VENTEVOGEL, MELISSA SAMO. Cytokine Modulation of Thymopoiesis. (Under the direction of Gregory D. Sempowski.)

The thymus is an organ derived from embryonic endoderm and mesoderm differentiation. It is located above the heart and is made up of two compartments, the thymic epithelial space and the perivascular space. The thymic epithelial space consists of the cortex and the medulla, which is where T cell development, maturation and induction of self tolerance occur in a process known as thymopoiesis. The thymus is susceptible to chronic and acute stressors that result in thymic involution. A consequence of thymic involution is reduced thymopoiesis, which affects the generation of a diverse T cell repertoire and establishment of central T cell tolerance. Many thymosuppressive and thymostimulatory cytokines are involved in thymopoiesis and thymic involution. Keratinocyte growth factor and IL-7 are two cytokines that function in driving early thymic progenitor proliferation and T cell development, respectively. We hypothesized that IL-7 and Keratinocyte growth factor, delivered via recombinant adenovirus, can improve thymopoiesis and T cell reconstitution in mice in an endotoxin model of acute thymic atrophy. Analysis of thymus weight, cellularity, phenotype and TCR gene rearrangement showed moderate increases in thymic function with delivery of IL-7 or Keratinocyte growth factor versus control. Taken together, these data suggested that IL-7 and Keratinocyte growth factor, delivered via recombinant adenoviruses, have thymostimulatory effects on the thymus in normal thymus or settings of acute thymic atrophy and maybe beneficial for future development as therapeutics.
DEDICATION

For
Roberto Samo, my father
BIOGRAPHY

Melissa Samo Ventevogel was born on February 17, 1974 in Chicago, Illinois. After receiving her diploma from Miramar High School in Florida, she completed a Bachelor of Science degree in Biological Science from Florida Atlantic University, Boca Raton, Florida. She then accepted a position as a Research Technician at the Duke Human Vaccine Institute, Duke University School of Medicine, Durham, NC where she has worked for the past five years. In her second year at Duke, she enrolled as a graduate student at North Carolina State University in the Immunology Program. Under the guidance of Dr. Gregory D. Sempowski (Duke University), she studied cytokine modulation of thymopoiesis via delivery of recombinant Adenovirus in models of thymic involution. Upon the completion of her Master's degree, Melissa will continue with her current research at Duke University in the capacity of Senior Research Analyst.
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LIST OF ABBREVIATIONS

APC: allophycocyanin
APCs: antigen presenting cells
BM: bone marrow
CCR7: chemokine receptor 7
CCR9: chemokine receptor 9
CD: cluster of differentiation
CMV: cytomegalovirus
COS: Cercopithecus aethiops (Vero Cells)
CPE: cytopathic effect
CsCl: cesium chloride
DN: double negative
DP: double positive
ELISA: enzyme-linked immuno-sorbent assay
ETP: early thymic progenitor
Fgf7: fibroblast growth factor 7
FITC: fluorescein isothyocynate
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
HEK: human embryonic kidney
HSC: hematopoietic stem cell
IL-2: interleukin 2
IL-7: interleukin 7
i.p.: intraperitoneal
i.t.: intrathymic
i.v.: intravenous
KGF: keratinocyte growth factor
LIP: lymphopenia-induced proliferation
LN: lymph node
LPS: lipopolysaccharide
MSC: multiple cloning site
MHC: major histocompatibility
MOI: multiplicity of infection
PCR: polymerase chain reaction
PE: phycoerythrin
PFU: plaque forming unit
qRT-PCR: quantitative reverse transcriptase PCR
RAG: recombinase-activating gene
RO: retro-orbital
spMHC: self-peptide MHC complex
SP: single positive
TCR: T cell receptor

TES: thymic epithelial space

TGF-β: transforming growth factor beta

TREC: t cell receptor excision circle
1. INTRODUCTION

1.1. Overall

The thymus is an organ derived from embryonic endoderm and mesoderm differentiation. It is located above the heart (1) and is made up of two compartments, the thymic epithelial space and the perivascular space. The thymic epithelial space consists of the cortex and the medulla, which is where T cell development, maturation and induction of self tolerance occur in a process known as thymopoiesis (2). The cortex is composed primarily of lymphoid cells in a network of epithelial reticular cells. The cortex is the location of early thymocyte development including T cell receptor gene rearrangement and positive selection (3). The medulla is the location of subsequent thymocyte development. Thymocytes that reach the medulla have successfully undergone T cell receptor gene rearrangement and positive selection. In the medulla, composed primarily of macrophages, B cells and dendritic cells, thymocytes undergo negative selection to remove auto-reactive T cells from the mature repertoire (3, 4). The perivascular space, composed of adipocytes, peripheral lymphocytes and stroma, is non-epithelial and non-thymopoietic. With age there is an expansion of the perivascular space with a simultaneous shrinking of the thymic epithelial space (5).

The thymus and its microenvironment change under the influence of many different types of intrinsic and extrinsic factors, including: puberty, pregnancy, disease, inflammation, bacterial
and viral infections, environmental conditions, exposure to corticosteroid therapy, and chemotherapy (6). The changes a thymus undergoes due to chronic or acute stress is termed thymic involution. Age associated involution (chronic) leads to a decrease in T cell responses. There is also a decrease in the proportion of T cells that express a naive phenotype (CD44 lo, CD45RB hi, CD62L hi) and an increase in those that express a memory phenotype (CD44 hi, CD45RB lo, CD62L lo) (7). An example of acute thymic involution involves lipopolysaccharide (LPS) an endotoxin produced by gram negative bacteria.

1.2. Thymopoiesis

T cells develop from self-renewing hematopoietic stem cells (HSCs) that are multipotent, with lineage potential for all blood cell types. HSCs lack expression of lineage markers expressed by differentiated cell types (8).

Hematopoietic stem cells travel from the bone marrow (BM) into the thymus cortex as early thymic progenitors (ETP). Trafficking to the thymus is a two-step process. Progenitors must first be released from their micro-environmental niches in the bone marrow into the blood through the process of mobilization. Progenitors then exit the circulation and settle in the thymus (9). ETPs enter at the sub-capsular region of the cortex and do not express CD3, alpha or beta-chains of the T cell receptor, nor CD4 and CD8 molecules. The ETP cell phenotype is CD4-/CD8- double-negative (DN). The movement of DN ETPs through the
cortex and into the sub-capsular region of the thymus is influenced by CCR7, CCR9 and CXCR4 signaling (10). When DN thymocytes differentiate into pre-DP thymocytes they migrate from the sub-capsular region returning to the cortex. This migration from sub-capsular region to cortex requires expression of adhesion molecules such as vascular-cell adhesion molecule 1 (VCAM-1) (11).

A factor that drives early thymic progenitor (ETP) proliferation and organization is fibroblast growth factor 7 (FGF7), which is also known as keratinocyte growth factor (KGF). KGF is produced by thymic stromal cells and mature T cells (10). The KGF receptor is FGFR2IIIb (10). Another molecule involved in driving T cell development is Interleukin 7 (IL-7). IL-7 is produced by ETPs and dendritic cells and is important for thymocyte proliferation (10).

Thymocyte maturation is an ordered process that occurs in multiple steps. As previously stated hematopoietic stem cells travel from the bone marrow (BM) into the thymus and arrive as CD4-/CD8- double-negative (DN) early thymic progenitors (ETP). ETP DN thymocytes undergo a series of phenotypical changes on their way to becoming CD4+/CD8+ double-positive (DP) thymocytes. DN thymocytes (CD4-/CD8-) first progress to DN 1 (CD44+/CD25-), then DN 2 (CD44+/CD25+), DN 3 (CD44-/CD25+) and finally to DN 4 (CD44-/CD25-) (5). DN 1 cells are multipotent, while DN 2 cells have lost the ability to become B cells. DN 2 cells, however, can still become T cells or dendritic cells (12). At the
DN 3 stage, thymocytes are committed to becoming T cells and their TCRβ chain genes then undergo extensive rearrangement (13). After the TCRβ chain genes have undergone rearrangement, they associate with an α surrogate chain forming the pre-TCR (14).

An interesting observation in aged mice shows that there is a significant increase in the percentage of DN1 cells, indicating that the primary age-specific block in thymopoiesis occurs at the DN1 stage. Transition from DN1 to DN2 stage requires signaling through the IL-7R (15). During DN1-DN2 transition, IL-7 up-regulates bcl-2, an anti-apoptotic protein, allowing for DN1 T cells to avoid apoptosis (15). In the aged mice, the block from DN1 to DN2 results in diminished thymopoiesis.

To complete thymocyte maturation, T cells must transition from the pre-TCR to a fully functional TCR by undergoing positive and negative selection. Positive selection occurs in the cortex and eliminates, by apoptosis, T cells that weakly bind to the major histocompatibility complex (MHC) allowing medium and high binding T cells to survive. Following positive selection, the TCRα chain undergoes rearrangement and T cells become CD4+/CD8+ DP. After positive selection and TCRα chain rearrangement in the cortex, CCR7 ligands guide the thymocytes towards the medulla (10). At the cortex-medulla border DP thymocytes undergo negative selection to eliminate thymocytes that react too strongly to self antigens (auto-reactive) (15). Thymocytes that clear negative selection migrate into the medulla and mature into CD8+ or CD4+ single-positive (SP) T cells, a process which takes 3
to 14 days (10). The now mature CD8+ and CD4+ SP T cells are exported to the periphery and travel to secondary lymphoid organs to function as cytotoxic and helper cells, respectively (2). Emigration from the medulla into the periphery is regulated by the expression of sphingosine-1-phosphate receptor 1 (S1P(1)) (16). Allende et al. found that S1P(1) receptor expression on T-cells controls their exit from the thymus and is up-regulated on mature thymocytes (17).

An immune response requires T cells to respond to an infinite number of antigens without attacking the host. It is this process of TCR-mediated positive and negative selection of T cells that ensures the selection of a diverse TCR repertoire able to react with foreign peptides and have a tolerance to self-antigens (10). Although mature, recently exported T cells in the periphery are considered naïve T cells with respect to antigenic exposure. Naïve T cells are activated by antigen presenting cells which present foreign antigen in association with appropriate MHC (14). A fundamental role of robust thymopoiesis is that it allows for the generation of a diverse T cell repertoire and establishment of central T cell tolerance. A heterogeneous availability of TCRs is critical to the robustness of host immunity (5).

1.3. Peripheral T cell homeostasis

Homeostasis is the ability of the body to preserve its internal steady-state when stressors are encountered (20). Peripheral T cell homeostasis is achieved when the number and diversity
of lymphocytes is maintained in the periphery. The peripheral T cell pool remains constant because naïve T cells barely proliferate while memory T cells proliferate at a slow but steady rate. The peripheral T cell pool is made up of two cellular compartments (naïve recent thymic emigrants and activated/memory T cells) whose homeostasis are independently regulated. T cell recovery and the size of the peripheral T cell pool are regulated by several mechanisms that bypass the reduced production of mature T cells by the thymus. The pool of peripheral naïve T cells, however, is dependent on a minimally functional thymus that produces a minimum number of competent DN precursors (18).

As previously mentioned, recent T cell emigrants from the thymus are naïve with respect to antigenic exposure, once they encountered antigen in the periphery, T cells will clonally expand and switch from naïve (CD44 low) to memory (CD44 high) T cells (19). Memory T cells proliferate, even in the absence of stimulation, to maintain the T cell repertoire in the steady-state. The constant proliferation of peripheral T cells is also known as basal or homeostatic proliferation (20).

The peripheral T cell pool can be disrupted by: ageing (naïve T cell output decrease), viral infections and acute stress-induced thymic atrophy. T cells can also be forced to proliferate if a stressor results in peripheral lymphopenia (T cell loss). Lymphopenia leads to space-driven expansion of T cells, also known as lymphopenia-induced proliferation (LIP) (20).
Lymphopenia can result, for example, from viral infections such as CD4+ T cell depletion due to HIV-1 infection (21). With ageing there is a shift in the balance of the peripheral T cell pool with respect to naïve and memory T cells. Ageing causes a decrease in the number of naïve T cells resulting in a transient lymphopenia that is eliminated when the appropriate cell signals result in an up-regulation of lymphopenia-induced proliferation and there is expansion of the T cell memory compartment. The increase in induced proliferation allows for peripheral T cell homeostasis (5). Marleau et al. report that current knowledge of lymphopenia-induced proliferation is due to studies in which T cells are adoptively transferred into immune deficient mice, lacking either recombinase-activating gene (RAG-1/RAG-2), for example (20). Irradiation serve as an additional animal model used to study lymphopenia-induced proliferation.

Homeostatic and lymphopenia-induced proliferations are regulated by the availability of resources required for T cell proliferation. It is this competition for T cell resources and thymic output that is crucial for maintaining T cell homeostasis. The most important cytokines involved in T cell proliferation are IL-7 and IL-15. T cells also require interaction with self-peptide MHC complex (spMHC) in a TCR-dependent manner (22). Specifically, it is the survival and proliferation of memory T cells that depend on the availability of IL-15, while naïve T cells require IL-7 and interaction with spMHC. To prevent memory T cells from becoming auto-reactive, they do not interact with spMHC thereby avoiding activation
by a specific antigen. CD4+ and CD8+ memory T cells do, however, interact with spMHC to maintain function (23-27). Kassiotis et al. have shown that memory CD4+ T cells deprived of MHC class II contact are functionally defective upon antigen re-encounter. Therefore, although not required for survival, memory T cell and spMHC interaction is required for a quality antigen response (27). In addition to memory T cells proliferating to maintain homeostasis, naïve T cells also proliferate when the body is in a lymphopenic state. During lymphopenia, naïve T cells differentiate into memory-like or “homeostatic” memory-like cells. The differentiation of naïve T cells into memory-like T cells requires IL-7 and spMHC (22).

Efforts to maintain peripheral homeostasis also involve T cell and cytokine interactions, specifically IL-7 and IL-15. Unlike the TCR-dependent interactions (determine T cell repertoire) of spMHC with memory and naïve T cells, cytokine and T cell interactions are TCR-independent (determine total T cell number) (22). The level of bcl-2 (an anti-apoptotic factor) increases in T cells by IL-7 and IL-15 (28). IL-7 and IL-15 work together in multiple functions that help maintain peripheral homeostasis. IL-7 is required for the proliferation of naïve CD8+ and CD4+ T cells in the presence of lymphopenia and for generating T cell memory (20). IL-15 plays a role in reconstitution of T cells during lymphopenia and is required for memory CD8+ T cell maintenance. Specifically, IL-15 induces the proliferation of memory CD8+ T cells (29). Homeostasis of naïve and memory CD8+ T cells are both
enhanced with IL-7. Because both naïve and memory compartments must be maintained to ensure proper immune responses, it is critical to examine not only thymopoiesis but also thymic output in studies of thymus involution (29).

1.4. Acute thymus involution, LPS septic shock

Involution of the thymus can be due to many factors, including environmental and physiological stresses. Of the numerous stressors the thymus, and subsequently the thymic environment encounters, there are two that are most prevalent: ageing and bacterial infections. Much research has been done on the effects of ageing and bacterial infections on the thymus and how these two events cause thymic involution. In this study, our focus has been on a model of bacterial infection, specifically thymic atrophy caused by lipopolysaccharide.

LPS is an endotoxin produced by *Escherichia coli*, a gram-negative bacterium, that induces toxicity, adjuvanticity, anti-complementary activity and interferon-inducing activity (30). LPS challenge induces a cascade of acute pro-inflammatory cytokines, such as TNF-alpha and IL-1, that result in acute thymic atrophy (31). Acute pro-inflammatory cytokines are known to cause tissue injury and sepsis, both of which result in septic shock, a common cause of mortality in clinical settings (32).
Sepsis occurs when there is an infection in the blood stream. Initially the host will try to control the infection by mounting an immune response. With all immune resources focused on clearing the infection, the subject shifts from immune overreaction to a state of anti-inflammatory immuno-suppression. Research suggests that apoptotic cell death, due to the LPS, causes this shift from immune overreaction to inflammatory immuno-suppression and it is this shift that leads to septic shock (33).

Other reports also examine thymocyte apoptosis due to LPS. Zhang et al. reported that LPS injections into mice induced DNA fragmentation in thymocytes, leading to thymocyte apoptosis. Thymocyte apoptosis was confirmed by visualizing DNA fragmentation (on agarose gels) of mice injected with increasing concentrations of LPS (30). Additionally, Zhang et al. demonstrated that no significant DNA fragmentation was seen in mice injected with 1 μg of LPS, while maximum DNA fragmentation was seen with 100 μg of LPS. A minimum of 10 μg was required to begin to visualize DNA fragmentation (30).

LPS has also been examined as a model of acute thymic atrophy by our lab and others. Our lab has demonstrated that LPS results in a decrease of thymus weight, cellularity, absolute number of CD4/CD8-double positive thymocytes and mTREC/mg thymus, and that this process is mediated by leukemia inhibitory factor, cytokines and steroids (34, 35).
In this study we have used an LPS-induced acute thymic atrophy model to induce acute thymic atrophy and examine the effect of thymostimulatory cytokines IL-7 and KGF in aiding the recovery of the thymus by increasing thymopoiesis and reversing thymic involution.

1.5. Thymostimulatory cytokines: IL-7 and KGF

Interleukin (IL)-7, a 15 kDa protein containing 129 amino acid residues, (15) is a cytokine necessary for the development of B and T lymphocytes. IL-7 is constitutively expressed in the thymus and in bone marrow, stromal, epithelial and dendritic cells (36). IL-7 acts through its receptor (IL-7R), a heterodimer of an α chain (CD127), specific for IL-7, and a γ common chain (CD132) shared by other cytokine receptors (36). The IL-7R is present on stromal cells and acts primarily as a growth and anti-apoptotic factor for B and T cell precursors in the BM (37).

Studies with transgenic IL-7R knockout mice reveal that this ligand is required for early T cell development, for later stages of T cell development and for T cell expansion in the periphery (15). For T cells to move from the DN1 to the DN2 stage of development, signaling through the IL-7R is required. IL-7 up-regulates bcl-2, an anti-apoptotic protein, which allows the uncommitted DN1 precursor to escape apoptosis (38). Oosterwegel et al. showed that stromal cells from fetal thymic organ cultures of wild type mice allowed for
stem cells to commit to the T cell developmental pathway, while stromal cells from IL-7 knockout mice did not (39).

Previous studies with IL-7 or IL-7R deficient mice have shown that by constitutive expression of bcl-2, thymopoiesis is restored (38). Phillips et al. transfected murine bone-marrow stromal cells with an IL-7-expressing plasmid, and then delivered these cells intrathymically to C57BL/6 mice. The injection of an IL-7 producing plasmid into the mice resulted in an increase of DN2 cells and bcl-2 expression (38).

Keratinocyte growth factor (KGF) is a 28-kDa cytokine and a member of the fibroblast growth factor (FGF) family (40). KGF supports thymocyte development and mediates epithelial cell proliferation and differentiation in gut epithelial cells, keratinocytes, and thymic epithelial cells (41). All functions by KGF take place by signaling through its receptor: FGFR2IIIb. The KGF receptor, FGFR2IIIb, is a membrane-spanning tyrosine kinase generated by alternative splicing of FGFR2 and is expressed on thymic epithelial cells (TEC). The extracellular domain of the KGF receptor is made up of Ig-like domains (40). In addition to KGF (FGF-7), FGFR2IIIb is also activated by other ligands: FGF-1, FGF3, and FGF-10 (41). Of all the FGF ligands, FGF-7 (KGF) is the most important because it is produced by thymic stromal cells and mature T cells (10).
Various experiments have shown that KGF is a critical component in T cell reconstitution via the thymus. A KGF knockout mouse (KGF−/−) model has demonstrated that KGF is required for T cell reconstitution as KGF−/− mice that had been sub-lethally irradiated (450 cGy) did not respond to syngeneic or allogeneic bone marrow transplants (41). It has also been shown that KGF plays a role in postnatal thymic regeneration because KGF treatment before bone marrow transplantation led to increased thymopoiesis and peripheral T cell numbers in mice that received allogenic bone marrow transplants (41).

1.6. Hypothesis and approach

Previous studies have shown that recombinant adenoviruses serve as efficient gene delivery vectors. Amalfitano et al. described replication-deficient adenovirus vectors as being able to deliver recombinant genes to many cell types and being capable of infecting non-dividing cells (eg. thymic stromal cells). Adenoviruses also have the advantage of being able to incorporate large genes, of up to 8.3 Kb in size (42).

Oberholzer et al. demonstrated that delivery of IL-10 via a recombinant adenovirus decreased thymic apoptosis in a model of mouse sepsis. IL-10 is an anti-inflammatory cytokine shown to reduce T cell apoptosis by up-regulating bcl-2, an anti-apoptotic protein that inhibits caspase-9 and caspase-3 activation (43). In this study, mice were injected by intravenous or intrathymic routes with 10⁵ or 10¹⁰ particles of recombinant adenovirus expressing human
IL-10. Twenty-four hours later cecal ligation and puncture (CLP) was performed, to induce generalized peritonitis, and the mice were monitored for six days. Mice without IL-10 treatment had a 30% survival rate while those receiving IL-10 had a 75% survival rate (43). Oberholzer et al. hypothesized that IL-10 in the thymus would increase bcl-2 expression and reduce caspase-3-dependent thymocyte apoptosis thereby improving survival of mice with peritonitis. They concluded from their results that IL-10 did eliminate caspase-3 activity resulting in decreased thymic apoptosis and increased animal survival.

We hypothesize that mIL-7 and mKGF/FGF-7 delivered via recombinant adenovirus can improve thymopoiesis and T cell reconstitution in mice using LPS, a model of acute thymic atrophy. To address this hypothesis, we created recombinant adenovirus vectors which express mIL-7 and mKGF. To test the functionality of our adenovirus constructs we performed multiple assays, including restriction enzyme analysis, DNA sequencing, mRNA expression (qRT-PCR), in vitro delivery into HEK293 cells and in vivo delivery (and confirming of beta-galactosidase production with the Lac Z construct). Once our recombinant adenovirus constructs were complete and confirmed, we moved into in vivo assays. We used LPS to induce acute thymic atrophy and then treated with our recombinant adenovirus constructs. We chose two methods of adenovirus delivery, retro-orbitally (RO) and intrathymically (i.t.). To monitor thymopoiesis we employed various techniques: flow cytometry for phenotyping thymocytes and splenocytes, real-time PCR to examine mTREC
numbers, quantitative reverse-transcriptase PCR to monitor for cytokine expression and cytokine profiling in the serum to screen for cytokine levels.
2. MATERIALS AND METHODS

2.1. Animals and treatments

Female BALB/c mice (6-120 weeks of age) were purchased from either Charles River Laboratories (Wilmington, MA) or from the National Institute on Aging Aged Rodent Colony (NIH, Bethesda, MD). Animals were housed in specific pathogen free conditions in the Duke University Barrier (Durham, NC). Mouse handling and experimental procedures were conducted in accordance with AAALAC guidelines for animal care and use under approved Duke University IACUC protocols. For the various in vivo experiments, mice were harvested at days 3, 7 or 21; serum or plasma was collected and organs collected at necropsy included thymus, spleen and liver. Serum, plasma and tissues collected were analyzed with various assays which are detailed in following sections within Materials and Methods.

Procedures done on live animals are listed here. BALB/c mice received intravenous (retro-orbital) or intra-thymic injections of saline, rAdeno-mIL-7, rAdeno-mKGF or rAdeno-LacZ (500 μL at 1x10^10 pfu/mL). For retro-orbital injections, mice were anesthetized with ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and xylazine (Lloyd Laboratories, Shenandoah, IA) (173/7 mg/kg ketamine/xylazine). Once adequately anesthetized, the mouse was laid on its side to allow eye exposure for injection. A 28½ G insulin syringe (BD Biosciences, Palo Alto, CA) was placed at a 45° angle to the mouse’s nose. The needle was
slowly inserted 1-2 mm into the anterior distal corner of the eye until resistance was met.

Needle content was then injected slowly and the mouse placed in a clean cage under a warm lamp for recovery (Technique provided by Francis J. Sun, DVM, DACLAM, Duke University Division of Laboratory Animal Research, Durham, NC).

For intra-thymic injections (32), mice were anesthetized as above. Once adequately anesthetized, the incision site was disinfected with an alcohol wipe and a 4–5 mm transverse incision was made in the skin at the height of the second intercostal space, perpendicular to the sternum. The thymic lobes were then accessed through the intercostal space on either side of the sternum with a 25G needle (BD Biosciences). The needle was placed at a 30° - 40° angle relative to the sternum and needle contents were injected. The incision was closed with Vetbond tissue adhesive (3M Canada, London, Ontario).

For sepsis/shock studies, animals received intraperitoneal injections of saline or lipopolysaccharide from *E. coli* (Sigma, St. Louis, MO) (100 μL at 10 mg/mL). Serum or heparinized whole blood collection was done by maxillary vein bleed. Briefly, the mice were restrained by scruffing the loose skin over the neck and shoulders. Using a 5 mm lancet (MEDIpoint, Mineola, NY), a puncture was made in the small dimpled area (near whiskers) on the side of the face (there is a vascular bundle at the rear of the jaw bone) (44).
Whole blood was collected and processed for serum or plasma by low speed centrifugation. Samples isolated were frozen for future study.

2.2. Cytokine gene cloning and recombinant adenovirus vector construction

Recombinant adenovirus vectors were constructed using standard molecular techniques to express mIL-7 and mKGF. RNA was extracted from pooled mouse thymus and spleen tissue with Trizol (Invitrogen, Carlsbad, CA) per the manufacturer’s protocol. cDNA was produced with iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to manufacturer’s protocol. To amplify the mIL-7 (Accession #NM_008371) and mKGF (Fgf7) (Accession #Z22703) genes for cloning from the cDNA pool, PCR with appropriate primers (IDT, Coralville, IA) and AccuPrime Pfx proof-reading, high-fidelity Taq polymerase (Invitrogen, Carlsbad, CA) was performed. Primer sequences used: Fgf7 F (5’-AAG GGA CCC AGG AGA TGA AGA A-3’), Fgf7 R (5’-GAA GTT GCA ATC CTC ATT GCA TT-3’), IL-7 F (5’-TGT ACT GAT GAT CAG CAT CGA TGA-3’) and IL-7 R (5’-GTA AGT GGA CAT TGA ATT CTT CAC TGA TAT-3’) (IDT, Coralville, IA). The resulting mIL-7 and mKGF amplicons were PCR-cloned into the pCR2.1 plasmid vector (Invitrogen, Carlsbad, CA) according to manufacturer’s protocol. pCR2.1 is a pre-linearized vector that has single 3’-deoxythymidine residues that allow for PCR inserts to ligate efficiently (TA cloning). One Shot Competent Cells (Invitrogen, Carlsbad, CA) were transformed by adding the ligation reactions of fresh amplicon plus open pCR2.1 to the cells and heat shocking for
30 seconds at 42°C. This mixture was then spread on LB agar plates containing X-Gal and 100 μg/mL ampicillin (Sigma, St. Louis, MO). White colonies were selected and plasmid isolation was done with Qiagen’s Plasmid Purification Mini/Midi Kits (Qiagen, Valencia, CA). Restriction analysis was then done with Apa I and Kpn I (New England Biolabs, Ipswich, MA) to confirm insert orientation. Sequence of final selected clones was confirmed by the Duke Cancer Center DNA Sequencing Facility (Durham, NC).

The plasmid vectors containing the mIL-7 and mKGF genes were then cloned into the mammalian expression cassette pShuttle2 (BD Biosciences, Palo Alto, CA) as per manufacturer’s protocol. pShuttle2 was selected because it contains the human cytomegalovirus immediate early promoter/enhancer (PCMV IE), a multiple cloning site (MCS), the SV40 polyadenylation signal (SV40 poly A) and a kanamycin resistance gene for selection (BD AdeonX Expression System). pShuttle-Lac Z provided by Dr. Ryan Hick (Sempowski lab) as a control. pShuttle2-mIL-7 and pShuttle2-mKGF were double digested with I-Ceu I and PI-Sce I (New England Biolabs, Ipswich, MA) and the expression cassettes excised. The expression cassettes were then purified to obtain plasmid DNA with Nucloespin Plasmid Kit (BD Palo Alto, CA) as per manufacturer’s protocol. The excised expression cassettes were sub-cloned into the BD Adeno-X Viral DNA by a ligation reaction that incubated overnight. The next day electro-competent E. coli were transformed with the ligation reaction (excised expression cassettes and adeno viral DNA).
One electroporation cuvette and one microfuge tube were placed on ice per sample to be transformed. The ligation reaction (1 μL) and competent cells (20 μL) were added to the chilled microfuge tube and mixed by tapping. Twenty microliters of the ligation reaction-cell mixture was transferred into a cold, electroporation cuvette. The cuvette was pulsed once using the Gene Pulser Xcell (Bio-Rad, Hercules, CA) and with the following parameters: 1.8 kV, 25 μF and pulse controller setting 200 Ω. Cuvette contents was added to 1 mL of room temperature SOC, mixed, and transferred to a 14 mL tube containing 5 mL of SOC media. The tubes were incubated at 37°C for 1 hour, shaking at 225–250 rpm. Spread 100 μL of the ligation reaction-cell culture on LB plates containing 100 μg/mL ampicillin.

The replication incompetent BD Adeno-X plasmid was derived from an adenovirus type 5 (Ad5) whose genome is replication deficient due to deletions of the E1 and E3 regions of the Ad5 genome. HEK293 packaging cells (ATCC, Manassas, VA) allow adenovirus propagation as they express the Ad5 E1 genes in trans needed for Adeno Viral DNA replication and transcription. The sub-cloning of the expression cassettes into the BD Adeno-X Viral DNA was confirmed by restriction analysis using PI-Sce I and I-Ceu I (New England Biolabs, Ipswich, MA) restriction enzymes according to the manufacturer’s protocol (BD, Palo Alto, CA). rAdeno-Lac Z was generously provided as a control by Dr. Zachary Hartman (Amalfitano Lab, Duke University).
2.3. **Generation of adenovirus high-titer stocks and purification**

High-titer stocks of replication-deficient adenovirus vectors were obtained by seeding 300 cm$^3$ cell culture flasks (BD Biosciences, Palo Alto, CA) with $7 \times 10^6$ HEK293 cells each (ATCC, Manassas, VA). When the HEK293 cells were approximately 80% confluent, in about 3 days, purified cell lysate ($5 \times 10^9$ total pfu) from a previous smaller-scale viral culture was added. When approximately 70% of the infected HEK293 cells had lifted off the bottom of the flask, due to cytopathic effect (CPE), the virus was harvested (42). To harvest the adenovirus, the attached and floating cells were collected by scraping the plates with a cell scraper and transferring the contents to 50 mL conical tubes. Three rounds of freeze/thaw were performed with an ethanol/dry ice bath and a beaker of 37°C water. Lysed cells were then transferred to 750 mL centrifuge bottles. The cells were centrifuged at 800xg for 15 minutes, supernatant was discarded and the cell pellet was resuspended in 100 mL of the remaining supernatant. This suspension was then transferred into 50 mL conical tubes and centrifuged at 800xg for 15 minutes. Supernatant was discarded and the cell pellet was resuspended in 15 mL of 0.1 M Tris pH 8.0. Adenovirus purification was then carried out by cesium chloride gradient (42). Briefly, sodium deoxycholate (FW: 65.01 g) (Sigma, St. Louis, MO) was added per 15 mL of cell suspensions, to a final concentration of 0.5% w/v, and incubated at room temperature for 30 min. Next, 150 uL of 2M MgCl$_2$ (Invitrogen, Carlsbad, CA) and 75 uL DNase I (New England Biolabs, Ipswich, MA) was added followed by incubation at 37°C for 1 hour with mixing every 10 minutes for a total of 60 minutes. The
cells suspensions were then centrifuged at 3,000xg for 15 minutes at 5°C.

Three different weights of cesium chloride (Invitrogen, Carlsbad, CA) were prepared as follows to generate a gradient: Heavy CcCl consisted of 1.5 g/mL of CsCl in 10mM Tris-HCl pH 8.0. Medium CsCl consisted of 1.35 g/mL of CsCl in 10mM Tris-HCl pH 8.0. Light CsCl consisted of 1.25 g/mL of CsCl in 10mM Tris-HCl pH 8.0. One cesium chloride gradient was prepared, per 15 mL of lysate and the gradients were prepared as follows: 8.5 mL of heavy CsCl was placed into a pollyallomer centrifuge tube (Beckman Coulter, Miami, FL), then 1.5 mL of heavy CsCl and finished with 8.5 mL of the light CsCl. The clarified viral supernatant was then layered onto the gradient. The tubes were centrifuged at 20,000 rpm for 2 hrs at 10°C.

The tubes were then removed from the centrifuge and the first virus band (white, opalescent layer near the inter-phase of the medium and light CsCl) was removed using a 21G needle inserted just below the virus band. The band was then placed in a quick-seal ultracentrifuge tube (Kendro, Newtown, CT) and topped off with medium CsCl. Tubes were then centrifuged at 63,000 rpm for at least 16 hours at 4°C. The final virus band was collected using a 21G 5 mL syringe (BD Biosciences, Palo Alto, CA) inserted just below the virus band. The band was then transferred to a 10,000 molecular weight Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) and dialyzed overnight at 4°C in 6 liters of 10 mM Tris,
pH 8.0. Dialyzed virus was collected from the cassette, measured, and 1/10 volume added of sterile 10% sucrose (Sigma, St. Louis, MO) in 10X PBS (Invitrogen, Carlsbad, CA). 100 uL aliquots were stored at –80°C.

Virus titer was determined by the end-point titer dilution assay. HEK293 cells (1x10^4 in 100 uL per well) were seeded into 96-well plates (BD Biosciences, Palo Alto, CA). In about 1-2 days, the HEK293 cells formed an even monolayer. Once an even monolayer of HEK293 cells was obtained, serial dilutions of sample viral stocks were created in a range of 10^{-3} to 10^{-10} and added to the monolayer of HEK293 cells. Ten days later, the wells were monitored for CPE. The fraction of CPE-positive wells in each row was calculated with the following equation: Titer (pfu/mL) = 10^(x + 0.8) where x is equal to the sum of the fractions of CPE-positive wells. In some studies the Rapid Titer assay (BD, Palo Alto, CA) was also used according to the manufacturer’s protocol. Typical yield of virus obtained, post purification by CsCl gradient, increased with each subsequent high titer stock grown and purified. Initial yields were about 1 mL of purified virus at 5x10^9 pfu/mL. Subsequent yields were 4 to 5 mLs with concentrations ranging from 4x10^{12} to 6.3x10^{13} pfu/mL for both constructs.

2.4. In vitro COS cells transfections

To confirm the delivery of the Lac Z gene, HEK293 cells (ATCC, Manassas, VA) were seeded into 6-well TC plates (BD, Palo Alto, CA) at a concentration of 0.6x10^6 cells/well.
The HEK293 cells were then transfected with Lac Z-pShuttle2 (5x10^{10} pfu total) at various ratios (1:1, 1:10, 1:20, 1:30 and 1:40) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The HEK/Lac Z-pShuttle2 cultures were then monitored for CPE. A similar transfection of COS cells (ATCC, Manassas, VA) with pShuttle2-mIL-7 and pShuttle2-mKGF was used to confirm mRNA expression of mIL-7 and mKGF.

2.5. Beta-galactosidase assay

Forty-eight hours post transient transfection of COS cells with Lac Z pShuttle2, beta-galactosidase production was measured at an optical density of 420 nm with an ELISA-based beta-galactosidase detection assay (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. To normalize the ELISA results, beta-gal production per mg of input protein was calculated by measuring the protein concentration of each sample with a spectrophotometer (Nanodrop Technologies, Wilmington, DE).

2.6. Murine lymphocyte isolation

Following euthanasia, mouse thymus and spleen were excised, cut in half and each half weighed. One-half of the organ was snap frozen in a dry ice/ethanol bath and stored at -80°C. The other organ half was teased into a single-cell suspension into RPMI 1640 (with L-glutamine) (Invitrogen, Carlsbad, CA) plus 5% FBS with a 1 mL syringe plunger (BD
Biosciences, Palo Alto, CA) through a 70 μm Falcon nylon screen (BD Biosciences, Palo Alto, CA). Red blood cells were removed from the cell suspensions with 1X ammonium chloride (8.29g NH₄Cl, 0.15M + 1g KHCO₃, 1mM + 37.2mg Na₂EDTA, 0.1mM + water to 1 liter and pH adjusted to 7.2-7.4) (45). Thymocytes and splenocytes were then counted by a particle size counter (Beckman Coulter, Miami, FL). Approximately 1x10⁶ thymocytes and splenocytes were then used per tube for flow cytometric phenotyping and the remaining cells cryo-preserved for additional studies in 90% FBS plus 10% dimethyl sulfoxide (45).

2.7. Flow cytometry/Immunophenotyping

Multi-color flow cytometric phenotypic analysis was performed on a BD LSRII (BD Biosciences, Palo Alto, CA) using fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), PE-Texas Red (PETXR) and peridinin chlorophyll protein conjugated to the cyanine dye 5.5 (PerCP-Cy5.5) as fluorescent dyes. Directly conjugated anti-mouse monoclonal antibodies were used against CD3, CD4, CD8, CD25 (PC61), CD44, CD45RB and CD62L (BD Pharmingen, San Jose, CA). Saturating amounts of antibody were used to stain approximately 1 × 10⁶ thymocytes or splenocytes. Cells were stained in PBS wash (1× PBS, 1% BSA, 0.1% NaN₃) (final volume of 100 μL) at 4°C for 30 min. All samples were washed with 3 mL of PBS wash and resuspended in 1 mL of PBS wash with 0.4% (w/v) paraformaldehyde (35). For each sample, forward and side angle light scatter profiles were used to acquire 300,000 events representing viable lymphocytes. Data were
saved as FCS 3.0 and analyzed with FlowJo software (Tree Star, Inc. Ashland, OR).

2.8. Real-time quantitative PCR for mouse signal joint TCR delta excision circles (mTREC)

Signal joint T cell receptor delta (TCRD) excision circles (TRECs) are episomal DNA circles generated by the TCRA locus recombination process that generates the repertoire of TCRαβ antigen-specific T cells (45). Molecules of mouse TCRD TREC from whole thymus DNA were quantified by real-time PCR using a standard curve of known number of molecules of mouse TREC according to our previously published work (45). Genomic DNA samples from whole thymus tissues were prepared by homogenization in 1 mL TRIZOL Reagent (Life Technologies/Invitrogen, Carlsbad, CA) using a homogenizer probe according to manufacturer’s protocol (Omni International, Warrenton, VA). Total genomic DNA was extracted as previously described (46) and quantified by spectrophotometry. One microgram of DNA was run per PCR reaction in duplicate. Real-time PCR reactions consisted of forward (5’-CAT TGC CTT TGA ACC AAG CTG-3’) and reverse (5’-TTA TGC ACA GGG TGC AGG TG-3’) DNA primers for the mTREC sequence, DNA probe (5’-FAM-CAG GGC AGG TTT TTG TAA AGG TGC TCA CTT-QSY-3’) conjugated to a fluorescent dye and genomic thymus DNA. The cycle conditions were set as follows: Cycle 1: (1x) Step 1, 95°C for 10 min, Cycle 2: (45x) Step 1, 95°C for 15 sec; Step 2, 60°C for 1 min and
Cycle 3: (1x) Step 1, 4°C hold. mTREC numbers per 1 μg of DNA were normalized to reflect levels per milligram of whole thymus tissue (45).

2.9. Quantitative reverse-transcriptase PCR for cytokine expression

To determine steady-state mRNA expression of the mIL-7, mKGF and GAPDH, quantitative reverse-transcriptase PCR (qRT-PCR) was performed on thymus, spleen and liver total RNA samples and COS cells with the iScript One-Step RT-PCR SYBR Green system from Bio-Rad (Hercules, CA). Total RNA from whole tissues was prepared by homogenization of ~100 mg of tissue or 1 x 10⁶ cells in 1 mL TRIZOL Reagent (Invitrogen, Carlsbad, CA) using a homogenizer probe (Omni International, Warrenton, VA). Total RNA was extracted as previously described (46) and quantified by spectrophotometry (OD 260/280). Five hundred ng of total RNA was run per PCR reaction. Each qRT-PCR reaction was done in duplicate in an optical thermocycler (iCycler, Bio-Rad, Hercules, CA) (47). The reaction volume was 50 μL consisting of: 25 μL of 2X SYBR Green Reaction Mix, 18 μL of nuclease-free water, 1 μL of iScript RT enzyme and 0.5 μL of each 10 μM primer. Primers used were: Fgf7 F (5’-AAG GGA CCC AGG AGA TGA AGA A-3’), Fgf7 R (5’-GAA GTT GCA ATC CTC ATT GCA TT-3’), IL-7 F (5’-TGT ACT GAT GAT CAG CAT CGA TGA-3’), IL-7 R (5’-GTA AGT GGA CAT TGA ATT CTT CAC TGA TAT-3’) GAPDH F (5’-AAT GTG TTC GTC GTG GAT CTG-3’) and GAPDH R (5’-CAA CCT GGT CCT
CAG TGT AGC-3’) (IDT, Coralville, IA). The cycle conditions were set as follows: 50°C for 10 min, 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 10 sec, and primer annealing/elongation at 55°C for 30 sec. Final dissociation curve was done at 95°C for 1 min, 55°C for 1 min and 95°C for 15 sec. To assess the specificity of the PCR products, a melt curve analysis was done starting at 55°C with the temperature increasing by 0.5°C every 10 sec. Baseline and threshold values of gene expression were calculated using the comparative C
\[ C_{t} \] method ($\Delta\Delta C_{t}$) with the Bio-Rad Gene Expression Macro (Hercules, CA).

To use the comparative C
\[ C_{t} \] method ($\Delta\Delta C_{t}$) you have to normalize the target RNA to another endogenous control and the amplification efficiencies of the target RNA and the endogenous control must be the same. The calculations for the $\Delta\Delta C_{t}$ method are: 1) Normalization to the endogenous control ($C_{t}$ target gene - $C_{t}$ endogenous control = $\Delta C_{t}$), 2) normalization to reference sample ($\Delta C_{t}$ sample - $\Delta C_{t}$ reference = $\Delta\Delta C_{t}$) and 3) calculate fold changes ($2^{\Delta\Delta C_{t}}$).

2.10. Cytokine profiling

Quantification of cytokines in mouse serum was determined using a commercial cytokine-specific Luminex bead kit according to the manufacturer’s protocol (Biosource/Invitrogen, Carlsbad, CA). Cytokines quantified included: FGF basic, GM-CSF, IL-1a, IL-1b, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17, IFNg, IP-10, KC, MCP-1, MIG, MIP-1a, TNFa and VEGF. Briefly, 50 uL of mouse serum was incubated with the capture antibody coated beads in the provided plate along with incubation buffer and assay diluent. The plate was shaken
on a platform shaker for 2 hours protected from light. Biotinylated detector antibody was added (100 uL) and shaken for 1 hour. The 1x Streptavidin-RPE solution was added next (100 uL) with an additional 30 minutes of shaking. Standard curves for each cytokine were generated by using the reference cytokine concentrations supplied by the kit.

2.11. Statistics

T-test for unpaired data was performed using GraphPad Software (San Diego, CA) to compare the means between data sets. Differences were considered significantly different with $p \leq 0.05$. 

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3. RESULTS

3.1. Cytokine gene cloning/recombinant adenovirus vector construction

To determine if mIL-7 and mKGF/FGF-7 delivered via recombinant adenovirus can modulate thymopoiesis and T cell reconstitution in mice, recombinant adenovirus vectors expressing these two cytokines genes were constructed. The construction of a recombinant adenoviral vector requires many manipulations (cloning, PCR, ligation, transformation and restriction analysis) that allow progression through various vectors (pCR2.1, pShuttle2 and Ad5 backbone) to obtain a functional recombinant adenovirus (replication deficient).

To begin the construction of recombinant adenoviruses, total RNA was extracted from pooled mouse thymus and spleen tissue to generate a cDNA pool. For cloning purposes, full-length mIL-7 and mKGF genes were PCR amplified from the cDNA pool with gene-specific primers and a proof-reading, high-fidelity Taq polymerase, as detailed in the methods. Restriction sites were engineered into the plasmid for directional cloning. The amplified mIL-7 and mKGF PCR products were ligated into the pCR2.1 plasmid vector (Figure 1). Chemically competent *E. coli* were transformed with the contents of the ligation reaction (mIL-7 or mKGF DNA and pCR2.1 vector). pCR2.1 has an ampicillin resistance gene so transformed *E. coli* were plated on LB agar plates containing 100 µg/mL of ampicillin. The plates also contained 40 µL of 40 mg/mL X-gal to allow for blue/white colony selection.
To screen for pCR2.1 vectors containing mIL-7 or mKGF inserts, white colonies (10-15) were selected, swirled into LB broth and allowed to grow in culture. DNA was isolated as per Qiagen’s plasmid purification protocol. PCR with mIL-7 or mKGF gene-specific primers amplified the purified DNA, needed to move forward in the strategy (Figure 1).

To next confirm the insertion of the mIL-7 and mKGF genes into the pCR2.1 vector, restriction enzyme analysis was performed on the purified DNA from selected colonies with Apa I and Kpn I. Briefly, 1μg of DNA (in 15 or 20 μL of PCR-grade water) were added to a restriction enzyme reaction containing the Apa I and Kpn enzymes plus appropriate buffer and BSA (supplied with the enzymes). After one hour at room temperature, the restriction enzyme reactions were run on a 1% agarose gel. Figure 2 shows digested clones containing the pCR2.1 vector (3.9 Kb) with the 500 bp mIL-7 insert (lanes 2 and 4). Uncut pCR2.1 is seen in lane 5. Figure 3 shows digested clones containing the pCR2.1 vector (3.9 Kb) with the 603 bp mKGF insert (lanes 2-5). A 1 Kb ladder is used as a size marker.

To further confirm the integrity of our promising clones, we next submitted selected mIL-7-pCR2.1 and mKGF-pCR2.1 clones for sequencing; a reaction consisting of 50 ng of DNA, 30ng of forward primer and PCR-grade water (up to 12 μL) was submitted to the Duke DNA Sequencing Facility. To establish homology between our clone sequences and consensus nucleotide sequences, BLAST analysis was performed. The inserts in the recombinant mIL-
7-pCR2.1 and mKGF-pCR2.1 clones selected were 98% and 99% homologous to consensus (Figures 4 and 5).

In Figures 4 and 5 the start and stop sites are in frame and aligned for the query and subject. Regions with low-complexity sequences are identified as lowercase grey characters. Low-complexity sequences have an unusual composition that can create problems in sequence similarity searching. Low-complexity sequences are recognized by visual inspection of the sequence and corresponding chromatograph. The nucleotide sequence AAATAAAAATAAAAAAT, for example, is a low-complexity sequence. BLAST automatically filters your query for low-complexity sequences to prevent alignments that many consider to be artifacts. The filter substitutes low-complexity sequences with lowercase grey characters allowing you to see the sequence that was filtered.
Figure 1. Cloning strategy of amplified mIL-7 and mKGF PCR products into pCR2.1 vector.
Figure 2. Restriction enzyme analysis of mIL-7-pCR2.1 clones. DNA from selected mIL-7-pCR2.1 clones was done with enzymes Apa I and Kpn I. Restriction digestion products were run on a 1% agarose gel. Clones 3 and 5 (lanes 2 and 4 respectively) contained the mIL-7 insert as seen with the 500 bp band. Gel lanes contained 1Kb ladder (lanes 1 and 6) and mIL-7-pCR2.1 clones 3, 4, 5 and 9 (lanes 2-5).
Figure 3. Restriction enzyme analysis of mKGF-pCR2.1 clones. DNA from selected mKGF-pCR2.1 clones was done with enzymes Apa I and Kpn I. Restriction digestion products were run on a 1% agarose gel. All clones (lanes 2-5) contained the mKGF insert as seen with the 603 bp band. Gel lanes contained 1Kb ladder (lane 1), mKGF-pCR2.1 clones 6.1-6.4 (lanes 2-5) and a 100 bp ladder (lane 6).
Figure 4. DNA sequence confirmation of mIL-7-pCR2.1. DNA sequence for mIL-7 insert was obtained from the Duke Cancer Center DNA Sequencing Facility was entered into the Entrez Nucleotide Database and BLAST (Basic Local Alignment Search Tool) analysis was performed resulting in an identity score of 98%. = start codon  = stop codon.
**Mus musculus Fibroblast Growth Factor 7 mRNA**

| Query: 7  | cctcgctctgtcctagtggcaactatatattctctagcttggcatgagctccggagc 66 |
| Sbjct: 406 | cctcgctctgtcctagtggcaactatatattctctagcttggcatgagctccggagc 464 |

| Query: 67  | cggctacgagtgtgaaactgtttnnnnnngancacacaccatataagttatgactacntg 126 |
| Sbjct: 465 | cggctacgagtgtgaaactgttccagcccccgtgacaacaccag-aagttatgactacatg 523 |

| Query: 127  | gaaggaggagatataagggtgagaagactgttctgtcnncccgagtgcacctgtgaggatt 186 |
| Sbjct: 524 | gaaggaggagatataagggtgagaagactgttctgtcncaccagttgtacccatgagatt 583 |

| Query: 187  | gaacaacgggcaaatggaagggacctttactaacaacacctatgtgga 246 |
| Sbjct: 584 | gaacaacgggcaaatggaagggacctttacataaacaacacctatgtgg 641 |

| Query: 247  | aaatcaggacctgt 259 |
| Sbjct: 642 | aaatcaggacctgt 654 |

**Figure 5. DNA sequence confirmation of mKGF-pCR2.1.** DNA sequence for mKFG (FGF7) was obtained from the Duke Cancer Center DNA Sequencing Facility was entered into the Entrez Nucleotide Database and BLAST (Basic Local Alignment Search Tool) analysis was performed resulting in an identity score of 99%. **Green** = start codon  **Red** = stop codon.
The vector containing the Ad5 genome backbone requires that genes be inserted as part of a mammalian expression cassette. To obtain gene-specific mammalian expression cassettes containing the mIL-7 and mKGF genes, the pShuttle2 vector was used. The pShuttle2 vector was selected because it constitutively expresses the human cytomegalovirus immediate early promoter/enhancer ($P_{\text{CMV\ IE}}$), allowing the gene insert to be expressed at high levels even though the Ad5 genes remain inactive. pShuttle2 vector also contains a multiple cloning site (MCS), a kanamycin resistance gene for selection, and most importantly, restriction sites that flank the expression cassette allowing for its excision.

Recombinant mIL-7-pCR2.1 and mKGF-pCR2.1 DNA were next cloned into the pShuttle2 transfer vector by digesting them and the pShuttle2 vector, with Apa I and Kpn I restriction enzymes and performing a ligation reaction (Figure 6). Chemically competent *E. coli* were transformed with the contents of the ligation reaction (mIL-7-pCR2.1 or mKGF-pCR2.1 DNA and pShuttle2 vector). pShuttle2 has a kanamycin resistance gene so transformed *E. coli* were plated on LB agar plates containing 50 μg/mL of kanamycin. To screen for pShuttle2 vectors containing mIL-7 or mKGF inserts, single colonies (10-15) were selected, swirled into LB broth and allowed to grow in culture. DNA was isolated as per Nucleospin’s plasmid purification protocol. PCR with mIL-7 or mKGF gene-specific primers amplified the purified DNA, needed to move forward in the strategy (Figure 6).
Figure 6. Cloning strategy of amplified mIL-7-pCR2.1 and mKGF-pCR2.1 into pShuttle2 transfer vector.
To next confirm the insertion of the mIL-7 and mKGF genes into the pShuttle2 vector, restriction enzyme analysis was performed on the purified DNA from selected colonies with Apa I and Kpn I. Briefly, 1 μg of DNA (in 15 or 20 μL of PCR-grade water) were added to a restriction enzyme reaction containing the Apa I and Kpn enzymes plus appropriate buffer and BSA (supplied with the enzymes). After one hour incubation at room temperature, the restriction enzyme reactions were run on a 1% agarose gel. **Figure 7** shows uncut pShuttle2 in lanes 2 and 3. Digested clones reveal the pShuttle2 vector (4 Kb) with the 500 bp mIL-7 insert (lanes 5 and 6). **Figure 8** shows digested clones containing the pShuttle2 vector (4 Kb) with the 603 bp mKGF insert (lanes 2-4). A 1 Kb ladder is used as a size marker.

The final step for constructing recombinant adenovirus plasmids is outlined in **Figure 9**. pShuttle2-mIL-7 and pShuttle2-mKGF were double digested with I-Ceu I and PI-Sce I to excise the expression cassette. The excised expression cassettes were sub-cloned into the adeno viral plasmid DNA by a standard ligation reaction. To reduce the frequency of non-recombinant clones, the ligation reactions were treated with the restriction enzyme Swa I to linearize self-ligated adeno viral plasmid DNA prior to transforming *E. coli*. Electro-competent *E. coli* were then transformed with the ligation reaction containing the excised expression cassettes and adeno viral plasmid DNA by electroporation. Transformed *E. coli* were plated on LB agar plates containing 100 μg/mL of ampicillin. Colonies were selected and DNA was isolated for restriction enzyme analysis (**Figure 9**).
Figure 7. Restriction enzyme analysis of mIL-7-pShuttle2 clones. DNA from selected mIL-7-pShuttle2 clones was done with enzymes Apa I and Kpn I. Restriction digestion products were run on a 1% agarose gel. Both clones, 7.3-2 and 7.3-5, contained the mIL-7 insert as seen with the 500 bp band. The pShuttle2 vector (3.9 Kb) is seen in the lanes containing the uncut samples as well as the digested samples. Gel lanes contained: 100 bp ladder (lane 1), 1 Kb ladder (lane 4), uncut mIL-7-pShuttle2 clones 7.3-2 and 7.3-5 (lanes 2 and 3) and digested mIL-7-pShuttle2 clones 7.3-2 and 7.3-5 (lanes 5 and 6).
Figure 8. Restriction enzyme analysis of mKGF-pShuttle2 clones. Restriction analysis of DNA from the selected mKGF-pShuttle2 clone was done with enzymes Apa I and Kpn I. Restriction digestion products were run on a 1% agarose gel. Clones 2, 6 and 10 contained the mKGF insert as seen with the 603 bp band. The partially digested pShuttle2 vector (4.0 Kb) is seen in lanes 2-4. Gel lanes contained: 1 Kb ladder (lane 1), cut mKGF-pShuttle2 clones 2, 6 and 10 (lanes 2-4) and 100 bp ladder (lane 5).
Figure 9. Strategy used for constructing recombinant adenoviruses.
To confirm the insertion of mIL-7 and mKGF expression cassettes into the adeno genome backbone, restriction enzyme analysis was performed on the DNA from selected colonies with I-Ceu I and PI-Sce I. Cloned mIL-7 (a 500 bp insert) and mKGF (a 603 bp insert) were excised from pShuttle2 as part of a larger expression cassette. The expression cassette is 1,136 bp in size therefore; promising clones containing the mIL-7 insert will display a 1,636 bp band while clones containing the mKGF insert will display a 1,739 bp band. In Figures 10 and 11 bands for the Adeno vector (31 Kb) and the mIL-7 and mKGF inserts within the expression cassette (1,636 and 1,739 respectively) are visualized. At this point mIL-7 and mKGF were successfully cloned into the adeno genome backbone, as confirmed with restriction enzyme analysis.
Figure 10. Restriction enzyme analysis of mIL-7-Adeno. DNA from a selected mIL-7-Adeno clone was done with enzymes P1-Sce I and I-Ceu I. Restriction digestion products were run on a 1% agarose gel. Clone mIL-7-Adeno 1-1 contained the mIL-7 insert plus the expression cassette as seen with the 1,636 bp band in lane 1. The partially digested adeno vector (31 Kb) is seen in lane 1 containing the insert. Gel lanes contained: 1 Kb ladder (lane 2) and digested mIL-7-Adeno clone 1-1 (lane 1).
Figure 11. Restriction enzyme analysis of mKGF-Adeno. DNA from a selected mKGF-Adeno clone was done with enzymes PI-Sce I and I-Ceu I. Restriction digestion products were run on a 1% agarose gel. Clone mKGF-Adeno 6.2-58 contained the mKGF insert plus the expression cassette as seen with the 1,739 bp band in lane 2. The partially digested adeno vector (31 Kb) is seen in lane 2. Gel lanes contained: 1 Kb ladder (lane 1) and digested mKGF-Adeno clone 6.2-58 (lane 2).
3.2. Expression of mIL-7 and mKGF in transiently transfected COS cells

To examine the relative IL-7 and KGF RNA expression of mIL-7-pShuttle2 and mKGF-pShuttle2 constructs, quantitative RT-PCR of transiently transfected COS cells was performed. COS cells were seeded into 6-well plates (0.8 x 10^6/well) and allowed to incubate overnight at 37°C, 5% CO₂. The next day, DNA (3 μg) from mIL-7- pShuttle2, mKGF- pShuttle2 and empty pShuttle2 vector were mixed with 20 μL of Qiagen’s PolyFect reagent and allowed to incubate. The COS cells were then transiently transfected with the PolyFect reactions. Twenty-four and forty-eight hours later, COS cells (and their supernatants) were harvested and RNA was extracted with Trizol as described in the methods (Figure 12). qRT-PCR was performed with gene-specific primers for mIL-7 and mKGF and 500 ng of RNA (Figure 13A). Empty pShuttle2 vector was used as a negative control (providing the baseline C_t value) and the housekeeping gene GAPDH was used as the normalizing reference gene. qRT-PCR software reports the threshold cycle (C_t). The threshold cycle is the cycle number at which a sample crosses the no template control threshold cycle, known as the baseline. The C_t values seen in the y-axis of the PCR amplification graph in Figure 13A are PCR Base Line Subtracted”. C_t values were then used to calculate relative mRNA expression of mIL-7 and mKGF using the comparative C_T method (ΔΔC_T), as described in the methods (48). Relative mRNA expression of mIL-7 and mKGF was then plotted on a log scale with empty pShuttle vector providing the baseline C_t value, 1.0 (Figures 13B and 13C).
Figure 12. Strategy to confirm expression of mIL-7-pShuttle2 and mKGF-pShuttle2 transcripts in transiently transfected COS cells.
Figure 13. Relative expression of mIL-7 and mKGF mRNA in transiently transfected COS cells. (A) qRT-PCR of RNA from transiently transfected COS cells with mIL-7-pShuttle2 and mKGF-pShuttle2. (B) Relative mIL-7 mRNA expression by COS cells transiently transfected with mIL-7-pShuttle2 and mKGF-pShuttle2 after 24 and 48 hours. (C) Relative mKGF mRNA expression by COS cells transiently transfected with mIL-7-pShuttle2 and mKGF-pShuttle2 after 24 and 48 hours. Relative expression in (B) and (C) were normalized using ΔΔCt and setting the pShuttle2 vector control to 1.0. Note the log10 scale.
**Figure 13B** shows the relative mRNA expression of mil7. As expected, relative mRNA expression of the mil-7 clones was higher than that for the mKGF clones since this graph included the data from wells amplifying for mil-7 (mil-7 primers) only. Similarly, relative mRNA expression of the mKGF clones was higher than that for the mil-7 clones in **Figure 13C** since this graph included the data from wells amplifying for mKGF (mKGF primers) only. Overall, the 48 hour time point resulted in higher mRNA expression with IL-7 clone 2 having the highest mRNA expression, at about 10 x 10⁶ above baseline while for mKGF; it was KGF clone 2 that had the highest mRNA expression at 10 x 10⁷ above baseline. Taken together, the qRT-PCR data confirms the function of our cloned genes as they express high levels of mRNA.

### 3.3. Packaging and generation of adenovirus in HEK293 cells

To validate and verify the quality of our procedures for generating recombinant adenoviruses, rAdenovirus-LacZ virions (generously provided by Dr. Hartman in the Amalfitano laboratory) and a pShuttle-LacZ construct (generated by Dr. Hick in the Sempowski laboratory) were used as controls for *in vitro* and *in vivo* studies. The adenoviral vector used to create the mil-7, mKGF and control Lac Z recombinant adenoviruses was derived from an adenovirus type 5 (Ad5). Ad5 is a replication deficient adenovirus. The replication deficiency is due to genome deletions of large portions of the Early Regions 1 (E1) and 3 (E3). Numerous packaging cell lines provide replication deficient adenoviruses
with the necessary genes to allow for replication. HEK293 cells express the Ad5 E1 and E3 genes required for complete adenovirus propagation. The HEK293 packaging cell line was used for packaging and generation of our recombinant adenoviruses.

**Figure 14A** outlines the procedure for recombinant adenovirus packaging into HEK293 cells. To linearize the genome and expose the inverted terminal repeats containing the origins of adenovirus DNA replication, recombinant adenoviruses mIL-7, mKGF and Lac Z (gene represented in green) were digested with the restriction enzyme Pac I. The digested recombinant adenovirus genomes were then chemically transfected into HEK293 cells as described in the methods. Successful transfection of recombinant adenovirus genomes into HEK293 cells is seen when HEK293 cells round and detach from the bottom of a culture flask after a few days of culture, representative uninfected and infected HEK293 cells are shown in **Figures 14B and C**, respectively. This rounding and detaching is known as cytopathic effect (CPE) and is due to the generation of infectious adenovirus virions. As described in the methods, when approximately 70% of the cells detached from the bottom of the flask, the cells and supernatant were collected and processed with commercial kit to obtain low-titer, purified viral stocks (1-2 mL at 1-2 x 10^6 pfu/mL).

Based on published doses for i.v. delivery of adenovirus **in vivo**, we anticipated needing large quantities of high-titer virus. Commercially available kits had stringent limits on the amount
of virus that could be purified at one time because most of them were filter-based. As described in the methods, high titer viral stocks were generated by using cesium chloride gradient to purify large-scale cultures. The generation of large-scale cultures, the procedure for cesium chloride purification and the end-point titer assay are described in detail in section 2.3 of the methods. Figure 15 shows the final steps of a large-scale CsCl purification: the viral bands obtained after the 16 hour, 63,000 rpm centrifuge step, the cassette used to dialyze the purified virus and a sample end-point titer dilution assay plate setup. The final viral titers obtained in this study are summarized in Table 1.
Figure 14. Strategy for recombinant adenovirus production. (A) Recombinant Lac A-Adeno, mIL-7-Adeno and mKGF-Adeno (inserted gene shown in green) were digested with Pac I to exposes the inverted terminal repeats (ITR) containing the origins of Adeno DNA replication. mIL-7-Adeno and mKGF-Adeno were transfected into HEK293 packaging cells using Qiagen Poly-Fect reagent. (B) HEK293 cells. (C) HEK293 with CPE.
Figure 15. Strategy for cesium chloride gradient virus purification and end-point titer assay.
Table 1. Recombinant adenovirus titers and batch yields.

<table>
<thead>
<tr>
<th>Batch #</th>
<th>mIL-7-Adeno</th>
<th>mKGF-Adeno</th>
<th>Lac Z-Adeno</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3 x 10^{10} pfu/mL</td>
<td>4.0 x 10^{8} pfu/mL</td>
<td>6.3 x 10^{13} pfu/mL</td>
</tr>
<tr>
<td></td>
<td>0.5 mL</td>
<td>0.8 mL</td>
<td>4 mL</td>
</tr>
<tr>
<td>2</td>
<td>1.3 x 10^{11} pfu/mL</td>
<td>6.3 x 10^{10} pfu/mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 mL</td>
<td>0.7 mL</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.0 x 10^{11} pfu/mL</td>
<td>6.3 x 10^{13} pfu/mL</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 mL</td>
<td>4 mL</td>
<td></td>
</tr>
</tbody>
</table>
3.4. In vitro validation studies of rAdenovirus Lac Z

To examine for functional expression of the inserted Lac Z gene in the recombinant Lac Z adenovirus, and to test our viral propagation protocols, an ELISA-based assay for beta-galactosidase production in infected HEK293 cells was performed. Figure 16A shows increasing cell to virus ratios and the average optical density (415nm) of each sample as an indicator of beta-galactosidase activity. The highest beta-galactosidase production was seen with a cell to virus ratio of 1:30. A decrease in beta-galactosidase production was seen with a 1:40 ratio, probably due to excessive CPE of the high viral titer on the cells. The Lac Z-rAdeno caused CPE in the HEK293 cell cultures, as seen with the reduced Lac Z production in the 1:40 cell:virus ratio, and produced high levels of beta-gal in the 1:30 cell:virus ratio. In vitro beta-galactosidase activity confirmed our technique for generating a functional recombinant adenovirus. We next decided to validate our rAdenovirus Lac Z with in vivo studies.

3.5. In vivo validation studies of rAdenovirus Lac Z

To confirm our technique, not only for generating a functional recombinant adenovirus, but also for delivering it in an in vivo setting, we conducted in vivo studies with our rAdenovirus Lac Z. BALB/c mice were injected retro-orbitally (RO) with Lac Z-rAdeno, saline was injected as a control. Three days later the mice were euthanized. Thymus, spleen and liver were removed, homogenized in PCR-grade water and tested for beta-galactosidase activity.
production with the ELISA-based assay used in the \textit{in vitro} validation studies. As shown in \textbf{Figure 16B}, beta-galactosidase production was highest in the liver, with the OD reading above the linear detection range of the assay (3.0). In the spleen (\textbf{Figure 16C}), beta-galactosidase activity was low, at 0.54 and 0.058 OD for Adeno prep 1 and Adeno prep 2, respectively. Unfortunately, little beta-galactosidase activity was also seen in the thymus (\textbf{Figure 16D}) (0.42 and 0.047 OD for Adeno prep 1 and Adeno prep 2 respectively), which was our target organ for delivering the recombinant adenoviruses expressing thymo-stimulatory cytokines, mIL-7 and mKGF.

\textbf{3.6. Refinement of intrathymic injection in mice}

Based on the lack of expression of Lac Z in the thymus, and studies with a recombinant adenovirus expressing IL-10 (43), we speculated that we would need to explore the possibility of delivering our recombinant adenoviruses, containing thymo-stimulatory cytokines, \textbf{directly} into the thymus. To do this we needed to develop an intrathymic, survival surgery technique such as work by de la Cueva et al. (32).

Our experiments with the cell tracking dye (CellTracker Orange) to evaluate intrathymic injection technique are outlined in \textbf{Figure 17}. Fresh thymocytes were stained with the CellTracker dye according to manufacturer’s protocol and mice were prepared for surgery as described in the methods. Mice were injected intrathymically with $10 \times 10^6$ total stained
thymocytes (in 100 μL of saline, 50 μL per lobe) or saline only as control. Mice survived and were ambulatory 15 minutes post-surgery. Ninety minutes post-surgery the mice were euthanized, thymus removed and processed for thymocytes as described in the methods.
Figure 16. Confirmation of Beta-galactosidase production by Lac Z-Adeno. (A) In vitro production of beta-galactosidase by Lac Z-Adeno was measured in HEK293 cell lysates and detected by ELISA. (B-D) In vivo production of beta-galactosidase was measured in tissue lysates from mice injected retro-orbitally (RO) with Lac Z-Adeno at concentrations of $0.7 \times 10^{11}$ and $1.5 \times 10^{11}$ pfu as described in Materials and Methods.
Experiments using the cell tracking dye were analyzed by flow cytometry (Figures 18A-D). Thymocytes were analyzed with flow cytometry. Shown are control un-labeled thymocytes (Figure 18A) and dye-labeled thymocytes (Figure 18B). Thymocytes from a saline-injected mouse (Figures 18C) and thymocytes from a labeled-thymocyte injected mouse (Figure 18D). The mice injected intrathymically with $10 \times 10^6$ dye-stained thymocytes (~10% of total thymus cellularity), revealed that approximately 8% of the recovered thymocytes were dye-labeled thymocytes injected into the thymus of the mouse could be recovered (Figure 18D). These results were consistent with those reported by de la Cueva et al. (32) and suggested our technique would work with adenovirus delivery.
Figure 17. Strategy for refinement and validation of live animal intrathymic injection.
Figure 18. Flow cytometric confirmation of intrathymic injection protocol. (A) Unlabeled thymocytes, negative control. (B) Dye-labeled thymocytes, positive control. (C) Thymocytes from mouse injected intrathymically with saline. (D) Thymocytes from mouse injected intrathymically with dye-labeled thymocytes. Percent dye-labeled isolated thymocytes (CellTracker) is indicated in the red gate.
3.7. Intravenous delivery of mIL-7 and mKGF in the setting of LPS-induced acute thymic atrophy

From previous endotoxin-induced thymic involution studies in our lab, we have shown that a single administration of *E. coli* LPS (100 μg i.p.) induces acute thymic atrophy within 3-5 days and an initiatory recovery 7 days post administration (Figure 19, courtesy of Hick et al.) (35).

To test our hypothesis mIL-7 and mKGF delivered via recombinant adenovirus improve thymopoiesis and T cell reconstitution in mice; we performed in vivo experiments utilizing the LPS acute thymic atrophy model and two forms of adenovirus delivery, intravenous and intrathymic. It is important to note that we used a lower dose (1.0 x 10^{10} pfu) for the intravenous and intrathymic experiments than what we used for the in vivo validation studies with rAdenovirus Lac Z. Oberholzer et al. reported that the goal of adenovirus studies is to achieve therapeutic levels with minimal adenovirus appearance in circulation and suggested 1 x 10^{10} pfu (43).

With functional vectors that 1) when transiently transfected into COS cells induce mRNA expression by qRT-PCR, 2) could be sub-cloned into Ad5 and cause CPE and 3) are functional in an in vivo setting as seen by beta-gal activity in the tissues of mice injected with
Figure 19. Endotoxin-induced thymic involution. A single administration of *E. coli* LPS induced acute thymic atrophy with subsequent recovery. Groups of female BALB/c mice were treated with saline or LPS (100 μg i.p.) on day 0, and three mice per group were euthanized on days 1, 3, 7, 11, 15, 21, and 28. Thymus weights analyzed as a measure of thymopoiesis and shown as mean ± SEM (n = 3 per group). Courtesy of Dr. Ryan Hick, Sempowski Lab (35).
Lac Z-rAdeno, we felt confident in moving forward with generating high titer viral stocks of rAdenovirus expressing Lac Z, IL-7 and KGF.

To investigate if delivery of mIL-7 and mKGF cytokines would improve thymopoiesis in a damaged thymus via the intravenous route (experimental plan outlined in Figure 20), BALB/c mice (n = 3) were injected retro-orbitally (i.v.) with 10^10 pfu of mIL7-rAdeno, mKGF-rAdeno, Lac Z-rAdeno, or saline as control on day 0. On day one the mice were given an i.p. injection of *E. coli* LPS (100 μg) or saline. On day three the mice were euthanized; thymus, spleen and liver were collected. The thymus and spleen were weighed, processed for thymocytes and splenocytes, respectively, and cells counted. Classic measures of thymopoiesis included: thymus weight, thymus cellularity, mTREC analysis and lymphocyte phenotype.

In Figures 21A and 21B, thymus weight and cellularity were reduced in LPS-treated when compared to saline-treated mice. Thymus weight and cellularity of LPS-treated mice that received mKGF was slightly higher than Lac Z control (p = 0.1672), but significantly higher than saline-treated mice (p = 0.0014). Thymus weight of saline-treated mice that received mKGF was significantly higher than mice that received saline (p = 0.022). CD4+/CD8+ double positive T cells are a marker of thymopoiesis. Flow cytometry allowed for the analysis of this T cell subset. As expected, percent and absolute number of CD4+/CD8+
double positive thymocytes (Figures 21C and 21D) were significantly decreased in LPS-treated mice. There was no significant improvement with mIL-7 or mKGF treatment in LPS-treated mice. In saline-treated mice, there were significant increases in the percent of CD4+/CD8+ double positive thymocytes in mice that received mIL-7 ($p = 0.0006$) and mKGF ($p = 0.0067$) when compared to Lac Z control mice. This is promising because it suggested that mIL-7 and mKGF improved thymopoiesis in a thymus without damage therefore mIL-7 and mKGF might improve thymopoiesis LPS-treated mice if the harvest time point was extended.
Figure 20. Schematic of experimental design to test intravenous method of adenovirus delivery in endotoxin-induced thymic involution model.
Figure 21. Measures of thymopoiesis, intravenous model. On day 0, BALB/c mice were injected retro-orbitally (i.v.) with $10^{10}$ virus particles of mIL-7-rAdeno, mKGF-rAdeno, Lac Z-rAdeno or saline as control. On day 1 mice were given an i.p. injection of *E. coli* LPS (100 μg) or saline. On day 3 mice were euthanized and thymus removed, weighed, processed thymus DNA and for thymocytes. Thymus weight (A), total number of thymocytes (B), % and absolute number of CD4+/CD8+ double positive thymocytes (C) (D) and TREC per μg of DNA (E) and per mg of thymus (F) are shown as mean ± SEM (n = 3 per group). ND = Not Determined, * = ($p = 0.05$ vs. Lac Z) and † = ($p = 0.05$ vs. Saline).
T cell receptor rearrangement is a measure of thymopoiesis. mTREC analysis of whole thymus genomic DNA is used to quantify thymopoiesis at the T cell receptor gene rearrangement level. **Figures 21E and 21F**, show the results of TREC/μg DNA and TREC/mg of whole thymus, respectively. At three days thymopoiesis was not significantly diminished in LPS-treated mice. Interestingly, there was a decrease in both TREC/μg DNA ($p = 0.274$) and TREC/mg thymus ($p = 0.695$) in LPS-treated mice that received Lac Z-Adeno when compared to LPS-treated mice that received saline control. Although not significant, these data suggested that adenovirus quickly causes a slight decrease in thymopoiesis and three days is not sufficient time for mIL-7 and m-KGF adenoviruses to establish their thymo-stimulatory effects when delivered i.v., which might explain why no benefit is seen in LPS-treated mice that received mIL-7 nor mKGF. There is a slight increase in TREC/μg of DNA of LPS-treated mice that received mIL-7 versus mice that received Lac Z, but it was not significant ($p = 0.771$).

We next asked if we could detect IL-7 or KGF mRNA expression in tissue from our *in vivo* intravenous experiment. Total RNA was isolated from thymus, spleen and liver as described in the methods. Relative IL-7 mRNA expression in the thymus (**Figure 22A**), spleen (**Figure 22C**) and liver (**Figure 22E**) as well as KGF mRNA expression in the thymus (**Figure 22B**), spleen (**Figure 22D**) and liver (**Figure 22F**) were determined by quantitative reverse-transcriptase PCR and the $\Delta\Delta C_t$ method as described in the methods.
At 4 days post adenovirus infection (3 days post LPS challenge), minimal IL-7 mRNA was detected over saline control in the liver and spleen (Figure 22C and 22E). No IL-7 mRNA was detected over saline control in the thymus (Figure 22A). In the setting of LPS-damage, IL-7 mRNA appeared to be induced by KGF treatment in the thymus (Figure 22A). These results suggested that perhaps over expression of KGF in the thymus was inducing IL-7 gene expression.

In LPS-treated mice, KGF expression in the thymus (of mice that received KGF) was significantly increased over saline control (Figure 22B). In the spleen of LPS-treated mice (that received KGF) KGF expression was slightly increased over saline control (Figure 22D). In saline-treated mice, KGF expression in the spleen was significantly increased over saline control (Figure 22D). In saline and LPS-treated mice (that received KGF) there was a tremendous induction of KGF in the liver (Figure 22F). This is to be expected because of the route of adenovirus delivery, intravenous.
Figure 22. Relative IL-7 and KGF mRNA expression in the thymus, spleen and liver, intravenous model. Thymus (A, B), spleen (C, D) and liver (E, F) from the experimental mice described in Figure 21 were processed for total RNA. Relative IL-7 mRNA (A, C and E) and KGF mRNA (B, D and F) expression were determined by quantitative reverse-transcriptase PCR and the ΔΔC\textsubscript{T} method as described in Methods. Saline/Saline samples were used to calibrate to 1.0. Note different scales throughout. ND = Not Determined, * = (p = 0.05 vs. Lac Z) and † = (p = 0.05 vs. Saline).
In the periphery, naïve and memory splenocyte subsets (Figure 23) were analyzed by flow cytometry as an indicator of thymic export and peripheral T cell reconstitution. There were no significant changes in either the CD4 or CD8 naïve or memory T cell compartments were detected.

Intravenous delivery of rAdenovirus had mild to no modulation on thymopoiesis in the LPS model at day three. The effect of LPS damage persists up to seven days; this fact allows us to speculate that perhaps more time is needed post-LPS challenge to allow cytokines IL-7 and KGF to have an impact on thymopoiesis. Therefore, for our next in vivo experiment, we chose to extend the harvest time point from three to seven days. Additionally, we decided on an alternative strategy to deliver the rAdenovirus directly into the thymus: intrathymic injection.
Figure 23. Naïve and memory splenocyte subsets, intravenous model. From the experimental mice described in Figure 21, spleen was removed and processed for splenocytes. Immunophenotyping was used to quantify naïve (A and C) and memory (B and D) splenocyte subsets.
3.8. Intrathymic delivery of mIL-7 and mKGF in the setting of LPS-induced acute thymic atrophy

To investigate if delivery of mIL-7 and mKGF cytokines would stimulate thymopoiesis in a damaged thymus via the direct intrathymic route, (experimental plan outlined in Figure 24), BALB/c mice (n = 5) were injected intrathymically (i.t.) with $10^{10}$ pfu of mIL7-rAdeno, mKGF-rAdeno, Lac Z-rAdeno, or saline as control on day 0. On day one the mice were given an i.p. injection of *E. coli* LPS (100 μg) or saline. On day seven the mice were euthanized; thymus, spleen and liver were collected. The additional time post LPS challenge allowed for thymus damage. Serum was collected six hours post adenovirus infection on day 0 of the experiment, six hours post LPS challenge on day one and on the day of harvest, day seven and analyzed for cytokine quantification with a bead-based ELISA as described in the methods.

The thymus and spleen were weighed, processed for thymocytes and splenocytes, respectively, and cells counted. In Figures 25A and 25B, the overall thymus weight and cellularity of LPS-treated mice was reduced when compared to saline-treated or Lac Z control mice. Thymus weight of LPS-treated mice that received mIL-7 was higher than Lac Z control ($p = 0.094$) and significantly higher than saline-treated mice ($p = 0.006$) (Figure 25A). Thymus cellularity of LPS treated mice that received mIL-7 was higher than mice that
received Lac Z control or saline, (Figure 25B) suggesting improved thymus function. In saline-treated mice thymus weight of mice that received mIL-7 or mKGF was significantly higher than mice that received saline. In Figures 25C and 25D, both the percent and absolute number of CD4+/CD8+ double positive thymocytes were decreased in LPS-treated mice, as expected. There was an increase in the absolute number of double positive thymocytes of LPS-treated mice that received mIL-7 versus Lac Z control.
Figure 24. Schematic of experimental design to test intrathymic method of adenovirus delivery in endotoxin-induced thymic involution model.
Figure 25. Measures of thymopoiesis, intrathymic model. On day 0, BALB/c mice were injected intrathymically (i.t.) with $10^{10}$ virus particles of mIL-7-rAdeno, mKGF-rAdeno, Lac Z-rAdeno or saline as control. On day 1 mice were given an i.p. injection of E. coli LPS (100 μg) or saline. On day 7 mice were euthanized and thymus removed, weighed, processed thymus DNA and for thymocytes. Thymus weight (A), total number of thymocytes (B), % and absolute number of CD4+/CD8+ double positive thymocytes (C) (D) and TREC per μg of DNA (E) and per mg of thymus (F) are shown as mean ± SEM (n = 5 per group). ND = Not Determined, * = (p = 0.05 vs. Lac Z) and † = (p = 0.05 vs. Saline).
mTREC analysis of genomic DNA (Figures 25E and 25F) revealed that LPS-treated mice that received Lac Z had significantly less TREC/μg of DNA than mice that received saline ($p = 0.03$) suggesting that recombinant adenovirus reduces mTREC levels. Conversely, LPS-treated mice that received mKGF had significantly higher TREC/μg of DNA than mice that received Lac Z control ($p = 0.026$) suggesting that KGF administration had a thymostimulatory effect in the setting of LPS-damage. LPS-treated mice that received mIL-7 had slightly higher TREC/μg of DNA than Lac Z control mice.

In the periphery, naïve and memory splenocyte subsets (Figure 26) were analyzed by flow cytometry. In the context of acute LPS thymus damage (0-7 days) there was no significant impact on the percent absolute number of the T cell subset compartments. There was however, a significant increase in the number of naïve CD4+ and CD8+ splenocytes in mice that received mKGF when compared to mice that received Lac Z, in the absence of LPS. These data thus suggested that KGF increases thymopoiesis in the thymus which is then stimulates export of naïve T cells into the periphery.
Figure 26. Naïve and memory splenocyte subsets, intrathymic model. From the experimental mice described in Figure 25, spleen was removed and processed for splenocytes. Immunophenotyping was used to quantify absolute number of naïve CD4 (A), memory CD4 (B), naïve CD8 (C), and memory CD8 (D) splenocyte subsets.
4. DISCUSSION

This study demonstrated that mIL-7 and mKGF, delivered via recombinant adenovirus (intravenously) had mild to no modulation of thymopoiesis in mice when tissues were collected at day three. The level of thymopoietic indicators such as thymus weight, cellularity and mTREC numbers were, however, slightly increased when mice received the rAdenovirus intrathymically and their tissues collected seven days after LPS challenge.

We hypothesize that mIL-7 and mKGF/FGF-7 delivered via recombinant adenovirus can improve thymopoiesis and T cell reconstitution in mice using LPS, a model of acute thymic atrophy.

The approach we took to test our hypothesis utilized methods familiar to our lab, such as the LPS model for acute thymic atrophy, and new methods previously employed by others, but new to our lab, (eg. adenovirus delivery system). Amalfitano et al. described some advantages of recombinant adenovirus delivery system such as: ability to infect dividing and non-dividing cells with high efficiency and to be able to carry genes up to 8.3 Kb in size (42). Because recombinant adenovirus technology was new in our lab, we confirmed our process and product at every step. We made recombinant adenoviruses that expressed mIL-7 and mKGF. We also used a recombinant adenovirus Lac Z which served as a viral vector.
control. Extensive confirmation of the functionality of our recombinant adenovirus constructs included qRT-PCR to confirm mRNA expression of mIL-7 and mKGF by our constructs. Results of the qRT-PCR revealed that one or our mIL-7 constructs had an expression of $10 \times 10^6$ above baseline (empty pShuttle2 vector) while for mKGF one clone had an expression of $10 \times 10^7$ above baseline. To confirm the functionality of our construct and our delivery technique, we used our Lac Z control construct in \textit{in vitro} and \textit{in vivo} quality control experiments. Beta-galactosidase activity was seen in the thymus, spleen and liver of mice injected with rAdeno-Lac Z confirming the functionality of our construct and confirming our delivery technique. As expected, the highest levels of beta-galactosidase activity were detected in the liver with much lower levels seen in the spleen and thymus.

Although our original plan was to inject the rAdenoviruses retro-orbitally (i.v.), low expression in the thymus prompted us to develop/refine our technique of intrathyemic injection so we could include this method of direct delivery into the thymus in our studies. What we needed was to develop an intrathyemic, survival surgery technique with a high hit rate on the thymus. To further develop and monitor our technique for intrathyemic injections, we used a cell-staining dye, CellTracker Orange. Mice were injected intrathymerically with $10^{10}$ dye-labeled thymocytes (CellTracker) or saline. Ninety minutes later the thymus was removed and analyzed by flow to reveal that approximately 10% of the dye-labeled cells could be recovered, which is consistent with the literature (32).
There are some general implications to consider when using recombinant adenovirus delivered intrathymically as a therapy. These implications are discussed below and include: 1) adenovirus infection, 2) possible damage to the thymus during intrathymic injections, 3) maximum volume of treatment the thymus lobes can accommodate and 4) duration of adenovirus-induced gene expression *in vivo*.

In our studies of attenuation of thymic function, we use the model of LPS-induced acute thymic atrophy. LPS is an endotoxin that induces the production of pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 and corticosteroids such as cortisol. These cellular mediators stimulate an inflammatory immune response that results in vasodilation, capillary permeability and low blood pressure. It is this acute pro-inflammatory cascade that causes tissue injury, sepsis and results in acute thymic atrophy and subsequent attenuation of thymic function (35).

The immune system recognizes virally infected cells and eliminates them. It is the activation of both the innate and adaptive immune systems, due to the toxicity associated with adenovirus administration *in vivo*, that is responsible for the elimination of the adenovirus infected cells (49). The problem with the elimination of the virally infected cells is that it results in only a transient expression of the genes encoded within the recombinant adenovirus. The transient expression of IL-7 and KGF might explain why no benefit is seen
in LPS-treated mice that received mIL-7 or mKGF and were harvested on day three.

Amalfitano et al. described an Ad vector with deletions that block viral DNA replication and decrease viral protein production, thereby allowing for longer periods of gene expression by the recombinant adenovirus, due to a weaker immune response mounted by the host. Normally adenovirus vectors used for gene delivery and expression in vivo are already replication deficient, so then, what is different about the adenovirus suggested by Amalfitano et al.? The difference lies in not only employing the usual deletions found in adenovirus vectors, such as deletion of the E1 and E3 early regions of viral replication, but to add on a gene that suppresses the constitutively expressed adenovirus DNA polymerase protein, a virally encoded protein essential for Ad genome replication. H5ts36, is an adenovirus with a temperature-sensitive mutation for polymerase protein production. H5ts36, with its mutation for polymerase protein production, allows for longer-lived gene expression and is a vector that we are considering for future studies (42).

The second issue to consider with recombinant adenovirus therapy is possible damage to the thymus during intrathymic injections. In the current literature there are no studies on the damages that can occur to the thymus during intrathymic injection. There was one study that used intrathymic injections and examined the condition of the thymus upon removal; however, it was only for the purpose of eliminating damaged thymuses from their study (50). Studies by de la Cueva et al. outline an intrathymic injection technique that is simple,
minimally invasive, reduces distress, and surgical complications, and maintains the
efficiency of previous approaches. The technique described by de la Cueva et al. is the one
we used for our intrathymic injections. (32). We feel that the damage caused by the
intrathymic injection procedure is controlled for by the saline and/or the Lac Z-Adeno.

The third issue to consider in our model of intrathymic injections is the maximum volume of
treatment the thymus lobes can accommodate. Previous studies have injected volumes of 0.5
to 50 μL into each thymus lobe (32, 50, 51). Unfortunately, previous studies employing
intrathymic injections have not examined the condition of the thymus upon harvest. In our
studies we injected 50 μL per thymus lobe. In future studies we will assess thymus histology
with various volumes to define the maximum volume possible.

The fourth and final point to consider in our model is that our recombinant adenovirus is
replication deficient. When examining our experimental data, we found that the beneficial
effects of mIL-7 and mKGF on thymopoiesis (in the setting of LPS-induced damage) were
greater in mice that were harvested seven versus three days post adenovirus infection (as
confirmed by thymus weight, thymus cellularity and mTREC analysis). However, gene
expression by recombinant adenovirus IL-7 and KGF was analyzed with qRT-PCR and
confirmed to have high levels of mRNA expression (10 x 10^6 and 10 x 10^7 above baseline for
IL-7-Adeno and KGF-Adeno respectively). By employing other methods, such as adeno-
associated virus (AAV) vectors, gene expression might be further extended and provide additional protection in this model.

Adeno-associated virus (AAV) vectors are an alternative to recombinant adenovirus construct for delivery of genes. AAV vectors provide several advantages over recombinant adenoviruses; first the vector only contains inverted terminal repeats required for replication, packaging, and integration. Since 96% of the viral genome is removed, no wild-type helper virus are generated, therefore, there is no immune response to residual viral gene expression. The deleted viral genome is replaced with a 4.4 Kb coding region that express non-viral genes. AAV, like recombinant adenovirus, infect both dividing and non-dividing cells, it will integrate into the host chromosome with a long-term transduction, up to 1.5 years (52). The use of AAV will be considered in our lab to obtain long-term gene expression.

In addition to all the challenges encountered by the thymus during acute stress-induced thymic atrophy, the peripheral T cell pool and how it is affected, must also be considered and examined. As discussed in the introduction, the peripheral T cell pool is disrupted by acute stress-induced thymic atrophy. In our model of acute stress-induced thymic atrophy, reconstitution of the peripheral T cell pool shifts from thymic output (polyclonal) to peripheral expansion (oligoclonal). The shift from a polyclonal to oligoclonal repertoire results in decreased TCR diversity and antigen specificity. The consequence of decreased
TCR diversity and antigen specificity is limited and restricted T cell immunity.

We examined the state of the peripheral T cell pool during LPS-induced acute thymic atrophy and subsequent thymostimulatory treatment delivered via recombinant adenovirus, by looking at naïve and memory splenocyte subsets with flow cytometry. We did not, however, see any significant conversion of peripheral homeostasis back to a naïve phenotype in either of our studies.

Another alternative approach, which we are currently working on to amplify/complement thymopoiesis in the setting of LPS-induced acute thymic atrophy is the use of short hairpin RNA (shRNA). shRNAs are delivered in a lentiviral vector. Lentiviral vectors are part of the Retroviridae family and can deliver a significant amount of genetic information into the DNA of the host cell resulting in long term knock down of a protein. Our goal with this approach would be to knock down signaling through gp130. gp130 is a shared signaling receptor for the IL-6 cytokine family, which includes leukemia inhibitory factor (LIF) and oncostatin-M (OSM). LIF and OSM are pro-inflammatory cytokines and suppress thymopoiesis. Previous studies by our lab, using a neutralizing polyclonal antibody that blocks the gp130 signaling, have shown that neutralization of the gp130 receptor promotes thymopoiesis (34).
To conclude, the approaches we have explored in our acute thymic atrophy model may not be therapeutic but they may be useful as tools to understand the mechanisms that drive age-induced thymic involution. Alternatively, they may be beneficial in the development and understanding of novel immune stimulants as adjuvants to vaccines for the elderly or individuals with damaged thymuses.
5. REFERENCES


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