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

transfected with a first-generation adenoviral construct following intrathymic injection, and that these transfections are associated with only modest inflammation. This study further demonstrates that thymic cells transfected with adenoviral recombinants are able to express an intracellular protein (β-galactosidase, green fluorescent protein), as well as secrete human interleukin 10 (hIL-10) in the local compartment. In addition, forced expression of the latter decreases thymic apoptosis during acute bacterial peritonitis.

4.1.2. Introduction

Dendritic cells are potent antigen-presenting cells. They play a critical role in the initiation of immune responses in diseases such as sepsis, autoimmunity, transplant rejection and anti-tumor response. Due to their important role in processing antigens and presenting them to cytotoxic T-cells as well as T-helper cells (126), dendritic cells represent a potential target to alter both the innate and acquired immune responses. Viral gene therapy directed towards dendritic cells has been used in the development of vaccines against infectious diseases and cancer. Transfer of foreign genes to dendritic cells by adenovirus has been shown to be effective in treating or even preventing cancer in rodent models (127-129). This has been achieved through increases in the cytotoxic T lymphocyte (CTL) response to the transgenes encoded by adenoviral vectors (130, 131). Not only can genes transferred to dendritic cells be presented by the major histocompatibility complex (MHC) class I molecule, but they can also influence the secretion of cytokines by the dendritic cells which may have dramatic effects on the acquired immune response (132, 133).

Dendritic cells in the thymus are primarily present in the medulla and at the cortico-medullary border (134). We initially chose to target the thymus, as it was our aim to target an organ rich in both dendritic cells and lymphocytes. Targeting of the spleen and preventing the vector from escaping into the systemic vasculature has been more difficult, in part, because of the spleen's high perfusion rate and vascularity. The majority of studies conducted to date have transfected dendritic cells *ex vivo*, derived mainly from bone marrow, which were then readministered to the

animals (129, 135). The *in vitro* transfections used multiplicity of infections (MOI) of 100 to 1000 to transduce between 70 to 90% of the dendritic cells (129, 136, 137).
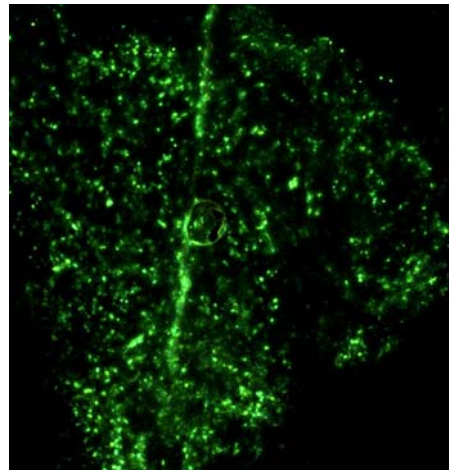
A. Adv/empty   **B. Adv/gfp**

Fig. 7: Histological section of gfp-positive cells. Sections of thymi of mice injected with either Adv/empty (A) or Adv/gfp (B) at particle dose of 10^{10}. Tissues were examined 24 hours postinjection for *gfp* fluorescence *(magnification 100x)*.

4.1.3. Results

Adenovirus transfects thymic cells. Previous studies have shown that adenovirus transfects dendritic cells *in vitro* (129, 136). We therefore analyzed thymic tissue sections of animals sacrificed 24 hours after intrathymic instillation of 10^{10} particles of the adenoviral construct containing either an empty casette (Adv/empty) or expressing gfp (Adv/gfp). We chose this dose based on our earlier studies, which showed efficient *in vivo* transfection in the lung at this particle dose (97). The majority of cells of the thymic sections that were gfp positive appeared to be accessory cells and not necessarily lymphocytes according to their location **(Fig. 7)**. Gfp positive cells were observed throughout the tissue, and interestingly, the highest density of gfp-

positive cells was seen following the injection path of the needle. To further evaluate if dendritic cells were among the adenovirus transfected cells, we performed flow cytometric analyses on dispersed thymi from an additional group of mice injected with the same vectors also into the left lobe of the thymus. Out of the 10^6 cells analyzed from one total thymus, 2.5% of the total number of cells was gfp positive (*data not shown*). 1.76% of the total cells were both gfp and CD11c$^+$ positive (dendritic cells), and 0.8% of the cells were both gfp and CD3$^+$ positive (pan lymphocyte) **(Fig. 8)**. Although only 1.76% of the cells were both gfp and CD11c$^+$ positive, this accounted for 42% of the total CD11c$^+$ cell population. Considering that the total thymus was analyzed and only one lobe received the adenoviral injection, the transfection rate of dendritic cells was relatively high.
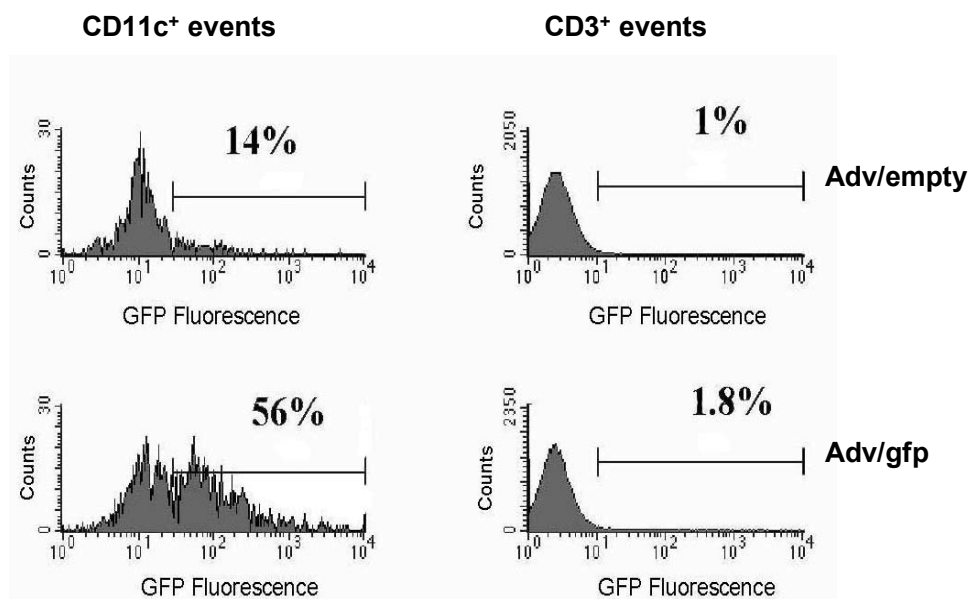

CD11c$^+$ events   **CD3$^+$ events**

Fig. 8: Flow cytometry of thymic cells. 24 hours after intrathymic Adv/empty or Adv/gfp instillation of 10^{10} particles, thymic cells were analyzed for gfp. Cells were stained with CD11c and CD3-antibodies.

Expression of transgenes in the thymus. We further wanted to investigate if other transgenes could be expressed by thymic cells following injection of adenoviral recombinants. Thymi of mice injected with 10^8, 10^9 or 10^{10} particles of Adv/β-gal were

sacrificed 24 hours postinjection and β-gal activity was determined. The activities measured were dose dependent, with expression being the highest in mice treated with 10^{10} particles **(Fig. 9A)**. For the highest particle dose, additional mice were sacrificed on days three and six. As expected β-galactosidase expression declined over the six days being nearly a 3 log lower on day six **(Fig. 9B)**.
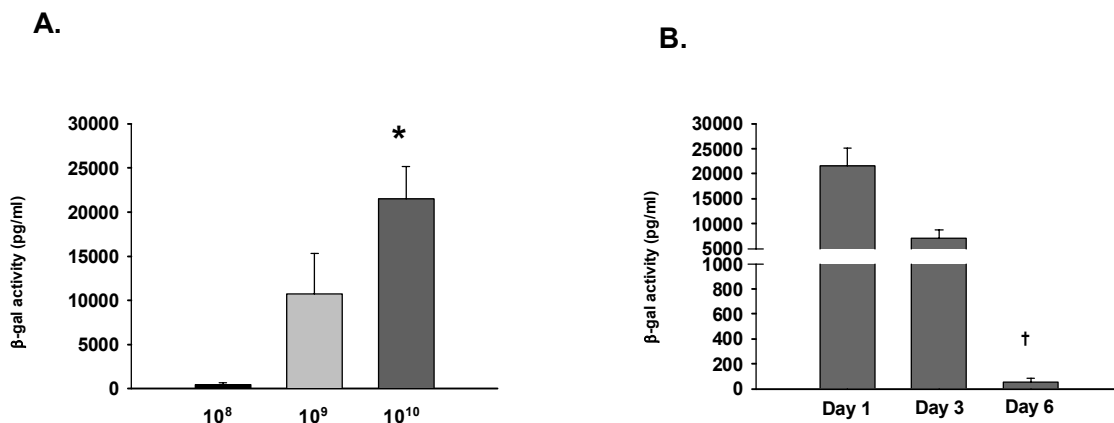
A.

B.

Fig. 9: ß-Galactasidose expression in the thymus. Thymi of mice injected with Adv/ß-gal 24 hours after injection of either 10^8, 10^9 or 10^{10} particles (A) were analyzed for ß-galactasidose expression. Figure B shows expression of ß-galactosidase on days 1, 3 and 6 postinjection of Adv/ß-gal at particle dose of 10^{10}. *$p<0.05$ 10^8 vs. 10^{10}. †$p<0.05$ day 1 vs. day 6.

After having shown that an intracellular protein could be expressed we examined expression of a secretable protein, such as IL-10. One and three days, respectively, after intrathymic instillation of 10^{10} particles of the adenoviral construct expressing hIL-10, mice were terminally bled by cardiac puncture and thymi were harvested. At both time points, hIL-10 levels were higher in the thymi compared to the plasma. Thymic levels were not significantly different at the two time points, whereas plasma levels decreased by a four-fold **(Table 3)**.

Table 3: Human IL-10 levels on days 1 and 3 after intrathymic injection of Adv/hIL-10 10^{10} particles/mouse.

	Thymus (ng/g wet wt.)	**Plasma** (ng/ml)
Day 1	85.64 ± 9.45	29.58 ± 0.71 [†]
Day 3	73.56 ± 38.18	6.79 ± 1.52 *

*$p<0.001$ plasma day 1 vs. day 3. [†]$p< 0.004$ thymus vs. plasma. Mann-Whitney Rank sum Test.

Immune response to adenovirus injections. Intravenous injections of adenoviral vectors have been shown to induce not only local inflammation and apoptosis, but also a systemic immune response, thus, limiting their application *in vivo* (138, 139). It was of interest to determine if our viral vector led to both an early local and a systemic
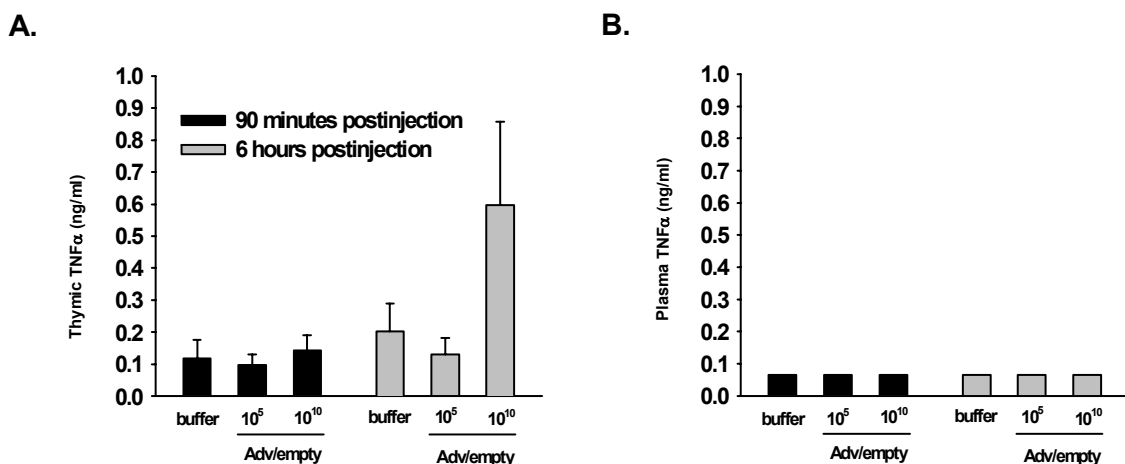
A.

B.

Fig. 10: TNFα levels in plasma and thymus. TNFα was measured at 90 minutes and 6 hours after intrathymic injection of Adv/empty (10^5 or 10^{10}) or buffer in healthy mice. Thymic TNFα levels were increased but not significantly different between the study groups (A). TNFα was below the detectablity level in the plasma (B).

inflammatory response after intrathymic injections of 10^5 or 10^{10} particles of the adenoviral construct with the empty cassette compared to buffer injections. Local and systemic cytokine levels were determined at 90 minutes and 6 hours postinjection as the early inflammatory response to the virus with increases in TNFα and IL-6 take place in the first hours postinjection (97, 140). In addition, caspase-3-like activity was measured at these two time points, as well as at 24 hours, which corresponds to the time point animals were usually sacrificed. Thymic caspase-3-like activity, an indicator of apoptosis, was not present at any of these time points *(data no shown)*. Local TNFα and IL-6 levels in the thymus 90 minutes postinjection were approximately 0.1 ng/g wet wt. for the former **(Fig. 10A)** and 5 ng/g wet wt. for the latter **(Fig. 11A)**. There were no significant difference between buffer and adenovirus treated groups. Similarly, plasma levels for these two cytokines at 90 minutes did not differ between the study groups indicating that the cytokine response seen may have been due to surgical procedure and not to the administration of the virus **(Fig. 10B and Fig. 11B)**. On the other hand, at six hours following intrathymic injection, both thymic TNFα as well as thymic and plasma IL-6 levels were increased, although not statistically different, in mice treated with the adenovirus with the empty cassette at particle dose 10^{10}, but not 10^5 **(Fig. 10 and Fig. 11)**.
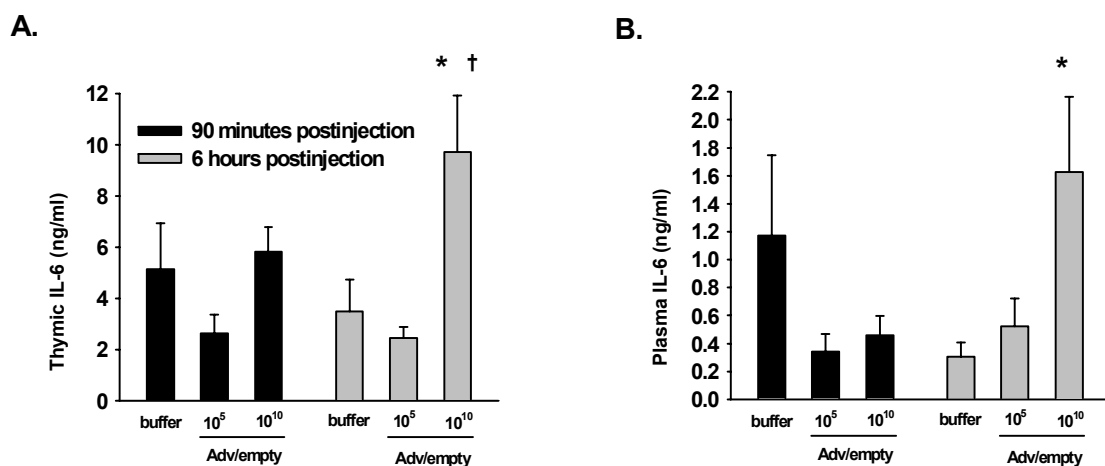

Fig. 11: IL-6 levels in plasma and thymus. IL-6 was measured at 90 minutes and 6 hours after intrathymic injection of Adv/empty (10^5 or 10^{10}) or buffer in healthy mice in the thymus (A) and the plasma (B). *p<0.05 buffer vs. Adv/empty. †p>0.05 10^5 vs. 10^{10}.

Thymic expression of IL-10 decreases thymic apoptosis induced by polymicrobial sepsis. Polymicrobial sepsis induced by cecal ligation and puncture increases apoptosis in lymphoid organs, such as the thymus. Similarly, we saw increases in thymic caspase-3-like activities 24 hours after cecal ligation and puncture **(Table 4)**. To investigate the therapeutic potential of intrathymic expression of hIL-10 with this vector, mice were subjected to cecal ligation and puncture. Mice injected with adenovirus expressing hIL-10 at a particle dose of 10^5 24 hours prior to cecal ligation and puncture had significantly decreased caspase-3-like activity. On the other hand, pretreatment with 10^5 particles of the adenovirus recombinant with the empty cassette also led to decreased caspase-3 activity, but not to the same extent **(Table 4)**.

Table 4: Thymic caspase-3-like activity (RFI) was determined 48 hours after pretreatment with intrathymic injections of 10^5 particles/mouse of either buffer, Adv/empty or Adv/hIL-10, and 24 hours after cecal ligation and puncture (CLP).

intrathymic injection		caspase-3-like acitivity
Buffer	sham	0
Buffer	CLP	1901 ± 212.1 *
Adv/empty	CLP	904.3 ± 181.6 *[†‡]
Adv/hIL-10	CLP	242 ± 130.8 [†]

*$p<0.05$ sham vs. CLP. [†]$p<0.05$ buffer injection and CLP vs. adenovirus and CLP. [‡]$p<0.05$ Adv/hIL-10 vs. Adv/empty. Student-Newman-Keuls test.

4.1.4. Discussion

This study demonstrates that dendritic cells can be readily transduced by recombinant adenoviral vectors administered *in vivo*. Depending on the dose or the

transgene employed, variances in expression as well as duration and localization of the gene product were seen. Although earlier studies have demonstrated transfection of dendritic cells *in vitro*, high MOIs were required (136, 141-143). Our study indicates that in the thymus with a dendritic cell population of 0.1% (144), transfection is possible in nearly half of the dendritic cells, and this is associated with a very modest acute inflammatory response. As we were primarily interested in dendritic cells we did not analyze other cell populations for their frequency of transduction with the adenovirus. Since adenovirus readily transfects epithelial cells we presume that these were also transfected, and their expression contributed to the β-galactosidase and human IL-10 detected in that organ (90).

In vivo treatment with adenoviral vectors is controversial due to the inflammatory response to the virus and production of antibodies, which eliminates procedures with multiple treatment schemes (96, 139, 145). Shortly (90 minutes and six hours) after intrathymic instillation, we could not observe differences in cytokine levels between buffer and both doses of adenovirus. TNFα was not detectable in the plasma at this time point. Although thymic TNFα was modestly increased and IL-6 levels locally and systemically were increased they did not differ significantly between treatments. In addition, caspase-3-like activity was not detectable at any of the time points out to 24 hours, indicating that increased apoptosis was not evident. The similar immune response in all study groups suggests that it might be the host reaction to the surgery (sternotomy) and not the virus instillation. Only the higher particle dose led to an increased cytokine response, reflecting a dose response between the applied virus dose and immune activation. A reason for the minimal immune reaction may be the unique and localized area of application, as previous studies have generally shown greater immune and inflammatory responses when the adenoviral vectors were administered systemically and not only locally (138, 145, 146).

This study proposes that *in vivo* application of recombinant adenovirus represents a feasible approach for targeting dendritic cells when applied locally and thus only leads to a minimal immune response to the vector. The failure to transduce lymphocytes directly eliminated the possibility of direct adenovirus gene transfer of anti-apoptotic proteins such as Bcl-2 to T-cells, and would require a "bystander" effect

to achieve increased expression of anti-apoptotic proteins. A next step towards treatment of patients with acute inflammatory processes will be to further evaluate the mechanism of blocking T-cell apoptosis by gene therapy using adenovirus expressing hIL-10 and to investigate the biological effect of preventing T-cell apoptosis in an acute inflammation model.

4.2.   Targeted adenovirus-induced expression of IL-10 decreases thymic apoptosis and improves survival in murine sepsis

4.2.1. Summary

In line with animal studies, which have suggested that increased lymphocyte apoptosis may contribute to sepsis-induced mortality, we report here that inhibition of thymocyte apoptosis by targeted adenovirus-induced thymic expression of human interleukin-10 reduces blood bacteremia and prevents mortality in sepsis. In contrast, systemic administration of an adenovirus expressing IL-10 is without any protective effect. Improvements in survival are associated with significant increases in Bcl-2 expession, and reductions in caspase-3 activity and thymocyte apoptosis. These studies demonstrate that thymic apoptosis plays a critical role in the pathogenesis of sepsis, and identifies a novel gene therapy approach for its therapeutic intervention.

4.2.2. Introduction

Recent studies in animal models of sepsis have demonstrated that inhibition of lymphocyte apoptosis increases survival. For example, transgenic mice overexpressing Bcl-2 are protected from lethality secondary to generalized peritonitis (71). Similarly, treatment of mice with a broad-acting caspase inhibitor can also protect mice from sepsis-induced mortality (35). Therefore, the current study was undertaken to directly test whether inhibition of thymocyte apoptosis could improve outcome in a model of generalized peritonitis. We hypothesized that targeted overexpression of interleukin-10 (IL-10) in the thymus might increase Bcl-2 expression, reduce caspase-3 dependent thymocyte apoptosis and hence improve survival. IL-10, an anti-inflammatory cytokine, has been previously shown *in vitro* to

reduce T-cell apoptosis in part through up-regulation of Bcl-2 (152). Bcl-2 inhibits the release of cytochrome c from the mitochondria, and thus, inhibits caspase-9 and -3 activation (153). To test this hypothesis, mice were injected intrathymically 24 hours prior to cecal ligation and puncture (CLP) with a recombinant adenovirus expressing human IL-10 (Adv/hIL-10). Because adenovirus does not readily transfect lymphocytes, but shows tropism for epithelial and mesenchymal cells, secreted protein by thymic accessory cells would act on adjacent T-cells through a local bystander effect.

4.2.3. Results

Adenovirus readily transfects thymic accessory cells and hepatocytes. To confirm the identity of adenovirus-transfected cells, thymi and livers were harvested 24 hours after intrathymic and intravenous injection of a recombinant adenovirus expressing *gfp* in healthy mice. In the thymus, the distribution of cells expressing *gfp* was
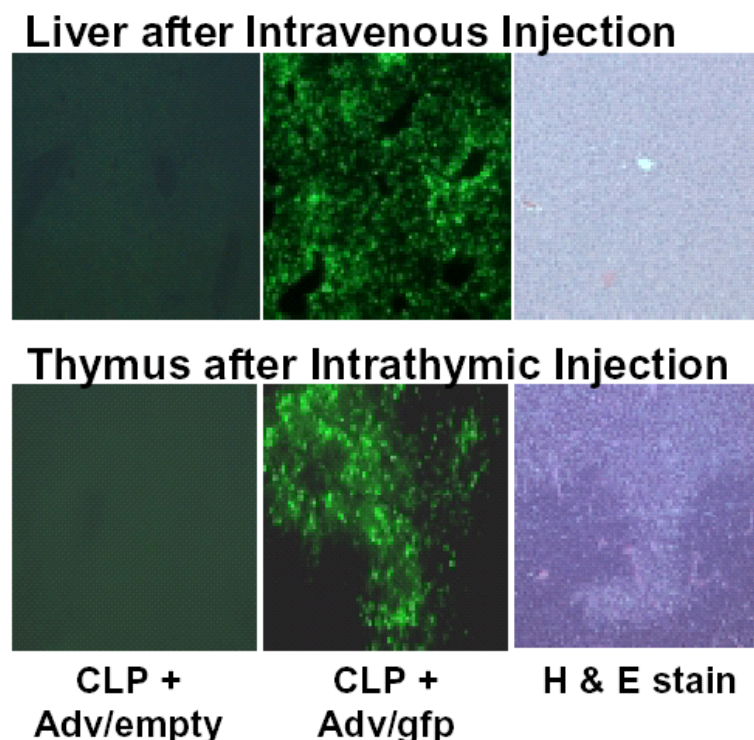

Fig. 12: Histological GFP (Green fluorescent protein) - positive cells. Mice were injected intravenously (*top panels)* or intrathymically (*lower panels*) with 10^{10} particles of an adenoviral vector containing an empty cassette or expressing gfp. Tissues were examined 24 hours later for *gfp* fluorescence or by hematoxylin and eosin staining *(magnification 100x).*

consistent with transduction limited primarily to accessory cells, and not to thymocyte populations, as determined by phenotype and localization **(Fig. 12)**. In contrast, following the intravenous injection of the same adenoviral recombinant, gfp expression could not be detected in the thymus, but was widespread in hepatocytes, although concentrated around perivascular spaces **(Fig. 12)**.

To identify the phenotype of thymic cells transduced with the adenovirus, flow cytometric analysis was performed. Only 0.8% of the total $CD3^+$ positive thymocytes were expressing gfp (Fig. 8), suggesting that the transfected cells were accessory cells in the thymus such as dendritic cells, natural killer cells or macrophages. In contrast, 25.3% of the $CD11c^+$ cells were expressing gfp indicating that dendritic-like cells were one target of the adenovirus. Thus, it was obvious that although adenovirus gene therapy could be used to target ectopic expression in the thymus, forced over-expression in a significant number of thymocytes could not be achieved with this adenoviral recombinant vector.

Because IL-10 is a secreted protein with anti-apoptotic properties on T and B lymphocytes (152, 154), it represents an ideal therapeutic approach to employ with adenovirus. Furthermore, biological responses to IL-10 are often seen with low concentrations, often in the range of 0.1 to 10 ng/ml or ng/g wet weight. In healthy mice, intrathymic injections of 10^5 and 10^{10} particles/mouse of the recombinant Adv/hIL-10 resulted in detectable tissue levels of human IL-10 **(Table 5)**. After 24 hours, tissue concentrations ranged from 7-800 ng/g wet weight, while plasma concentrations were more than a log less. As anticipated, mice treated with 10^{10} particles had higher IL-10 concentrations than levels seen in mice treated with 10^5 particles. When treated intravenously with the same doses of adenovirus, expression in the thymus was markedly reduced. Mice treated intravenously with 10^5 particles/mouse had no detectable hIL-10 in either plasma or thymus, whereas at the higher dose (10^{10} particles), concentrations were significantly higher in the plasma than in the thymus **(Table 5)**.

Table 5: Human IL-10 levels in plasma and thymus of healthy and septic mice depending on dose and route of administration (n = 5). The time intervals chosen represent 24 and 48 hours post adenoviral transduction.

route of injection	dose	24 hrs post transduction		48 hrs, 24 hrs post CLP	
		plasma (ng/ml)	thymus (ng/g wet weight)	plasma (ng/ml)	thymus (ng/g wet weight)
intrathymic	10^5	0.55 ± 0.27	7.81 ± 2.19 *	≤ 0.03	0.85 ± 0.23 *
	10^{10}	65.6 ± 13.3 [†]	825.4 ± 327.4 *[†]	3.2 ± 0.2 [†]	38.1 ± 7.8 *[†]
intravenous	10^5	≤0.03	≤0.45	ND	ND
	10^{10}	232.8 ± 17.2 [†]	2.26 ± 0.84 *[†]	ND	ND

Data are presented as mean ± standard error of the mean. *$p<0.05$ plasma vs. thymus. [†]$p<0.05$ 10^5 vs 10^{10}. ND = not determined.

Twenty four hours later, corresponding to 48 hours after adenovirus transduction and 24 hours after induction of CLP, plasma hIL-10 expression had significantly ($p<0.05$) declined in mice transduced intrathymically with the Adv/hIL-10 **(Table 5)**. Although the values represent the means for the entire group, nondetectable levels (≤0.45 ng/g wet wt) were observed in approximately one-third of the mice 24 hours after CLP.
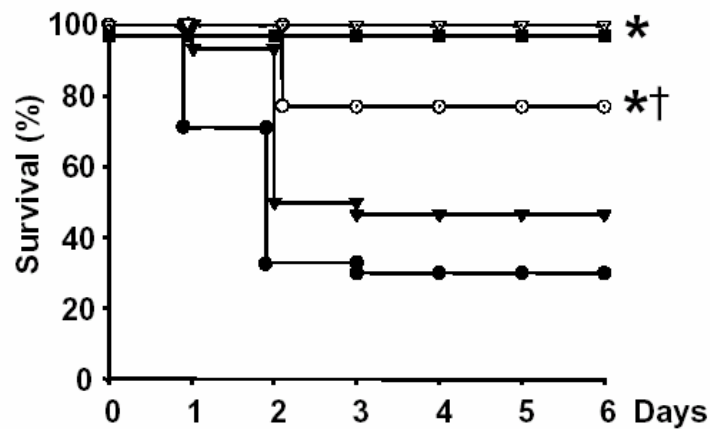
Fig. 13: Survival rate of different treatment and non-treatment groups. Survival rate of mice undergoing CLP without treatment (●) was compared to that of animals pretreated intrathymically with 10^5 particles of an adenoviral vector expressing human IL-10 (Adv/hIL-10) (○) 24 hours prior to CLP and mice pretreated with an equivalent number of adenoviral particles containing an empty cassette (▼). Transgenic mice overexpressing Bcl-2 in T-cells (■) as well as sham mice (∇) had a survival of 100%. *p<0.05 CLP vs. treatment or sham, †p<0.05 Adv/hIL-10 vs. Adv/empty, by Kaplan-Meier, log rank.

Intrathymic expression of human IL-10 improves survival and reduces bacteremia in septic mice. 10^5 particles of adenovirus were chosen as a therapeutic dose since the goal of these studies was to achieve therapeutic levels in the thymus (1-10 ng/ml) without significant appearance in the systemic circulation. However, as a control, similar doses of adenovirus were administered intravenously. Mice were pretreated with the intravenous or intrathymic instillation of adenoviral vectors, and 24 hours later, the animals underwent CLP, and were observed for six days. Animals receiving no pretreatment had a CLP survival rate of 30% (9/30) **(Fig. 13)**. In contrast, mice pretreated intrathymically with 10^5 particles of the Adv/hIL-10 had a significantly increased survival rate of 75% (15/20,p<0.05) compared to the untreated group. Intrathymic administration of a recombinant Adv/empty also improved outcome (14/30, 47%), but the degree of improvement was significantly less (p<0.05) than compared to mice treated intrathymically with the Adv/hIL-10, and was not statistically different than the untreated CLP group. Transgenic mice overexpressing Bcl-2 primarily in lymphocytes demonstrated no mortality to CLP (10/10 survived).

To confirm that the beneficial effect seen with intrathymic administrations of adenovirus was not due to systemic appearance of hIL-10, but was due to targeted expression in the thymus, additional mice were injected intravenously with 10^5 particles of both recombinant adenovirus vectors and compared to septic mice receiving no treatment. There was no significant difference in survival among the three study groups: 3/11 mice (27%) survived CLP without treatment, 5/11 (45%) and 5/12 (42%) survived in groups receiving Adv/empty or Adv/hIL-10, respectively.

The increased outcome in mice receiving intrathymic injections of Adv/hIL-10 was associated with a reduced blood bacteremia. Blood bacteria counts were 2.7 x 10^4 and 5.6 x 10^4 colonies/ml in the untreated septic (n=10) and Adv/empty treated septic mice (n=9), respectively. In contrast, blood bacteremia levels were reduced greater than ten fold in septic mice pretreated with Adv/hIL-10 (1.3 x 10^3 colonies/ml; n=9, p<0.05 by ANOVA and Fischer's LSD post hoc versus both groups). Levels were essentially undetectable in healthy controls.
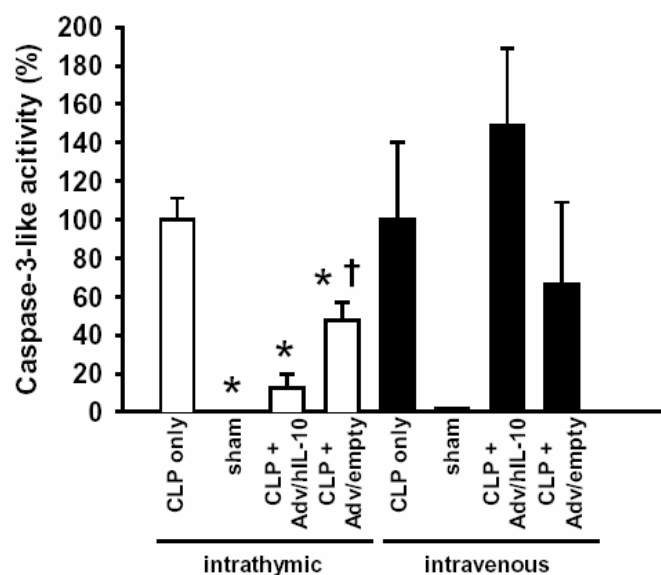

Fig. 14: Caspase-3-like activity in the thymus of septic mice. Caspase-3-like activity was determined in the thymus from septic mice (n=10/group) 24 hours after CLP and 48 hours after intrathymic or intravenous pretreatment. Data from several experiments were combined and the rate of caspase-3 activity in the thymus of septic mice was normalized to 100%. Statistical comparisons could not be performed between groups of mice treated intrathymically and intravenously because of the effect of a prior surgery (thoracotomy) on subsequent thymic caspase activity. Data were normalized (100%) for values from sham, septic mice (2,329 relative fluorescence intensity (RFI) for intrathymic and 300.9 RFI for intravenous injections). *p<0.05 CLP vs. treatment or sham, †p<0.05 Adv/hIL-10 vs. Adv/empty, by one way ANOVA and Fischer's LSD post-hoc test.

Increased survival is associated with reduced thymic apoptosis. Reductions in thymic apoptosis were associated with improved outcome in mice receiving intrathymic instillation of Adv/hIL-10. Septic mice were sacrificed 24 hours after CLP, and 48 hours after adenovirus instillation. As expected, sepsis induced by CLP increased caspase-3 activity in the thymus of these mice greater than 50-fold **(Fig. 14)**. Pretreatment of mice with intrathymic injections of 10^5 particles of adenovirus significantly reduced caspase-3 activity in a pattern similar to that seen for survival. The recombinant Adv/empty significantly decreased caspase-3 activity (56% reduction), but the increased caspase-3 activity was completely abolished by intrathymic treatment of 10^5 particles of Adv/hIL-10 (92% reduction). The reduction in caspase-3 activity in mice treated with Adv/hIL-10 was significantly greater than the reductions seen in the mice treated with the Adv/empty vector **(Fig. 14)**. Transgenic mice over-expressing Bcl-2 also demonstrated no increase in caspase-3 activity in the thymus (*data not shown*).

In addition, thymic caspase-3 was determined in mice pretreated with intravenous injections of the adenovirus vectors. Caspase-3 activities among CLP mice receiving no pretreatment, or pretreatment with Adv/empty or Adv/hIL-10 were not significantly different **(Fig. 14)**.
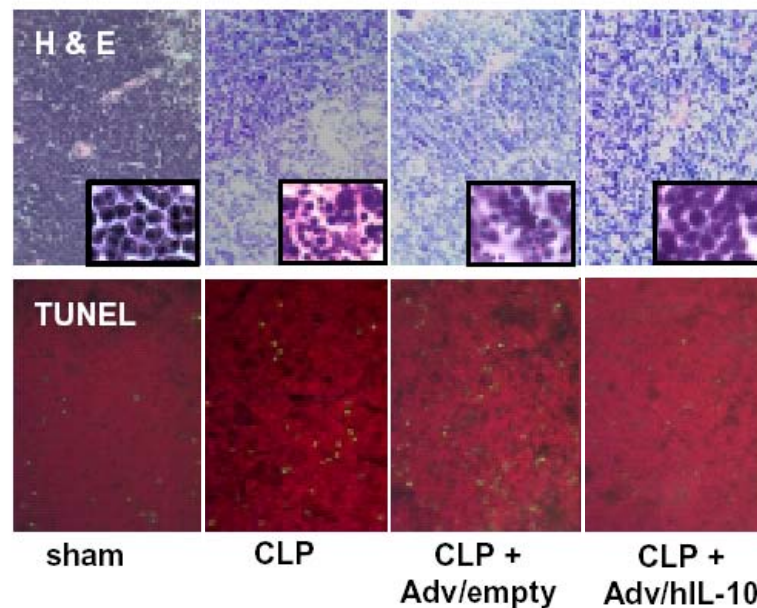
Fig. 15: *In situ* **TUNEL staining and histological examination of thymi from mice following cecal ligation and puncture.** Thymi were harvested from mice 24 hours after a cecal ligation and puncture, and tissues were stained with hematoxylin & eosin, or by 3' end labeling of apoptotic nuclei (TUNEL) staining as described in the Materials and Methods section. Increased numbers of cells undergoing apoptosis were seen in mice following cecal ligation and puncture. Mice pretreated intrathymically with 10^5 particles of Adv/empty had similar numbers of apoptotic cells, as determined by TUNEL staining. In mice pretreated with 10^5 particles of Adv/hIL-10, there was a marked reduction in the numbers of apoptotic cells. In the hematoxylin and eosin stained sections (see inset for greater detail), apoptotic cells (fragmented and pyknotic) were seen primarily in the thymus of untreated mice and mice pretreated with Adv/empty (*magnification 100 x and 1000x for insert*)

These findings were confirmed with *in situ* 3' end labeling and hematoxylin and eosin staining. *In situ* 3' end-labeling, although not quantitative, indicated a similar number of apoptotic cells in the untreated septic mice, as well as in the septic mice treated with intrathymic instillation of Adv/empty **(Fig. 15)**. Conversely, in mice treated with 10^5 particles of an Adv/hIL-10, there was a dramatic reduction in the number of apoptotic cells. The physical characteristics of apoptosis, such as shrinking and fragmentation of cells, were evident in the hematoxylin and eosin staining. These apoptotic cells were found predominantly in the cortex. In contrast, mice receiving intravenous administration of Adv/empty or Adv/hIL-10 showed no significant reduction of either caspase-3 activity or the numbers of apoptotic nuclei (*data not shown*).

Bcl-2 is upregulated in the thymus of hIL-10 treated mice. Transgenic mice overexpressing Bcl-2 were protected from the lethality associated with CLP **(Fig. 13)**. We therefore examined whether the decreases in apoptosis seen in the thymi of mice
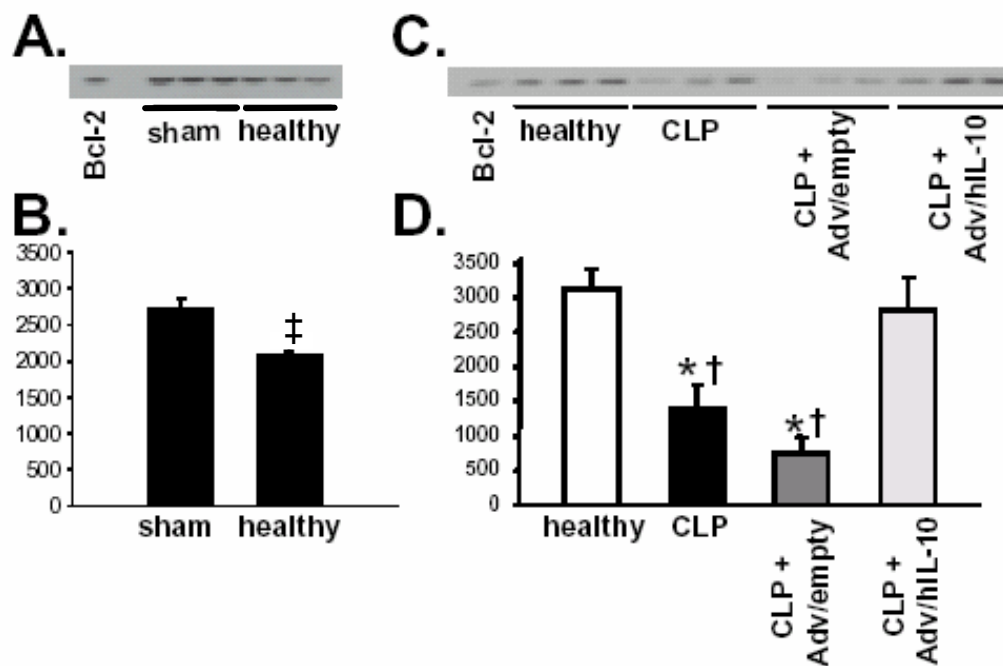

Fig. 16: Bcl-2 expression in thymus as determined by Western blot analysis. Bcl-2 levels were examined in the thymus of healthy mice and mice following CLP. Mice were pretreated intrathymically with either Adv/hIL-10 or Adv/empty at a dose of 10^5 particles. Bcl-2 levels were determined by Western blot analysis (panel A and C), and the relative quantities of Bcl-2 were determined by densitometric analysis (panel B and D). *$p<0.05$ healthy vs. CLP, †$p<0.05$ Adv/hIL-10 vs. Adv/empty or CLP, ‡$p<0.05$ healthy vs. sham. As a positive control for detecting murine Bcl-2, M1 cell lysate (ATCC TIB 192), was used.

treated intrathymically with adenoviral constructs expressing hIL-10 were also associated with an upregulation of the anti-apoptotic protein Bcl-2. Six mice per group underwent the procedures as earlier described. Samples were obtained from three representative mice in the groups receiving intrathymic administration of Adv/empty or Adv/hIL-10, as well as in CLP group not receiving a pretreatment. In both the untreated septic mice, as well as in septic mice pretreated with the Adv/empty, Bcl-2 expression was significantly reduced compared to both the healthy group as well as

to mice pretreated with the Adv/hIL-10 **(Fig 16 C and D)**. Interestingly, there was no apparent difference in thymic Bcl-2 levels between this latter group and the healthy animals. To rule out any reduction in Bcl-2 expression secondary to the surgical procedure, Bcl-2 expression between healthy animals and animals undergoing sham procedure were compared. Surprisingly, Bcl-2 expression appeared to be increased in the thymus of mice following a modest sham treatment, when compared to that of healthy mice **(Figure 16 A and B)**.

Thymic expression of hIL-10 is associated with a generalized reduction in the magnitude of the systemic inflammatory response. Plasma proinflammatory cytokine concentrations were determined 24 hours after induction of sepsis in an effort to determine whether the magnitude of the systemic inflammatory response was abrogated by intrathymic therapies that suppressed apoptosis. Plasma TNFα concentrations in mice treated with 10^5 particles of an Adv/hIL-10 could not be detected (<110 pg/ml), and were no different from sham mice or mice pretreated with Adv/empty. Only in the untreated septic mice was TNFα detectable in the plasma (1,344 ± 776 pg/ml). Interestingly, mice pretreated with both adenoviral constructs intravenously also did not show any detectable plasma TNFα level.

There was also evidence that intrathymic administration of the recombinant Adv/hIL-10 reduced the magnitude of more distal markers of the systemic inflammatory response. For example, both plasma IL-6 and murine IL-10 concentrations were significantly reduced by intrathymic instillation of Adv/hIL-10 **(Table 6)**. Moreover, plasma IL-6 concentrations were decreased by greater than 90% in septic mice treated with Adv/hIL-10 **(Table 6)**. Pretreatment with the Adv/empty also produced reductions in plasma IL-6, although not to the degree seen in animals treated with the Adv/hIL-10. Similarly, plasma IL-6 concentrations were also significantly decreased in mice treated intravenously with the Adv/empty, as well as in mice receiving the viral construct expressing hIL-10.

Plasma murine IL-10 concentrations were markedly reduced in animals receiving the intrathymic instillation of Adv/hIL-10 compared to CLP group **(Table 6)**. Although not significant, murine IL-10 concentrations were also decreased in the plasma of mice pretreated intrathymically with the Adv/empty. Murine IL-10

concentrations in the thymus actually followed a different pattern. The animals treated intrathymically with the adenoviral construct expressing hIL-10 had the highest murine IL-10 concentrations and the untreated CLP group the lowest. The mice receiving the Adv/empty had levels intermediate to these two groups.

Table 6: Plasma IL-6 and murine IL-10 levels in plasma and thymus of septic mice depending on dose and route of administration (n = 7). The time intervals chosen represent 24 hours post cecal ligation and puncture.

treatment group	Plasma IL-6 (ng/ml)		Murine IL-10 *Intrathymic administration*	
	intravenous administration	*intrathymic administration*	*plasma (pg/ml)*	*thymus (pg/g wet wt)*
sham	≤0.07		< 32	1126 ± 42
CLP	512 ± 264		4157 ± 2609*	1063 ± 125
CLP + Adv/empty	139 ± 111*	99 ± 63*	2240 ± 889*	2283 ± 494*
CLP + Adv/hIL-10	18 ± 12	34 ± 32	339 ± 149*	2845 ± 315*†

Data are presented as mean ± standard error of the mean. *$p<0.05$ versus sham, †$p<0.05$ CLP vs. CLP + Adv.

4.2.4. Discussion

Sepsis syndrome is often characterized by both a systemic proinflammatory response, as well as defects in antigen presentation, macrophage "paralysis", and reduced T-cell proliferation to mitogenic stimulation (155). Current therapeutic approaches aimed at blocking the proinflammatory response have generally failed to show any improvements in outcome in either mice with CLP (156) or in patients with sepsis syndrome (147, 148). Rather, recent studies have focused on the observation that increased apoptosis of lymphoid cells is also seen in animal models of sepsis, burn injury and trauma (32). Buchmann and Hotchkiss have recently reported the presence of increased numbers of apoptotic lymphocytes in the spleen, as well as in intestinal epithelial cells of patients dying from sepsis (36).

Although increased apoptosis in lymphoid and epithelial tissues has been demonstrated to accompany sepsis syndromes, increased apoptosis may also directly contribute to the adverse outcome, and be a potential therapeutic target for intervention. For example, the role of caspase-dependent apoptosis in the survival response to sepsis has recently been clarified. Hotchkiss and colleagues observed that systemic injection of Z-VAD, a broad acting caspase inhibitor, into mice receiving a similar CLP improved survival, and reduced apoptosis in the thymus (35). Although such studies confirm a direct involvement of caspase activation in outcome to sepsis syndrome, the systemic administration of a broad acting caspase inhibitor, like Z-VAD, can not identify either the specific target (such as the individual caspase) or anatomical site of action (such as the thymus).

Caspase-dependent apoptosis is regulated in part by a family of mitochondrial proteins of the Bcl-2 family. Bcl-2 and its other family members, such as Bcl-xL and the proapoptotic proteins, Bid, Bad and Bax, are important regulators of cellular apoptosis (71, 72, 157-159). Members of this family can inhibit or promote apoptosis via homo- and heterodimerization (160). Thymocytes overexpressing Bcl-2 are resistant to apoptotic stimuli *in vitro*, such as dexamethasone and γ-irradiation, and they survive apoptosis induced by the withdrawal of growth factors (158). In animal models performed with Bcl-2 overexpressing mice, such as the ischemia-reperfusion-injury model as well as in the CLP model, decreased numbers of apoptotic intestinal cells or lymphocytes of spleen and thymus have been observed, and a survival benefit was demonstrated in the latter group (71, 72). Furthermore, mice deficient in the proapoptotic protein Bid, are resistant to Fas-induced liver injury and have an increased survival (159).

In the present report, we have demonstrated that ectopic overexpression of IL-10 in the thymus of septic mice also improves survival. Improvements in outcome were associated with increases in thymic Bcl-2 expression and reductions in thymic apoptosis and caspase-3 activity. We have employed an adenoviral construct to deliver human IL-10 to the thymus of mice prior to the induction of generalized peritonitis or sepsis syndrome. Adenovirus readily transduces epithelial cells, fibroblasts and dendritic cells, but has only a limited propensity for transducing thymocytes or other lymphocyte populations. This difficulty in transducing thymocytes

with adenovirus necessitated an indirect approach to target thymocyte apoptosis. We chose to transduce thymic accessory cells with the cDNA for a secretable protein, hIL-10, and affect adjacent lymphocytes through a paracrine, bystander process.

Although IL-10 is a pleiotropic cytokine with both anti-inflammatory and immunosuppressive properties, IL-10 has been shown to suppress T-cell apoptosis through a Bcl-2 dependent process. For example, Cohen and colleagues reported that IL-10 suppressed peripheral blood T-lymphocyte apoptosis *in vitro* by increasing Bcl-2 expression (152). Similarly, Taga and colleagues observed that IL-10 prevents T-cell apoptosis following IL-2 withdrawal and EBV infections through upregulation of Bcl-2 expression (161).

In the present report, intrathymic expression of hIL-10 markedly increased Bcl-2 levels in the thymus, and this was associated with reductions in thymic caspase-3 activity and the numbers of apoptotic cells. The improved outcome in mice receiving intrathymic administration of adenoviral vectors expressing hIL-10 was due to the local intrathymic expression, since mice injected intravenously with the same vector did not have improved outcome compared to untreated septic mice. This confirms that systemic hIL-10 expression can not explain the improved outcome in this model, or the reductions in thymocyte apoptosis. Rather, the beneficial effects appear to require localized hIL-10 expression in the thymus. In a recent report, Takakuwa systemically administered an adenovirus expressing IL-10 and saw improved outcome to a bacterial bolus (161). However, this peritoneal administration of live bacteria is predominantly a model of an exaggerated proinflammatory response and hypovolemic shock where exogenous administration of IL-10 protein is known to be effective. In contrast, we have used a more clinically relevant model of sepsis and one in which systemic administratition of IL-10 or other cytokine inhibitors have not been effective (162).

To further demonstrate the influence of apoptosis on outcome in this model, we compared the results to those obtained from transgenic mice over-expressing Bcl-2 under a T-cell specific promoter (158). These mice were completely resistant to a CLP induced mortality, suggesting that the improvements in outcome in mice receiving intrathymic instillation of Adv/hIL-10 were potentially through upregulation of Bcl-2.

Unfortunately, IL-10 is a pleiotropic cytokine, and improvements in outcome may have been secondary to IL-10 activities not involving Bcl-2 expression and apoptosis. Although IL-10 has anti-inflammatory properties, the improvements in outcome cannot be easily attributed to any reductions in the magnitude of the systemic inflammatory response induced by IL-10. The attenuation of the plasma TNFα and IL-6 responses following CLP were also seen when the mice were treated intravenously with the same dose of adenovirus vectors, but similar improvements in outcome were not observed. Thus, the proinflammatory cytokine response was attenuated by all of these treatments, but did not appear to be associated with any differences in survival. This latter finding is consistent with the inability of anti-TNFα therapies (147, 156) or systemically administered IL-10 (163) to improve outcome in this model of sepsis syndrome.

One surprising aspect of the current study was the observation that intrathymic, but not intravenous, instillation of a recombinant Adv/empty vector modestly improved outcome and reduced thymic caspase-3 activity. This beneficial effect may be secondary to activation of the innate or acquired immune response to the adenoviral expression (72, 159). Consistent with this latter hypothesis is the observation that intrathymic levels of murine IL-10 were significantly increased in septic mice pretreated with the Adv/empty when compared to sham-treated animals. Intrathymic adenovirus instillation may have induced a sufficient local IL-10 expression to partially attenuate the increases in caspase-3 activity.

In conclusion, intrathymic expression of hIL-10 with adenovirus leads to a reduction in thymocyte apoptosis, and significant increases in survival following CLP. These results appear to be mediated at least in part through up-regulation of Bcl-2 and inhibition of caspase-3 dependent apoptosis. The study provides strong evidence that lymphocyte apoptosis plays a critical role in sepsis, indicating a potentially new therapeutical approach for treatment of sepsis syndrome. Further studies are required to demonstrate the feasibility of gene therapy in T-cell rich organs other than the thymus to prevent T-cells apoptosis in humans.

4.3. Increased survival in sepsis by *in vivo* adenovirus-induced expression of IL-10 in dendritic cells through footpad injection

4.3.1. Summary

The dendritic cell is the most potent antigen presenting cell of the immune system, capable of stimulating naive T cells to proliferate and differentiate into effector T cells. Recombinant adenovirus readily transduces DCs *in vitro* allowing directed delivery of transgenes that modify DCs function and immune responses. Here we demonstrate that footpad injection of a recombinant adenovirus readily targets transduction of myeloid and lymphoid DCs in the draining popliteal lymph node, but not in other lymphoid organs. Popliteal DCs transduced with an empty recombinant adenovirus undergo maturation, as determined by high MHC class II and CD86 expression. However, transduction with vectors expressing human IL-10 limit DC maturation and associated T-cell activation in the draining lymph node. The extent of IL-10 expression is dose dependent; transduction with low particle numbers (10^5) yields only local expression, while transduction with higher particle numbers (10^7, 10^{10}) leads to additional IL-10 appearance in the circulation. Furthermore, local DC expression of human IL-10 at low particle numbers (10^5) significantly improves survival following cecal ligation and puncture, suggesting that compartmental modulation of DC function profoundly alters the sepsis-induced immune response.

4.3.2. Introduction

Dendritic cells are potent antigen-presenting cells, distinguished by their ability to induce naive T cells to proliferate and differentiate into effector T cells in the absence of exogenous stimuli (164-166). Immature DCs exist in all peripheral tissues, where they phagocytose and process soluble and particulate antigen. After exposure to an activating stimulus, such as LPS, IL-1α, CpG motifs, or apoptotic cell fragments, the immature DC will begin a maturation process involving modulation of chemokine receptor expression, migration to the draining lymph node, increased processing and presentation of peptides on both Class I and Class II MHC molecules, upregulation of costimulatory molecules such as CD80 and CD86, and increased production of soluble cytokines such as IL-12 (76, 166). Within the lymph node, the mature DC

secretes chemokines such as MIP-3β to attract large numbers of naive T cells to the area, allowing the presented peptide on MHC to interact with a wide variety of TCR complexes.

Dysregulation of the immune response occurs during sepsis and often leads to the development of multi-organ failure. Although the pathogenesis of the sepsis response to bacterial pathogens has been extensively studied, the mechanisms leading to high morbidity and mortality in patients remain unclear. Possible explanations include an unregulated production of pro-and anti-inflammatory cytokines that lead to endothelial injury, as well as increased activation-induced cell death (apoptosis) of T- and B- lymphocytes that limit acquired immune response-mediated clearance of pathogens (149, 167).

The effects of sepsis on DC function, however, are largely unknown. Bacteremia, endotoxemia and generalized peritonitis, all of which promote extensive inflammatory cytokine release (e.g. TNFα), may induce unregulated and widespread activation and maturation of DCs. In the latter phases of sepsis, DCs may globally lose their ability to produce IL-12 and generate protective T_H1 immune responses against the primary and secondary infectious organisms (168, 169). Alternatively, extensive activation of DCs at early time points could promote responses that limit the capacity of these cells to contain the sepsis response. Controlling the extent of the inflammatory response and DC activation may provide novel therapeutic approaches to limit the untoward outcomes of the sepsis response.

The goal of the present study was to determine whether modification of DCs toward a less activated state would influence the outcome of sepsis. Previous studies demonstrated that IL-10 potently inhibits DC maturation, antigen presentation and IL-12 production, and maintains macropinocytosis and endocytosis (170, 171). Furthermore, DCs treated with an anti-IL-10 antibody show an increased capacity to activate and prime naive T-cells to a more prominent T_H1 polarization, suggesting autocrine regulation of DCs by this cytokine is important (172).

We hypothesized that transduction of DCs to induce transient expression of human IL-10 would inhibit DC maturation in the setting of sepsis, and might modify the outcome to the septic response. To accomplish this, we considered the use of recombinant viral vectors. DCs have been shown recently to be permissive to

adenovirus infection *in vitro* at high particle concentrations (136). Due to the relative short duration of transgene expression, adenovirus appears promising as a vector for transient gene therapy (173, 174), appropriate for acute inflammatory processes like sepsis.

4.3.3. Results

Adenovirus infects DCs in vivo. 2×10^5 or 2×10^{10} particles of recombinant adenovirus expressing green fluorescent protein (gfp), or containing an empty cassette, were injected into both hind footpads of mice. Twenty-four hours later, the draining popliteal
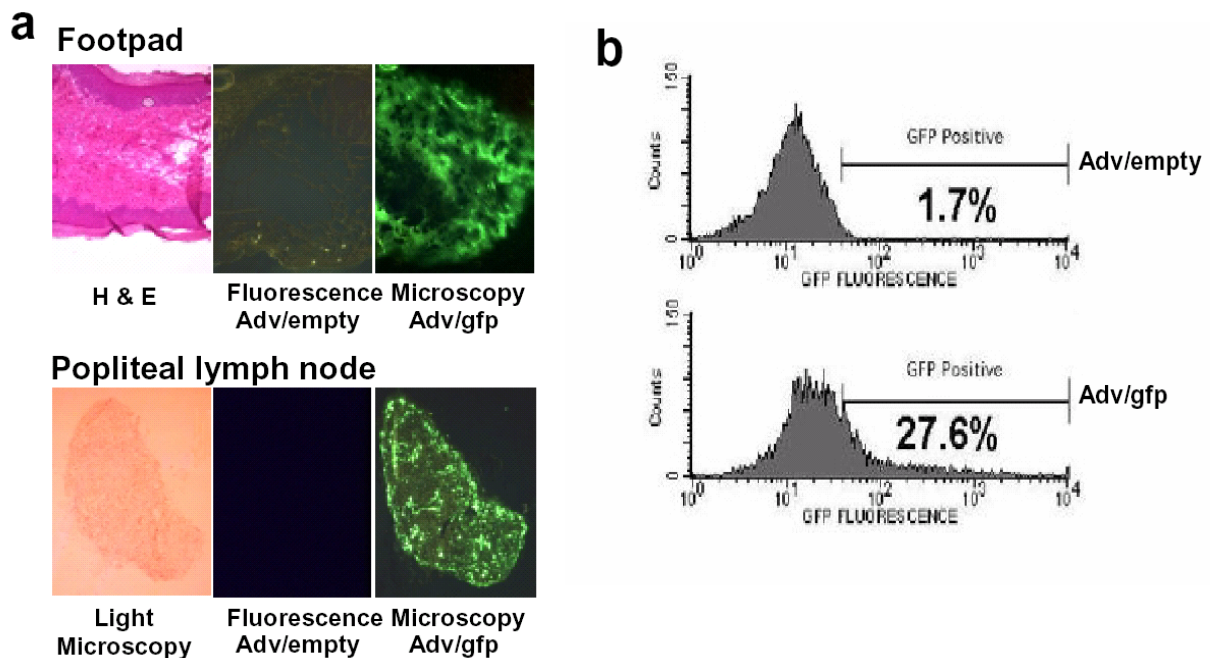

Fig. 17: Detection of gfp positive cells in the footpad and popliteal lymph node (one representative figure from four repetitions). **a)** Fluorescent microscopy of frozen section of footpad as well as popliteal lymph node were examined 24 hours following the footpad injection of 2×10^{10} particles of an adenoviral vector expressing either gfp (Adv/gfp) or containing an empty cassette (Adv/empty). **b)** Flow cytometric analysis was performed on the popliteal lymph node, and gated on live CD11c+ and high MHC Class II cells, revealing that 26% of these cells had been transduced with the adenoviral vector expressing gfp.

lymph nodes were harvested and examined for green fluorescence by either frozen section or flow cytometry. Frozen sections of the popliteal lymph nodes showed fluorescent cells distributed in the subcapsular and T cell areas of the lymph node **(Fig.17a)**. Analysis of the lymph nodes by flow cytometry revealed that approximately

26% of CD11c$^+$, MHC class II expressing cells were gfp positive, indicating that DCs had been transduced with the recombinant adenovirus and were expressing the transgene **(Fig. 17b)**. Additional flow analysis revealed that there were essentially no gfp positive cells in the CD3$^+$, CD4$^+$ and CD8$^+$ cell populations, confirming that T-cells are not readily transduced by adenovirus (*data not shown*). Examining the DC population in greater detail revealed that lymphoid DCs (CD8$^+$ CD11c$^+$, MHC class II^{hi}, CD86^{hi}) were transduced to a greater degree than were myeloid DCs (CD8$^-$, CD11c$^+$, MHC class II^{hi}, CD86^{hi}). Three percent of all lymphoid DCs in the lymph node were gfp positive following injection of 2 x 10^5 particles of recombinant adenovirus, whereas nearly 30% were gfp positive following injection of 2 x 10^{10} particles of the same vector. In contrast, 0.5% and 10% of myeloid DCs were also gfp positive following administration of 2 x 10^5 and 2 x 10^{10} particles, respectively (*data not shown*).

Adenovirus expressing human IL-10 decreases DC maturation and lymphocyte activation. Because DCs are activated by adenovirus *in vitro* (174), we examined the status of DC maturation in CD8$^+$ and CD8$^-$ subsets 24 hours after footpad administration of a recombinant adenovirus containing an empty cassette (Adv/empty). We also administered adenovirus expressing human IL-10 (Adv/hIL-10) to determine if the expression of this anti-inflammatory cytokine blocked DC maturation as reported with administration of the recombinant protein. In addition, because DCs can stimulate T cell activation, we determined whether human IL-10 expression in DCs within the popliteal lymph node would regulate CD4$^+$ or CD8$^+$ lymphocyte activation by assessing CD69 or CD25 expression following viral exposure. Treatment with 2 x 10^{10} particles of the adenovirus containing an empty cassette led to maturation of both lymphoid and myeloid DCs in the popliteal lymph node, as demonstrated by upregulation of CD86 surface antigen **(Fig. 18a)**. Interestingly, treatment of mice with the recombinant vector expressing human IL-10 at the same dose (10^{10} particles) reversed this effect with CD86 expression below the levels seen in DCs from mice injected with buffer alone.

Injection of 2 x 10^5 particles of the adenoviral vector with an empty cassette led to only a minimal activation of CD4$^+$ and CD8$^+$ T cells **(Fig. 18b)**. Approximately 2%
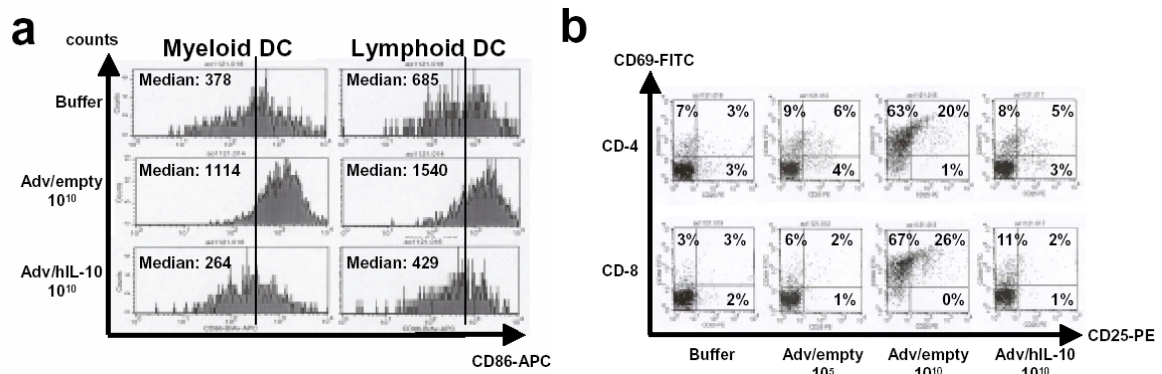
Fig. 18: Adenovirus-induced DC and T-cell activation. Flow cytometric analysis of popliteal lymph node (one representative analysis from four repetitions). 24 hours after the footpad injection of either 2×10^5 or 2×10^{10} particles of adenovirus expressing either hIL-10 (Adv/hIL-10) or containing an empty cassette (Adv/empty). **a)** Dendritic cells were stained and gated for either the myeloid population as CD11c+-PE, CD8-PerCp- and Class-II-FITC high, or the lymphoid population as CD11c+-PE, CD8-PerCp+ and Class-II-FITC high, and activation was measured by the upregulation of CD86+-APC. **b)** T-cells were stained for CD4+-APC and CD8+-PerCp, and their activation was determined by up-regulation of the early activation marker CD69-FITC, and late activation marker CD25-PE.

more CD4$^+$ and CD8$^+$ cells expressed CD69 after adenoviral injection compared to buffer treatment. Injection of a higher particle dose (2×10^{10}) led to nearly a six-fold increase in the number of activated CD4$^+$ and CD8$^+$ T cells. Interestingly, administration of 2×10^{10} particles of the vector expressing human IL-10 (Adv/hIL-10) reversed the effect leading to a similar degree of activation as seen with the lower particle dose **(Fig. 18b)**.

Local versus systemic appearance of IL-10 is dose dependent. One possible advantage of utilizing IL-10 therapies for the treatment of inflammatory disease is the capacity to achieve only local expression, as systemic exposure to this cytokine often leads to unwanted immunosuppressive effects (175). As demonstrated by Steinhauser et al., systemic administration of IL-10 suppressed the immune response to *Pseudomonas* pneumonia following a cecal ligation and puncture (176). Therefore, one of the goals of these studies was to establish whether local adenoviral expression of IL-10 could be obtained. We, therefore, administered different quantities of adenovirus expressing human IL-10 into the footpad of healthy mice to determine whether local production in the draining lymph nodes, in the absence of systemic

appearance in the circulation, could be achieved. At 24 hours, we analyzed plasma for human IL-10, and lymph nodes for human IL-10 cDNA. With a dose of 2×10^5 particles, we could not detect human IL-10 in the plasma of recipient animals **(Fig. 19a)**. In marked contrast, higher doses of adenovirus expressing IL-10 (2×10^7 particles and 2×10^{10} particles, respectively) showed a dose-dependent increase in the plasma appearance of human IL-10.
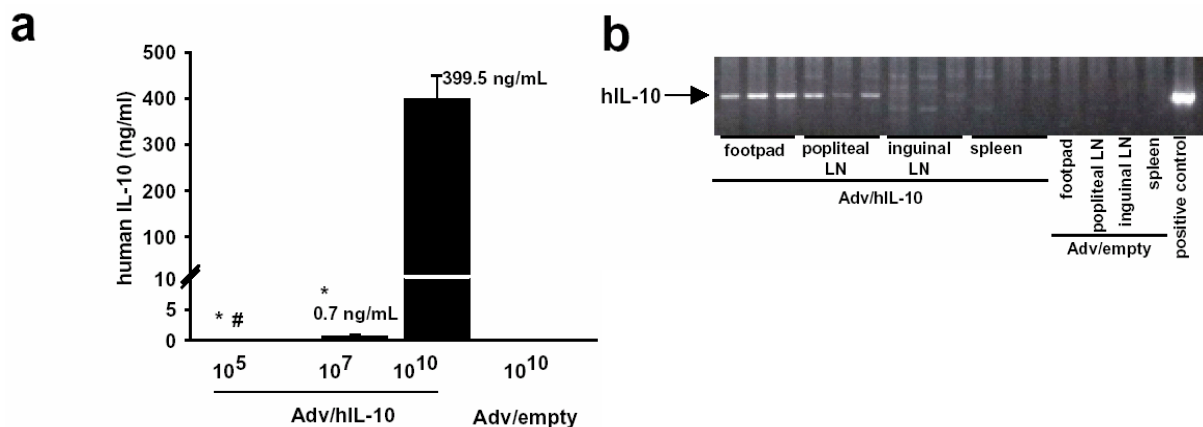

Fig. 19: Plasma human IL-10 concentrations and expression in the lymph node. a) Human IL-10 was measured in the plasma 24 hours after injection in each hind footpad of either 2×10^5, 2×10^7 or 2×10^{10} particles of Adv/hIL-10 or Adv/empty (*n*=5). **b)** PCR analysis for human IL-10 cDNA was performed on the popliteal and inguinal lymph nodes, spleen and footpad 24 hours after footpad injection of 2×10^5 particles of either Adv/hIL-10 or Adv/empty. *, $P<0.05$ 10^5 and 10^7 verus 10^{10}; #, $P<0.05$ 10^5 verus 10^7.

To confirm that the adenoviral delivery of IL-10 at a dose of 2×10^5 was indeed localized to the draining lymph nodes, we examined human IL-10 cDNA in the popliteal lymph node and at the site of injection (footpad), as well as at distant sites, such as the inguinal lymph node, spleen, thymus, lung and liver. Human IL-10 cDNA could be detected in the popliteal lymph nodes as well as in the injected footpad on post-injection days one and three **(Fig. 19b)**. On the other hand, in only one out of three replicates could human IL-10 cDNA be recovered from the more distant inguinal lymph node. No human IL-10 cDNA could be recovered from any other organ, as determined by PCR (*data not shown*).

Local expression of human IL-10 leads to an increased survival of septic mice. Having demonstrated that local expression of IL-10 could be achieved, we next determined whether the compartmentalized expression of this cytokine in peripheral lymph nodes would affect the survival of mice developing polymicrobial sepsis
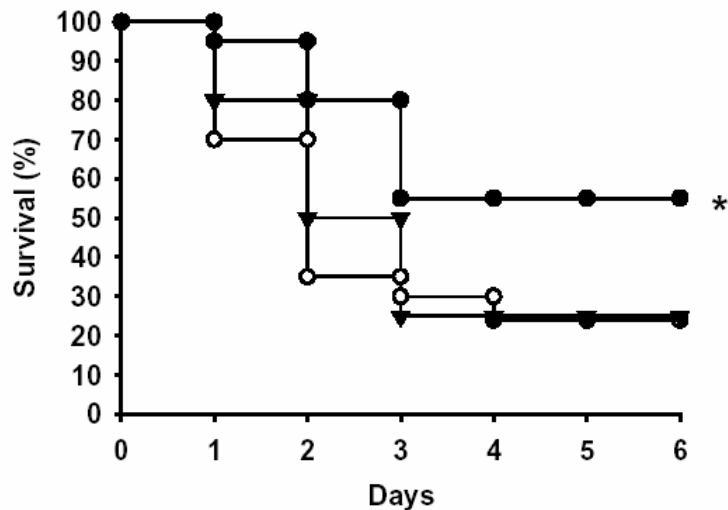

Fig. 20: Survival rate of septic mice pretreated with adenovirus. A survival study (*n*=20 for each group) was performed by pretreating mice with footpad injections in both footpads of either 2 x 10^5 particles of Adv/hIL-10 (●) or Adv/empty (○) or buffer (∇). 24 hours later, mice underwent cecal ligation and puncture and were observed for 6 days.
*$P < 0.05$ Adv/hIL-10 versus Adv/empty or buffer.

following a cecal ligation and puncture. To obtain local expression of IL-10, mice were pretreated with footpad injections of 2 x 10^5 particles of adenovirus expressing human IL-10 or containing an empty cassette, respectively, 24 hours prior to a cecal ligation and puncture. Mice pretreated with the recombinant expressing human IL-10 had a significant increase in survival (55%, 11/20), whereas animals receiving the empty vector as a control (Adv/empty), died at the same rate as mice injected with buffer (both 25%, 5/20) **(Fig. 20)**. Furthermore, this experiment was repeated with the highest particle dose of 2 x 10^{10} of the respective adenovirus vectors (empty as well as expressng human IL-10), as well as a group treated with buffer. In contrast to the

former study, there was no difference in outcome among the treatment groups (Adv/empty 4/20; Adv/hIL-10 5/20; buffer control 4/20).

Plasma IL-6, murine IL-10 and thymic caspase-3 activity are not associated with an improved outcome. Twenty-four hours following cecal ligation and puncture, and thus 48 hours after footpad injection, plasma IL-6 and murine IL-10 levels, as well as thymic caspase-3 activity, were determined. Earlier studies have shown that increased levels of these cytokines, and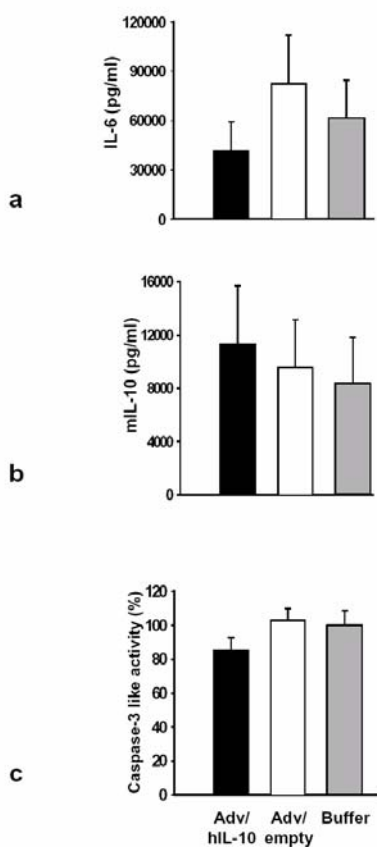 increased thymic apoptosis, correlate with an adverse outcome in similar animal models, and in patients with sepsis (46, 177). At this time point, however, no differences among the three treatment groups were seen, despite significant differences in outcome **(Fig. 21)**. There was, however, a trend towards reduced plasma IL-6 and increased murine IL-10 levels as well as decreased thymic caspase-3 activity in the group of mice treated with footpad injection of adenovirus expressing human IL-10 **(Fig. 21)**.

Fig. 21: Cytokine response and thymic apoptosis. Pretreated mice (n = 5) injected in both hind footpads with either 2×10^5 particles of Adv/hIL-10, Adv/empty or buffer underwent cecal ligation and puncture 24 hours later. After an additional 24 hours, mice were euthanized and IL-6 (**a**) and mIL-10 (**b**) were measured in plasma, as well as thymic caspase-3 activity (**c**).

4.3.4. Discussion

In the present report, we demonstrate that, following subcutaneous injection, recombinant adenoviral vectors naturally target DCs in the draining lymph nodes (174). We found that within 24 hours, footpad injection with recombinant adenovirus

targets both myeloid and lymphoid DCs in the draining popliteal lymph node, and results in protein expression. The appearance of transduced popliteal lymph node DCs could be explained by lymphatic trafficking of adenovirus from the footpad to the lymph node where both lymphoid-related and myeloid-related DCs are directly transduced. Alternatively, myeloid DCs and other antigen presenting cells residing in the interstitial tissue of the footpad may be transduced directly, and then migrate to the draining lymph node. If so, then adenovirus transduction of CD8$^+$ DCs may occur as a result of phagocytosis of myeloid DCs by these cells (178). Regardless of how each DC population is transduced, these findings are of significant potential interest as DCs can be targeted *in vivo* in a lymph node compartment for expression of genes that may regulate their function and immune responses.

This study also demonstrates a dose effect of adenovirus on *in vivo* DC activation, such that higher particle numbers of adenovirus (10^{10}) promote DC maturation, while lower numbers (10^5) do not cause significant DC maturation *in vivo*. Although only 30% of all DCs in the lymph node are transduced following administration of 10^{10} particles, the majority of both CD8$^+$ and CD8$^-$ DC populations in the popliteal lymph node are mature at 24 hours following injection. In addition, popliteal lymph node T cells are also activated with this dose of adenovirus. This suggests that at higher doses, recombinant adenovirus may act as an adjuvant of transduced as well as uninfected DCs, and subsequent T cell activation. Dendritic cell maturation may be induced directly by viral DNA or proteins derived from the virus (165). Alternatively, maturation may result from the paracrine actions of IFNγ produced as a consequence of transduction-mediated induction of inflammatory cytokines produced by infected DCs, macrophages or activated T cells (165). Importantly, DC maturation as well as T cell activation is efficiently blocked *in vivo* when recombinant adenovirus expresses human IL-10. Previous *in vitro* studies suggest that exposure of immature DCs to IL-10 blocks maturation and makes them resistant to maturation stimuli applied at later time points (179). Although we have determined that DCs expressing the IL-10 transgene are resistant to subsequent maturation stimuli, we find that *in vitro* transduction of DCs with adenovirus expressing IL-10 will still undergo phenotypic maturation with LPS. However, the production of IL-12 by Adv/hIL-10 transduced cells in response to LPS-induced

maturation remains suppressed. These results when considered in the context of Steinbrink's findings (179) suggest that the adenoviral vector itself may modify the DC response to IL-10, and IL-10 may modify the DC response to adenoviral vector transduction *in vivo*.

Perhaps one of the most interesting findings is that targeted expression of IL-10 in popliteal lymph node DCs provides significant protection from the lethal effects of generalized peritonitis. Based upon our studies reported here, only 3% of the DCs in the draining lymph node were transduced following injection with 10^5 particles of adenovirus, and IL-10 expression was localized to the popliteal lymph node and foot pad. Expression of human IL-10 cDNA was not reproducibly observed in any other lymphoid structures or organs, and IL-10 protein was not detected in the circulation.

A central question is why compartmentalized local expression of IL-10 improves the outcome in sepsis, while systemic production does not. Previous studies examining the role of systemic endogenous IL-10 in the pathogenesis of sepsis are somewhat confounding, but some suggest that systemic expression of this cytokine contributes to immune suppression and mortality following sepsis (163, 176). Other studies with systemic administration of exogenous IL-10 have also demonstrated increased mortality (180). As described in the previous studies, we demonstrated that intravenous administration of recombinant adenovirus expressing IL-10 failed to protect mice from mortality in an identical cecal ligation and puncture model of sepsis. In contrast, when the same vector was administered intrathymically, survival was significantly improved (114). In these same studies, intrathymic injection of adenovirus led to local expression primarily in DCs, whereas the non-protective intravenous administration of adenovirus resulted in hepatocyte transduction and the systemic appearance of IL-10. We are, therefore, lead to postulate that local, contained expression of IL-10 in certain primary and secondary lymphoid organs may promote host survival during the sepsis response in contrast to the systemic expression of this cytokine. This hypothesis is consistent with studies where high systemic levels of endogenous IL-10 predict adverse outcome in septic and trauma patients (181), presumably by causing generalized immune suppression and a predominant Th_2 response, as is seen after thermal injuries and generalized peritonitis (182).

Beyond the issue of local expression, the capacity to target DCs and the ability of IL-10 to maintain an immature DC phenotype in a lymphoid structure may be of critical importance in regulating the sepsis response. Previous studies suggest that DCs are lost from tissues during sepsis (24, 67), perhaps due to activation-induced cell death by systemic TNFα, compromising the acquired immune responses to pathogens. Our *in vitro* studies suggest that IL-10 expression in Adv/hIL-10 transduced DCs does not suppress increases in CD86 and MHC class II antigens following LPS stimulation, but does limit IL-12 production. Therefore, the presence of immature or mature DC without the capacity to produce normal amounts of IL-12 could promote the generation of regulatory T cells (183) that may limit aspects of the acquired or innate immune responses in the setting of sepsis.

As we did not assess the cell phenotype from the footpad of adenovirus injected mice, these studies may underestimate the total number of DCs which were transduced. If tissue myeloid DCs in the footpad were transduced at the site of injection, IL-10 expression may have interfered with the maturation of these cells, limiting their expression of chemokine receptors, and their ability to migrate to the draining lymph node. In fact, Takayama and colleagues demonstrated that exposure of immature DCs to IL-10 prevents their expression of CCR7, and impairs the homing of these cells to secondary lymphoid tissues (184). Furthermore, IL-10 exposure of DCs results in their expression of chemokine decoy receptors that fail to signal and elicit migration, and serve only as a sink for locally-produced inflammatory chemokines (185).

Overall, these studies provide a new *in vivo* approach to modify murine DC function that allows direct manipulation of the immune response. Our data also demonstrate that gene targeting of DCs with IL-10 may also be useful for acute infectious diseases, such as sepsis syndromes. Although the mechanism of action is not resolved, compartmentalized expression of IL-10 in DCs clearly alters the response to sepsis. Further studies will be required to more fully delineate the cellular mechanisms by which compartmentalized modification of DC function impacts host immunity and outcome in sepsis.

4.4. Reinjection of novel dendritic cell phenotype by adenoviral expression of interleukin-10 improves survival in sepsis

4.4.1. Summary

Control of dendritic cell (DC) maturation and function is critical for strategies to modulate innate and acquired immune responses. We examined whether transduction of murine DCs with adenoviral vectors expressing IL-10 could alter their responsiveness to inflammatory stimuli. Murine bone marrow-derived DCs were transduced *in vitro* with either an empty adenoviral vector, or one expressing GFP, or human IL-10. *In vitro* transduction of immature DCs with adenovirus expressing gfp resulted in dose-dependent maturation. Transduction with adenovirus expressing human IL-10 (Adv/IL-10) maintained DCs in an immature state with low MHC class II, CD86, and IL-12 expression. Although these DCs were responsive to additional maturation stimuli, the resulting DCs were phenotypically unique, characterized by a continued ability to endocytose antigen, a reduced production of IL-12, and the failure to drive Th1 or Th2 responses. Administration of these DCs to mice prior to a generalized peritonitis improved outcome. We conclude that murine DCs are readily transduced with adenoviral vectors *in vitro*, and their maturation state can be modulated by autocrine production of IL-10. Modifying DCs by *in vitro* adenoviral expression of IL-10 creates a hybrid DC that may provide a novel *ex vivo* approach to modulating acute and chronic inflammatory diseases.

4.4.2. Introduction

Immature myeloid dendritic cells (DCs) readily endocytose antigens and respond to various self and foreign antigens which subsequently modulate their development and function. Unless activated by environmental signals that promote maturation, immature DCs that migrate from tissue sites to draining lymph nodes are likely to be non-immunogenic, as they express low levels of MHC class II and co-stimulatory molecules, and present little peptide antigen (76). Because of their limited capacity to present antigen and to stimulate T cell responses, immature DCs may promote tolerance. In contrast, upon receiving an activating stimulus, immature DCs migrate to the draining lymph nodes and lose their capacity to process antigen. As they proceed

through the maturation process, DCs present antigen efficiently and readily activate T lymphocyte responses as they increase surface expression of MHC-peptide complexes, upregulate costimulatory molecules, and produce essential cytokines (164). Various stimuli are capable of triggering DC maturation, including proinflammatory cytokines, bacterial cell products, viral agents and CD40 ligand (186, 187). Furthermore, DC maturation and function are modulated by other factors from the microenvironment that influence the ability of these cells to stimulate specific lymphocyte responses (e.g. Th1 vs. Th2).

Given their central role in directing immune responses, manipulation of DCs is a promising avenue for the development of cellular therapeutics for inflammatory diseases, autoimmunity, and other immune-mediated processes. Recent studies have demonstrated the feasibility of adenoviral gene transfer and expression to manipulate both human and murine DCs (173, 174, 188, 189). Adenoviral transduction of DCs with specific tumor genes resulted in increased immunogenicity and inhibition of tumor growth and metastases (129). In addition, adenovirus induced gene transfer can be used to directly modulate DC function, and thus, T cell responses, allowing regulation of autoimmune diseases and transplant rejection.

IL-10 is an important cytokine that regulates DC function and development. In addition to its effects on suppressing inflammatory and Th1 responses (190), IL-10 strongly inhibits DC maturation and IL-12 production (171, 191), and can promote the ability of these antigen presenting cells to promote Th2 responses. Certain populations of DCs appear to produce IL-10 which may promote the Th2 responses, or the generation of regulatory T cell populations, (e.g. Tr1) (192). Importantly, localized and regulated IL-10 expression appears to be critical as the systemic presence of this cytokine has unwanted immunosuppressive effects in a number of disease processes. Therefore, localized expression of IL-10 from DCs may provide a more direct therapeutic approach for diseases of exaggerated pro-inflammation or Th1-type responses, such as in autoimmune diseases, transplant rejection, acute bacterial infections, and sepsis.

4.4.3. Results

Adenovirus transfects immature and mature DCs. Past reports vary as to the extent that DCs can be transduced *in vitro* (129, 188). We, therefore, sought to determine the transduction rate of immature (MHC class II and CD86 low expressing) and mature (MHC class II and CD86 high expressing) DCs. Both unstimulated and LPS-matured bone-marrow-derived GM-CSF and IL-4 treated DCs were transduced by a first generation E1, E3 deleted recombinant adenovirus vector with the reporter gene gfp (Adv/gfp) at doses ranging from 10^6 to 10^{10} particles/ml. The number of transduced DCs increased with the number of virus particles, with less than ten percent of the cells transduced at particle concentrations less than 10^8 particles/ml **(Fig. 22A and 22B)**. At concentrations of 10^9 and 10^{10} particles/ml, transduction rates were approximately 30% and 70%, respectively. Surprisingly, there was no significant difference in the transduction rates between DCs that were either immature or had been previously matured with LPS, although it is important to note that the proportion
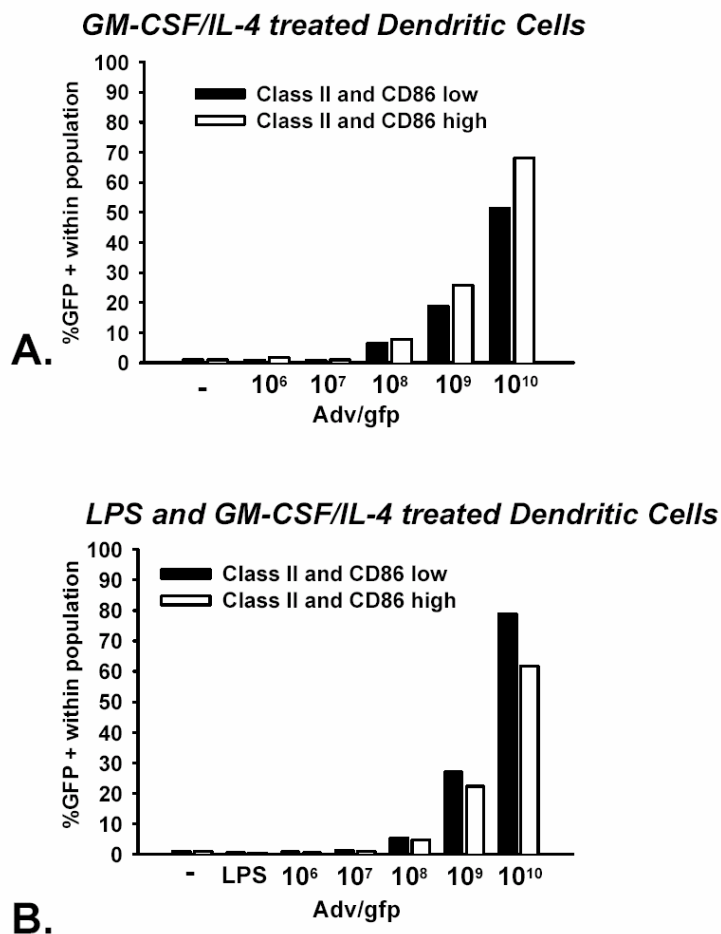

Fig. 22: Transduction efficiency of class II and CD86 low and class II and CD86 high DCs. GM-CSF/IL-4 treated (A) and 24 hour LPS stimulated (B) DCs were incubated with increasing quantities of Adv/gfp. Mature cells were determined by high MHC class II and CD86+ expression whereas cells were considered immature when the expression of both surface markers was low. Intracellular green fluorescent protein (GFP) was measured by flow cytometry gated on live cells. The reported percentage of GFP positive cells represents the percent of that class II and CD86 high or low cell population.

of immature and mature cells were not identical, with over 80% of the DCs being mature following LPS stimulation **(Table 7)**.

Table 7: Transduction efficiency of mature (class II and CD86 high) and immature (class II and CD86 low) DC (n = 5) induced with 10^{10} particles/ml of Adv/gfp. To induce maturation, DCs were stimulated with 1 μg/ml LPS 24 hours prior to transduction with the Adv/gfp.

GM-CSF/IL-4 DCs	Phenotype	% of cells	% gfp pos. cells	gfp MCF[a]
Controls	Mature	36.2 ± 2.7	1.0 ± 0.1	32.6 ± 7.0
	Immature	62.9 ± 3.1	1.0 ± 0.1	12.0 ± 1.0
+ Adv/gfp	Mature	80.7 ± 3.2[b]	69.2 ± 3.2 [b]	329.9 ± 103.7 [b]
	Immature	19.4 ± 3.3	66.3 ± 6.8 [b]	157.9 ± 72.1 [b]
+ LPS	Mature	80.7 ± 4.4[c]	1.1 ± 0.1	16.4 ± 1.4
	Immature	19.4 ± 4.3	1.1 ± 0.1	6.2 ± 1.0
+ LPS + Adv/gfp	Mature	89.8 ± 2.4[c]	53.8 ± 1.0 [b,c]	187.8 ± 67.7 [b]
	Immature	10.3 ± 2.6	63.8 ± 9.7 [b]	79.5 ± 26.7 [b]

[a]mean channel fluorescence; [b]$p<0.05$ DC versus transfected DC; [c]$p<0.05$ unstimulated DC versus stimulated DC

Human IL-10 expression inhibits adenovirus induced maturation of DCs. We next sought to determine whether transduction with the adenovirus itself would drive DC maturation, and whether this process could be influenced by expression of human IL-10. Untreated and LPS-stimulated bone marrow-derived DCs were incubated with a recombinant adenovirus containing an empty cassette (Adv/empty) or with the same vector expressing human IL-10 (Adv/IL-10). As the transduction dose of Adv/empty virus increased, the number of transduced cells that underwent maturation (MHC class II ^{high} and CD86$^+$ expression) also increased **(Fig. 23A)**. Interestingly, transduction with the recombinant expressing human IL-10 blocked this adenovirus-induced maturation. The percent of mature DCs decreased from 50-80% to 20-30% when transduced with Adv/IL-10 at concentrations of 10^9 particles/ml or greater **(Fig. 23A)**. As anticipated, LPS stimulation increased the percentage of mature DCs **(Fig. 23C)**, and subsequent infection with Adv/empty only modestly enhanced further
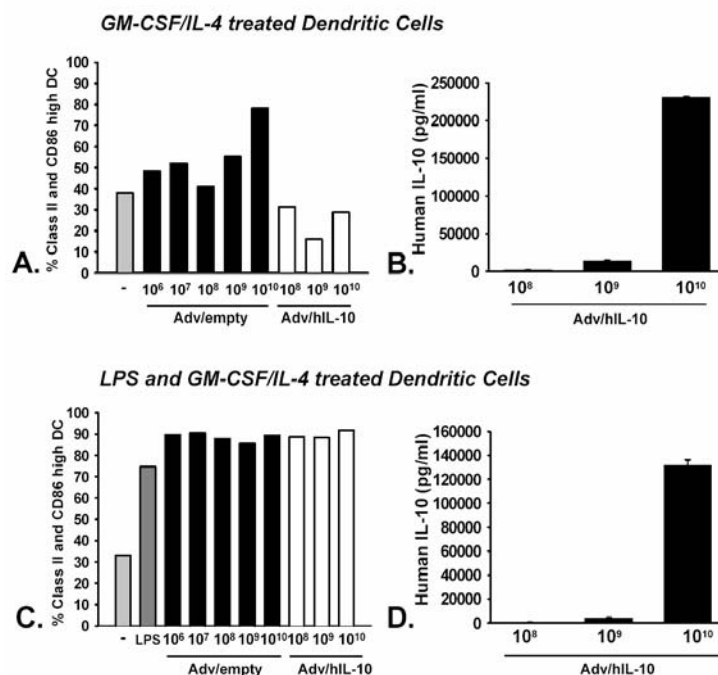

Fig. 23: DC maturation induced by adenovirus transduction and human IL-10 expression. GM-CSF/IL-4 treated (A) and 24 hour LPS stimulated (C) DCs were incubated with increasing quantities of Adv/empty and Adv/hIL-10 for two hours. DC maturation was determined by flow cytometry, gated on live cells. Mature DCs were determined by high MHC class II and CD86 expression. Twenty four hours following transduction with Adv/hIL-10 for 2 hours, human IL-10 secretion was determined in the supernatants of DCs (B, D). GM-CSF/IL-4 treated DCs were either unstimulated (A,B) or stimulated with LPS (C,D), and transduced with 10^8, 10^9 and 10^{10} particles/ml.

maturation. DC maturation induced by LPS was not affected by subsequent transduction of DCs with Adv/IL-10 **(Fig. 23C)**, suggesting that IL-10 expression could not reverse maturation induced by LPS.

To assess the degree of transgene expression in DCs transduced by Adv/IL-10, human IL-10 secretion was determined in the DC supernatants **(Fig. 23B and 23D)**. In both unstimulated DCs, as well as in LPS-stimulated DCs, there was a dose dependent increase in human IL-10 release in the cell supernatants. Levels in the former group were approximately two-fold increased (230'300 ± 1'400 pg/ml), compared to the LPS stimulated cells (131'800 ± 4'500 pg/ml), despite similar transduction rates **(Fig. 22)**.
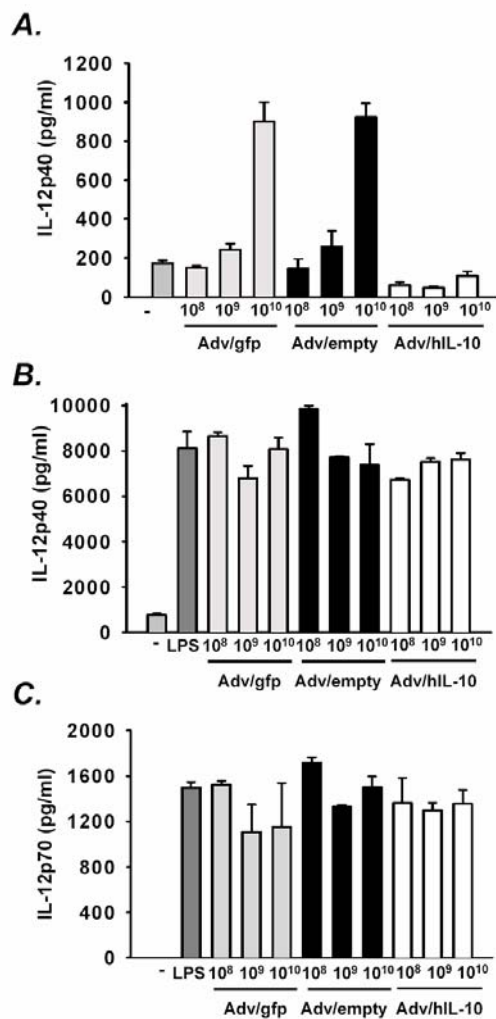

Fig. 24: IL-12 secretion by DCs following adenoviral transduction. Unstimulated (A) and LPS stimulated (B) GM-CSF/IL-4 treated DCs were transduced with Adv/gfp, Adv/empty and Adv/hIL-10 at increasing particles/ml. IL-12p40 was determined in supernatants of unstimulated and stimulated DCs, whereas IL-12p70 (C) was evaluated in only stimulated DCs 24 hours following incubation with the respective vectors.

DC function is modulated by IL-10 expression. IL-12p70 is produced by mature DCs (193) whereas p40, a component of the IL-12p70 heterodimer is produced by both immature and mature DCs (194). As anticipated, robust production of IL-12p40 (7'000 – 10'000 pg/ml) was noted in the supernatants of LPS treated DCs, and adenovirus transduction did little to increase this **(Fig. 24B)**. In contrast, markedly lower levels of p40 were observed in unstimulated DCs, which increased in a dose dependent manner in cultures of DCs transduced with increasing quantities of adenovirus **(Fig. 24A)**. Accordingly, unstimulated GM-CSF/IL-4 treated DCs transduced with Adv/IL-10 showed reduced IL-12p40 release, with concentrations ranging from 47-108 pg/ml **(Fig. 24A)**. Transduction of DCs with Adv/IL-10 after maturation with LPS, however, did not affect IL-12 p40 or p70 production, which is consistent with previous reports of an absence of IL-10 regulation of IL-12 production in mature DCs (179) **(Fig. 24B and 24C)**.

Next, we studied the effects of adenovirus transduction and IL-10 expression on the ability of DCs to activate CD3$^+$ T lymphocyte responses. Mixed lymphocyte reactions (MLR) were established in which DCs were transduced with 10^{10} particles/ml of either Adv/empty or Adv/IL-10, washed and then placed in culture with purified syngeneic CD3$^+$ T cells. Previous studies demonstrated that IL-12, produced by DCs, promotes a Th1 cell response (e.g. IFN-γ production) (186, 195). As IL-12 production was decreased following Adv/IL-10 transduction, it was anticipated that Th1 cytokine production by syngeneic T-cells would also be decreased. Indeed, IFN-γ, as well as IL-2 production, were significantly reduced in the co-cultures containing DCs transduced with Adv/IL-10 **(Fig. 25A and 25B)**. Surprisingly, although IL-4 levels were only slightly above the assay sensitivity, T-cells co-cultured with DCs transduced with Adv/empty produced more IL-4, a Th2 cytokine, than T-cells co-cultured with DCs transduced with Adv/IL-10 **(Fig. 25C)**.
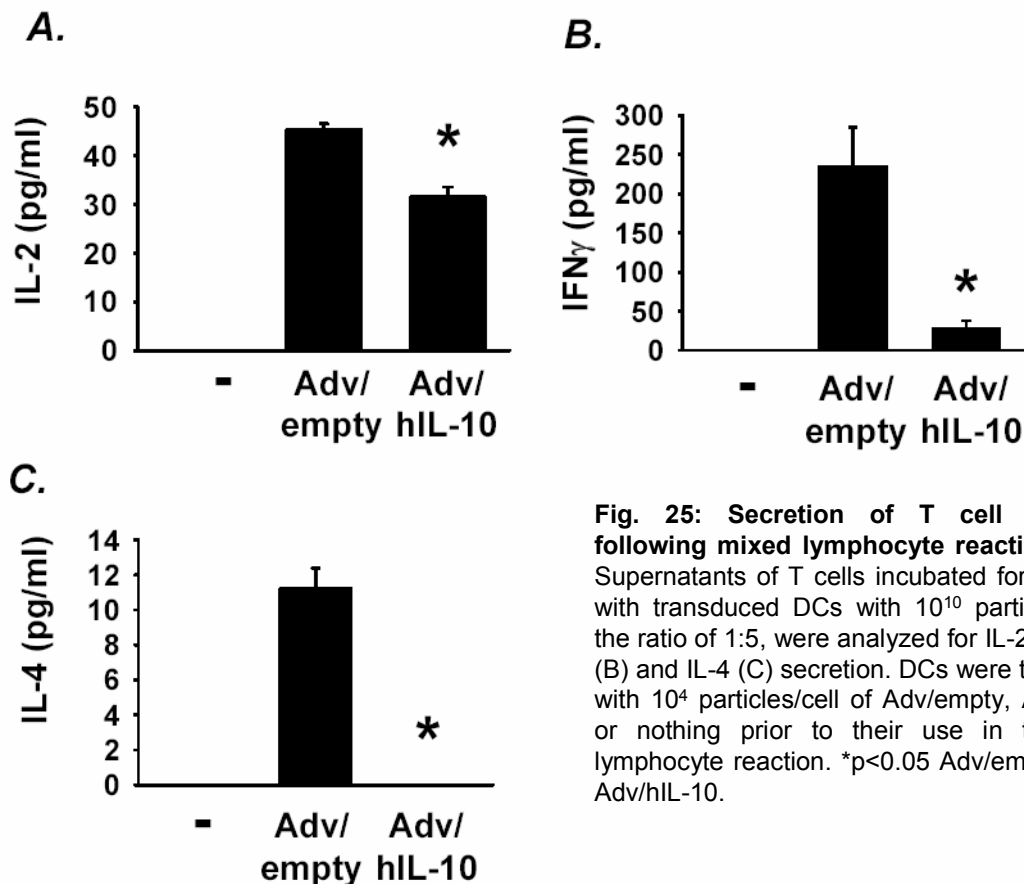
A.

B.

C.

Fig. 25: Secretion of T cell cytokines following mixed lymphocyte reaction (MLR). Supernatants of T cells incubated for four days with transduced DCs with 10^{10} particles/ml, at the ratio of 1:5, were analyzed for IL-2 (A), IFN-γ (B) and IL-4 (C) secretion. DCs were transduced with 10^4 particles/cell of Adv/empty, Adv/hIL-10 or nothing prior to their use in the mixed lymphocyte reaction. *p<0.05 Adv/empty versus Adv/hIL-10.

Production of human IL-10 and endogenous IL-12p40 were confirmed in this experiment. Human IL-10 concentrations were 126 ± 2 ng/ml, whereas IL-12p40 concentrations were 520 ± 150 pg/ml in co-cultures containing DCs transduced with the Adv/IL-10 vector. In contrast, IL-12p40 concentrations were 1'700 ± 350 pg/ml in the co-cultures containing DCs transduced with the Adv/empty vector.

Pre-exposure of DCs to adenovirus expressing IL-10 does not influence subsequent phenotypic maturation to LPS/antiCD40, but alters their function. As a potential therapeutic use of *ex vivo* manipulated DCs is being considered for modulation of inflammatory diseases, we wished to determine whether DCs transduced with Adv/IL-10 are resistant to further maturation with factors they may be exposed to *in vivo*, such as LPS or CD40L. Initially, the transduction efficiency and

then the maturation state of the DCs were determined. Following *ex vivo* transduction of DCs with 10^{10} particles/ml of Adv/gfp **(Fig. 26)**, cells were stimulated with LPS or anti-CD40 antibody. Stimulation with anti-CD40 and LPS induced maturation (MHC class II [high] and CD86^{+} expression) in over 80% of the cells **(Fig. 26A)**. There was no difference in the percentage of mature cells between LPS and anti-CD40 stimulus, nor were there any differences in the proportion of mature cells with regard to whether cells were transduced with Adv/empty or Adv/IL-10. Therefore, unlike previous studies where exogenous IL-10 added to DC cultures blocked the maturation responses to LPS (179), production of this cytokine by DCs transduced with adenovirus could not prevent subsequent maturation when these cells were exposed to activating stimuli.
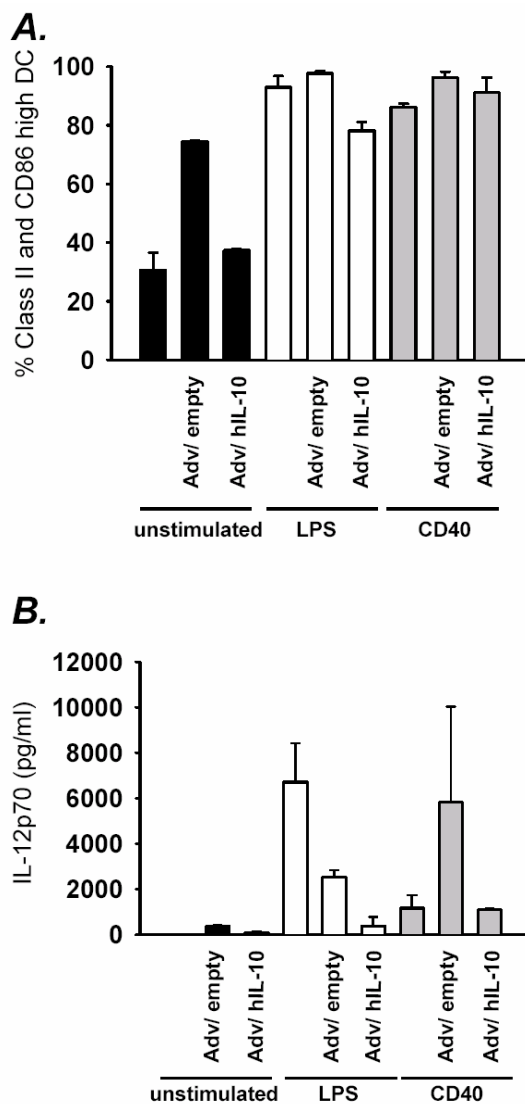

Fig. 26: Transduction and maturation of DCs stimulated with LPS or anti-CD40 antibody post-incubation. 10^{10} particles/ml of Adv/gfp, Adv/empty and Adv/hIL-10 were incubated with GM-CSF/IL-4 treated DCs for two hours, and then stimulated with either LPS or anti-CD40. Maturation status (A) was determined by flow cytometric analysis and IL-12p70 was determined in the supernatants (B).

However, the DCs first transduced with adenovirus expressing IL-10 and then matured with either LPS or anti-CD40 remained functionally immature. For example, IL-12p70 secretion by the mature DCs exposed to LPS or anti-CD40 was suppressed by human IL-10 expression, although slightly increased compared to baseline **(Fig. 26B)**. Of interest, there appeared to be a general inhibitory effect of adenovirus transduction on IL-12p70 production when stimulated with LPS, which was not seen with a CD40 stimulus. The lack of IL-12p70 secretion is consistent with an immature DC phenotype.

Furthermore, we wanted to determine whether other characteristics of immature DCs were maintained. Therefore, we examined the endocytic capacity of DCs transduced with Adv/IL-10 and then exposed to maturation stimuli. Specifically, we examined dextran uptake mediated by the mannose receptor typically expressed on immature but not mature DCs (125). DCs were cultured at either 37° C or at 4° C
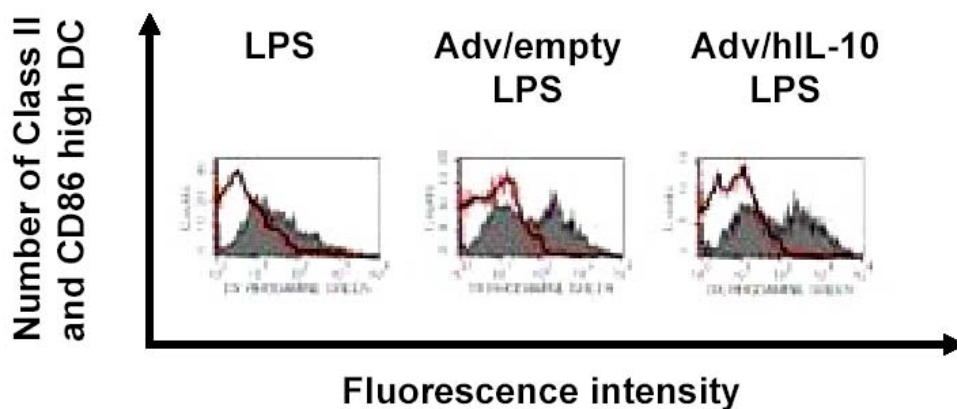

Fig. 27: Endocytic capacity of DCs. Endocytosis of rhodamine-green dextran was determined in GM-CSF/IL-4 derived DCs that were stimulated with LPS, following transduction with adenovirus vectors. Live cells were gated and DC populations were defined by high CD86 and high MHC class II expression (mature), or low CD86 and low MHC class II (immature) expression. Full gray histogram represents cells incubated at 37°C and white histogram background (4°C incubation).

(background), and the dextran-rhodamine green uptake was determined after four hours of incubation. Transduction of DCs with both adenoviral vectors increased the endocytic capacity of DCs as compared to non-transfected LPS stimulated DCs, which otherwise typically have reduced endocytic activity **(Fig. 27)**. Once again, the adenovirus infected cells demonstrate immature DC functional activity. Interestingly, the endocytic capacity of adenovirus transfected cells was also increased compared to that of immature cells (*data not shown*).

To evaluate the function of these unique DCs on T cell activation, syngeneic mixed lymphocyte reactions were performed. DCs were first incubated with 10^{10} particles/ml of one of the two viral vectors, and thereafter, stimulated with LPS for 24 hours and then extensively washed. After four days of co-culture of DCs with syngeneic splenocytes, the T cell cytokines IL-2, IL-4 and IFN-γ were determined in the cell supernatants **(Fig. 28)**. IL-12p70 was also measured in the cell supernatants as a parameter for DC maturation. Interestingly, production of all of the cytokines was reduced in conditions where stimulator cells were DCs that had been transduced with Adv/IL-10 prior to the mixed lymphocyte reaction. Similar results were also seen using CD40 activated DCs (*data not shown). As anticipated, IL-12p70 concentrations were also reduced. These data suggest that DCs transduced with an adenoviral vector expressing IL-10 and then matured with either LPS or CD40 do not stimulate either type of Th response.
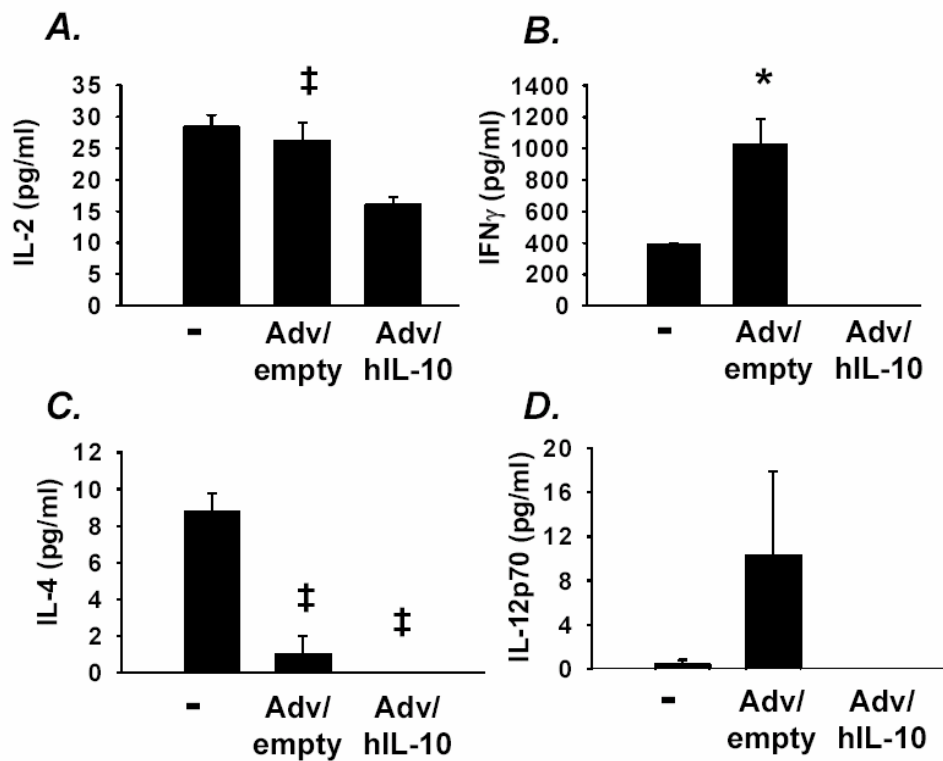
Fig. 28: Secretion of T cell cytokines following the mixed lymphocyte reaction (MLR). GM-CSF/IL-4 treated DCs were first transduced with the adenoviral vectors and then stimulated with LPS for 24 hours. After being washed, they were co-cultured with T cells for four days and analyzed for IL-2 (A), IL-12p40 (B), IFN-γ (C) and IL-4 (D) secretion. *$p < 0.05$ Adv/empty versus Adv/hIL-10. ‡$p < 0.05$ LPS alone (-) versus the adenovirus groups.

We have previously demonstrated that DCs migrate to the draining lymph node following injection into the footpad, and potently modulate immune responses (117, 196). In this study, we examined the effect of DCs transduced with Adv/IL-10 on the outcome of an acute polymicrobial sepsis. DCs were either transduced with 10^{10} particles/ml of Adv/IL-10 or Adv/empty, or were incubated with buffer alone. These cells were washed and then injected into the hind footpad
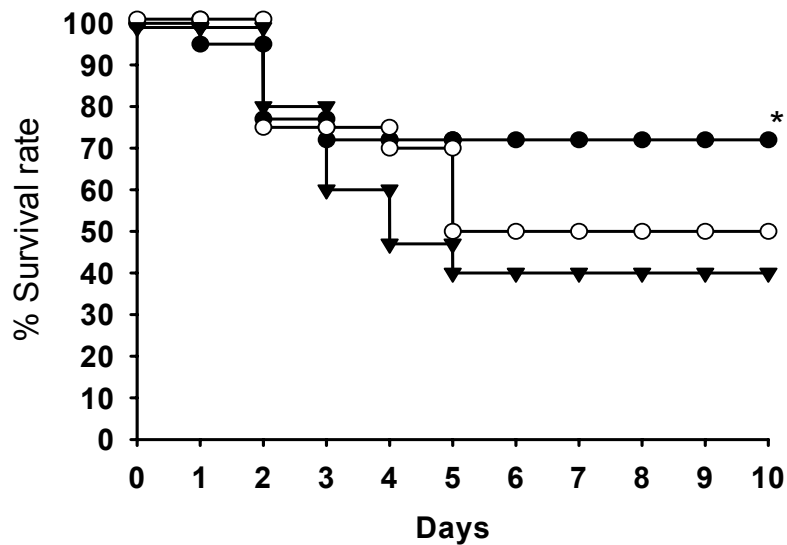
Fig. 29: Survival rate of mice injected with transduced DCs. 3.2 x 105 DCs were transduced with 10^{10} particles/ml of Adv/empty (○), Adv/hIL-10 (●) or incubated with buffer (▼), and were readministered into the hind foot pad of mice (n = 20 per study group). Shortly thereafter, cecal ligation and puncture was performed. Animals receiving DCs transduced with Adv/hIL-10 had the highest survival rate of 70% compared to the other two groups. *p<0.05 Adv/hIL-10 versus Adv/empty and buffer.

(3.2 x 10^5 DCs/recipient) of recipient mice that immediately underwent cecal ligation and puncture. Animals receiving DCs transduced with Adv/IL-10 had significantly improved outcome compared to animals receiving DCs transduced with Adv/empty or just buffer **(Fig. 29)**. The production of human IL-10 was apparently restricted to the lymph nodes, since human IL-10 was not recovered from the plasma of septic animals pretreated with Adv/IL-10 transduced DCs (*data not shown*).

4.4.4. Discussion

The present study explored the capacity of adenovirus to transduce murine bone marrow-derived DCs, and whether adenoviral-induced expression of human IL-10 could alter DC phenotype in response to an *ex vivo* maturation and inflammatory challenge, and improve outcome to an acute inflammatory challenge. First, we have demonstrated that adenoviral induced expression of human IL-10 prevents

adenovirus-induced maturation of DCs. Second, we have shown that transduction of DCs with adenoviral vectors expressing human IL-10 can also modify their subsequent maturation induced by either LPS or anti-CD40, yielding a DC population with characteristics of both immature and mature cells. Transduced DCs expressing human IL-10 are poor stimulants of a syngeneic T cell mixed lymphocyte reaction, preventing both a Th1 and Th2 type secreted cytokine response. And finally, we have shown that when these cells are administered to animals prior to an acute, lethal, inflammatory challenge, outcome is improved.

Recent studies have reported decreased transduction rates of mature compared to immature DCs (136, 173). Here, we demonstrate that both immature and mature murine bone-marrow-derived DCs can be transduced with similar efficiencies by a first generation E1, E3 deleted recombinant adenovirus. Even after exposure of the cells to the maturation stimulus leading to over 70% mature DCs, the majority of these cells could still be transduced with an adenoviral vector. Interestingly, in the study of Rea, the transduction efficiency of mature DCs was dependent on the nature of the maturation stimulus (136). Maturation induced by TNFα, for example, led to a transduction rate of approximately 75%, whereas DCs matured with LPS demonstrated transduction rates of only 25%. These differences may be due to the different origin of DCs, as earlier studies employed DCs obtained from human blood. Moreover, relative adenovirus particle to cell numbers were required to be high ($>10^3$ particles/cell) to achieve transduction rates in excess of 30% (188, 197).

In addition, we have now shown that transduction of DCs with the adenoviral vector increased the maturation status of the cells, as determined by high MHC class II and CD86 expression, and the increase in the proportion of mature cells was adenovirus dose-dependent. Other studies have also noted that transduction of adenovirus induces maturation of DCs with upregulation of surface molecules, such as CD54, CD86 and MHC class II, via an NF-κB dependent pathway (136, 174, 198). In contrast, Zhong et al. concluded that adenovirus is a non-perturbing vector (173), but these studies were performed with DCs of human origin. In the present study, the percent of DCs with high level CD86 and MHC class II expression increased with increasing viral number. This is in line with the findings of Morelli and colleagues who

also examined the expression of these two surface molecules, and demonstrated similar results (174).

Importantly, our results further indicate that this effect can be blocked by transduction with 10^{10} particles/ml of the adenoviral vector expressing human IL-10, with nearly 40% fewer mature cells. This inhibition of adenovirus-induced DC maturation might be of relevance in situations where the immunogenicity towards virally transfected DCs is not desired. Although this was not the goal of the present study, the findings suggest that DC maturation and adenoviral antigen presentation may be inhibited by IL-10 expression.

Exogenous IL-10 administration has been shown to decrease expression of MHC class II and costimulatory molecules such as CD58 and CD86 on antigen presenting cells (179, 197). This study confirms this observation, demonstrating that transduction with Adv/IL-10 and production of human IL-10 limited DC maturation in response to the adenoviral vector itself. However, the effects of this cytokine are dependent upon the maturation state of the DC, as human IL-10 expression does not inhibit DC function (e.g IL-12p70 production) when mature cells are transduced with virus. These results are consistent with the lack of previously described effects of IL-10 on mature DCs (199).

DC expression of human IL-10 also affects the stimulatory capacity and ability of DCs to polarize T cell responses, as IL- 2, IL-4 and particularly IFN-γ production were suppressed in syngeneic mixed lymphocyte reactions. This is in line with previous studies demonstrating reduced expression of IL-12 mRNA and IL-12p40 secretion when DCs were exposed to exogenous IL-10 (171). Furthermore, in these same studies, IFN-γ production was also reduced following incubation of IL-10 treated DCs with lymph node cells, but in contrast to our results, IL-2 and IL-4 were increased. These differences are possibly due to the origin of the DCs, as they used splenic DCs, and not DCs derived from bone-marrow. In addition, IL-10 expression in the context of immunostimulatory recombinant adenoviral infection may alter the phenotype of the resulting DC subpopulation. In contrast to our study, DCs propagated in GM-CSF and TGF-β, which are transduced with an adenovirus expressing murine IL-10, have increased allostimulatory activity leading to a strong alloantigen specific cytotoxic T cell activation with increased transplant rejection,

despite modestly decreased DC surface costimulatory molecules (200). Our findings are not consistent with these results, and these differences again may be due to differences in the culture of the DC populations (TGF-β versus IL-4), as well as the transgene expressed (human versus murine IL-10).

We were also interested in determining whether the phenotype of immature DCs transduced with Adv/IL-10 was resistant to maturation stimuli. Under *in vitro* experimental conditions, prior exposure of DCs to adenovirus vectors expressing human IL-10 did not prevent DC maturation by LPS or anti-CD40. Although the cells appeared phenotypically mature (high MHC class II and CD86 expression), they had functional characteristics more consistent with an immature state. For example, IL-12p70 production was reduced following LPS stimulation independent of cytokine gene expression (e.g. Adv/empty or Adv/hIL-10); however, IL-12p70 production was further reduced in DCs transduced with the adenovirus vector expressing IL-10 when stimulated with anti-CD40 or LPS.

Previous studies demonstrated that IL-10 maintains other functional aspects of immature DCs, such as sustaining the capacity of these cells to macropinocytose and endocytose particles (191). In these studies, we demonstrate that this functional characteristic of DCs was perturbed. We found that adenovirus transduction sustained the capacity of DCs for uptake of dextran particles following a maturation stimulus. These data further suggest that recombinant adenoviral vectors can modify the LPS stimulated developmental programs of murine DCs. These results suggest that adenoviral transduction and expression of human IL-10 can induce a phenotypically unique DC population with reduced IL-12 production and increased endocytosis consistent with an immature phenotype, but increased expression of costimulatory surface molecules associated with mature phenotypes.

Furthermore, our data support the hypothesis that inhibition of full functional DC maturation may be of importance for modulating disease processes associated with an exaggerated immune response, such those occurring in acute infections. Septic mice pretreated with *ex vivo* transduced DCs expressing IL-10 demonstrated a significant reduction in mortality. In two previous studies, we observed that footpad or direct thymic injection of the same adenoviral recombinant expressing human IL-10 also improved survival in this model of cecal ligation and puncture (114, 117). In both

cases, DCs were a primary *in vivo* target for the adenoviral recombinants, and following footpad injection of the adenovirus expressing human IL-10, DC maturation in the draining lymph node was inhibited (114, 117). Following thymic injection of adenovirus expressing human IL-10, the increases in sepsis induced lymphocyte apoptosis and caspase-3 activation were significantly attenuated, suggesting that transduction of DCs, and presumably modification of their phenotypic response to sepsis, attenuated activation induced cell death and apoptosis of resident lymphocyte populations (114, 117). The current studies extend these findings by demonstrating that comparable improvements in survival can be obtained when the DC populations are transduced *ex vivo* and readministered to the animal, thus providing a more direct association between DC transduction and improved outcome.

Taken together, these studies highlight a central role that DC subpopulations may play to regulate T cell responses in septic hosts and to alter clinical outcome. It is interesting that pretreatment of DCs with adenoviral recombinants expressing IL-10 prior to LPS stimulation was required to modify their response to this potent activation stimulus. This effect on DCs was very similar to animal studies where anti-cytokine therapies had to be administered prior to the induction of sepsis (12), indicating that prophylactic treatment for sepsis is more effective than treatments applied subsequent to sepsis induction. Although mortality from polymicrobial sepsis is complex and multifactorial, recent studies have suggested that increased activation induced cell death of CD4^{+} T-helper cell populations may contribute to this process (149). It was suggested that increased apoptosis of lymphoid cell populations is responsible for an adverse outcome in this model (1). One possible explanation for these results from both direct *in vivo* administration of adenovirus expressing IL-10 (117) or *ex vivo* transduction of DCs with the same adenoviral recombinants may be that by interfering with DC maturation and the ability to present antigens to T-cells, mice are protected from an exaggerated acquired immune response, and deletion of CD4^{+} T cells through activation induced cell death (apoptosis). An alternative explanation is that the interaction between IL-10 expressing DCs and resting T cells results in a T cell population phenotypically distinct from either Th1 or Th2 cells that regulate the inflammatory process. This may explain our findings where incubation of DCs expressing IL-10 with splenocytes resulted in a secreted cytokine profile

consistent with a decrease in both Th1 and Th2 responses. Jonuleit et al. have shown that repeated stimulation of T cells with immature DCs induces a T cell response different from mature DCs, and consistent with the generation of a regulatory, Tr1-like cell population (201). If the transferred DC population maintained an immature state, then these Tr1-like mechanisms may have been active. Our experiments suggest that the presence of this unique DC population, induced by IL-10 expressing adenovirus transduction, may be essential in this model. Recent studies support this suggestion as pulmonary DCs with high co-stimulatory molecule and MHC expression that produce IL-10, similar to our Adv/IL-10 population, induce regulatory T cell responses (192). These T cells may be responsible for maintaining peripheral tolerance during the healthy state, and may have played a critical role in modulating the magnitude of the inflammatory response to the microbial infection. The generation of these Tr1-like cells has been proposed as a potential mechanism for the treatment of experimental colitis (202), and as a means to directly modulate the inflammatory response (203).

In conclusion, these studies provide important information regarding the effects of adenoviral vectors on DC biology that have essential implications for host-vector interactions. In addition, they also demonstrate that the expression of human IL-10 by DCs can alter their phenotype and function, such that they strongly modulate lymphocyte responses.