Abstract

Immunotherapy is not a new concept for veterinary medicine; however, adoptive T cell therapy is a new area of research in humans and canines alike. In humans, T cell therapy has been used against many different tumor histologies, including lymphoma, melanoma, and colon cancer. Although in dogs this approach has currently only been applied to lymphoma, other tumor types are under investigation. There are many different strategies used to take advantage of cell-mediated antitumor properties of T cells. This review will discuss many of the current strategies used in both humans and canines in regards to adoptive T cell therapy.

Key Words: adoptive T cell therapy; canine; chimeric antigen receptor; lymphoma; T cell receptor

Canine B Lineage Lymphoma

Canine B lineage lymphoma (BSA), which accounts for up to 24% of all reported neoplasms and 85% of diagnosed hematological malignancies (Kaiser 1981; Moulton and Harvey 1990). Similar to humans, approximately 60–80% of canine LSA arises from malignant B cells (Christensson et al. 1983; Valli et al. 2010). The most common presentation is a generalized lymphadenopathy corresponding to stages III to V as described by the World Health Organization staging system, where tumor cells may be observed in the lymph nodes, spleen, liver, blood, bone marrow, and other organ systems (Kaiser 1981). World Health Organization classification incorporates the categorization of lymphoid malignancies according to cellular maturation with the accompanied type of prognosis (Valli et al. 2013). During a historical pathological review of 992 canine LSA cases, samples were subdivided into three major groups and then graded based on mitotic index, general cell type observed histologically, and general tissue architecture (Valli et al. 2013). Subtype diagnosis may be further correlated with aggressive disease and grading. This study grouped subtypes into low-, intermediate-, and high-grade LSA. The low-grade LSAs included diffuse large B cell low grade immunoblastic, diffuse large B cell low grade centroblastic, T cell rich large, B cell small lymphocytic, B cell chronic lymphocytic leukemia, and diffuse intermediate B cell. The intermediate-grade lymphomas include diffuse large B cell mid CB, diffuse large B cell mid IB, plasmacytoma, and lymphoplasmacytoid lymphoma. High-grade B cell lymphoma subsets include diffuse large B cell high grade centroblastic, diffuse large B cell high grade immunoblastic, Burkitt-like, B anaplastic large cell, B cell lymphoblastic, B cell lymphoblastic lymphoma cleft, and plasmablastic lymphoma (Valli et al. 2013). Three commonly observed high-grade subtypes are histologically diagnosed as diffuse large cell (20%), immunoblastic (24.9%), and small noncleaved (24.2%) (Carter et al. 1986; Valli et al. 2010). The etiology of this spontaneous disease in dogs is analogous to humans, as both arise from genetic abnormalities, predisposition (breed, in the case of dogs), and common environmental exposures (Modiano et al. 2005). Tremendous efforts have been made to delineate the risk factors associated with non-Hodgkin lymphoma (NHL) diagnosis in humans. Possible, but not completely conclusive, factors that may attribute to increased risk of NHL diagnosis include smoking, ambient ultraviolet radiation exposure, occupation, time and intensity of exposure to solvents, pesticides, immune deficiencies or dysfunction, gene translocations, errant DNA repair mechanisms, and genetic variations in several cellular signaling pathways (Bassig et al. 2013).

The current standard of care for B lineage LSA is a combination chemotherapy regimen of cyclophosphamide, vincristine, doxorubicin, and prednisone (CHOP), which induces a temporary remission (9–11 months) in approximately 85% of dogs (Moulton and Harvey 1990; Vail and Young 2007; Valerius et al. 1997). Attempts to improve the duration of first remission have been only moderately successful with a high-dose CHOP protocol, improving the median disease-free interval (DFI) from 197 days to 300 days (Chun et al. 2000), and the addition of mitoxantrone maintenance to the CHOP protocol improved the median DFI in this group of dogs to 302 days (Daters et al. 2010). Both of these studies used...
historical controls as a basis of comparison for their results. The toxicity in the first protocol has led to treatment delays or discontinuation in more than 44% of cases. Because of this toxicity, this protocol is not widely used today. Several alternative combination chemotherapeutic rescue protocols, such as lomustine, L-asparaginase, CCNU (lomustine), DTIC (dacarbazine), MOPP (methchlorathamine, vincristine, procarbazine, and prednisone), doxil, and prednisone alone, have been yielded from attempts to improve survival in dogs with refractory disease. However, these protocols have mostly proven unsuccessful in significantly increasing (progression-free and tumor-free) survival beyond 2 to 3 months (Back et al. 2013; Fahey et al. 2011; Flory et al. 2008; Zandvliet et al. 2013). With the majority of articles in the veterinary literature reporting DFI for CHOP-based chemotherapy protocols ranging from 94 to 302 days and median survival times ranging from 220 to 397 days, there is an urgent and unmet need in veterinary medicine to develop new treatment strategies for refractory disease (Back et al. 2013; Fahey et al. 2011; Flory et al. 2008; Zandvliet et al. 2013).

Recent promising advances in human cancer treatments have focused specifically on chemotherapies and biological therapies. Immunotherapy, a specific genre of biological therapy, harnesses the power of the body’s own immune system to provide a durable antitumor response. The veterinary profession is beginning to develop similar immunotherapy treatments. Strategies ranging from the addition of exogenous cytokines to immune-modulating radiation doses and chemotherapeutic regimens to tumor antigen-specific monoclonal antibody therapy to cell-based treatments are currently used alone or in combination for the treatment of human and canine cancers under the term immunotherapy (Flory et al. 2008; Impellizzeri et al. 2006; Mackall et al. 2011; Milner et al. 2006; O’Connor et al. 2012; Ott et al. 2013; Tobinai et al. 2010; Vose et al. 2005; Voulgarelis, Giannouli, Anagnostou et al. 2004).

**Adaptive Immune System Dysfunction and LSA**

A functional immune system effectively maintains the integrity of self by destroying and removing diseased and infected cells, while a complex network of checks and balances ensures healthy cells and tissues are not targeted (Ruella and Kalos 2014). These checks and balances are provided through collaborations between the adaptive and innate arms of the immune system, which include cell-to-cell interaction/cross-talk, as well as the release of soluble factors, such as cytokines. A dysfunction in the intact immune system can lead to increased risk of infections, autoimmune diseases, and the progression or development of malignancies, such as LSA. In humans diagnosed with a malignancy, such as head and neck squamous cell carcinoma, immune system dysfunction can be characterized by, among other things, alterations in cytokine production, antigen presentation defects mediated by dendritic cells, and T cell dysfunction characterized by ratio imbalances, signaling issues, and gene modulation (O’Connor et al. 2012; Varilla et al. 2013).

Tumors can produce strong immunosuppressive cytokines, such as interleukin 10 (IL-10), interleukin 6 (IL-6), and transforming growth factor β (TGF-β). IL-10 and TGF-β can inhibit the function of cytotoxic T cells (CTLs), generate T regulatory cells, and create tumor resistance to apoptosis (Jarnicki et al. 2006; Rabinovich et al. 2007). IL-6 has been shown to downregulate MHC II on dendritic cells and significantly inhibit dendritic cell maturation, resulting in decreased antitumor immunity through aberrant antigen presentation (Jarnicki et al. 2006; Narita et al. 2013; Rabinovich et al. 2007). In human colorectal patients, elevated serum levels of IL-10 and IL-6 can be correlated to poor prognosis (Galizia et al. 2002). Patients with pancreatic cancer expressed increased serum levels of interleukin 8, IL-10, interleukin 12, interleukin 18, and TGF-β compared with healthy control subjects (Bellone et al. 2006). In this same study, pancreatic carcinoma patients with lower serum levels of IL-6, interleukin 18, and TGF-β survived longer (Bellone et al. 2006).

Dendritic cells are a subset of specialized antigen presenting cells (APCs), which mediate tolerance and immunity. Proper regulation of dendritic cell function is essential for sustaining an activated antitumor T cell response (Narita et al. 2013). This regulation encompasses dendritic cell maturation, upregulation of costimulatory molecules (CD86, CD80, CD40), increased expression of proinflammatory cytokines (tumor necrosis factor alpha), capture and presentation of antigen, and migration from tissues to the lymphoid organs (Rabinovich et al. 2007). Tumors may modulate the maturation of dendritic cells through the secretion of IL-10 and TGF-β (Varilla et al. 2013). These cytokines can convert immature dendritic cells into a tolerogenic state in the presence

![Figure 1](https://academic.oup.com/ilarjournal/article-abstract/55/1/169/848587) Canine T cells cocultured with artificial antigen presenting cells. Elongated cells are T cells, whereas the larger circular cells are artificial antigen presenting cells.
of malignancies, such as head and neck squamous cell carcinoma. Antigen-specific T cell tolerance is induced through the interaction with tolerized dendritic cells, leading to activation and differentiation of T regulatory cells (Varilla et al. 2013). Tumors may also mediate escape and cause further immune system dysfunction by inhibiting the myeloid differentiation into dendritic cells through the STAT3 pathway (Farren et al. 2014).

The adaptive immune system has both effector and regulatory T cells, which can influence tumor growth and metastases (Malhotra et al. 2013). γδ T cells comprise approximately 95% of the human peripheral blood T cell pool and are primarily regarded as effector cells (CD3+CD8αβ) or helper cells (CD3+CD4αβ) (Deniger et al. 2013). CD3+CD8αβ CD25+Foxp3+ T cells are regarded as regulatory cells (Tregs) (Ruella and Kalos 2014). The vast majority of T cell therapies for human and dogs incorporate only γδ T cells. The remaining 1–5% of the T cell pool are regarded as γδ T cells, a rare population of T cells that do not express the αβ T cell receptor nor are subdivided into CD8αβ or CD4αβ categories (Deniger et al. 2013). γδ T cells are predominantly located in areas of mucosal immunity and directly recognize tumor-associated antigens (TAA) such as heat shock proteins, MHC I–related gene A/B, F1-ATPase, and intermediates in cholesterol metabolism in an MHC unrestricted manner (Deniger et al. 2013; Ferreira 2013). These particular T cells have a broad recognition of tumor antigen and potent antitumor immunity. However, expansion techniques to achieve clinically significant numbers have returned limited results in humans. Using this subset as a T cell therapy for human malignancies has recently been explored in a xenograft mouse model using γδ T cells genetically modified to express a CD19-specific chimeric antigen receptor (CAR) (Deniger et al. 2013).

The T cell interacts with tumors and antigen-presenting cells by interrogating the surface with their T cell receptor (TCR). The TCR is a heterodimer of two chains, αβ or γδ (Essand and Loskog 2013). The major histocompatibility complex located on the surface of the affected cell, such as a dendritic cell, interacts with the TCR by presenting it with the specific antigenic peptide for which the TCR is programmed. The TCR is associated with a CD3 complex made up of a γ, δ, ε, and ζ signaling endodomains. When the TCR interacts with an MHC antigen complex, it triggers the CD3 complex to mediate signal transduction through immunotyrosine-activating motifs. This interaction leads to proinflammatory cytokine production, T cell mediated lysis, and apoptosis (Essand and Loskog 2013). Cellular death is mediated by the release of perforins from the T cell, which allows granzymes to enter into the target cells and induce apoptosis.

In human head and neck squamous cell carcinoma, as well as other neoplasia, tumor-induced T cell dysfunctions have been observed. Increased numbers of Tregs in the lymph nodes and tumor were correlated with poor prognosis. Peripheral blood lymphocyte counts were lower in patients diagnosed with malignancies than in healthy control subjects (De Costa et al. 2012). This included a decrease in CD8+ T cells, natural killer cells, and natural killer T cells (Varilla et al. 2013). Decreased expression of CD3 zeta chain resulted in loss of cytotoxicity and expansion impairment in the presence of antigen or costimulatory molecules. Diminished interleukin 2 (IL-2) and interferon γ (IFN-γ) production, as well as increased apoptotic features, were observed in samples from patients diagnosed with malignancies (Varilla et al. 2013).

**Peripheral and Central Tolerance Effect on LSA and Cellular Therapy**

The primary obstacles faced by cellular-based immunotherapies are overcoming both peripheral and central tolerance issues as the tumor evolves through immuno-editing pressures to become recognizable immunologically and develops the ability to suppress antitumor/proinflammatory responses. Central tolerance focuses on the deletion of self-antigen–recognizing T cells with high-affinity TCRs during development in the thymus. To evade immunosurveillance and destruction by the immune system, tumors routinely overexpress or aberrantly express self-antigens. Central tolerance prevents the endogenous targeting of these tumor antigens through the deletion of self-reactive and high-affinity TCRs in the thymus during T cell maturation. Therefore, the immune system loses
a specific antitumor TCR repertoire (Falkenburg et al. 2011; Schmitt et al. 2009; Schmitt et al. 2013). Conversely, peripheral tolerance is the development of a dysfunctional immune system through the formation of an immunosuppressive condition. Tumor cells commonly take advantage and promote this condition. Peripheral tolerance can be overcome by blocking inhibitory immune-modulating molecules such as CTLA-4 and PD-1 or creating a proinflammatory environment around the tumor. Human phase II and phase III trials involving monoclonal antibodies against cytotoxic T-lymphocyte antigen 4 (Ott et al. 2013) and programmed death 1 (Ott et al. 2013) alone or in combination with other therapies have resulted in potent, tolerable antitumor responses in melanoma, non–small cell lung cancer, and head and neck squamous cell carcinoma (Delecrin and Vansteenkiste 2014; Ott et al. 2013).

Adoptive cellular therapy (ACT) and TCR gene therapy are treatments that have shown promise against central tolerance. To circumvent central tolerance and reintroduce thymic-deleted TCR repertoires, T cells can be genetically engineered to express deleted high-affinity TCRs, which are generally more effective in the elimination of tumor cells as compared with lower-affinity TCRs against the same antigen (Schmitt et al. 2013). Wilms Tumor gene 1 (WT1) is one such tumor target for human TCR adoptive therapy that has been tested in preclinical murine models and subsequent targeted human immunotherapy clinical trials. The WT1 antigen is expressed highly on several hematological and solid tumors such as glioma, leukemia, renal cell carcinoma, and gynecological malignancies (Coosemans et al. 2013; Lee and Haber 2001; Schmitt et al. 2013; Uttenhal et al. 2014). WT1 has also been detected at low levels on normal cell types (Coosemans et al. 2013; Lee and Haber 2001; Park et al. 1993; Schmitt et al. 2013). In a recent murine study, Schmitt and colleagues (2013) transduced peripheral T cells with two high-affinity variants of the WT1 TCR. These variants were not expressed in wild-type mice. The WT1-specific TCRs responded significantly to WT1 tumor targets without causing autoimmune activation or associated pathology. Responses against low-expressing WT1 on normal cells and new off-target effects were not observed. Their conclusions support the overcoming of central tolerance to mediate antitumor effects through the infusion of WT1-specific CD8+ T cells, which can differentiate between low levels on self-tissue and higher levels expressed by tumor cells (Schmitt et al. 2013).

ACT is a biological treatment that harnesses the natural ability of the body’s lymphocytes to effectively recognize and eliminate pervasive target cells while initiating and managing a systemic immune response against advanced disease. Donor lymphocyte infusions (and allogeneic hematopoietic stem cell transplantation (HSCT) combinations after myeloablative therapies are other methods used to circumvent central tolerance with success (Deniger et al. 2013; Ruella and Kalos 2014). HSCT in dogs has been shown to provide an immune-tolerant platform, allowing for a break in central tolerance (Kuhr et al. 2002; Kuhr et al. 2007; Niemeyer et al. 2005; Parker et al. 2008). A study by Parker and colleagues (2008) demonstrated the ability of dogs with Duchenne muscular dystrophy to accept injections of freshly isolated muscle-derived cells from the HSCT donor. These dogs showed improved engraftment and function of donor muscle-derived cells (Parker et al. 2008). Several other studies have demonstrated long-term tolerance to kidney allotransplants in dogs pretreated with HSCT before kidney allo-transplantation (Kuhr et al. 2002; Kuhr et al. 2007; Niemeyer et al. 2005). Recipients maintained normal kidney function for up to 5 years after transplantation with only short-term immunosuppressive therapy at the time of engraftment (Kuhr et al. 2007). Further work by this group has shown that mixed chimerism in dogs receiving HSCT for purpose of kidney allotransplants can be intentionally reverted to host hematopoiesis without rejection of the kidney graft even though these dogs then later rejected their donor hematopoietic graft. Protocol limitations, such as donor matching/availability and graft versus host disease, have restricted the broad use of allogeneic HSCT in the dog. (Lupu et al. 2006; Willcox et al. 2012). Autologous HSCT have been used with some success in the dog also (Lupu et al. 2006; Willcox et al. 2012).

Autologous ACT is another promising approach involving the infusion of ex vivo manipulated expanded T cells after chemotherapy or radiation therapy. The foundation for ACT was formed on two early human trials infusing (1) rapidly expanded tumor infiltrating lymphocytes from melanoma patients and (2) cytomegalovirus -specific T cell clones into cancer patients with a high-risk of cytomegalovirus infection. Both were shown to induce vigorous antitumor immune responses (Chinnasamy et al. 2011; Greenberg et al. 1991). The data from these initial ACT trials revealed three important points that would need to be taken into account for ACT to be clinically successful: (1) adoptive T cell immunotherapy could influence endogenous antitumor activity; (2) future treatment strategies should incorporate the immunological contact between T cells and tumor cells; and (3) the manufactur of clinically significant numbers of efficacious T cell phenotypes and infusion protocols would need to be advanced (Ruella and Kalos 2014).

Tumor Microenvironment Manipulation to Increase ACT Efficacy

The ACT approach to tumor destruction incorporates cell-to-cell interactions and the production of soluble factors from said interactions. The tumor microenvironment (TME) is a complex milieu of tumor cells, endothelial cells, stromal cells, fibroblasts, myeloid cells, natural killer cells, effector and memory T cells, monocytes/macrophages, and Tregs. Tumors can regulate their growth based on signals from these cells, leading to the production of IL-10 and TGF-β, chemotactic factors to recruit tumor-associated macrophages (Schaft et al. 2003) and growth factors, such as proangiogenic factors (VEGF). In addition to the TME providing attractive targets for monoclonal antibody therapy and prognostic information, its disruption may also potentiate T cell–based therapy.
Targets include TAM, vasculature cells, and stromal cells, which provide a foundation for the tumor. Bevacizumab is a humanized monoclonal antibody that targets human VEGFA. It has been approved in the United States for treatment of human metastatic colorectal cancer, nonsquamous non-small cell lung cancer, metastatic renal carcinoma, and glioblastoma (Mortimer et al. 2012). Bevacizumab has demonstrated efficacy in murine models of canine mesenchymal neoplasms (hemangiopericytoma and osteosarcoma) by inhibiting intratumoral angiogenesis (Michishita et al. 2013). In both studies, tumor growth was inhibited as a direct result of limited angiogenesis and suggests cross-reactivity of the antibody between species (Michishita et al. 2013; Scharf et al. 2013). Therefore, a canine-derived monoclonal antibody similar to bevacizumab, which specifically targets canine VEGFA, may be useful in veterinary oncology alone or in combination with other immunotherapies.

The immunophenotype of the TME lymphocyte population can be directly correlated with prognosis and survival rates in both human and dogs with cancer. The presence of tumor-infiltrating lymphocyte CD8⁺ T cells correlates with improved tumor control and prognosis in human colorectal cancer, cervical cancer, and non–small cell lung carcinoma (Al-Shibli et al. 2008; Galon et al. 2006; Piersma et al. 2007). The increased infiltration of Tregs and TAMs into the TME and peripheral blood is directly correlated with negative prognosis and poor outcome in many human cancer subtypes such as non-Hodgkin lymphoma, cervical malignancies, colorectal carcinoma, and non–small cell lung carcinomas (Bayer and Schultzte 2009; Eljaszewicz et al. 2013; Galon et al. 2006; Shimizu et al. 2010). These observations have translated into veterinary oncology. Decreased peripheral blood CD4/CD8 ratios were linked to longer survival and better prognoses in canine LSA, whereas an increased CD4/CD8 ratio suggested poor prognosis (O’Connor et al. 2012). The increased CD4/CD8 ratio was also linked to relapse of LSA before clinical manifestations were detected and served as a biomarker for engraftment after ex vivo activated T cell infusions. Estrela-Lima and colleagues (2010) observed in 51 dogs diagnosed with mixed mammary carcinomas that tumors with increased numbers of CD4⁺ T cells, decreased total tumor infiltrating T cells, and increased CD4/CD8 ratios were significantly correlated with distant metastases and negative prognosis. However, increased populations of CD8 effector T cells were observed intratumorally, as well as lower CD4/CD8 ratios, in the peripheral blood of dogs without metastases (Estrela-Lima et al. 2010). Another study evaluating the lymphocyte subpopulations of aged dogs and dogs with cancer discovered a similar trend. The authors observed that dogs diagnosed with malignancies had a significantly higher CD4/CD8 ratio than healthy older dogs, which reported lower CD4/CD8 ratios. The ratio increase was directly correlated with poorer disease staging (Watabe et al. 2011). Furthermore, Biller and colleagues (2010) discovered that a decreased percentage of CD8⁺ T cells or increased CD4/CD8 ratio led to a shortened survival in dogs with osteosarcoma. These data, coupled with the human historical data, suggest a role for the clinical use of significant CD4/CD8 ratios changes for monitoring and prognostic information.

To increase efficacy of T cell therapy, the TME and immunosuppressed immune system can be altered through nonmyeloablative, lymphodepleting chemotherapy and radiotherapy regimens before ACT (O’Connor et al. 2012; Vose 2005; Voulgarelis, Giannouli, Ritis et al. 2004). Chemotherapies have also been shown to increase the expression of neoantigens by residual tumor, thereby allowing the tumor to be recognized as “foreign” to the immune system (Aquino et al. 2004; Gattinoni et al. 2006; Hinrichs et al. 2006; Kaiser 1981; Kaneno et al. 2011; Klebanoff et al. 2006; Paulos 2007; Tongu et al. 2010). In a study by Mitchell et al. (2012), tumor associated T lymphocytes were evaluated in dogs with B cell malignancies. They observed high numbers of Tregs and low numbers of CTLs in untreated affected lymph nodes compared with lymph nodes of healthy dogs. The CTLs became much more active and more adept at killing autologous tumor cells after doxorubicin therapy. Tumor cell destruction with chemotherapy likely exposes the CTLs to new TAAs, priming the immune system for an antitumor response. Other studies have demonstrated improved engraftment and expansion of infused T cells using preconditioning regimens. These regimens destroy endogenous lymphocytes, therefore decreasing competition between infused ex vivo and endogenous T cells for cytokines and growth factors (Dudley 2002; Wrzesinski 2010; Wrzesinski and Restifo 2005). Lymphodepletion is a necessary, but beneficial, consequence of radiation and chemotherapy which creates space in the lymphoid compartment for the infused T cells. Because of the increased number of circulating Tregs and immunosuppressive environment, chemotherapy preferably reduces this number to create a proinflammatory, antitumor environment. Chemotherapy further reduces the myeloid suppressor cells in the TME, which can inhibit CTL cytotoxicity and aid in tumor growth, while activating APCs. TGF-β is an anti-inflammatory cytokine whose secretion is elevated during malignancies. In the companion canine study of adoptive T cell therapy after CHOP, TGF-β serum concentrations decreased after CHOP and continued to diminish after each infusion, supporting the need for preconditioning to allow the redevelopment of an antitumor, intact immune system (O’Connor et al. 2012).

Canine Adoptive T Cell Therapy for B Lineage LSA

More recently, adoptive transfer of nonspecific ex vivo expanded T cells has shown promise as an anticancer therapy in dogs, especially when used in combination with chemotherapy. The phenomenon of chemotherapy’s immune and tumor-modulating capabilities, as well as the associated iatrogenic lymphodepletion observed after therapy, prompted the testing of repeated infusions containing activated autologous nonspecific T cells after CHOP to treat dogs diagnosed with LSA (O’Connor et al. 2012). In humans, the increased rate
of T cell recovery after chemotherapy has been directly correlated with progression-free and overall survival times in malignancies such as colorectal cancer, acute myeloid leukemia, cervical carcinomas, acute lymphoblastic leukemia, non–small cell lung carcinoma, and NHL (De Angulo et al. 2008; Lenschow et al. 1996; Li et al. 2005; Pagès et al. 2005; Pagès et al. 2010; Porrata et al. 2008; Siddiqui et al. 2006). As previously stated, human patients have better prognosis with faster lymphoid recovery after chemotherapy, increased CD3+CD8+ effector T cells trafficking to the tumor site, and circulating peripheral blood Tregs are decreased. A recent study by O’Connor and colleagues (2012) showed a significant benefit to survival with the administration of nonspecific T cell infusions after CHOP protocol to dogs diagnosed with LSA. In this study, ex vivo expanded T cells were infused three times using an intrapatient dose-escalating scheme. O’Connor and colleagues administered nonspecific autologous T cells to eight dogs with spontaneously occurring B cell LSA in the published trial but have infused five additional dogs on this protocol since publication. The infused product was primarily CD3+CD8+ T cells that expressed lymph node homing molecule CCR7 and cytolytic enzyme granzyme B (O’Connor et al. 2012) (Figure 1). Like humans, dogs diagnosed with LSA exhibited increased numbers of CD3+CD4+ helper T cells circulating in the peripheral blood and at the sites of tumor at the time of diagnosis until complete remission was achieved. The adaptive immune system was further characterized as dysfunctional by the significant decrease in circulating CD3+ T cells compared with healthy donors. Multiplex digital profiling using nCounter analysis system quantified T cell–specific gene expression change between peripheral blood CD3+ and the expanded infusion product. Gene products associated with T cell activation (IL-21R, IL-2R), cytotoxicity (KLR, GZMH, GZMB, PRF-1, IFN-γ, CD8), proliferation (EOMES, DPP4, TNFRSF9, LCK, ZAP70), inflammation, and trafficking (CCL3, CCL4, CCL5 receptors) were upregulated in expanded T cells. mRNA sequences coding for chemokines to allow for tumor trafficking LEF1, TGFβ1, FOXP3, CD4, and hypoxia-related genes were upregulated in the nonexpanded peripheral blood preinfusion samples. The expanded CD3+CD8+ products secreted the proinflammatory cytokine protein IFN-γ, which supported the mRNA findings.

During cryopreservation, the expanded T cells were labeled with a red fluorescent dye, PKH-26, to allow ex vivo tracking by flow cytometry and immunohistochemistry. Infused T cells persisted greater than 49 days, as supported by PKH-26 detection, increased CD3+CD8+ peripheral blood population, and decreased CD4/CD8 ratios. In normal dogs, there was a higher proportion of CD8+ T cells in circulation than CD4+ T cells. However, in dogs with LSA, this ratio and the levels of circulating CD4+ cell were increased. Infusions of CD3+CD8+ T cells were able to convert this ratio to a pre-cancer homeostatic level, which was determined from healthy control dogs. In this study, dogs whose ratios significantly improved demonstrated prolonged survival over those treated with chemotherapy alone. Clinical evaluation of disease relapse could be correlated with increasing CD4/CD8 ratios over time, as remission was correlated with lower, stable CD4/CD8 ratios. This study demonstrated the importance of CD4/CD8 T cell ratios as a prognostic factor or assay to determine relapse. Within 3 hours and at 10 days after infusion, infused T cells could be observed in the lymph node. In dogs with tumor burden, the percentage of lymph node CD3+CD8+ T cells decreased with the influx of CD3+CD8+ expanded T cells (data not published). This observation suggested that the infused CD3+ T cells were targeting malignant and normal B cells to decrease tumor burden and increase survival. Other factors that were noted to be linked with improved prognosis in this study were peripheral blood neutrophil/lymphocyte ratios less than 2.2:1 and elevated granzyme B protein levels of the expanded T cells before infusion. Supporting human observations, dogs that received too three doses of expanded T cells had significantly longer tumor-free and overall survival compared with age- and stage-matched historical control dogs treated with CHOP chemotherapy alone. Immune reconstitution with effector T cells was able to improve the median overall survival to 392 days versus 167 days and tumor-free survival to 338 days versus 71 days (O’Connor et al. 2012). This implied that the combination therapy of CHOP and activated T cells provides more clinical benefit to dogs than CHOP alone.

Based on these data, a second canine activated T cell therapy trial has been initiated by the group. The immunosuppressive regimen of cyclosporine, methotrexate, and prednisolone was more effective in preventing graft versus host disease grades II to IV after allogeneic HSCT and peripheral blood progenitor cell (Chao et al. 1993; Hoyt et al. 2008; Ruutu et al. 2000). However, immunosuppression can dampen potent anti-tumor immunity necessary after lymphodepleting treatments and, furthermore, impede the reconstitution of effectors cells and antitumor immunity. Thus, developing an immunosuppressive-tolerant CTL may improve donor lymphocyte infusions after HSCT. In the dogs diagnosed with LSA, owners can elect to treat their dog with prednisolone/dexamethasone as a single-agent therapy. Clinically significant doses of prednisolone may continue systemic effector T cell dysfunction while increasing numbers of negative prognostic-related Tregs. Therefore, the development and infusion of steroid-resistant T cells for these dogs could inform on the human situation. Autologous T cells have been expanded either normally or on a clinically relevant dose of dexamethasone that still yields a predominantly CD3+CD8+ product. The dexamethasone treatment does increase the percentage of CD3+CD4+ helper T cells as well as Tregs. (Chao et al. 1993; Hoyt et al. 2008; Ruutu et al. 2000; Vail and Young 2007). The normal T cells are labeled with PKH-67, a green fluorescent dye, whereas the dexamethasone-treated fractions are labeled with PKH-67, a green fluorescent dye. Both T cell populations are monitored in vivo using flow cytometry of the peripheral blood and fine needle aspirates of tumor-involved lymph nodes. Currently, three patients have been infused under this protocol (data not published as trial is still currently enrolling at the time of publication).
The attractive benefits of adoptive polyclonal T cell therapy range from the ability to produce long-lasting memory T cells to the reconstitution of a lympho-depleted immune system after chemotherapy or radiotherapy. The use of chemotherapy and radiotherapy before infusion may allow for the expression of neoantigens by the remaining tumor cells, thus providing new TAAs to stimulate and clonally expand the infused T cell population, which may explain the increased efficacy observed in dogs treated with combination immunotherapies (O’Connor et al. 2012; Peggs et al. 2007; Shurin et al. 2009; Tongu et al. 2010).

Genetically Modified T Cell Therapy to Treat Canine LSA

Increasing the efficacy of ACT has long been a goal of immunologists. New technology has allowed the detection of novel and ubiquitously expressed tumor antigens. Combined with recent advances in gene transfer and safety, T cells can be engineered to engage in target-specific killing. Stable genetic modifications may occur with the use of retroviral vectors, lentiviral vectors, TALENS, zinc fingers nucleases, and transposon/transposase systems. mRNA transfections are currently being tested as an alternative method to stable DNA integration (Barrett et al. 2013).

Originally, genes were transferred into T cells using γ-retroviral vector. These vectors are not widely used for transfection in clinical settings because concerns have been raised that these viruses may allow for malignant transformation in transfected primary T cells, though this would be a rare occurrence in mature T cells. More recently, the HIV-1–based self-inactivating lentiviral vectors have been used to insert genes into the genome of both proliferating and quiescent T cells and has been approved for human clinical use (Kalos et al. 2011). There are four major disadvantages of lentiviral based delivery systems: (1) they are time-consuming and expensive to prepare because high titers are required for gene therapy; (2) virus preparation may create risks of infectious agent contamination; (3) viral vectors may produce adverse cellular reactions; and (4) propagation of vectors may limit the size of the insert and require additional replication regulation sequences (Hackett et al. 2010).

Nonviral vectors such as the Sleeping Beauty (SB) (Ito et al. 2011) transposon system have also been investigated and have been approved for clinical use (Singh et al. 2013). The SB system is comprised of two components, the transposon, which carries the gene of interest between two inverted terminal repeat sequences, and the SB transposase, which is a hyperactive enzyme that recognizes the thymidine-adenine (TA) repeat sequences. Once electroporated into the cell, the transposase excises the gene of interest out of the transposon and inserts it randomly into the genome between TA sites (Deniger et al. 2013; Hackett et al. 2010). The SB system is attractive for gene therapy use because of the low cost to generate vectors and low copy number of integrations. However, larger transposon size may inhibit genome integration.

Although DNA genetically modified T cells can be efficacious and have potential long-term persistence, stable expression of the CAR or TCR have led to concerns about the chronic toxic effects associated with targeting TAAs, such as the B cell antigen CD19 or epidermal growth factor receptor, which are also expressed on normal cells. Temporary CAR expression may allow the targeting of ubiquitous TAAs. mRNA can be efficiently transfected into T cells to provide transient CAR or other gene expression without DNA integration because mRNA is degraded by endogenous RNases quickly in the cytoplasm and protein expression only lasts for several days. Current data show that CAR expression by RNA is efficacious and target specific and may be clinically safer to use against widely expressed targets (Barrett et al. 2013).

T cells can also be modified genetically to demonstrate a high affinity for TAAs through expression of specific TCRs. The first instance of successful TCR gene transfer was in human T cells in 1999. The TCR was designed to be specific for the MART-1 antigen expressed on malignant melanoma cells (Clay et al. 1999). Since then, many TCRs have been developed against multiple TAAs, and several successful human clinical trials have been performed with response rates varying 13–30% (Chinnasamy et al. 2011; Cohen et al. 2005; Frankel et al. 2010; Hillerdal et al. 2012; Johnson et al. 2006; Johnson et al. 2009; Morgan et al. 2003; Morgan et al. 2006; Parkhurst et al. 2009; Schaft et al. 2003; Stanislawski et al. 2001; Struetsmans et al. 2012).

Many techniques have been used to optimize the TCR affinity for TAAs. These techniques include the use of human leukocyte antigen (HLA)-A2 transgenic mice to produce the TCRs. Another strategy is to use an HLA mismatched donor to generate the high avidity HLA-A2 restricted T cell clones. However, this technique is quite cumbersome. The HLA complex in humans is polymorphic, and HLA-A2 is most commonly expressed; therefore most of the therapies using a TCR are designed to interact with this specific HLA. Soluble TCRs have also been developed. These TCRs are called ImmTACs (immune-mobilizing monoclonal TCRs against cancer) and are made up of a high affinity TCR fused to a CD3-specific single chain antibody fragment. These TCRs bind their TAAs and drive naturally occurring T cells to bind and destroy tumor cells.

One of the biggest impediments to TCR therapy is the ability of tumor cells to downregulate MHC expression preventing the TCR interaction, effectively allowing the tumor to evade immune surveillance. To overcome this limitation, CAR-modified T cells were developed to induce target cell apoptosis upon binding with the TAA independently of MHC expression. The CAR is a transmembrane molecule created using the binding domain of a single chain antibody fragment that is specific for the desired TAA, a modified hinge to allow flexibility during binding, extracellular stalk, and a transmembrane domain (Singh et al. 2008; Singh et al. 2011) (Figure 2). The CAR also contains various combinations of signaling endodomains (CD3ζ, OX40L, 4-1BBL, CD28) allowing for optimal T cell activation and CAR anchoring (Jena et al. 2010; Milone
Research is focusing on developing more efficacious CARs by altering the structure.

There are currently more than 30 human clinical trials investigating the application of genetically modified T cells for the treatment of cancer (Jena et al. 2010; Marcela et al. 2014). Most of these trials are focused on B cell malignancies; however, trials implementing this technique for solid tumors also exist, with limited success and significant side effects. The most successful trials have used a CD19-specific CAR in T cells for B cell malignancies. These CARs are second generation (i.e., they incorporate CD3ζ and CD28). The CD19-specific CAR was used in a small clinical study in three human patients with chemotherapy-resistant B cell chronic lymphocytic leukemia (Kalos et al. 2011; Porter et al. 2011). There were two clinical remissions and one long-lasting partial remission. The T cells expanded out to 1000 times the original engraftment dose and persisted at these levels for 6 months. The only significant toxicity associated with this trial was the development of B cell aplasia and hypogammaglobulinemia in these patients (Porter et al. 2011).

Because the goal of this therapy is engraftment of the modified T cells with long-term survival in the host, there is always the concern for toxicity. Additionally, the toxicity may become worse over time as the T cells expand in number. These cells are engineered to resist the normal signals for downregulation that cancers can secrete. For these reasons, it is important to consider a suicide gene that can allow the T cells to be eliminated in the event of a severe toxicity. One such strategy that has been used is using herpes simplex virus thymidine kinase. Incorporation of this gene allows the genetically modified T cells to be sensitized to gancyclovir or acyclovir therapy. This provides a safety net for uncontrolled cytotoxicity and subsequent inactivation of the immune response as required. The main drawback to herpes simplex virus thymidine kinase is that it can be immunogenic and lead to immune destruction of the T cells by the host. Other strategies include using a humanized caspase 9 dimer to induce apoptosis in the effector cells.

**Alternative Immunotherapies in Veterinary Oncology**

Monoclonal antibody–mediated immunotherapy, as opposed to cell-based immunotherapy, is not a new concept for veterinary medicine. It was one of the first successful techniques used to treat cancer in humans and dogs. Drugs such as trastuzumab and rituximab have been used to successfully treat breast cancer and B-cell lymphoma, respectively, in humans (Scott et al. 2012). One of the more successful uses of monoclonal antibodies to treat human B cell malignancies, such as NHL and diffuse large B cell lymphoma, is the addition of rituximab to other traditional chemotherapy protocols. Rituximab specifically targets surface expression of CD20 on normal and malignant B cells and has proven to significantly increase overall survival when used in combination with CHOP protocols in humans (Anderson et al. 1997; Maloney et al. 1997; McLaughlin et al. 1998). The lack of homology between dog and human CD20, which rituximab recognizes, limits the clinical utility of this antibody’s use in canine lymphoma (Impellizzeri et al. 2006; Jubala et al. 2005). Several companies are working on other monoclonal antibody treatments targeting canine LSA. Another possible canine tumor target is interleukin 13 receptor alpha 2. Promising results have been yielded from in vitro studies, which could lead to treatments for dogs with brain tumors (Debinski et al. 2013). However, therapeutic T cells have emerged as a novel immunotherapy with several advantages over monoclonal antibody therapy. These cells have the ability to traffic to and through tumor sites against interstitial pressures, expand after stimulation, create an immunological memory, and persist in the body for long periods of time after a single infusion.

Tumor vaccines have been developed for canine lymphoma, melanoma, hemangiosarcoma, and meningioma (Andersen et al. 2013; Denies and Sanders 2012; Gavazza et al. 2013; Liao et al. 2006). Oncept, which targets canine malignant melanoma, is the first USDA-licensed tumor vaccine in veterinary medicine. The efficacy of this vaccine is controversial. Groesenbaugh and colleagues (2011) reported improved survival for 58 dogs with stage II or stage III malignant melanoma after treatment with the vaccine when compared with 53 historical control dogs. However, in a recent retrospective study of dogs with oral malignant melanoma and loco-regional cancer control, the use of Oncept failed to provide a significant survival advantage compared with dogs not receiving the vaccine (Ottnod et al. 2013). This has prompted the investigation of other techniques to more robustly stimulate the immune system.

Another technique used to improve immune competence in tumor-bearing dogs is the use of biological response modifiers such as Bacillus Calmette-Guerin (BCG), often in combination with other chemotherapies (e.g., vincristine). BCG is a tuberculosis bacterial vaccine that produces a nonspecific proinflammatory response. The use of a BCG immune-modulator has been applied to canine transmissible venereal tumors, bladder carcinomas, mammary carcinomas, primary bone tumors, and others (Henry et al. 2007; Meyer et al. 1982; Mukaratiwa et al. 2009; Parodi et al. 1983). Several cell-based therapies to treat cancer use the immune system’s components such as dendritic cells, B cells, and T cells, as discussed earlier. Dendritic cell vaccines can promote robust immune responses in patients with cancer or immune-mediated diseases; however, this technique has been limited by a lack of standardized isolation techniques, as well as a lack of detailed information regarding dog-specific properties of these cells (Qeska et al. 2013).

Finally, Sorenmo and colleagues (2011) have used a cell-based vaccine against LSA in which RNA-loaded CD40L-activated B cells serve as highly efficient APCs. In this therapy, tumor RNA was used as the antigenic stimulus. The benefit of this approach is that it allows for an MHC-independent stimulation of the T cells and multiple antigen targeting. The RNA-loaded activated B cells elicited an in vitro T cell response using T cells isolated from healthy dogs and dogs diagnosed with LSA. The stimulated T cells had increased secretion of IFN-γ and upregulated expression
of the lymphoid homing molecule CCR7. A clinical trial performed in dogs diagnosed with LSA determined that even though the CD40L B-cell vaccine plus chemotherapy did not improve time to progression or overall survival, it did improve the rate and duration of second remissions (Sorenmo et al. 2011).

Concluding Remarks

Although the adoptive cellular treatment arm of immunotherapy is new to veterinary medicine, the successes and advances of knowledge into the intricacies of the immune system have renewed interest in clinical use for humans and dogs alike. Comparative oncology and immunology research has better classified canine T cells and their role in tumor prevention, progression and treatment. Clinical trials are currently being developed to implement CAR-specific T cell therapy in dogs with LSA and other neoplasms. Because of their ability to infiltrate and destroy solid tumor masses, nonspecific and tumor-specific T cell therapies may be able to improve survival outcomes, whether used alone or in combination with chemotherapies and radiotherapies. Additionally, using these strategies to program T cells to specifically target B cells may allow for treatment of immune-mediated or allergic diseases. This type of approach has been used with rituximab in humans with much success for many different diseases ranging from immune-mediated hemolytic anemia to asthma (Birgens et al. 2013; Cambridge et al. 2014; Listing et al. 2013; Sánchez-Ramón et al. 2013).

Adoptive T cell therapy provides a new and exciting approach to cancer therapy in humans and pet animals. This new technique, although cumbersome to manufacture, may provide the necessary therapeutic approach to push progression-free survival in our patients beyond 1 year and possibly even provide long-term immune monitoring through immunologic memory in successfully treated patients.

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