Review

Novel immunotherapies for immune-mediated haemolytic anaemia in dogs and people

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Abstract

Therapy of autoimmune diseases in dogs and people currently relies on use of broad-spectrum immunosuppressive drugs, which are associated with unacceptable adverse effects in some patients. Detractions of broad-spectrum immunosuppressive drugs are particularly apparent in people and animals with autoimmune haemolytic anaemia (AIHA), in whom such therapy is often required at high doses and for prolonged periods. Greater understanding of the immune aberrations that occur in patients with AIHA has permitted development of several forms of novel immunotherapy, which are intended to re-establish tolerance of self-antigens rather than suppress all parts of the immune system. Such therapies should be efficacious while still permitting normal responses to pathogens and inoculation. Immunotherapies of particular interest include monoclonal antibodies that produce selective depletion of the B cell compartment to decrease autoantibody production, administration of peptide antigens by subcutaneous or sublingual routes to establish tolerance, adoptive transfer of regulatory T cells (Tregs), and administration of low dose recombinant interleukin 2 to encourage proliferation and activation of Tregs. These therapies are in variable stages of development, with some being trialled in people and client-owned dogs, and others undergoing validation in experimental murine models. Continued development of these immunotherapies is likely to lead to the introduction of several novel products for the management of autoimmune disease in veterinary practice in the future.

Keywords: autoimmunity, AIHA, IMHA, dog, Treg

Introduction

Autoimmune diseases are caused by the development of inappropriate immune responses directed against host antigens. Ordinarily, the immune system remains tolerant of self-antigens through a number of mechanisms, beginning with deletion of autoreactive T cells in the thymus and B cells in bone marrow (Mouchess and Anderson, 2014). This process is insufficient to maintain immune tolerance because not all autoreactive cells are deleted and because mature lymphocytes may be exposed to cryptic antigens, such as after entry into the eye, brain, testis or other immunologically privileged sites (Forrester et al., 2008). The activity of mature T cells emigrating from the thymus is therefore regulated to limit development of responses to self-antigens, while permitting differentiation of effector cells capable of responding to exogenous insults. This regulation, synonymous with maintenance of peripheral tolerance, is manifest in the complex interaction of physical barriers, soluble signalling molecules and synapses between cells of the innate and adaptive immune systems, of which one important participant is the regulatory T cell (Treg) (Sakaguchi et al., 2009).

Regulatory T cells: The term Treg is now generally applied to a group of $CD4^+$ T cells recognised in mice and people by their surface expression of the interleukin (IL)-2 receptor alpha chain, CD25, and by expression of the transcription factor Forkhead box Protein 3 (FoxP3), which is required for their differentiation and function (Brunkow et al., 2001; Hori et al., 2003). The presence of a functional thymus is required for development of these thymic Tregs (Kojima and Prehn, 1981), though similar cells can differentiate in the periphery (Yadav et al., 2013). As in other species, a group of $CD4^+$ T cells that expresses FoxP3 and displays CD25 has been described in dogs (Garden et al., 2011; Knueppel et al., 2011; Pinheiro et al., 2011), in which they were also capable of suppressing non-specific proliferation of T cells *in vitro*. A more recent study of the markers expressed by human $CD4^+CD25^+FOXP3^+$ T cells suggests that many different subdivisions of this group will be recognised in future using emerging methodologies such as flow spectrometry, though the functional characteristics of these sub-groups have yet to be investigated (Mason et al., 2015).

Regulatory T cells are able to suppress the activation and proliferation of effector T cells ($CD4^+$ cells with Th1, Th2, Th9, Th17 and other phenotypes) and cytotoxic T cells *in vitro* when the latter cells are activated by either polyclonal or specific antigenic stimuli (Thornton et al., 1998; Dieckmann et al., 2001; Wing et al., 2003). Corresponding effects are observed *in vivo*, with suppression of transplant rejection and cessation of deleterious autoimmune responses observed after adoptive transfer of Tregs into experimental animals (Graca et al., 2002; Hoffmann et al., 2002; Buckner, 2010). The importance of Tregs is also demonstrated by mice that have undergone thymectomy (Kojima and Prehn, 1981; Sakaguchi et al., 1985), adult mice in which Tregs have been depleted pharmacologically (Ellis et al., 2013; Kim et al., 2007) and people and mice that are unable to express FOXP3/foxp3 in Tregs (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001), all of which develop multisystemic autoimmune diseases.

Investigations of Tregs in people with spontaneous autoimmune diseases have yielded results that are more equivocal, possibly because findings may depend on the types of samples collected and the gating strategies used to define Tregs using flow cytometry. For example, in people with rheumatoid arthritis, the frequency of Tregs in blood has been reported to be normal (Aerts et al., 2008), increased (Han et al., 2008) or decreased (Cao et al., 2004) in different studies, whereas Tregs are consistently increased in synovial fluid of inflamed joints (Miyara et al., 2011). Changes in peripheral blood Tregs in various human autoimmune diseases have been summarised elsewhere (Miyara et al., 2011; Grant et al., 2015).

Preliminary investigations of Tregs in canine immune-mediated diseases have yielded similarly mixed results. Decreased frequencies of Tregs were described in the blood of dogs with primary immune-mediated thrombocytopaenia and chronic enteropathies compared to healthy control dogs in a small pilot study (Volkmann et al., 2014), but frequencies were not different from healthy dogs after the clinical signs of both diseases were controlled. In a separate study, the proportion of T cells that expressed both CD4 and FoxP3 did not differ between healthy dogs and dogs with primary hypothyroidism (Miller et al., 2015).

Immune-mediated haemolytic anaemia in people and dogs: One autoimmune disease in which the role of Tregs is not fully defined is autoimmune haemolytic anaemia (AIHA), which was first described in a human patient by Vanlair and Masius in 1871 (Packman, 2001). The disease is characterised by production of antibodies directed against normal glycoprotein antigens on the surface of erythrocytes. Anti-erythrocyte antibodies are produced normally to assist in clearance of senescent cells (Lutz and Wipf, 1982); in people with AIHA, the antibodies facilitate complement-mediated intravascular haemolysis or phagocytosis of opsonised erythrocytes in the liver and spleen (Berentsen and Tundic, 2015), resulting in anaemia that is often severe. Autoimmune haemolytic anaemia is classified according to the activity of autoantibodies at different temperatures: the most common form is 'warm' AIHA, in which antibodies are capable of causing haemolysis at 37^{o}C, which differentiates the disease from several forms of cold agglutinating disease (CAD), in which antibodies are most active at 3-4^{o}C (Berentsen and Tundic, 2015). Warm AIHA bears strong resemblance to canine primary immune-mediated haemolytic anaemia (IMHA), which is considered to be the most common autoimmune disease of dogs (McCullough, 2003). Both diseases cause severe anaemia that typically develops acutely and may be accompanied by pre-hepatic icterus (Swann and Skelly, 2011; Berentsen and Tundic, 2015).

Studies of people with warm AIHA indicate that the aberrant autoimmune response may have a Th17 phenotype; the frequency of Th17 cells in peripheral blood was increased in people with AIHA compared to healthy controls and these and the serum concentration of IL-17 were correlated with the severity of clinical disease (Hall et al., 2012; Xu et al., 2012). A further study indicated that the frequency of Tregs was decreased in people with warm AIHA compared to healthy volunteers, and this also correlated with some markers of disease severity (Ahmad et al., 2011). There are no published studies describing numbers or suppressive function of Tregs in dogs with primary IMHA, but this is an area of active investigation in our own laboratory. A single abstract described an unusually high average frequency of Tregs as a proportion of total lymphocytes (24.84%) in seven affected dogs (Baek et al., 2013).

Therapy of autoimmune diseases: In clinical practice today, autoimmune diseases are treated with immunosuppressive agents that frequently have an effect on many parts of the immune system simultaneously. While often effective, these drugs may be associated with adverse effects because they suppress immune responses directed at all antigens, including exogenous pathogens. Also, because these drugs do not induce tolerance of self-antigens, there is a continued risk of relapse during and after treatment, such that a very long or indefinite course of treatment is often required. Owing to the detractions inherent in immunosuppressive treatment, much current research is directed at generation of immunomodulatory therapies that induce or re-instate peripheral tolerance of self-antigens, either by altering responses of effector T cells or by increasing numbers or activity of Tregs (Figure 1).

Rationale for and implications of immunosuppressive treatment: There has been a pleasing and saleable symmetry about the use of immunosuppressive drugs for the treatment of autoimmune diseases in people and animals since the discovery and widespread production of the synthetic corticosteroids in the 1950s (Herzog and Oliveto, 1992). Indeed, glucocorticoids remain the only group of drugs licensed for the treatment of autoimmune diseases in dogs and cats in the UK[1] and, in a recent systematic review of evidence relating to the treatment of IMHA in dogs, we found that glucocorticoids had been administered to all of the 843 dogs from which data were derived (Swann and Skelly, 2013). Use of prednisolone (or prednisone) alone seems to result in survival rates of up to 65% at six months after diagnosis (Swann and Skelly, 2013), with similar response rates reported in people (Zanella and Barcellini, 2014). There are concerns that long-term administration of these drugs may result in unacceptable adverse effects, largely due to iatrogenic recapitulation of Cushing's syndrome and increased risk of thromboembolic disease (Rose et al., 2011).

[1] See: http://www.noahcompendium.co.uk

In order to facilitate a polypharmaceutical approach to the management of autoimmune disease, several other immunosuppressive drugs have been used in medical and veterinary practice, as has been reviewed elsewhere (Whitley and Day, 2011; Zanella and Barcellini, 2014). Adoption of combination therapies for treatment of dogs with IMHA is conceptually attractive but is not proven to result in improved survival or decreased prevalence of glucocorticoid-associated adverse effects (Swann and Skelly, 2013). Indeed, concurrent use of immunosuppressive drugs that act on different components of the immune response may produce additional risks, such as development of cutaneous fungal infections in dogs receiving ciclosporin and glucocorticoids (Dowling et al., 2015).

Greater understanding of the immunological changes that occur in people and animals with autoimmune diseases has also informed the use of some immunosuppressive drugs. Epidemiological studies revealed that the alkylating agent cyclophosphamide had a paradoxical effect, causing immune stimulation at low doses and immunosuppression at high doses, as reviewed elsewhere (Heylmann et al., 2013). Investigations in people and mice have shown that Tregs are particularly sensitive to the lymphotoxic effects of cyclophosphamide at low doses, favouring increased activity of effector components of the immune system (Brode and Cooke, 2008). While this effect is beneficial to prevent immune evasion of neoplastic cells in patients receiving metronomic chemotherapy (Schabowsky et al., 2007), it is interesting to note that administration of cyclophosphamide was associated with a poorer outcome in dogs with IMHA in two small epidemiological studies (Reimer et al., 1999; Grundy and Barton, 2001), possibly related to its effects on Tregs. In contrast, the same drug has been used at high doses in people to achieve control of AIHA that has not responded to glucocorticoids or other forms of conventional treatment, resulting in clinical remission in five of eight patients in one study (Moyo et al., 2002).

Monoclonal antibody therapy: People with AIHA who have failed treatment with glucocorticoids are frequently treated with rituximab, a human/murine chimeric monoclonal antibody specific for the human CD20 molecule. Exclusive expression on the surface of B cells makes this molecule an attractive target in diseases characterised by autoantibody production; binding of rituximab facilitates

complement-mediated destruction and antibody-mediated cell cytotoxicity, resulting in rapid depletion of the B cell compartment in blood, lymphoid tissue and bone marrow (Reff et al., 1994). Since the first report of its use in people with warm AIHA in 2002 (Zaja et al., 2002), more than 20 studies have evaluated its effects. A recent meta-analysis of data from 409 people concluded that the overall response rate was 79% (95% confidence interval [CI] 60-90%) in people with warm AIHA, and that the rate of overall and complete response was higher in this group of patients compared to those with other forms of AIHA in univariable meta-regression analysis (Reynaud et al., 2015). In one study, the majority of patients making a complete response remained in remission for at least six months, with responses more likely in younger patients and those with a shorter duration of disease prior to receiving rituximab (Penalver et al, 2010; Barcellini et al, 2013; Reynaud et al, 2015).

Monoclonal antibodies have a more restricted immunosuppressive effect than glucocorticoids and other broad-spectrum immunosuppressive drugs, but they still increase the risk of opportunistic infections. Adverse effects were observed in approximately 14% (95% CI 9-21) of people treated with rituximab in the meta-analysis described in the preceding paragraph (Reynaud et al., 2015), with severe infections, neutropaenia and *Pneumocystis jirovecii* pneumonia representing the most inimical. Monoclonal antibodies, though usually humanised or composed of chimeric murine and human elements, may still be recognised as foreign antigens by the immune system, resulting in development of responses that neutralise their effects (Keiserman et al., 2014). Finally, depletion of the B cell compartment may also create niches in the spleen that are conducive to survival of long-lived autoreactive plasma cells, which would not otherwise persist in the face of conventional immunosuppressive treatment. This phenomenon has been demonstrated in studies of people with warm AIHA and immune-mediated thrombocytopaenia that received rituximab before undergoing splenectomy owing to failure to control their clinical signs (Mahevas et al., 2013; 2015), though this has not been associated with increased risk of relapse.

Rituximab was manufactured for specificity to epitopes on the extracellular domain of human CD20. The structure of this domain varies among mammalian species (Polyak and Deans, 2002), but the

functional importance of this diversity is unknown because the physiological ligand for CD20 has yet to be identified. Consequent to these differences in structure, rituximab does not cross react with the extracellular domain of canine molecules (Jubala et al., 2005) and has no apparent therapeutic potential in this species, as demonstrated by failure to deplete B cells in an *ex vivo* model of B cell lymphoma (Impellizeri et al., 2006). Nevertheless, established methodologies have been applied to develop monoclonal 'caninised' antibodies that bind CD20 and deplete B cells in dogs (Ito et al., 2015; Rue et al., 2015). One such product has been licensed in the United States, and clinical trials are currently ongoing to evaluate its efficacy in the management of canine lymphoma[2] (Rodriguez et al., 2015).

Autoantigen-specific immunotherapy: Production of antibodies with high affinity for antigen does not occur without provision of stimulatory signals from T helper cells, which premise has led to renewed interest in the role of these cells in autoimmune diseases. Naïve T cells become activated when they recognise their cognate antigens presented in the context of major histocompatibility (MHC) molecules, but mounting evidence suggests that the concentration, preparation and route of entry of the antigen into the body can have a major impact on the nature of the immune response that develops (Verhagen et al., 2015). For example, experiments in the early twentieth Century showed that administration of pollen extracts to patients with pollen hypersensitivity (hay fever) by subcutaneous injection could alleviate or completely resolve their clinical signs, whereas the same allergens would be detrimental if encountered naturally (Ring and Gutermuth, 2011). These observations led to the development of allergen-specific immunotherapy protocols for use in this and other hypersensitivity diseases (Jutel et al., 2015), and delivery of allergens by subcutaneous or sublingual routes is also widely practiced in veterinary medicine for management of canine atopic dermatitis (Olivry et al., 2015; Mueller et al., 2015).

[2] See: http://www.aratana.com/for-veterinarians/clinical-studies/

Several experimental models suggest that the same process could be applied to control established autoimmune responses (Sabatos-Peyton et al., 2010). This autoantigen-specific immunotherapy is based primarily on the ability to induce Th1, Th2 and Th17 cells to adopt a regulatory phenotype characterised by production of IL-10 (Meiler et al., 2008) and possibly by display of the surface markers CD49b and LAG-3 (Gagliani et al., 2013) in response to their cognate antigens. Effector T cells are more likely to differentiate into these so-called Tr1 cells (also described as $_{IL\text{-}10}$Tregs cells) when exposed to repeated, high doses of their cognate antigens delivered by subcutaneous injection, though, in patients with clinically active diseases, there are concerns that such dosing schedules could exacerbate of the autoimmune response.

The latter phenomenon was observed in three patients with multiple sclerosis who received subcutaneous injections of a peptide derived from the immunodominant myelin basic protein. These patients suffered more severe neurological abnormalities and brain inflammation (as indicated by magnetic resonance imaging) but were subsequently rescued by administration of glucocorticoids (Bielekova et al., 2000). Elucidation of immunological changes revealed that administration of the autoantigen was associated with increased numbers of Th1 effector cells in the cerebrospinal fluid of some patients, rather than induction of anergy or differentiation of Tr1 cells. While notably this study used an altered peptide antigen, in which some amino acids had been artificially substituted to modify the interaction at the synapse between T cell and MHC-peptide complex, this study demonstrates the risks inherent in autoantigen-specific immunotherapy and the need for a dosing schedule that minimises them.

Autoantigen-specific immunotherapy has not been attempted in people with AIHA or dogs with IMHA, but this form of treatment could be feasible in both species because previous studies have identified several of the immunodominant self-antigens targeted by autoimmune responses. In people, autoantibodies are most commonly specific for epitopes on the Rhesus polypeptides (Barker et al., 1992), and T cells from patients with AIHA showed a proliferative response *in vitro* to peptides derived from the Rhesus D molecule (Barker et al., 1997). In dogs with IMHA, autoreactive T and B

cells are more likely to be specific for glycophorin molecules, though patterns of reactivity to erythrocyte antigens do not appear to be as consistent between individuals as in people (Barker et al., 1991; Corato et al., 1997).

In people with warm AIHA, T cells that produce IL-10 in response to Rhesus D molecules have also been identified in the spleen and peripheral blood. These cells were able to suppress production of the Th1-associated cytokine interferon gamma (IFNγ) in peripheral blood mononuclear cell cultures *in vitro*, contingent on their expression of CTLA-4 (Hall et al., 2002; Ward et al., 2008). These studies suggest that Tr1 cells are present in the blood of people with AIHA, where they could be activated or induced by administration of autoantigen in an appropriate form.

Cell-based therapy: Whether or not defects in Treg frequency or function contribute to development of autoimmune diseases, there is growing evidence to suggest that they could be used as a therapeutic agent to re-establish tolerance of self-antigens. In support of this notion, adoptive transfer of splenic $CD4^+CD25^+$ Tregs prevented production of anti-erythrocyte antibodies in mice that were subsequently injected repeatedly with rat erythrocytes to generate an autoimmune response (in the Playfair Marshall-Clarke model of AIHA)(Mqadmi et al., 2005). Studies in other murine models of autoimmune disease have shown that similar adoptive transfers can ameliorate established autoimmune diseases and delay or prevent rejection of transplanted organs, as reviewed elsewhere (Singer et al., 2014).

There have been numerous obstacles in the path to use Tregs as therapy for people with autoimmune diseases, including the need to isolate Tregs from peripheral blood using reliable phenotypic markers, expand the number of Tregs in *ex vivo* culture systems that subscribe to Good Manufacturing Practice and characterise their suppressive abilities prior to infusion into patients (Putnam et al., 2013; Haase et al., 2015). Concerns have also arisen that some Tregs might lose FOXP3 expression and adopt an effector Th17 phenotype after infusion (Hori, 2011; Komatsu et al., 2014), possibly worsening the autoimmune disease. Nevertheless, the process of isolating, expanding and re-infusing autologous

Tregs has been completed in people with type 1 diabetes mellitus and Crohn's disease. Of the twelve children with diabetes mellitus who received autologous Tregs, eight achieved clinical remission, with no significant adverse events reported in any patient and appropriate and sustained responses to routine vaccination (Marek-Trzonkowska et al., 2014). Administration of Tregs to patients with Crohn's disease resulted in a greater frequency of adverse effects (including exacerbation of gastrointestinal disease (n=11) and thrombosis/thrombophlebitis (n=2)), with some form of response in eight of twenty patients overall (Desreumaux et al., 2012).

There are many preliminary steps that would also need to be taken before Treg cell therapy could become a reality in dogs, including more thorough phenotyping of the Treg population, demonstration of its stability in response to an inflammatory setting and generation of a protocol that could be used reliably and safely to expand the population *ex vivo*. In people, expansion of the Tregs to a number considered suitable for infusion also generally requires several weeks of repeated stimulation with anti-CD3 and anti-CD28 antibodies in a medium enriched with IL-2 (Putnam et al, 2013); this timescale may not be compatible with treatment of an autoimmune disease that often has an acute onset, though it could be useful to curtail regimens involving broad-spectrum immunosuppressive drugs.

Low dose interleukin 2 therapy: Interleukin 2 was traditionally considered to be a cytokine that stimulated effector T cell proliferation and differentiation, but mice that could not produce the cytokine were unexpectedly found to develop spontaneous autoimmune diseases (Sadlack et al., 1993). Interestingly, IL-2 gene knockouts on the Balb/c mouse background caused fatal AIHA within five weeks, which was related to deficiency of the Treg compartment, uncontrolled proliferation of effector T cells and production of autoantibodies (Sadlack et al., 1995). Similar abnormalities were detected in mice lacking the IL-2 receptor alpha chain (CD25), which is constitutively expressed at high levels by Tregs (Willerford et al., 1995), and in people with missense mutations in the equivalent gene (Sharfe et al., 1997).

Since the recognition that IL-2 is required for maintenance of Treg numbers and activity, there has been interest in the use of the recombinant human cytokine for treatment of autoimmune diseases (Klatzmann and Abbas, 2015). Administration of IL-2 at high doses has been associated with adverse effects, such as excessive production of other cytokines and increased capillary permeability (Rosenstein et al., 1986; Baluna and Vitetta, 1997), but low dose IL-2 therapy appears to be more promising in clinical practice.

Low dose IL-2 therapy has been used in 10 people with vasculitis induced by hepatitis C infection, in whom it caused only mild and transient reactions, with no signs of vascular leak syndrome. Clinical signs improved in eight of ten patients, with an increase in the average number of Tregs across the whole group (Saadoun et al., 2011). In a separate open pilot study, IL-2 was administered at a low dose to five people with alopecia areata, with improvements in clinical disease score and immunohistochemical findings of scalp biopsies in four of five patients (Castela et al., 2014).

Additional clinical trials are currently ongoing to evaluate low dose IL-2 in other human autoimmune diseases (Waldron-Lynch et al., 2014; Humrich et al., 2015), and this treatment could also be developed for canine use. So far, only recombinant human IL-2 is available, and, while this product has been administered intralesionally for treatment of several types of cancer in dogs without apparent adverse effects (Konietscke et al., 2012; Haagsman et al., 2013; Ziekman et al., 2013; Den Otter et al., 2015), there are no reports of its systemic administration. An appropriate dose for treatment of autoimmune disease would also need to be established, as doses of IL-2 required to stimulate Tregs appear to differ between species (Klatzmann and Abbas, 2015).

Emerging alternative therapies: Several other forms of immunotherapy that modulate the signals determining survival of circulating lymphocytes are in varying stages of development, and these could also have application in the treatment of AIHA. Maturation and survival of B cells in the periphery is dependent on interactions between the soluble molecule B lymphocyte stimulator (BLyS) and its

receptor, BLyS receptor 3 (BR3), which is expressed on their surface (Cancro et al., 2009). The concentration of BLyS regulates the size and nature of the mature B cell compartment, with higher concentrations resulting in greater numbers of B cells and also permitting B cells with autoreactive potential to survive (Cancro et al., 2009). The serum concentration of BLyS was greater in people with AIHA compared to healthy controls in two studies; in one of these, the concentration correlated with indicators of clinical disease activity (Zhao et al., 2015) and decreased after treatment with glucocorticoids (Xu et al., 2015).

These findings suggest that autoreactive B cells escaping suppression or deletion may be important in development of AIHA, providing further recourse for treatment using therapies that modulate serum concentrations of BLyS. One such treatment is belimumab, a human monoclonal antibody product that binds to and inhibits soluble BLyS, resulting in decreased B cell proliferation, depletion of B cells (Baker et al., 2003) and improved control of disease activity in people with systemic lupus erythematosus (SLE)(Ginzler et al, 2013).

Preservation of a functioning apoptotic pathway is essential to maintenance of tolerance in T cells because it permits cell death during selection in the thymus and after receipt of inhibitory signals from APCs (Tischner et al, 2010). Conversely, overexpression of anti-apoptotic regulators in lymphocytes may contribute to development of SLE in people (Andre et al, 2007). The Bcl family of cell signalling molecules includes pro- and anti-apoptotic members: Bcl-2 and Bcl-x_L are major members of the latter group (Tischner et al., 2010). Inhibitors of these molecules were developed primarily for treatment of lymphoma, but administration of the Bcl-2 family antagonist ABT-737 has also resulted in clinical improvements in murine models of SLE and rheumatoid arthritis (Bardwell et al, 2009).

Finally, differentiation of activated T cells could be modulated using drugs that alter the activity of the transcription factors that determine commitment to a particular subset. Recent efforts have focused on development of small molecule inhibitors of retinoic acid receptor-related orphan nuclear receptor (RORγt), the central transcription factor involved in differentiation of Th17 T cells. So far,

published work has shown clinical improvement in a murine model of multiple sclerosis after administration of a candidate molecule (Xiao et al., 2014), but similar products could be useful in people with AIHA as Th17 cells are present at increased frequency in these patients (Xu et al., 2012).

Conclusions

Several forms of novel immunotherapy are currently in active development, largely based on greater understanding of the regulatory processes that usually control autoimmune responses. Some of these forms of therapy warrant considerable testing before they could be applied in client-owned animals in veterinary practice, but others are undergoing clinical trials at present, raising the exciting prospect of novel immunotherapies for treatment of canine IMHA in the future.

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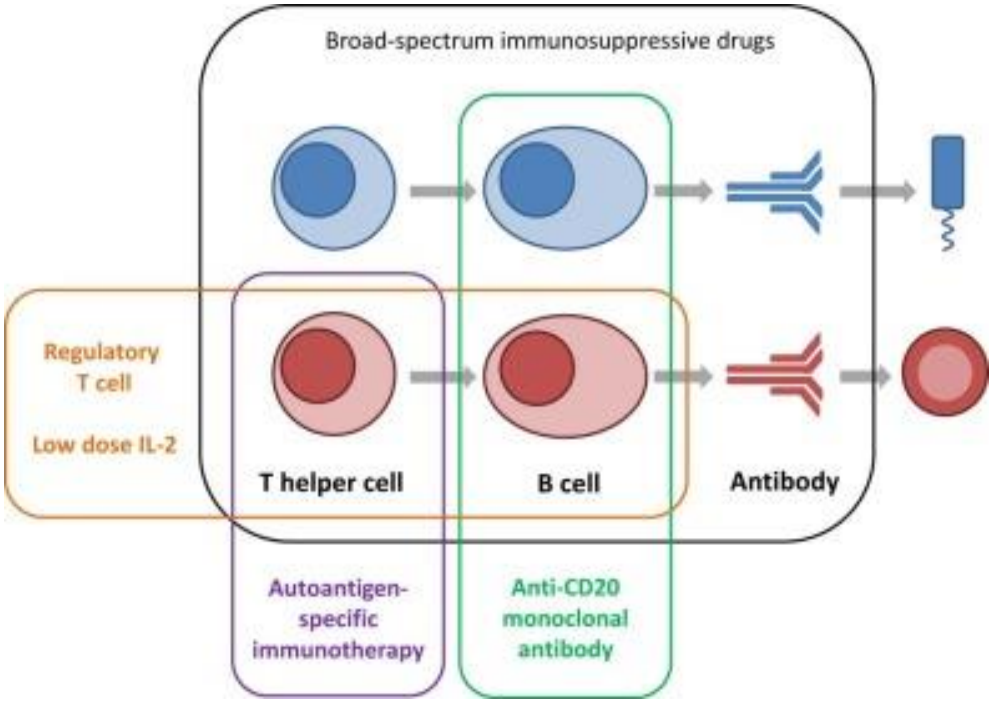
Figure 1: Schematic diagram to indicate parts of the immune response that are targeted by different forms of therapy. Blue section indicates normal immune response against pathogenic bacteria; red section indicates autoimmune response against erythrocytes. Broad-spectrum immunosuppressive agents affect many parts of the immune response (including several not shown), whereas emerging immunotherapies have a more specific action.



Review

Novel immunotherapies for immune-mediated haemolytic anaemia in dogs and people

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Abstract

Therapy of autoimmune diseases in dogs and people currently relies on use of broad-spectrum immunosuppressive drugs, which are associated with unacceptable adverse effects in some patients. Detractions of broad-spectrum immunosuppressive drugs are particularly apparent in people and animals with autoimmune haemolytic anaemia (AIHA), in whom such therapy is often required at high doses and for prolonged periods. Greater understanding of the immune aberrations that occur in patients with AIHA has permitted development of several forms of novel immunotherapy, which are intended to re-establish tolerance of self-antigens rather than suppress all parts of the immune system. Such therapies should be efficacious while still permitting normal responses to pathogens and inoculation. Immunotherapies of particular interest include monoclonal antibodies that produce selective depletion of the B cell compartment to decrease autoantibody production, administration of peptide antigens by subcutaneous or sublingual routes to establish tolerance, adoptive transfer of regulatory T cells (Tregs), and administration of low dose recombinant interleukin 2 to encourage proliferation and activation of Tregs. These therapies are in variable stages of development, with some being trialled in people and client-owned dogs, and others undergoing validation in experimental murine models. Continued development of these immunotherapies is likely to lead to the introduction of several novel products for the management of autoimmune disease in veterinary practice in the future.

Keywords: autoimmunity, AIHA, IMHA, dog, Treg

Introduction

Autoimmune diseases are caused by the development of inappropriate immune responses directed against host antigens. Ordinarily, the immune system remains tolerant of self-antigens through a number of mechanisms, beginning with deletion of autoreactive T cells in the thymus and B cells in bone marrow (Mouchess and Anderson, 2014). This process is insufficient to maintain immune tolerance because not all autoreactive cells are deleted and because mature lymphocytes may be exposed to cryptic antigens, such as after entry into the eye, brain, testis or other immunologically privileged sites (Forrester et al., 2008). The activity of mature T cells emigrating from the thymus is therefore regulated to limit development of responses to self-antigens, while permitting differentiation of effector cells capable of responding to exogenous insults. This regulation, synonymous with maintenance of peripheral tolerance, is manifest in the complex interaction of physical barriers, soluble signalling molecules and synapses between cells of the innate and adaptive immune systems, of which one important participant is the regulatory T cell (Treg) (Sakaguchi et al., 2009).

Regulatory T cells: The term Treg is now generally applied to a group of $CD4^+$ T cells recognised in mice and people by their surface expression of the interleukin (IL)-2 receptor alpha chain, CD25, and by expression of the transcription factor Forkhead box Protein 3 (FoxP3), which is required for their differentiation and function (Brunkow et al., 2001; Hori et al., 2003). The presence of a functional thymus is required for development of these thymic Tregs (Kojima and Prehn, 1981), though similar cells can differentiate in the periphery (Yadav et al., 2013). As in other species, a group of $CD4^+$ T cells that expresses FoxP3 and displays CD25 has been described in dogs (Garden et al., 2011; Knueppel et al., 2011; Pinheiro et al., 2011), in which they were also capable of suppressing non-specific proliferation of T cells *in vitro*. A more recent study of the markers expressed by human $CD4^+CD25^+FOXP3^+$ T cells suggests that many different subdivisions of this group will be recognised in future using emerging methodologies such as flow spectrometry, though the functional characteristics of these sub-groups have yet to be investigated (Mason et al., 2015).

Regulatory T cells are able to suppress the activation and proliferation of effector T cells ($CD4^+$ cells with Th1, Th2, Th9, Th17 and other phenotypes) and cytotoxic T cells *in vitro* when the latter cells are activated by either polyclonal or specific antigenic stimuli (Thornton et al., 1998; Dieckmann et al., 2001; Wing et al., 2003). Corresponding effects are observed *in vivo*, with suppression of transplant rejection and cessation of deleterious autoimmune responses observed after adoptive transfer of Tregs into experimental animals (Graca et al., 2002; Hoffmann et al., 2002; Buckner, 2010). The importance of Tregs is also demonstrated by mice that have undergone thymectomy (Kojima and Prehn, 1981; Sakaguchi et al., 1985), adult mice in which Tregs have been depleted pharmacologically (Ellis et al., 2013; Kim et al., 2007) and people and mice that are unable to express FOXP3/foxp3 in Tregs (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001), all of which develop multisystemic autoimmune diseases.

Investigations of Tregs in people with spontaneous autoimmune diseases have yielded results that are more equivocal, possibly because findings may depend on the types of samples collected and the gating strategies used to define Tregs using flow cytometry. For example, in people with rheumatoid arthritis, the frequency of Tregs in blood has been reported to be normal (Aerts et al., 2008), increased (Han et al., 2008) or decreased (Cao et al., 2004) in different studies, whereas Tregs are consistently increased in synovial fluid of inflamed joints (Miyara et al., 2011). Changes in peripheral blood Tregs in various human autoimmune diseases have been summarised elsewhere (Miyara et al., 2011; Grant et al., 2015).

Preliminary investigations of Tregs in canine immune-mediated diseases have yielded similarly mixed results. Decreased frequencies of Tregs were described in the blood of dogs with primary immune-mediated thrombocytopaenia and chronic enteropathies compared to healthy control dogs in a small pilot study (Volkmann et al., 2014), but frequencies were not different from healthy dogs after the clinical signs of both diseases were controlled. In a separate study, the proportion of T cells that expressed both CD4 and FoxP3 did not differ between healthy dogs and dogs with primary hypothyroidism (Miller et al., 2015).

Immune-mediated haemolytic anaemia in people and dogs: One autoimmune disease in which the role of Tregs is not fully defined is autoimmune haemolytic anaemia (AIHA), which was first described in a human patient by Vanlair and Masius in 1871 (Packman, 2001). The disease is characterised by production of antibodies directed against normal glycoprotein antigens on the surface of erythrocytes. Anti-erythrocyte antibodies are produced normally to assist in clearance of senescent cells (Lutz and Wipf, 1982); in people with AIHA, the antibodies facilitate complement-mediated intravascular haemolysis or phagocytosis of opsonised erythrocytes in the liver and spleen (Berentsen and Tundic, 2015), resulting in anaemia that is often severe. Autoimmune haemolytic anaemia is classified according to the activity of autoantibodies at different temperatures: the most common form is 'warm' AIHA, in which antibodies are capable of causing haemolysis at 37^{o}C, which differentiates the disease from several forms of cold agglutinating disease (CAD), in which antibodies are most active at 3-4^{o}C (Berentsen and Tundic, 2015). Warm AIHA bears strong resemblance to canine primary immune-mediated haemolytic anaemia (IMHA), which is considered to be the most common autoimmune disease of dogs (McCullough, 2003). Both diseases cause severe anaemia that typically develops acutely and may be accompanied by pre-hepatic icterus (Swann and Skelly, 2011; Berentsen and Tundic, 2015).

Studies of people with warm AIHA indicate that the aberrant autoimmune response may have a Th17 phenotype; the frequency of Th17 cells in peripheral blood was increased in people with AIHA compared to healthy controls and these and the serum concentration of IL-17 were correlated with the severity of clinical disease (Hall et al., 2012; Xu et al., 2012). A further study indicated that the frequency of Tregs was decreased in people with warm AIHA compared to healthy volunteers, and this also correlated with some markers of disease severity (Ahmad et al., 2011). There are no published studies describing numbers or suppressive function of Tregs in dogs with primary IMHA, but this is an area of active investigation in our own laboratory. A single abstract described an unusually high average frequency of Tregs as a proportion of total lymphocytes (24.84%) in seven affected dogs (Baek et al., 2013).

Therapy of autoimmune diseases: In clinical practice today, autoimmune diseases are treated with immunosuppressive agents that frequently have an effect on many parts of the immune system simultaneously. While often effective, these drugs may be associated with adverse effects because they suppress immune responses directed at all antigens, including exogenous pathogens. Also, because these drugs do not induce tolerance of self-antigens, there is a continued risk of relapse during and after treatment, such that a very long or indefinite course of treatment is often required. Owing to the detractions inherent in immunosuppressive treatment, much current research is directed at generation of immunomodulatory therapies that induce or re-instate peripheral tolerance of self-antigens, either by altering responses of effector T cells or by increasing numbers or activity of Tregs (Figure 1).

Rationale for and implications of immunosuppressive treatment: There has been a pleasing and saleable symmetry about the use of immunosuppressive drugs for the treatment of autoimmune diseases in people and animals since the discovery and widespread production of the synthetic corticosteroids in the 1950s (Herzog and Oliveto, 1992). Indeed, glucocorticoids remain the only group of drugs licensed for the treatment of autoimmune diseases in dogs and cats in the UK[1] and, in a recent systematic review of evidence relating to the treatment of IMHA in dogs, we found that glucocorticoids had been administered to all of the 843 dogs from which data were derived (Swann and Skelly, 2013). Use of prednisolone (or prednisone) alone seems to result in survival rates of up to 65% at six months after diagnosis (Swann and Skelly, 2013), with similar response rates reported in people (Zanella and Barcellini, 2014). There are concerns that long-term administration of these drugs may result in unacceptable adverse effects, largely due to iatrogenic recapitulation of Cushing's syndrome and increased risk of thromboembolic disease (Rose et al., 2011).

[1] See: http://www.noahcompendium.co.uk

In order to facilitate a polypharmaceutical approach to the management of autoimmune disease, several other immunosuppressive drugs have been used in medical and veterinary practice, as has been reviewed elsewhere (Whitley and Day, 2011; Zanella and Barcellini, 2014). Adoption of combination therapies for treatment of dogs with IMHA is conceptually attractive but is not proven to result in improved survival or decreased prevalence of glucocorticoid-associated adverse effects (Swann and Skelly, 2013). Indeed, concurrent use of immunosuppressive drugs that act on different components of the immune response may produce additional risks, such as development of cutaneous fungal infections in dogs receiving ciclosporin and glucocorticoids (Dowling et al., 2015).

Greater understanding of the immunological changes that occur in people and animals with autoimmune diseases has also informed the use of some immunosuppressive drugs. Epidemiological studies revealed that the alkylating agent cyclophosphamide had a paradoxical effect, causing immune stimulation at low doses and immunosuppression at high doses, as reviewed elsewhere (Heylmann et al., 2013). Investigations in people and mice have shown that Tregs are particularly sensitive to the lymphotoxic effects of cyclophosphamide at low doses, favouring increased activity of effector components of the immune system (Brode and Cooke, 2008). While this effect is beneficial to prevent immune evasion of neoplastic cells in patients receiving metronomic chemotherapy (Schabowsky et al., 2007), it is interesting to note that administration of cyclophosphamide was associated with a poorer outcome in dogs with IMHA in two small epidemiological studies (Reimer et al., 1999; Grundy and Barton, 2001), possibly related to its effects on Tregs. In contrast, the same drug has been used at high doses in people to achieve control of AIHA that has not responded to glucocorticoids or other forms of conventional treatment, resulting in clinical remission in five of eight patients in one study (Moyo et al., 2002).

Monoclonal antibody therapy: People with AIHA who have failed treatment with glucocorticoids are frequently treated with rituximab, a human/murine chimeric monoclonal antibody specific for the human CD20 molecule. Exclusive expression on the surface of B cells makes this molecule an attractive target in diseases characterised by autoantibody production; binding of rituximab facilitates

complement-mediated destruction and antibody-mediated cell cytotoxicity, resulting in rapid depletion of the B cell compartment in blood, lymphoid tissue and bone marrow (Reff et al., 1994). Since the first report of its use in people with warm AIHA in 2002 (Zaja et al., 2002), more than 20 studies have evaluated its effects. A recent meta-analysis of data from 409 people concluded that the overall response rate was 79% (95% confidence interval [CI] 60-90%) in people with warm AIHA, and that the rate of overall and complete response was higher in this group of patients compared to those with other forms of AIHA in univariable meta-regression analysis (Reynaud et al., 2015). In one study, the majority of patients making a complete response remained in remission for at least six months, with responses more likely in younger patients and those with a shorter duration of disease prior to receiving rituximab (Penalver et al, 2010; Barcellini et al, 2013; Reynaud et al, 2015).

Monoclonal antibodies have a more restricted immunosuppressive effect than glucocorticoids and other broad-spectrum immunosuppressive drugs, but they still increase the risk of opportunistic infections. Adverse effects were observed in approximately 14% (95% CI 9-21) of people treated with rituximab in the meta-analysis described in the preceding paragraph (Reynaud et al., 2015), with severe infections, neutropaenia and *Pneumocystis jirovecii* pneumonia representing the most inimical. Monoclonal antibodies, though usually humanised or composed of chimeric murine and human elements, may still be recognised as foreign antigens by the immune system, resulting in development of responses that neutralise their effects (Keiserman et al., 2014). Finally, depletion of the B cell compartment may also create niches in the spleen that are conducive to survival of long-lived autoreactive plasma cells, which would not otherwise persist in the face of conventional immunosuppressive treatment. This phenomenon has been demonstrated in studies of people with warm AIHA and immune-mediated thrombocytopaenia that received rituximab before undergoing splenectomy owing to failure to control their clinical signs (Mahevas et al., 2013; 2015), though this has not been associated with increased risk of relapse.

Rituximab was manufactured for specificity to epitopes on the extracellular domain of human CD20. The structure of this domain varies among mammalian species (Polyak and Deans, 2002), but the

functional importance of this diversity is unknown because the physiological ligand for CD20 has yet to be identified. Consequent to these differences in structure, rituximab does not cross react with the extracellular domain of canine molecules (Jubala et al., 2005) and has no apparent therapeutic potential in this species, as demonstrated by failure to deplete B cells in an *ex vivo* model of B cell lymphoma (Impellizeri et al., 2006). Nevertheless, established methodologies have been applied to develop monoclonal 'caninised' antibodies that bind CD20 and deplete B cells in dogs (Ito et al., 2015; Rue et al., 2015). One such product has been licensed in the United States, and clinical trials are currently ongoing to evaluate its efficacy in the management of canine lymphoma[2] (Rodriguez et al., 2015).

Autoantigen-specific immunotherapy: Production of antibodies with high affinity for antigen does not occur without provision of stimulatory signals from T helper cells, which premise has led to renewed interest in the role of these cells in autoimmune diseases. Naïve T cells become activated when they recognise their cognate antigens presented in the context of major histocompatibility (MHC) molecules, but mounting evidence suggests that the concentration, preparation and route of entry of the antigen into the body can have a major impact on the nature of the immune response that develops (Verhagen et al., 2015). For example, experiments in the early twentieth Century showed that administration of pollen extracts to patients with pollen hypersensitivity (hay fever) by subcutaneous injection could alleviate or completely resolve their clinical signs, whereas the same allergens would be detrimental if encountered naturally (Ring and Gutermuth, 2011). These observations led to the development of allergen-specific immunotherapy protocols for use in this and other hypersensitivity diseases (Jutel et al., 2015), and delivery of allergens by subcutaneous or sublingual routes is also widely practiced in veterinary medicine for management of canine atopic dermatitis (Olivry et al., 2015; Mueller et al., 2015).

[2] See: http://www.aratana.com/for-veterinarians/clinical-studies/

Several experimental models suggest that the same process could be applied to control established autoimmune responses (Sabatos-Peyton et al., 2010). This autoantigen-specific immunotherapy is based primarily on the ability to induce Th1, Th2 and Th17 cells to adopt a regulatory phenotype characterised by production of IL-10 (Meiler et al., 2008) and possibly by display of the surface markers CD49b and LAG-3 (Gagliani et al., 2013) in response to their cognate antigens. Effector T cells are more likely to differentiate into these so-called Tr1 cells (also described as $_{IL\text{-}10}$Tregs cells) when exposed to repeated, high doses of their cognate antigens delivered by subcutaneous injection, though, in patients with clinically active diseases, there are concerns that such dosing schedules could exacerbate of the autoimmune response.

The latter phenomenon was observed in three patients with multiple sclerosis who received subcutaneous injections of a peptide derived from the immunodominant myelin basic protein. These patients suffered more severe neurological abnormalities and brain inflammation (as indicated by magnetic resonance imaging) but were subsequently rescued by administration of glucocorticoids (Bielekova et al., 2000). Elucidation of immunological changes revealed that administration of the autoantigen was associated with increased numbers of Th1 effector cells in the cerebrospinal fluid of some patients, rather than induction of anergy or differentiation of Tr1 cells. While notably this study used an altered peptide antigen, in which some amino acids had been artificially substituted to modify the interaction at the synapse between T cell and MHC-peptide complex, this study demonstrates the risks inherent in autoantigen-specific immunotherapy and the need for a dosing schedule that minimises them.

Autoantigen-specific immunotherapy has not been attempted in people with AIHA or dogs with IMHA, but this form of treatment could be feasible in both species because previous studies have identified several of the immunodominant self-antigens targeted by autoimmune responses. In people, autoantibodies are most commonly specific for epitopes on the Rhesus polypeptides (Barker et al., 1992), and T cells from patients with AIHA showed a proliferative response *in vitro* to peptides derived from the Rhesus D molecule (Barker et al., 1997). In dogs with IMHA, autoreactive T and B

cells are more likely to be specific for glycophorin molecules, though patterns of reactivity to erythrocyte antigens do not appear to be as consistent between individuals as in people (Barker et al., 1991; Corato et al., 1997).

In people with warm AIHA, T cells that produce IL-10 in response to Rhesus D molecules have also been identified in the spleen and peripheral blood. These cells were able to suppress production of the Th1-associated cytokine interferon gamma (IFNγ) in peripheral blood mononuclear cell cultures *in vitro*, contingent on their expression of CTLA-4 (Hall et al., 2002; Ward et al., 2008). These studies suggest that Tr1 cells are present in the blood of people with AIHA, where they could be activated or induced by administration of autoantigen in an appropriate form.

Cell-based therapy: Whether or not defects in Treg frequency or function contribute to development of autoimmune diseases, there is growing evidence to suggest that they could be used as a therapeutic agent to re-establish tolerance of self-antigens. In support of this notion, adoptive transfer of splenic $CD4^+CD25^+$ Tregs prevented production of anti-erythrocyte antibodies in mice that were subsequently injected repeatedly with rat erythrocytes to generate an autoimmune response (in the Playfair Marshall-Clarke model of AIHA)(Mqadmi et al., 2005). Studies in other murine models of autoimmune disease have shown that similar adoptive transfers can ameliorate established autoimmune diseases and delay or prevent rejection of transplanted organs, as reviewed elsewhere (Singer et al., 2014).

There have been numerous obstacles in the path to use Tregs as therapy for people with autoimmune diseases, including the need to isolate Tregs from peripheral blood using reliable phenotypic markers, expand the number of Tregs in *ex vivo* culture systems that subscribe to Good Manufacturing Practice and characterise their suppressive abilities prior to infusion into patients (Putnam et al., 2013; Haase et al., 2015). Concerns have also arisen that some Tregs might lose FOXP3 expression and adopt an effector Th17 phenotype after infusion (Hori, 2011; Komatsu et al., 2014), possibly worsening the autoimmune disease. Nevertheless, the process of isolating, expanding and re-infusing autologous

Tregs has been completed in people with type 1 diabetes mellitus and Crohn's disease. Of the twelve children with diabetes mellitus who received autologous Tregs, eight achieved clinical remission, with no significant adverse events reported in any patient and appropriate and sustained responses to routine vaccination (Marek-Trzonkowska et al., 2014). Administration of Tregs to patients with Crohn's disease resulted in a greater frequency of adverse effects (including exacerbation of gastrointestinal disease (n=11) and thrombosis/thrombophlebitis (n=2)), with some form of response in eight of twenty patients overall (Desreumaux et al., 2012).

There are many preliminary steps that would also need to be taken before Treg cell therapy could become a reality in dogs, including more thorough phenotyping of the Treg population, demonstration of its stability in response to an inflammatory setting and generation of a protocol that could be used reliably and safely to expand the population *ex vivo*. In people, expansion of the Tregs to a number considered suitable for infusion also generally requires several weeks of repeated stimulation with anti-CD3 and anti-CD28 antibodies in a medium enriched with IL-2 (Putnam et al, 2013); this timescale may not be compatible with treatment of an autoimmune disease that often has an acute onset, though it could be useful to curtail regimens involving broad-spectrum immunosuppressive drugs.

Low dose interleukin 2 therapy: Interleukin 2 was traditionally considered to be a cytokine that stimulated effector T cell proliferation and differentiation, but mice that could not produce the cytokine were unexpectedly found to develop spontaneous autoimmune diseases (Sadlack et al., 1993). Interestingly, IL-2 gene knockouts on the Balb/c mouse background caused fatal AIHA within five weeks, which was related to deficiency of the Treg compartment, uncontrolled proliferation of effector T cells and production of autoantibodies (Sadlack et al., 1995). Similar abnormalities were detected in mice lacking the IL-2 receptor alpha chain (CD25), which is constitutively expressed at high levels by Tregs (Willerford et al., 1995), and in people with missense mutations in the equivalent gene (Sharfe et al., 1997).

Since the recognition that IL-2 is required for maintenance of Treg numbers and activity, there has been interest in the use of the recombinant human cytokine for treatment of autoimmune diseases (Klatzmann and Abbas, 2015). Administration of IL-2 at high doses has been associated with adverse effects, such as excessive production of other cytokines and increased capillary permeability (Rosenstein et al., 1986; Baluna and Vitetta, 1997), but low dose IL-2 therapy appears to be more promising in clinical practice.

Low dose IL-2 therapy has been used in 10 people with vasculitis induced by hepatitis C infection, in whom it caused only mild and transient reactions, with no signs of vascular leak syndrome. Clinical signs improved in eight of ten patients, with an increase in the average number of Tregs across the whole group (Saadoun et al., 2011). In a separate open pilot study, IL-2 was administered at a low dose to five people with alopecia areata, with improvements in clinical disease score and immunohistochemical findings of scalp biopsies in four of five patients (Castela et al., 2014).

Additional clinical trials are currently ongoing to evaluate low dose IL-2 in other human autoimmune diseases (Waldron-Lynch et al., 2014; Humrich et al., 2015), and this treatment could also be developed for canine use. So far, only recombinant human IL-2 is available, and, while this product has been administered intralesionally for treatment of several types of cancer in dogs without apparent adverse effects (Konietscke et al., 2012; Haagsman et al., 2013; Ziekman et al., 2013; Den Otter et al., 2015), there are no reports of its systemic administration. An appropriate dose for treatment of autoimmune disease would also need to be established, as doses of IL-2 required to stimulate Tregs appear to differ between species (Klatzmann and Abbas, 2015).

Emerging alternative therapies: Several other forms of immunotherapy that modulate the signals determining survival of circulating lymphocytes are in varying stages of development, and these could also have application in the treatment of AIHA. Maturation and survival of B cells in the periphery is dependent on interactions between the soluble molecule B lymphocyte stimulator (BLyS) and its

receptor, BLyS receptor 3 (BR3), which is expressed on their surface (Cancro et al., 2009). The concentration of BLyS regulates the size and nature of the mature B cell compartment, with higher concentrations resulting in greater numbers of B cells and also permitting B cells with autoreactive potential to survive (Cancro et al., 2009). The serum concentration of BLyS was greater in people with AIHA compared to healthy controls in two studies; in one of these, the concentration correlated with indicators of clinical disease activity (Zhao et al., 2015) and decreased after treatment with glucocorticoids (Xu et al., 2015).

These findings suggest that autoreactive B cells escaping suppression or deletion may be important in development of AIHA, providing further recourse for treatment using therapies that modulate serum concentrations of BLyS. One such treatment is belimumab, a human monoclonal antibody product that binds to and inhibits soluble BLyS, resulting in decreased B cell proliferation, depletion of B cells (Baker et al., 2003) and improved control of disease activity in people with systemic lupus erythematosus (SLE)(Ginzler et al, 2013).

Preservation of a functioning apoptotic pathway is essential to maintenance of tolerance in T cells because it permits cell death during selection in the thymus and after receipt of inhibitory signals from APCs (Tischner et al, 2010). Conversely, overexpression of anti-apoptotic regulators in lymphocytes may contribute to development of SLE in people (Andre et al, 2007). The Bcl family of cell signalling molecules includes pro- and anti-apoptotic members: Bcl-2 and Bcl-x_L are major members of the latter group (Tischner et al., 2010). Inhibitors of these molecules were developed primarily for treatment of lymphoma, but administration of the Bcl-2 family antagonist ABT-737 has also resulted in clinical improvements in murine models of SLE and rheumatoid arthritis (Bardwell et al, 2009).

Finally, differentiation of activated T cells could be modulated using drugs that alter the activity of the transcription factors that determine commitment to a particular subset. Recent efforts have focused on development of small molecule inhibitors of retinoic acid receptor-related orphan nuclear receptor (RORγt), the central transcription factor involved in differentiation of Th17 T cells. So far,

published work has shown clinical improvement in a murine model of multiple sclerosis after administration of a candidate molecule (Xiao et al., 2014), but similar products could be useful in people with AIHA as Th17 cells are present at increased frequency in these patients (Xu et al., 2012).

Conclusions

Several forms of novel immunotherapy are currently in active development, largely based on greater understanding of the regulatory processes that usually control autoimmune responses. Some of these forms of therapy warrant considerable testing before they could be applied in client-owned animals in veterinary practice, but others are undergoing clinical trials at present, raising the exciting prospect of novel immunotherapies for treatment of canine IMHA in the future.

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Figure 1: Schematic diagram to indicate parts of the immune response that are targeted by different forms of therapy. Blue section indicates normal immune response against pathogenic bacteria; red section indicates autoimmune response against erythrocytes. Broad-spectrum immunosuppressive agents affect many parts of the immune response (including several not shown), whereas emerging immunotherapies have a more specific action.