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Swann, JW and Garden, OA (2015) Novel immunotherapies for immune-mediated haemolytic anaemia in dogs and people. VETERINARY JOURNAL.

The final version is available online at <http://dx.doi.org/10.1016/j.tvjl.2015.10.022>.

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The full details of the published version of the article are as follows:

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

AUTHORS: Swann, JW and Garden, OA

JOURNAL TITLE: VETERINARY JOURNAL

PUBLISHER: Elsevier

PUBLICATION DATE: 22 October 2015 (online)

DOI: 10.1016/j.tvjl.2015.10.022

1 Review

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

3

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29 Abstract

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

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48 Keywords: autoimmunity, AIHA, IMHA, dog, Treg

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56 Introduction

57

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

70

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

83 Regulatory T cells are able to suppress the activation and proliferation of effector T cells (CD4⁺ cells
84 with Th1, Th2, Th9, Th17 and other phenotypes) and cytotoxic T cells *in vitro* when the latter cells
85 are activated by either polyclonal or specific antigenic stimuli (Thornton et al., 1998; Dieckmann et
86 al., 2001; Wing et al., 2003). Corresponding effects are observed *in vivo*, with suppression of
87 transplant rejection and cessation of deleterious autoimmune responses observed after adoptive
88 transfer of Tregs into experimental animals (Graca et al., 2002; Hoffmann et al., 2002; Buckner,
89 2010). The importance of Tregs is also demonstrated by mice that have undergone thymectomy
90 (Kojima and Prehn, 1981; Sakaguchi et al., 1985), adult mice in which Tregs have been depleted
91 pharmacologically (Ellis et al., 2013; Kim et al., 2007) and people and mice that are unable to express
92 FOXP3/*foxp3* in Tregs (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001), all of which
93 develop multisystemic autoimmune diseases.

94

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

103

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

111

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

128

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

139

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

150

151 ***Rationale for and implications of immunosuppressive treatment:*** There has been a pleasing and
152 saleable symmetry about the use of immunosuppressive drugs for the treatment of autoimmune
153 diseases in people and animals since the discovery and widespread production of the synthetic
154 corticosteroids in the 1950s (Herzog and Oliveto, 1992). Indeed, glucocorticoids remain the only
155 group of drugs licensed for the treatment of autoimmune diseases in dogs and cats in the UK¹ and, in
156 a recent systematic review of evidence relating to the treatment of IMHA in dogs, we found that
157 glucocorticoids had been administered to all of the 843 dogs from which data were derived (Swann
158 and Skelly, 2013). Use of prednisolone (or prednisone) alone seems to result in survival rates of up to
159 65% at six months after diagnosis (Swann and Skelly, 2013), with similar response rates reported in
160 people (Zanella and Barcellini, 2014). There are concerns that long-term administration of these
161 drugs may result in unacceptable adverse effects, largely due to iatrogenic recapitulation of Cushing's
162 syndrome and increased risk of thromboembolic disease (Rose et al., 2011).

163

¹ See: <http://www.noahcompendium.co.uk>

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

172

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

187

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

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

202

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

217

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

220 functional importance of this diversity is unknown because the physiological ligand for CD20 has yet
221 to be identified. Consequent to these differences in structure, rituximab does not cross react with the
222 extracellular domain of canine molecules (Jubala et al., 2005) and has no apparent therapeutic
223 potential in this species, as demonstrated by failure to deplete B cells in an *ex vivo* model of B cell
224 lymphoma (Impellizeri et al., 2006). Nevertheless, established methodologies have been applied to
225 develop monoclonal 'caninised' antibodies that bind CD20 and deplete B cells in dogs (Ito et al.,
226 2015; Rue et al., 2015). One such product has been licensed in the United States, and clinical trials
227 are currently ongoing to evaluate its efficacy in the management of canine lymphoma² (Rodriguez et
228 al., 2015).

229

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

244

² See: <http://www.aratana.com/for-veterinarians/clinical-studies/>

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

254

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

266

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

273 cells are more likely to be specific for glycoprotein molecules, though patterns of reactivity to
274 erythrocyte antigens do not appear to be as consistent between individuals as in people (Barker et al.,
275 1991; Corato et al., 1997).

276

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

283

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

293

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

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

308

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

318

319 ***Low dose interleukin 2 therapy:*** Interleukin 2 was traditionally considered to be a cytokine that
320 stimulated effector T cell proliferation and differentiation, but mice that could not produce the
321 cytokine were unexpectedly found to develop spontaneous autoimmune diseases (Sadlack et al.,
322 1993). Interestingly, IL-2 gene knockouts on the Balb/c mouse background caused fatal AIHA within
323 five weeks, which was related to deficiency of the Treg compartment, uncontrolled proliferation of
324 effector T cells and production of autoantibodies (Sadlack et al., 1995). Similar abnormalities were
325 detected in mice lacking the IL-2 receptor alpha chain (CD25), which is constitutively expressed at
326 high levels by Tregs (Willerford et al., 1995), and in people with missense mutations in the equivalent
327 gene (Sharfe et al., 1997).

328

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

335

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

342

343 Additional clinical trials are currently ongoing to evaluate low dose IL-2 in other human autoimmune
344 diseases (Waldron-Lynch et al., 2014; Humrich et al., 2015), and this treatment could also be
345 developed for canine use. So far, only recombinant human IL-2 is available, and, while this product
346 has been administered intralesionally for treatment of several types of cancer in dogs without apparent
347 adverse effects (Konietscke et al., 2012; Haagsman et al., 2013; Ziekman et al., 2013; Den Otter et al.,
348 2015), there are no reports of its systemic administration. An appropriate dose for treatment of
349 autoimmune disease would also need to be established, as doses of IL-2 required to stimulate Tregs
350 appear to differ between species (Klatzmann and Abbas, 2015).

351

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

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

363

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

370

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

379

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

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

387

388 Conclusions

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

394

395 Conflict of interest statement: None of the authors of this paper has a financial or personal
396 relationship with other people or organisations that could inappropriately influence or bias the
397 content of the paper.

398

399 Acknowledgements: The authors' work is generously funded by the Kennel Club Charitable Trust, the
400 European College of Veterinary Internal Medicine, PetSavers and the Petplan Charitable Trust.

401 James Swann is in grateful receipt of an International Canine Health Award (made possible by a grant
402 from Vernon and Shirley Hill) from the Kennel Club Charitable Trust to extend his research on canine
403 IMHA.

404

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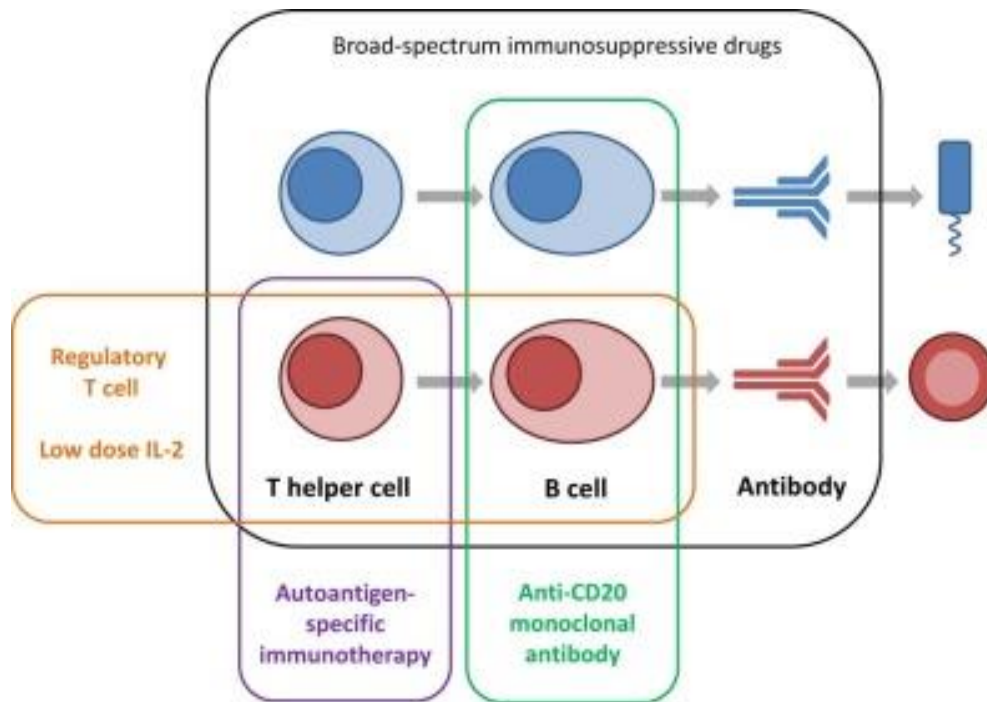
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722

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



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1 Review

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

3

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29 Abstract

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

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48 Keywords: autoimmunity, AIHA, IMHA, dog, Treg

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56 Introduction

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

70

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

83 Regulatory T cells are able to suppress the activation and proliferation of effector T cells (CD4⁺ cells
84 with Th1, Th2, Th9, Th17 and other phenotypes) and cytotoxic T cells *in vitro* when the latter cells
85 are activated by either polyclonal or specific antigenic stimuli (Thornton et al., 1998; Dieckmann et
86 al., 2001; Wing et al., 2003). Corresponding effects are observed *in vivo*, with suppression of
87 transplant rejection and cessation of deleterious autoimmune responses observed after adoptive
88 transfer of Tregs into experimental animals (Graca et al., 2002; Hoffmann et al., 2002; Buckner,
89 2010). The importance of Tregs is also demonstrated by mice that have undergone thymectomy
90 (Kojima and Prehn, 1981; Sakaguchi et al., 1985), adult mice in which Tregs have been depleted
91 pharmacologically (Ellis et al., 2013; Kim et al., 2007) and people and mice that are unable to express
92 FOXP3/foxp3 in Tregs (Bennett et al., 2001; Brunkow et al., 2001; Wildin et al., 2001), all of which
93 develop multisystemic autoimmune diseases.

94

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

103

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

111

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

128

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

139

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

150

151 ***Rationale for and implications of immunosuppressive treatment:*** There has been a pleasing and
152 saleable symmetry about the use of immunosuppressive drugs for the treatment of autoimmune
153 diseases in people and animals since the discovery and widespread production of the synthetic
154 corticosteroids in the 1950s (Herzog and Oliveto, 1992). Indeed, glucocorticoids remain the only
155 group of drugs licensed for the treatment of autoimmune diseases in dogs and cats in the UK¹ and, in
156 a recent systematic review of evidence relating to the treatment of IMHA in dogs, we found that
157 glucocorticoids had been administered to all of the 843 dogs from which data were derived (Swann
158 and Skelly, 2013). Use of prednisolone (or prednisone) alone seems to result in survival rates of up to
159 65% at six months after diagnosis (Swann and Skelly, 2013), with similar response rates reported in
160 people (Zanella and Barcellini, 2014). There are concerns that long-term administration of these
161 drugs may result in unacceptable adverse effects, largely due to iatrogenic recapitulation of Cushing's
162 syndrome and increased risk of thromboembolic disease (Rose et al., 2011).

163

¹ See: <http://www.noahcompendium.co.uk>

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

172

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

187

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

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

202

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

217

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

220 functional importance of this diversity is unknown because the physiological ligand for CD20 has yet
221 to be identified. Consequent to these differences in structure, rituximab does not cross react with the
222 extracellular domain of canine molecules (Jubala et al., 2005) and has no apparent therapeutic
223 potential in this species, as demonstrated by failure to deplete B cells in an *ex vivo* model of B cell
224 lymphoma (Impellizeri et al., 2006). Nevertheless, established methodologies have been applied to
225 develop monoclonal 'caninised' antibodies that bind CD20 and deplete B cells in dogs (Ito et al.,
226 2015; Rue et al., 2015). One such product has been licensed in the United States, and clinical trials
227 are currently ongoing to evaluate its efficacy in the management of canine lymphoma² (Rodriguez et
228 al., 2015).

229

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

244

² See: <http://www.aratana.com/for-veterinarians/clinical-studies/>

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

254

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

266

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

273 cells are more likely to be specific for glycoprotein molecules, though patterns of reactivity to
274 erythrocyte antigens do not appear to be as consistent between individuals as in people (Barker et al.,
275 1991; Corato et al., 1997).

276

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

283

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

293

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

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

308

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

318

319 ***Low dose interleukin 2 therapy:*** Interleukin 2 was traditionally considered to be a cytokine that
320 stimulated effector T cell proliferation and differentiation, but mice that could not produce the
321 cytokine were unexpectedly found to develop spontaneous autoimmune diseases (Sadlack et al.,
322 1993). Interestingly, IL-2 gene knockouts on the Balb/c mouse background caused fatal AIHA within
323 five weeks, which was related to deficiency of the Treg compartment, uncontrolled proliferation of
324 effector T cells and production of autoantibodies (Sadlack et al., 1995). Similar abnormalities were
325 detected in mice lacking the IL-2 receptor alpha chain (CD25), which is constitutively expressed at
326 high levels by Tregs (Willerford et al., 1995), and in people with missense mutations in the equivalent
327 gene (Sharfe et al., 1997).

328

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

335

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

342

343 Additional clinical trials are currently ongoing to evaluate low dose IL-2 in other human autoimmune
344 diseases (Waldron-Lynch et al., 2014; Humrich et al., 2015), and this treatment could also be
345 developed for canine use. So far, only recombinant human IL-2 is available, and, while this product
346 has been administered intralesionally for treatment of several types of cancer in dogs without apparent
347 adverse effects (Konietscke et al., 2012; Haagsman et al., 2013; Ziekman et al., 2013; Den Otter et al.,
348 2015), there are no reports of its systemic administration. An appropriate dose for treatment of
349 autoimmune disease would also need to be established, as doses of IL-2 required to stimulate Tregs
350 appear to differ between species (Klatzmann and Abbas, 2015).

351

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

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

363

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

370

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

379

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

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

387

388 Conclusions

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

394

395 Conflict of interest statement: None of the authors of this paper has a financial or personal
396 relationship with other people or organisations that could inappropriately influence or bias the
397 content of the paper.

398

399 Acknowledgements: The authors' work is generously funded by the Kennel Club Charitable Trust, the
400 European College of Veterinary Internal Medicine, PetSavers and the Petplan Charitable Trust.

401 James Swann is in grateful receipt of an International Canine Health Award (made possible by a grant
402 from Vernon and Shirley Hill) from the Kennel Club Charitable Trust to extend his research on canine
403 IMHA.

404

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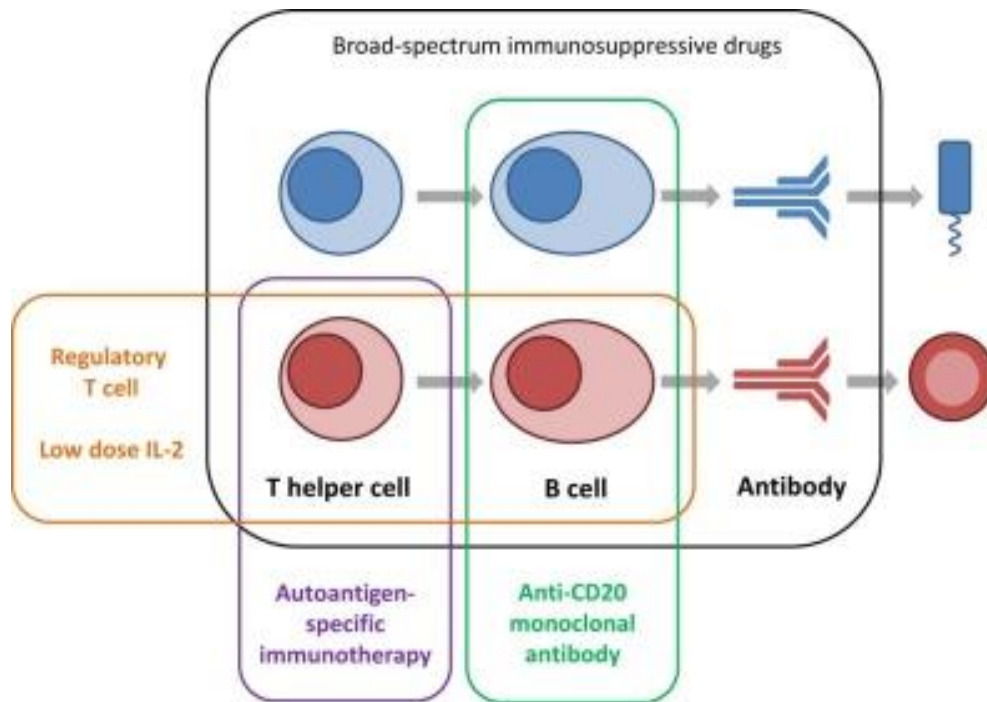
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723 Figure 1: Schematic diagram to indicate parts of the immune response that are targeted by different
724 forms of therapy. Blue section indicates normal immune response against pathogenic bacteria;
725 red section indicates autoimmune response against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



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