung tumors following different cytokine-gene transduced A-NK cell treatments and found that FoxP3+ cells are present in the lung sections. The levels of these cells were unaffected with A-NK_{12} cell treatment compared to untreated mice. We found, however, a slight decrease in the presence of Tregs following A-$NK_{12/TNF\alpha}$ cell treatment. We are not certain if this slight difference between treatment groups is functionally important or whether the levels of Tregs found in the tumors are able to prevent the successful induction and implementation of adaptive host immunity. We do believe, however, that the influence of Tregs on the therapeutic efficacy of the A-NK_{12} treatment should be taken into account in the future.

6.3 FUTURE APPLICATION OF CYTOKINE GENE-TRANSDUCED A-NK CELLS

We have shown here that transduced A-NK cells can be a powerful anti-tumor tool by delivering the right combinations of cytokines, and possibly in the future, chemokines and/or danger signals also. Determining which agents should be delivered and to what extent will continue to become a major focus in this lab. It may be a matter of providing additional injections of transduced A-NK cells. As described above, the presence of a cytokine (or any other agent) delivered by A-NK cells may not persist long enough to continue to promote immunity if it even has been initiated. Therefore, multiple injections of transduced A-NK cells could supply these agents at critical time points to boost levels that have diminished.

It may be that the initiation of an effective immune response requires injections of A-NK cells delivering various cytokines at different time points in the development of the response. This may be an important consideration for coordinating the immunologic processes by way of key regulators. For example, the first dose of A-NK cells might need to be transduced with A-NK cell promoting factors that maintain their survival and cytolytic function such as IL-12, which has been thoroughly tested in this project. However, the addition of other cytokines (such as GM-CSF, IL-2 or IL-15) and chemokines, that can draw immune cells into the tumors, will establish an appropriate immune cell population consisting of both innate effector cells and antigen presenting cells. Once this has happened, a second dose of A-NK cells, this time delivering cytokines involved in generating adaptive immunity (i.e. IL-12, TNFα, CD40L), may secure initiation of a Th1-biased T cell response. In addition, removing regulatory cells or immunosuppressive cytokines followed by the delivery of danger signals (e.g. TLR ligands such as CpGs) that can trigger immune activation against the tumor, should further stimulate T cell priming.

We feel that what has been accomplished through this dissertation work, albeit without the induction of long-term adaptive immune reactivity, remains a remarkable finding in that A-NK_{12} cells consistently provide significant anti-tumor therapy, with both the A-NK cells and host playing a vital part. Importantly, the application of A-NK_{12} cells has lead to a reduction in exogenous IL-2 support, and a subsequent elimination of IL-2-induced toxicity. Thus, we are confident that with the right combination of additional cytokines and proper treatment timings, even greater anti-tumor responses can be generated from the advances we have already made based on the A-NK_{12} cells.

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