Renal transplantation is the most successful treatment option for cats with end-stage renal disease, but its success is dependent on the use of global immunosuppression to prevent allograft rejection. Immunosuppressive treatment currently used in cats consists of the calcineurin inhibitor cyclosporine in combination with dexamethasone, or human CTLA4-Ig (10 g/mL). Cells from transplant recipients were stimulated with Con A alone. An ELISA was performed to measure production of interferon (IFN)-γ, granulocyte macrophage–colony stimulating factor (GM-CSF), interleukin (IL)-2, IL-4, and IL-10. Proliferation of CD4+ and CD8+ T cells from immunosuppressed cats were also evaluated. Pairwise comparisons were performed via a Wilcoxon signed rank test or Wilcoxon rank sum test.

Results—Cyclosporine, dexamethasone, cyclosporine-dexamethasone combination, and CTLA4-Ig caused a significant decrease in IL-2, IFN-γ, and GM-CSF production. Cyclosporine and cyclosporine-dexamethasone, but not human CTLA4-Ig, caused a significant decrease in IL-10 production. High basal concentrations of IL-2 and IL-10 were identified in transplant recipients, and IL-10 was significantly increased in stimulated cultures. In immunosuppressed cats, there was a decrease in frequency of responders and proliferative capacity of CD4+ and CD8+ T cells.

Conclusions and Clinical Relevance—CTLA4-Ig successfully inhibited proinflammatory cytokines while sparing cytokines critical for allograft tolerance. These data may be useful for developing better strategies to prevent rejection while sparing other immune functions.

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The clinical and histologic features of renal allograft rejection have been characterized in cats.\(^\text{14}\) Immunohistochemical analysis performed on rejected kidneys revealed that similar to humans, the inflammatory infiltrate was dominated by lymphocytes with subpopulations consisting mostly of CD4+ (Th) cells and a few CD8+ (T-cytotoxic) cells. A sparse number of macrophages was also evident in areas of inflammation. Activated Th cells can be subdivided into multiple categories (Th1, Th2, Th17, and Treg) on the basis of function and the cytokines they produce.\(^\text{15-22}\) Several cytokines (eg, IFN-\(\gamma\), IL-2, IL-4, IL-10, and GM-CSF) have been implicated in experimental animals and in humans as playing a role in acute and chronic allograft rejection.\(^\text{23-28}\) The Th1 cytokines (including IFN-\(\gamma\) and IL-2) contribute to rejection by activating the cellular immune response (including cytotoxic T cells, monocytes, and natural killer cells). It is believed that IL-2 is a driving force for acute cellular rejection; however, it is only 1 component of the rejection process. In contrast, IL-4 and IL-10 are pivotal in humoral responses and may play a role in the vasculopathy and fibrosis associated with chronic rejection or allograft tolerance.\(^\text{24,25,29}\)

With the many drawbacks associated with the immunosuppressive treatments currently used, research in the field of transplantation immunology has focused on the development of strategies that would prevent graft rejection but that do not cause global immunosuppression associated with increased susceptibility to infection. It currently is proposed that naive T cells require 2 distinct signals for activation. The first signal is through the interaction of the T-cell receptor with its cognate antigen presented in the context of the major histocompatibility complex on the surface of APCs.\(^\text{30-32}\) The second, or costimulatory, signal is not antigen specific and is between various receptors and their ligands.\(^\text{33}\) Many molecules have been identified that can serve as receptors for costimulatory signals, including the CD28 molecule on T cells and its counter receptors, which include the B7-1 (CD80) and B7-2 (CD86) molecules on APCs.\(^\text{31,32}\) The interaction between B7 on APCs with CD28 on T cells is one of the most important costimulatory interactions for T-cell activation. Activated T cells then direct immune responses through the production of cytokines, which in turn induce the activation and growth of other immune effector cells. The T cells that receive the first signal without receiving the second signal become anergic and are not able to respond to their cognate antigen. Therefore, 1 promising strategy to limit T-cell responses is the transient blockage of costimulatory signals that are essential for activation of naive T cells required for graft rejection.\(^\text{30}\)

The novel immunosuppressant human CTLA4-Ig is a potent antagonist of the B7-CD28 interaction. In animals experimentally used to evaluate transplantation, human CTLA4-Ig has been found to inhibit acute rejection and significantly prolong survival of transplanted organs.\(^\text{33-35}\) Additionally, in a study\(^\text{36}\) conducted by our research group, we found that compared with the effects of cyclosporine, human CTLA4-Ig had a sparing effect on antigen-specific proliferation of both CD4+ and CD8+ memory cells. These results suggest that human CTLA4-Ig may prevent immune responses to novel antigens such as grafts but that it may leave memory responses to previously encountered pathogens intact. In the aforementioned study\(^\text{37}\) conducted by our research group, we focused on T-cell proliferation in cats; however, to our knowledge, the effects of immunosuppressive drugs on cytokine production have not been evaluated. The purpose of the study reported here was to determine the effect of immunosuppressive drugs on cytokine production by lymphocytes of clinically normal cats and to assess whether these variables are altered in cats that have undergone renal transplantation.

**Materials and Methods**

**Animals**—Twenty-one healthy domestic shorthair cats that ranged from 1 to 3 years of age and 5 client-owned cats that underwent renal transplantation at the University of Pennsylvania School of Veterinary Medicine because of chronic renal failure were included in the study. The 5 cats that underwent renal transplantation included 3 castrated male domestic shorthair cats, 1 castrated male Siamese, and 1 spayed female domestic shorthair cat; these cats ranged from 9 to 15 years of age, and all 5 of them were receiving immunosuppressive treatment consisting of administration of cyclosporine in combination with prednisolone. Approval by the Institutional Animal Care and Use Committee at the University of Pennsylvania for the study as well as owner consent for participation of the 5 renal transplant recipients was obtained.

Blood samples were obtained via jugular venipuncture. Samples (3 to 4 mL) were collected into tubes containing EDTA, and PBMCs were isolated via density gradient centrifugation by use of a technique described elsewhere,\(^\text{44}\) with slight modifications. Briefly, PBMCs were isolated by use of Ficoll gradient solution\(^\text{4}(\text{density, 1.077})\) and centrifugation at 250 \(\times\) \(g\) for 20 minutes at 22°C. The mononuclear cell layer was washed twice in DPBSS without calcium and magnesium\(^\text{4}\) and centrifuged at 184 \(\times\) \(g\) for 10 minutes. The pellet was resuspended in 800 \\(\mu\)L of DPBSS.

**Lymphocyte culture and analysis**—Culture medium consisted of RPMI medium\(^\text{2}(250 \text{ mL})\) supplemented with 2.5% fetal calf serum\(^\text{2}(50 \text{ mL})\) as well as 100 U of penicillin/mL and 0.1 mg of streptomycin/mL. Stock solutions of a costimulation-dependent mitogen (Con A\(^\text{4}(200 \mu\text{g/mL})\) and the immunoglobulin fusion protein human CTLA4-Ig\(^\text{4}(1 \text{ mg/mL})\) were used at a concentration of 10 \(\mu\text{g/mL}\). Cyclosporine\(^\text{4}(50 \text{ mg/mL})\) was used at a concentration of 0.05 \(\mu\text{g/mL}\), and dexamethasone (4 \(\mu\text{g/mL}\)) was used at a concentration of 1 \(\times\) \(10^{-4}\)M. Dosages were based on results of other studies.\(^\text{45,46}\) The PBMCs obtained from the clinically normal cats were plated in triplicate at a concentration of 2 \(\times\) \(10^6\) cells/mL; 100 \(\mu\text{L}\) (2 \(\times\) \(10^7\) cells) was placed in each well. Cells were cultured in medium alone, stimulated with Con A alone, or stimulated with Con A and human CTLA4-Ig, cyclosporine, dexamethasone, or a combination of cyclosporine plus dexamethasone. Cells from cats that underwent renal transplantation were labeled with CFSE\(^\text{4}(0.8\text{mM})\), as described in another study.\(^\text{47}\) These cells were plated in triplicate at concentrations of < 1 \(\times\) \(10^9\) cells/mL (4 cats) or 2 \(\times\) \(10^9\) cells/mL (1 cat). Cells were plated with medium alone or were stimulated with Con A.

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**Notes**

Flow cytometry—Cells from cats that underwent renal transplantation were labeled with mouse anti-feline CD4-R-phycocerythrin conjugate (0.1 mg/mL) or mouse anti-feline CD8-R-phycocerythrin conjugate (0.1 mg/mL). Briefly, cells were washed with FACS buffer (1% BSA and 0.01% NaN₃ in 1X PBS solution) and then incubated to block Fc receptors (5 µL of rat immunoglobulin, 890 µL of FACS buffer, 5 µL of mouse serum, and 100 µL of an FcR blocker [24G2]) for 30 minutes at 4°C. Cells were labeled with anti-CD4+ or anti-CD8+ monoclonal antibodies by incubation for 20 minutes at 4°C; cells then were washed once and analyzed with an FACS cytometer. The percentage of cells proliferating in culture (responder frequency) and the mean number of daughter cells produced per proliferating cell (proliferative capacity) were determined for both CD4+ and CD8+ T cells, as described elsewhere.

Cytokine measurement—A feline-specific ELISA was used to measure concentrations of IFN-γ, IL-2, IL-10, IL-4, and GM-CSF in tissue culture supernatants. An ELISA plate for each antibody was coated with a capture antibody diluted in 1X PBS solution specific for each cytokine. Wells containing the capture antibody were allowed to incubate overnight at 4°C; then, each plate was washed 5 times with wash buffer. Each plate was blocked by the addition of a block buffer (1% BSA in PBS solution) specific for each cytokine and incubation for 2 hours at 37°C. Plates were washed as previously described. Samples (100 µL) and standards (100 µL) in reagent diluent (1% BSA in PBS solution for IL-2, IL-10, and GM-CSF; 0.1% BSA and 0.05% Tween 20 in tris-buffered saline solution for IL-4 and IFN-γ) were added to appropriate wells, and plates were incubated overnight at 4°C. Plates then were washed as previously described. Detection antibody (100 µL) diluted in reagent diluent was placed in each well, and plates were incubated for 2 hours at 22°C. Plates were washed as previously described. Streptavidin–horseradish peroxidase (100 µL) was added to each well, and the plates were covered for 30 minutes at 22°C. Plates then were washed as previously described. Substrate solution (100 µL) was added to each well, and plates were incubated in the dark for 20 minutes at 22°C. Stop solution (100 µL) was added to each well. Plates were evalu-
ated by use of an ELISA plate reader and commercial software. Optimal time for cytokine evaluation was 24 hours after start of culture for IL-2 and 96 hours after start of culture for IFN-γ, IL-10, IL-4, and GM-CSF.

**Statistical analysis**—Pairwise comparisons were performed by use of a Wilcoxon signed rank test or a Wilcoxon rank sum test. Significance was set at values of \( P < 0.05 \). For the first part of the study, results for blood obtained from 21 cats were used. Power analysis revealed that for 21 cats, a difference of 1 SD could be detected between groups (99% power).

**Results**

To assess the effects of the currently used immunosuppressive treatment as well as human CTLA4-Ig on cytokine production, lymphocytes from 21 clinically normal cats were incubated in medium alone (unstimulated cultures) or in medium containing Con A with or without cyclosporine, dexamethasone, a combination of cyclosporine plus dexamethasone, or human CTLA4-Ig. In unstimulated cultures, basal concentrations of all cytokines ranged from 0 to 0.088 ng/mL. Stimulation with Con A alone did not result in significant production of IL-4 (data not shown). However, stimulation with Con A alone induced the production of IFN-γ (mean, 0.609 ng/mL), IL-2 (mean, 0.615 ng/mL), IL-10 (mean, 0.256 ng/mL), and GM-CSF (mean, 0.685 ng/mL). The addition of cyclosporine, the combination of cyclosporine plus dexamethasone, or human CTLA4-Ig to these cultures led to significant suppression of IFN-γ, IL-2, and GM-CSF concentrations. Addition of dexamethasone alone caused significant suppression of only GM-CSF concentrations.

In contrast, although the addition of human CTLA4-Ig led to significant suppression of IFN-γ, IL-2, and GM-CSF concentrations, human CTLA4-Ig did not significantly suppress IL-10 concentrations. Moreover, whereas cyclosporine and the combination of cyclosporine plus dexamethasone caused a significant decrease in IL-10 production, addition of dexamethasone alone significantly enhanced production of IL-10 from feline lymphocytes (Figure 1). Cyclosporine alone and the combination of cyclosporine plus dexamethasone had a significantly greater effect, compared with the effect for human CTLA4-Ig, on decreasing the production of IL-10, GM-CSF, IFN-γ, and IL-2 in the cell cultures in response to Con A. These results are consistent with the hypothesis that the immunosuppressive treatment currently in use in cats has global inhibitory effects on cytokine production.

Analysis of the aforementioned results revealed the effect of short-term exposure of these immunosuppressants on in vitro cytokine production. To determine whether these inhibitory effects were reflected in vivo, the PBMC response and cytokine production of the 5 cats that underwent renal transplantation because of chronic renal failure and were receiving cyclosporine in combination with prednisolone were compared with results for the 21 clinically normal cats. All transplant recipients were receiving cyclosporine (dose range, 1.2 to 8.9 mg/kg, q 24 h) and prednisolone (0.18 to 0.75 mg/kg, q 24 h) to provide immunosuppression and aid in prevention of transplant rejection, and 4 of the 5 cats had cyclosporine blood concentrations of 158 to 324 ng/mL (therapeutic range, 250 to 500 ng/mL). One cat had a blood concentration of > 3,000 ng/mL.

**Figure 1**—Box-and-whiskers plots for concentrations of IFN-γ (A), GM-CSF (B), IL-2 (C), and IL-10 (D) in lymphocytes (obtained from 21 clinically normal cats and 5 cats that received a renal transplant) cultured with medium alone (left) or medium that contained Con A (right). Notice that the scale on the y-axis differs between culture conditions for each cytokine. *Within a culture condition, median value differs significantly (\( P < 0.05 \)) from the median value for the clinically normal cats. See Figure 1 for remainder of key.
of immunosuppressive treatment for these cats ranged from 3 to 25 months. A CFSE dilution was used to assess lymphocyte proliferation. Following staining with CFSE, cells were plated with medium alone or were stimulated with Con A, and cytokine concentrations were evaluated in the supernatant at 24 (IL-2) or 96 (IFN-γ, IL-10, IL-4, and GM-CSF) hours after the start of culture. Because of differences in the number of lymphocytes available for plating, data of all cats were normalized and expressed as pg of cytokine/1,000 cells. High basal concentrations of IL-2 were identified in cats that underwent transplantation; however, for stimulation with Con A, there was no significant difference between clinically normal and immunosuppressed cats (Figure 2). Additionally, compared with results for clinically normal cats, IL-10 production was significantly increased in both unstimulated and stimulated cultures for cats that underwent renal transplantation. Finally, there was no significant differences between clinically normal and immunosuppressed cats with regard to the production of IFN-γ and GM-CSF in both unstimulated and stimulated cultures.

In addition to the cytokine data, CFSE labeling and flow cytometry were used to assess CD4+ and CD8+ T-cell responses for cats receiving long-term immunosuppressive treatment. For comparison purposes, information from the 5 transplant recipients was compared with results for clinically normal cats reported in another study. In the 5 immunosuppressed cats, the CD4+ subset comprised 18.2% to 31.9% (median, 22.9%; reference range, 17.4% to 49.5% [median, 32.3%]) and the CD8+ subset comprised 12.1% to 31.9% (median, 21.5%; reference range, 6.5% to 39.3% [median, 18.7%]) of the total lymphocytes collected (Figure 3). Although percentages for both CD4+ and CD8+ subsets were within the respective reference ranges, a mean of only 15.6% (range, 8.4% to 20%) of the CD4+ T cells and 19.1% (range, 9.2% to 44.8%) of the CD8+ T cells proliferated in response to stimulation with Con A (Figure 4). Additionally, for stimulation with Con A, there was a decrease in the responder frequency and proliferative capacity of both CD4+ (responder frequency, 8.5%; proliferative capacity, 2 daughter cells) and CD8+ (responder frequency, 13.3%; proliferative capacity, 2 daughter cells) T cells in immunosuppressed cats, compared with values for CD4+ (responder frequency, 34%; proliferative capacity, 3 daughter cells) and CD8+ (responder frequency, 33%; proliferative capacity, 6 daughter cells) T cells in clinically normal cats. The effect on both CD4+ and CD8+ subsets suggested that immunosuppressive treatment currently used for feline renal transplant recipients resulted in global immunosuppression.

Discussion

It has been established in human medicine and through the use of experimental models that cytokines play an important role in immune defense, immunopathologic processes, and allograft rejection. Although information exists for animals used in transplantation experiments as well as for in vivo and in vitro studies in humans regarding the effects of immunosuppressive treatment on cytokines critical to the rejection process, limited information currently exists for these effects in cats. In 1 report, the effects of cyclosporine on the expression of inflammatory cytokines for PBMCs in 5 cats was evaluated by the use of Con A as a stimulus. Real-time PCR assay was used to determine that expression of mRNA for IL-2, IL-4, IFN-γ, and tumor necrosis factor-α was inhibited by cyclospo-
Cyclosporine in combination with corticosteroids has been an essential component in many immunosuppressive protocols for organ transplantation in humans and cats. Cyclosporine acts by inhibiting calcineurin, thus preventing the activation of transcription factors that regulate the expression of cytokines (including IL-2, IL-4, IFN-γ, tumor necrosis factor-α, and GM-CSF) and genes with a role in allograft rejection.53–57 Although these cytokines are also inhibited by corticosteroids, the exact mechanism of action of corticosteroids is not fully understood. The cytokines IFN-γ, IL-2, and GM-CSF are potential therapeutic targets for renal transplantation. Interferon-γ and IL-2 are Th1 cytokines that are thought to play a critical role in acute allograft rejection in humans. In addition, GM-CSF is a cytokine that functions as a WBC growth factor that stimulates the proliferation and differentiation of hematopoietic stem cells into granulocytes and macrophages responsible for responding to foreign tissue (eg, an allograft). Inhibition of these cytokines by immunosuppressive treatment is thought to be critical to graft survival. In the study reported here, which involved the use of lymphocytes from clinically normal cats, cyclosporine caused a significant decrease in the production of IFN-γ, IL-2, and GM-CSF, compared with results for Con A alone. Similar to results for humans, our data support a similar mechanism of action for cyclosporine in cats. Dexamethasone alone suppressed the production of only GM-CSF but not the production of IFN-γ or IL-2. These latter results are consistent with results in another study43 conducted by our research group in which it was found that dexamethasone alone did not significantly suppress lymphocyte proliferation in cats. Results from that study43 and the study reported here suggest that a different mechanism of action for dexamethasone may exist in cats. Immunosuppressive treatment used in the cats of the present report consisted of prednisolone in combination with cyclosporine. When dexamethasone was combined with cyclosporine in vitro, a significant decrease in the production of IFN-γ, IL-2, and GM-CSF was detected.

Compared with results for other cytokines, the results for IL-10 did not match with an expected profile. Cyclosporine caused a significant decrease in IL-10 production in the cats, although not to the degree of the aforementioned cytokines. This is in contrast to the finding in many reports49–52 in humans that indicated cyclosporine does not suppress IL-10 production. Interleukin-10 is a cytokine with immunosuppressive
and anti-inflammatory properties that, in some transplantation studies, has been thought to play a role in allograft tolerance. The ability to suppress proinflammatory factors while sparing IL-10 production would be beneficial for renal transplant recipients. However, an increase in IL-10 expression in allografts undergoing rejection has been identified in some studies. Confllicting information also exists in the literature with regard to corticosteroids; some studies have revealed an inhibition of IL-10 production, whereas other studies have found the opposite effect. In the study reported here, dexamethasone alone did not suppress IL-10 production; however, when dexamethasone was combined with cyclosporine, there was a significant decrease in the production of IL-10.

Similar to findings reported in the human literature, the addition of human CTLA4-Ig to feline lymphocytes resulted in a significant decrease in the production of IL-2, IFN-γ, and GM-CSF but not IL-10. These results support findings in another study conducted by our research group in which we found a greater effect of cyclosporine alone and cyclosporine in combination with dexamethasone on lymphocyte proliferation, compared with the effects for human CTLA4-Ig. Because of the potential role in allograft tolerance, sparing of IL-10 in feline renal transplant recipients while suppressing proinflammatory cytokine production may be beneficial to allograft survival and provides justification for evaluation of human CTLA4-Ig as an immunosuppressant for cats. It is unclear as to how sparing of IL-10 would affect the immune response to opportunistic infections in feline renal transplant recipients. Studies in human transplant patients as well as patients with HIV have detected elevated serum IL-10 concentrations in patients with disease progression or those with stable disease.

Currently, human CTLA4-Ig (abatacept) is being used in humans for the treatment of rheumatoid arthritis. In a study conducted to evaluate the efficacy of neonatal gene therapy for the treatment of mucopolysaccharidosis I in cats, short-term (4 doses over a 2-week period) administration of human CTLA4-Ig to kittens at the time of gene therapy had no adverse effects and resulted in long-term tolerance to the canine enzyme α-L-iduronidase. Additionally, cats with mucopolysaccharidosis I that were treated with human CTLA4-Ig prior to vaccination with a killed parvovirus vaccine still developed a 16-fold increase in anti-parvovirus titers. These results suggest an appropriate memory response was generated with vaccination in the face of CTLA4-Ig. In contrast to the fact that the currently used immunosuppressive treatment caused global immunosuppression, human CTLA4-Ig appears to have a sparing effect on antigen-specific proliferation of memory CD4+ and CD8+ T cells. Although unproven, the suppression of both naïve T-cell function as well as memory T-cell function by current immunosuppressive treatment may be associated with an increased incidence of opportunistic infections and neoplasia in cats that have undergone renal transplantation.

Cyclosporine and corticosteroids are immunosuppressive drugs used commonly in veterinary medicine to treat a variety of disorders; however, minimal information currently exists regarding their mechanisms of action in patients. Renal transplantation has been performed for > 20 years in cats, and cyclosporine and corticosteroids are the mainstay of immunosuppressive treatment to prevent allograft rejection. It might be expected that cytokine production for cats receiving long-term immunosuppressive treatment would mirror results from the first part of the present study in which the combination of cyclosporine and dexamethasone was added to naïve cells from clinically normal cats; however, this was not the case. Concentrations of Th1 cytokines, including IL-2 and IFN-γ, that are thought to play a key role in acute allograft rejection were not significantly decreased in cats receiving long-term immunosuppressive treatments. Interestingly, IL-10 concentrations were significantly increased in both unstimulated and stimulated cultures. The increase in expression of IL-10 identified in our stable allograft patients suggests that IL-10 may be associated with allograft tolerance in cats. Our results do not support findings of elevated IL-10 concentrations in patients having a rejection episode.

Finally, compared with results for clinically normal cats, evaluation of CD4+ and CD8+ T-cell responses for cats receiving long-term immunosuppressive treatment revealed a decrease in both the CD4+ and CD8+ T-cell subsets. Additionally, there was a decrease in the responder frequency of CD4+ but not CD8+ T cells. These results support findings in humans that the immunosuppressive agents cyclosporine and prednisolone have an additive effect in suppression of T lymphocytes and their subsets and that cyclosporine more selectively inhibits CD4+ T cells than it does CD8+ T cells.

Results of the study reported here further elucidate the mechanism of action of immunosuppressive treatment currently used in feline renal transplant recipients. The immunosuppressive treatment currently used has many shortcomings and can result in an increase in morbidity and mortality rates as well as potentially decreasing life expectancy of the graft and recipient. Strategies targeted at blocking T-cell costimulation are novel approaches to immunoregulation. In the present study, human CTLA4-Ig was successful in inhibiting cytokines critical to the rejection process and sparing cytokines potentially critical for allograft tolerance. Data from this study support further investigation of human CTLA4-Ig in clinical patients.

References


