3. Materials and Methods

3.1. Mice

Specific pathogen-free female C57BL/6 mice between five to eight weeks of age were obtained from The Jackson Laboratory (Bar Harbor, ME). Additionally, transgenic mice overexpressing Bcl-2 primarily in T lymphocytes (C57BL/6-TgN(Bcl2)25 Wehi; Jackson Laboratory) were also employed. In this latter case, heterozygotic mice were bred to wild-type C57BL/6 mice and offspring carrying *bcl-2* on the SV 40 promoter were identified by PCR analysis. All animals were provided water and rodent food ad libitum. The studies were approved by the Institutional Animal Care and Use Committee at the University of Florida, College of Medicine prior to initiation of these studies.

3.2. Construction of the recombinant adenovirus expressing the E. coli gene β-galactosidase (β-gal), human IL-10 (hIL-10) or green fluorescent protein (gfp)

A derivative of human adenovirus serotype 5 was used as the source of viral DNA backbone (118). The construct was deleted in early region 1, polypeptide IX and early region 3. The adenoviral vector contains a deletion of base pairs 355 to 3325 to eliminate E1a and E1b functions, a deletion of base pairs 3325 to 4021 to eliminate protein IX function and a deletion of base pairs 28592 to 30470 to eliminate E3 functions (119). The Adenovirus was constructed using standard homologous recombination methods as described by Graham and Prevec (120). Expression of transgenes (β-gal, hIL-10 and gfp) was driven using a human cytomegalovirus immediate early enhancer/promoter (CMV) as recently described (97).

3.3. Intrathymic, intravenous and footpad gene therapy

For **intrathymic injections**, animals were anesthetized and placed in a supine position. A 1 - 2 cm midline incision was made from the angle of the mandible to the level of the fourth rib. An upper median sternotomy was created from the incision to the second rib. Using a 50 μl Hamilton syringe and a 30 gauge needle, intrathymic

injections of 20 μl were made into the left lobe by direct visualization. Mice received injections of 10^5 to 10^{10} particles of a recombinant adenovirus construct expressing β-gal (Adv/β-gal), human interleukin-10 (Adv/hIL-10), gfp as a reporter gene (Adv/gfp), or an identical recombinant adenovirus vector (E1a, E1b, E3 deleted) containing an empty cassette (Adv/empty) in buffer (1x PBS, 2 mM $MgCl_2$ and 3% sucrose).

Intravenous injections (100 μl) were applied in the same doses of adenovirus to the tail vein using a tuberculin syringe.

Footpad gene therapy. Following anesthesia, mice were injected into both hind footpads with 50 μl of adenovirus or buffer using an insulin syringe and a 29-gauge needle (Becton Dickinson, NJ). Mice received injections of either 2 x 10^5, 2 x 10^7 or 2 x 10^{10} particles of a recombinant adenovirus construct expressing human IL-10 (Adv/hIL-10), gfp as a reporter gene (Adv/gfp), or an identical recombinant adenovirus containing an empty cassette (Adv/empty) in buffer (phosphate-buffered saline (PBS) supplemented with 2 mM $MgCl_2$ and 3% sucrose).

3.4. Cecal ligation and puncture model for polymicrobial sepsis

Mice underwent a cecal ligation and puncture as described earlier (35, 121). In brief, mice were anaesthetized and a 1-2 cm median laparotomy was performed. After visualization, the cecum was ligated 1cm from the distal end and punctured with a 22 gauge needle. The wound was then closed with sterile skin clips.

Sham treated animals (sham) received anesthesia and a laparotomy, but the cecum was neither ligated nor punctured.

3.5. Detection of gfp expression

Mice were anesthetized and a midline incision was made to expose the thorax. The right atrium was cannulated, and mice were perfused through the left ventricle first with phosphate buffered saline (PBS) and then with 50 ml of 4% paraformaldehyde (40 g/L paraformaldehyde in phosphate buffered saline (1x PBS), pH 7.0) until the animals became rigid. Organs were harvested and suspended in 30% sucrose (in PBS). The tissues were then sectioned at 20 microns and the sections were photographed using a fluorescent microscope (Zeiss Axioskop wide-field flourescent microscope).

3.6. β-Galactosidase activity in the thymus

β-Galactosidase activity was measured using a chemiluminescent reporter gene assay system (Tropix, Bedford, MA). Baseline β-galactosidase activity was determined from thymi of untreated mice, which were analysed simultaneously and were then subtracted from activities measured in treated mice.

3.7. Flow cytometric analysis

Fresh thymi were placed into Hanks balanced salt solution (HBSS) and then resuspended in 100 U/ml collagenase D (Boehringer Mannheim) and dissected. After transfer of tissue fragments into 400 U/ml collagenase D samples were incubated in a 37°C water bath for 30 minutes. The single cell suspension was washed twice in calcium-free HBSS (1% BSA, 0.5mM EDTA, 0.1 NaMg in HBSS) and then centrifuged at 400-500 x g for ten minutes. Residual erythrocytes were lysed in ice cold hypotonic ammonium chloride. After two washes cells were adjusted to $1x10^6$ cells per ml and stained with fluorescent indicators. T-cells were identified using anti-CD3 antibodies and dendritic cells with anti-CD11c antibodies, both conjugated with either (phycoerythrin) PE or (allophycocyanin) APC (PharMingen, San Diego, CA). Samples were analyzed by FACSCalibur® and Lysis II Software (Becton Dickinson Systems, San Jose, CA).

Lymph node*.* The popliteal lymph node was harvested at 24 hours after footpad injection. Lymph nodes were first dissected with 30-gauge needles, filtered through a 70 μm cell strainer (Falcon, Becton-Dickinson) and then resuspended in 400 U/mL collagenase D (Boehringer-Mannheim, Norwich, Ct) and incubated for 30 minutes in a 37^o C water bath (122). After washing twice with buffer (1% bovine serum albumin (BSA), 1mM EDTA (Fisher Scientific, Pittsburgh, Pa) and 0.1% sodium azide (NaN_3: Sigma) in Hanks' balanced salt solution without phenol red, calcium and magnesium (Cellgro, Mediatech, Herndon, Va)) cells were resuspended in 4% BSA flow buffer and blocked with CD16/CD32 Fc blocking buffer followed by staining. DCs were identified using anti-CD11c and anti-CD-8 antibodies (Immunotech-Coulter, Miami, Fl). Maturation of DCs was assessed by the levels of MHC class II and CD86. T-cells were identified with anti-CD3, anti-CD4 or anti-CD-8

antibodies. T-cell activation was assessed by expression of CD69 and CD25. Directly conjugated antibodies were used for FITC, PerCp and PE, whereas APC staining was achieved by an anti-biotinylated antibody labeled with APC (Moleculor Probes, Eugene, Or). Samples were acquired and analyzed on a FACSCalibur® with Lysis II Software (Becton Dickinson Systems, San Jose, Ca).

Bone marrow-derived DCs: Twenty four hours following adenoviral transduction with or without stimulation by LPS or anti-CD40 antibodies, respectively, cells were washed twice with flow buffer (1% bovine serum albumin (BSA), 1mM EDTA (Fisher Scientific, Atlanta, GA) and 0.1% sodium azide (NaN_3: Sigma Chemical Co., St. Louis, MO) in Hanks' balanced salt solution without phenol red, calcium and magnesium (Cellgro, Herndon, VA)), and were resuspended in 4% BSA flow buffer and blocked with CD16/CD32 Fc antibodies (PharMingen, San Diego, CA) followed by staining, as described below. DCs were identified using anti-CD11c and anti-CD8 antibodies, and DC maturation was determined based on the relative levels of CD86 and MHC Class II (Immunotech, Miami, FL) expression. Antibodies were either directly conjugated with FITC, PerCp or PE (PharMingen, San Diego, CA), or indirectly conjugated to an anti-biotinylated antibody labeled with APC (Moleculor Probes, Eugene, OR). Samples were analyzed on a FACSCalibur® instrument with Lysis II® Software (Becton Dickinson Systems, San Jose, CA).

3.8. Cytokine measurements

Mouse: Human and murine IL-10 and murine IL-6 in plasma and homogenized thymus were measured by specific ELISA using commercially available reagents (human IL-10 and IL-6 by Endogen, Woburn, MA, mouse IL-10 by R&D, Minneapolis, MN). The sensitivity of the IL-6 assay was 60 pg/ml plasma and 300 pg/g organ wet wt., and for hIL-10 30 pg/ml for plasma, and 450 pg/g wet wt. for organ homogenates. Bioactive TNF was also measured in the thymus and plasma using the TNF-sensitive WEHI 164 clone 13 murine fibrosarcoma cell line (123). The standard curve was generated with recombinant human TNFα. The assay sensitivity was 13.2 pg/ml plasma and 66 pg/g organ wet wt.

Supernatants: Human IL-10, as well as murine IL-2, IL-4, IL-12p40 and p70, and IFN-γ in cell supernatants were measured by specific ELISA using commercially

available reagents (Endogen, Inc. for hIL-10, and PharMingen Inc., for IL-2, IL-4, IFN-γ, and both IL-12 subunits).

3.9. Caspase-3 activity assay

Protein extracts were prepared by homogenization of tissues, and caspase-3 activity was determined by a fluorogenic assay (Enzyme Systems Products, Livermore, CA). Harvested organs were homogenized in 1 ml of 25 mM HEPES buffer (pH 7.5) containing 5 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 μg/ml leupeptin and aprotinin. After centrifugation at 13,500 rpm for 15 minutes the supernatants were collected. Protein concentrations in the supernatant were assayed using Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA). Forty micrograms of the extracted proteins were incubated with the synthetic fluorescent substrates benzylooxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Z-DEVD-AFC) for the caspase-3 activity assay at concentration of 30mM in 0.1 M HEPES buffer (pH 7.4) containing 2 mM dithiothreitol, 0.1% CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) and 10% sucrose. The kinetics of the proteolytic cleavage of the substrates were monitored in a fluorescence microplatereader using an excitation wave length of 360 nm and an emission wave length of 535 nm. The fluorescence intensity was calibrated with a standard concentration of AFC, and the caspase-3 activity was calculated from the slope of the recorded fluorescence and expressed in relative fluorescence intensity (RFI).

3.10. Histological examination of apoptosis by 3' end labeling (TUNEL)

At sacrifice, the organs from additional mice were fixed in 10% buffered formalin and embedded in paraffin. Five-micron sections were affixed to slides and deparaffinized. In situ terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay was performed using an in situ apoptosis detection kit (Apoptag®, Oncor Corp., Gaithersburg, MD) as described earlier (32).

Hematoxylin and eosin stained tissue sections were also examined by light microscopy.

3.11. Western blot analysis for Bcl-2

After harvesting organs, thymi were placed in lysis buffer (10 mM Tris/HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 170 mg/ml PMSF, 2 mg/ml leupeptin, 2 mg/ml aprotinin) for ten minutes and then homogenized and sonicated. Cellular protein (40 mg) was fractionated by SDS-PAGE, and transferred electrophoretically to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Blots were blocked (0.05% Tween, 5% non-fat dry milk in PBS) and then incubated in the same buffer for two hours with anti-mouse Bcl-2 antibody (Pharmingen). After washing (0.3% Tween in PBS), the membrane was incubated in HRP (horseradish-peroxidase) conjugated anti-hamster IgG (Pharmingen), for one hour. Western blot analysis was conducted according to standard procedures using enhanced chemiluminescence detection.

3.12. Blood bacteremia

Twenty-four hours after induction of generalized peritonitis, mice were euthanized and bled aseptically by cardiac puncture. Heparinized whole blood was log-serially diluted in sterile PBS and 100 μl aliquots cultured overnight at 37° C on Trypticase™ soy agar, 5% sheep blood plates (Becton-Dickinson, Inc). Colonies were counted, and data presented as colonies/ml of whole blood.

3.13. DNA isolation and PCR for hIL-10

DNA was isolated from popliteal and inguinal lymph nodes, spleen and footpad tissue using the Qiagen DNeasy Tissue Kit (Qiagen Inc., Valencia, Ca). PCR was performed with reagents from the GeneAmp PCR Core Kit (Applied Biosystems, Foster City, Ca). For each sample, 1μg of DNA was added to a 50 μl PCR reaction containing 2 mM $MgCl_2$, 1 x PCR buffer II, 0.2 mM dNTPs, 1.25 U AmpliTaq DNA Polymerase, and 50 μmol of specific oligonucleotide primers. The primer sequences of hIL-10 were 5'-CGGCCGCTCGAGTCTAGAC-3' and 5'-GTGGATAGCGGTTTGACTCAC-3'. The PCR temperature profile consisted of a single cycle at 94° C for 2 minutes, 35 cycles with one minute at 94° C (denaturing), one minute at 60° C (annealing), and two

minutes at 72° C (extension), and a final cycle at 72° C for seven minutes. The PCR products were electrophoresed on a 2% agarose gel containing 0.4 μg/ml ethidium bromide.

3.14. Generation of bone marrow-derived DCs

DCs derived from murine bone marrow were generated as previously described (124). Briefly, bone-marrow cells harvested from the femur and tibia of C57BL/6 female mice were depleted of red blood cells by lysis with ammonium chloride. Thereafter, 10^6 cells were cultured on 24-well plates (Costar, Corning, NY) in RPMI 1640 (Cellgro, Herndon, VA) with 10% heat inactivated fetal calf serum, 0.000375% 2-mercaptoethanol (Sigma Chemical Co., St. Louis) and 1% penicillin-streptomycin-neomycin (Gibco, Grand Island, NY), pH 7.2-7.4, supplemented with 500 U/ml of recombinant murine GM-CSF (R&D Systems, Minneapolis, MN) and 1,000 U/ml of recombinant murine IL-4 (PharMingen, San Diego, CA), and incubated in a 5% CO_2 atmosphere at 37°C for a total of four to five days. The medium was replaced on day two with additional recombinant cytokines.

3.15. Adenoviral transduction of bone marrow-derived DCs

Depending on the experiment, bone marrow-derived DCs were incubated on day four or day five with varying quantities of the recombinant adenoviral vectors for two hours. The number of adenovirus particles varied from 10^6 to 10^{10} particles/ml for 10^6 DCs, yielding an approximate particle per cell transduction number ranging from 10 through 10^4. Thereafter, the plate was centrifuged (Beckman GPR centrifuge) at 1,000 rpm for five minutes and the media was completely replaced and supplemented with recombinant GM-CSF and IL-4. For experiments in which maturation of DCs was induced, 1 μg/ml of lipopolysaccharide (LPS; *Escherichia coli* 0111:B4; Sigma Chemical Co., St. Louis, MO) or 5 μg/ml of anti-CD40 antibody (no azide/low endotoxin levels < 0.01ng/μg of protein, PharMingen, San Diego, CA) were added to the DCs on either day four (24 hours prior to adenovirus transduction) or on day five (24 hours after adenovirus transduction).

3.16. Syngeneic mixed lymphocyte reactions

$CD3^+$ lymphocytes from spleens of syngeneic mice were isolated with magnetic beads (StemCells, Vancouver, Canada). Thereafter, 2 x 10^6 $CD3^+$ cells were incubated for four days with 4 x 10^5 DCs, which had been previously transduced with either 10^{10} particles/ml of Adv/empty or Adv/hIL-10, and depending on the experiment, were additionally stimulated with LPS. We used a high ratio of DC to T cells (1:5) to achieve maximal effect of transduced DCs on T-cell activation to maximize T-cell responses. The appearance of Th1 (IL-2, IFN-γ) and Th2 cytokines (IL-4) in the supernatants was determined by immunoassay.

3.17. Measurement of endocytosis

DC uptake of rhodamine-green dextran was evaluated, as previously described (125). Very briefly, DCs were resuspended in flow tubes (Fisher Scientific, Altlanta, GA) at a concentration of 5 x 10^5 cells in 100 μl RPMI containing 25 mM HEPES (Cellgro, Herndon, VA), and preincubated either at 4°C (background) or 37°C for 15 minutes. Thereafter, 100 μg of dextran labeled with rhodomine-green (10,000 D, Molecular Probes, Eugene, OR) per sample was added for four hours. The uptake was stopped, and the remaining rhodamine-green dextran was removed by washing the cells twice with cold flow buffer. Thereafter, the maturation state of the DCs was determined with CD86-PE and Class II-APC as described above. Viability was confirmed by 7-AAD (7-amino-actinomycin D, PharMingen, San Diego, CA). Endotocytosis in living MHC class II and CD86 low, as well as in MHC class II and CD86 high expressing DCs was determined by evaluating the difference in rhoadamine-green dextran uptake at 37°C and 4°C cell incubation.

3.18. Readministration of adenovirus-transduced DCs

DCs were transduced for two hours with adenoviral vectors containing either an empty cassette, or expressing gfp or human IL-10, as described above. Twenty-four hours later, the cells were subjected to density gradient centrifugation (Ficoll-Histopaque, Sigma Chemical Co., St. Louis, MO, d=1.083) to eliminate dead cells. Thereafter, the interfacial cells were washed twice with sterile PBS. In addition, some

DCs transduced with the adenoviral vector expressing gfp were analyzed by flow cytometry to quantitate transduction efficiency. Following anesthesia, 50 μl containing 3.2 x 10^5 DCs transduced with either 10^{10} particles/ml of either Adv/empty or Adv/hIL-10 were injected into the hind footpad of mice. Approximately, 1-8 x 10^4 infectious viral particles were associated with the administration of the transduced DCs. Shortly thereafter (5 minutes), mice were challenged with a cecal ligation and puncture.

3.19. Presentation of data and statistics

Results are presented as the mean and standard error of the mean (mean ± SEM). Differences between two experimental groups were considered significant at $p < 0.05$ as determined by the Mann-Whitney Rank sum test or Student's t-test. For multiple comparisons, a one way ANOVA was used. Post-hoc analyses were performed with Tukey's multiple range test. Differences in the survival study were determined with Kaplan-Meier log-transformed survival analysis.

4. Results

4.1. Adenovirus transfects thymic dendritic cells but not lymphoctes *in vivo*

4.1.1. Summary

Dendritic cells represent a potential target for gene therapy. As initiators of the immune response by antigen processing and presentation to T-cells, dendritic cells play a unique role in both innate and acquired immunity. *In vitro* studies have shown the feasibility of transfection of dendritic cells by adenoviral recombinants. In cancer therapy, targeting of dendritic cells by adenovirus has proven to be effective in inhibiting tumor growth as well as reducing the number of tumor metastases. It was our aim to evaluate the feasibility of *in vivo* transfection of dendritic cells in a lymphocyte rich compartment as a treatment for acute inflammatory diseases. We were able to demonstrate that nearly 50% of the total thymic dendritic cells can be