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Review

Challenges and opportunities for monoclonal antibody therapy in veterinary oncology

Breno C.B.Beirão [a]; Teresa Raposo [a,b]; Saurabh Jain [c]; Ted Hupp [c]; David Argyle [a,*]

[a] *The Royal (Dick) School of Veterinary Studies and Roslin Institute, The University of Edinburgh, EH25 9RG, UK*
[b] *Department of Veterinary Sciences, Universidade de Trás-os-Montes e Alto Douro, 5001-801, Portugal*
[c] *Edinburgh Cancer Research Centre, The University of Edinburgh, EH4 2XR, UK*

* Corresponding author. Tel.: +44 0131 6506241
E-mail address: david.argyle@roslin.ed.ac.uk

Abstract

Monoclonal antibodies (mAbs) have come to dominate the biologics market in human cancer therapy. Nevertheless, in veterinary medicine very few clinical trials have been initiated using this form of therapy. Some of the advantages of monoclonal antibody therapeutics over conventional drugs are high specificity, precise mode of action and long half-life which favours infrequent dosing of the antibody. Further advancement in the field of biomedical sciences has led to the production of different forms of the antibody such as single chain antibody fragment (scFv), Fab, bi-specific and drug conjugates for use in diagnostic and therapeutic purposes. In this review we describe the potential for monoclonal antibodies in veterinary oncology patients in supporting both diagnosis and therapy of cancer. We explore the technical and financial hurdles to facilitate clinical acceptance of mAbs and offer insights into novel technologies and targets that could support more rapid clinical development.

Keywords: Monoclonal antibodies; Veterinary oncology; Therapy; Idiotype; Biologicals

Introduction

The history of monoclonal antibody (mAb) therapy can be traced back to the start of the hybridoma technology, in 1975 (Köhler and Milstein, 1975). Fig. 1 describes the chronology of mAb development.

The greatest advances in monoclonal antibody therapeutics began when recombinant DNA technology could be applied to antibody design. This facilitated the reduction in their immunogenicity and optimisation of antibody half-life and effector functions. Despite that, mAbs have taken a long time since the early days of discovery until the current "golden phase" of cancer therapies using biologicals (Waldmann, 1991). Since then, anti-cancer mAbs – and also mAbs for other therapeutic purposes – have become some of the highest selling medicines ever (King, 2013).

The importance of monoclonal antibodies as therapeutics is increasing, and currently there is an average of four new molecules reaching the human therapeutics market every year. This class of treatment is seen as critical for the future of the pharmaceutical industry, especially in a time when the development of new drugs and the discovery of new targets is declining (Munro et al., 2011; Arrowsmith, 2012; Ecker et al., 2014).

Mechanisms of action

Several mechanisms of action of the monoclonal antibodies have been identified. They are summarised in Table 1 per the molecular mechanism to which they are relevant (Glennie and Johnson, 2000; Adams and Weiner, 2005).

69 Many antibody functions shown in Table 1 are dependent on the Fc region of the
70 mAb. The interaction between the Fc region of the mAb and Fc receptors (FcR) is crucial for
71 the development of immune-mediated functions of the antibody, such as antibody-dependent
72 cell-mediated cytotoxicity (ADCC). Complement-dependent cytotoxicity (CDC) can also be
73 activated by interaction with the Fc fraction of the mAb. This interaction is specific, such that
74 not all antibody isotypes interact with the same intensity with the FcR, even within the IgG
75 subclass in a single species, for instance (Bergeron et al., 2014; Strietzel et al., 2014).
76 Therefore, the choice of Fc region during the chimerisation process (described below) affects
77 the effector functions of the therapeutic speciated mAb. In dogs, IgGB and IgGC are capable
78 of driving ADCC and CDC, while IgGD is only capable of activating the complement
79 cascade (for CDC), but not of binding to FcγRs (for ADCC or phagocytosis). The other IgG
80 isotypes of dogs, "A" and "D" are not drivers of antibody-mediated immune responses.
81 Human IgG1 and mouse IgG2a also bind to canine FcγRI, and are potentially useful as
82 therapeutics in dogs (Helfand et al., 1994; Bergeron et al., 2014). Indeed, mAb 231, a murine
83 antibody previously used against canine lymphoma (described below in detail), is reported to
84 show antibody-mediated cytotoxicity and complement activation in dogs (Rosales et al.,
85 1988). In cats, feline IgG1a and IgG1b bind to Fc receptors and also seem capable of
86 mediating CDC, while IgG2 does not (Strietzel et al., 2014).
87
88 IgE-Fc receptor binding is also being explored as a potential mechanism to treat
89 cancer. There are two views on the use of IgE antibodies against cancer: 1) IgE seems to have
90 a role in preventing cancer; 2) IgE can be used to target FcεR-expressing mast cell tumours.
91 As for the first approach on the use of IgE, this isotype is more effective in inducing ADCC
92 against cancer cells, as well as inducing stronger responses (Singer and Jensen-Jarolim,
93 2014). The second approach takes advantage of the high expression of IgE-binding Fc

receptors (FcεR) on mast cell tumours; thus, IgE can be used as a platform for the delivery of therapies to these cells (Elders et al., 2014).

Monoclonal antibody therapy strategies for cancer in veterinary medicine

The past of mAb therapy in veterinary medicine: mAb 231

One of the first clinical monoclonal antibody licenced for cancer treatment, denominated mAb231, was for use in canine lymphoma, although it was later discontinued. (Jeglum, 2009; USDA, 2016). Three years before the licencing of the first cancer-targeted monoclonal antibody in Germany (Panorex), and five years before the first monoclonal antibody was licenced by the FDA for the treatment of cancer in humans (Rituximab), a monoclonal antibody received approval in the US to treat lymphoma in dogs, called mAb 231. The selection of the monoclonal antibody was carried out against tumour cells and the molecular target of this antibody was never unveiled. Immunoprecipitation using the antibody did not display any protein. The antibody was nevertheless able to bind to 73% of the formalin-fixed and paraffin-embedded lymphomas tested (Steplewski et al., 1987; Jeglum, 2009). The antibody is of the IgG2aκ subclass, with a detailed study of its structure having already been performed (Harris et al., 1997). Its mechanisms of action were reported to be due to antibody-dependent cell-mediated cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) (Rosales et al., 1988) and inhibition of tumour growth (Steplewski et al., 1987).

The antibody was to be administered to dogs in remission after two four weeks' cycles of vincristine, cyclophosphamide, L-asparaginase and doxorubicin (modified VCAA) and a three-week rest. The total dose was 100mg/m^2, to be divided into 5 sequential days and to be administered over two hours. If lymphoma recurred after the administration of the Mab

119 231, a new 4-weeks cycle of the modified VCAA was to be administered, followed by a 3
120 weeks' rest and a new round of antibodies (Jeglum, 1996, 2009).
121
122 The complete remission of the 215 dogs tested was 80.9%, with a mean survival time
123 of 624.28 days for the responders (in complete remission) against 153.67 days for the non-
124 responders. Non-responders tended to be from the group of animals to which mAb231 does
125 not bind on immunohistochemistry (Jeglum, 1996). Anecdotally, the administration of mAb
126 231 showed a disproportional rate of achieving indefinite remission when compared to
127 chemotherapeutics alone (Crow, 2008).
128
129 The drug was discontinued in 1996, apparently due to lack of demand (Crow, 2008;
130 Kaplan, 2008). This mAb is no longer licenced for use by the USDA (USDA, 2016). Yet, it
131 must be brought to attention that the drug was marketed in a time when antibody therapy to
132 cancer was non-existent in human medicine, nor had the subject the same attention it receives
133 currently (Reichert, 2012).
134
135 *The present of mAb therapy in veterinary medicine: antibodies reaching the market*
136 The veterinary market is currently going through its first intense wave of interest in
137 monoclonal antibodies (Harvey, 2015; Ledford, 2016). Two product launches by Aratana
138 Therapeutics, against B (product AT-004, anti-CD20) and T cell lymphomas (AT-005, anti-
139 CD52) were widely regarded in the market as great promises for veterinary oncology. AT-
140 004 was shown to increase median progression-free survival of dogs with B cell lymphoma
141 (Anderson and Modiano, 2015). However, recent post-USDA licencing trials have not shown
142 encouraging results; in randomized clinical trials combining AT-005 to two different
143 chemotherapy protocols, mAb therapy did not seem to improve clinical outcomes compared

144 to placebo. Aratana indicated that both AT-004 and AT-005 showed poor specificity to their
145 respective targets. Despite this, both antibodies are currently still commercially available[1].
146
147 While other commercially available options of mAbs for the treatment of cancer in
148 animals are not yet available, there are candidates approaching or at market launch for other
149 clinical conditions. Zoetis has recently been granted a conditional licence by the USDA for
150 lokivetmab, to be used for treatment of canine atopic dermatitis. This mAb blocks IL-31,
151 which is important for the development of pruritus (Michels et al., 2016), with the intent of
152 interrupting the cycle of itching and inflammation typical of atopic dermatitis (Zoetis, 2015)[2].
153 Nexvet Biopharma is investigating a target for chronic pain, with an anti-nerve growth factor
154 mAb. Interestingly, they have already demonstrated the efficacy of this mAb both in
155 caninised and in felinised formats (Gearing et al., 2013, 2016)[3].
156
157 *The future of mAb therapy in veterinary medicine I: possible targets*
158 There is a limited number of publications evaluating the effects of monoclonal
159 antibodies or related molecules in veterinary species. Shown below in Table 2 are antibodies
160 that have been trialled or used in domestic animals, as well as future potential targets for
161 these patients.
162
163 *The future of mAb therapy in veterinary medicine II: 'speciation'*
164 Veterinary medicine has always borrowed tools from human practice. However,
165 contrarily to chemical drugs, biologicals such as mAbs are in most cases species-specific.

[1] See: http://aratana.investorroom.com/2015-09-24-Aratana-Therapeutics-Provides-Product-Updates (accessed 15 June 2016).
[2] See: http://news.zoetis.com/press-release/companion-animals/zoetis-receives-conditional-license-usda-canine-atopic-dermatitis-im (acessed 16 June 2016)
[3] See: http://ir.nexvet.com/phoenix.zhtml?c=253841&p=irol-newsArticle&ID=2176442 (accessed 16 June 2016).

166 Although the process of antibody caninisation is now becoming more widely used, it is still a
167 time-consuming method (Gearing et al., 2013). Also, producing mAbs exclusively for one
168 animal species may not be always commercially viable, and may impede further
169 developments of new candidates. This is clearly illustrated by the fact that while there are
170 now tens of monoclonal antibodies approved worldwide for cancer therapy in humans
171 (Modjtahedi et al., 2012; Reichert, 2012; Ecker et al., 2015), in veterinary oncology,
172 however, therapeutic antibodies are only just reaching the market (USDA, 2016).
173
174 The use of antibodies that have been developed for human therapy in animals is an
175 attractive approach. Some groups have explored the potential use of human mAbs in dogs.
176 Stein and colleagues, for instance, successfully tested an anti-human leukocyte antigen DR in
177 dogs. While it was then concluded that dogs were validated as good models for the trial of an
178 antibody, the authors also note how the study and the patients would have benefited from a
179 species-specific antibody (Impellizeri et al., 2006; Stein et al., 2011; Biller et al., 2014).
180
181 Monoclonal antibody 231, the best example of mAb therapies in veterinary medicine,
182 may have had reduced efficacy due to its xenogeneic origin. The canine anti-mouse antibody
183 (CAMA) levels were raised after therapy with this antibody, and, as expected, further
184 applications resulted in quicker and higher production of CAMA. No clinical disease seemed
185 to arise from the use of this murine antibody in dogs. The investigators believed that length of
186 remissions and survival times were not affected by the murine nature of the antibodies, but it
187 was never directly compared to a 'caninised' version of the antibody (Jeglum, 1996, 2009).
188 Possible side effects were occasionally observed, including facial oedema, erythema, fever,
189 pruritus, vomiting and/or diarrhoea, joint pain and myalgia. Although the antibody seemed to

190 provide clinical benefits, it is possible that its murine origin was part of the reasons why it
191 was later discontinued (Jeglum, 1996, 2009).
192
193 Much more likely to be successful is the interchange of therapeutic antibodies
194 between dogs and cats, for instance. Fc receptors, which are crucial for antibody half-life and
195 effector functions, are very similar between these two domestic species (tested using Ensembl
196 Genome Browser and ClustalOmega[4]) (Lobo et al., 2004; Strietzel et al., 2014). This could
197 prove to be an important feature for veterinary practitioners when new mAbs become
198 available for either species, since development of mAbs for these patients is slow and
199 receives relatively little attention, especially for cats, for whom no mAb is available currently
200 in the market.
201
202 Even though the majority of the literature indicates that anti-globulin responses limit
203 the function of mAbs, as discussed above, there are still licensed antibodies for use in humans
204 that are not humanised. Understanding how they are effective may set new parameters that
205 may be useful for the production of innovative antibodies for domestic animals.
206
207 At least two mouse antibodies are still currently in use for the treatment of
208 malignancies in humans. Both of these target CD20, and both are conjugated to
209 radionuclides. The use of murine antibodies in humans is permitted in these cases because the
210 mAbs are clinically useful with single dose schemes. Rituximab, a chimeric mAb that also
211 targets CD20 in humans, is used in a course of several doses, in comparison (Witzig et al.,
212 2002; Burdick and Macklis, 2009). Further possible explanations for the low seroconversion
213 from the use of these antibodies include the occurrence of immune suppression. It is expected

[4] See: http://www.ensembl.org/index.html and http://www.ebi.ac.uk/Tools/msa/clustalo/ (accessed 6 March 2015).

214 that both normal and malignant B cells are depleted by the treatment, therefore limiting the
215 capacity of the host to react to the mAbs (Mirick et al., 2004). Therefore, these results leave
216 open the possibility of using non-speciated mAbs in animals if these are administered in
217 single-dose regimens; mAbs coupled to radionuclides are potential candidates.
218

219 **The process of speciation**

220 As should be expected, xenogeneic antibodies are more immunogenic than syngeneic
221 ones. Therefore, speciation was probably the single most important step towards the success
222 of mAbs in clinical practice, since anti-mAb responses are responsible for rapid clearance of
223 circulating mAbs as well as for several side effects (Glennie and Johnson, 2000).
224 Chimerisation was the first stage towards antibody speciation (Hwang and Foote, 2005). In
225 this technique, after the antibody is raised and selected from mouse hybridomas, the murine
226 variable regions are cloned with human (or from any other species of interest) constant
227 regions by fusing the immunoglobulin genes in an expression vector (Fig. 2). This is then
228 expressed in a host system, such as Chinese hamster ovary (CHO) cells (Köhler and Milstein,
229 1975; Persic et al., 1997).
230

231 Humanisation is a further step in the engineering of the antibodies. This technology
232 starts from the chimerised version of the mAb, and attempts to further reduce the
233 immunogenicity of the antibody. In speciation, the framework regions of the variable fraction
234 (the parts of the variable fraction which do not contact the antigen) of murine antibodies are
235 substituted with human germline sequences. In this approach of speciation, CDRs are
236 retained from the original murine mAb sequence.
237

238 To completely avoid murine sequences, additional strategies have been developed to
239 generate fully speciated monoclonal antibodies through display technologies (such as phage
240 display) and transgenic mice (Deckert, 2009; Bradbury et al., 2011). In fully human
241 antibodies, all of the mAb molecule, including the CDRs, which bind to the antigen, are from
242 the species of interest (Thie et al., 2008). Phage display relies on libraries of antibody
243 fragments or whole IgGs (reviewed by Miersch & Sidhu, 2012) (Fig. 3). These can be
244 constructed from samples of the species of interest; thus, a display library can contain the
245 virtually the entirety of the antibodies being expressed in a blood sample, or a spleen, for
246 instance. The ultimate aim of the display technology is the selection of the recombinant
247 antibody that can bind to the antigen with highest affinity from the immense number of non-
248 specific antibodies in the library. After the construction of a display library putatively
249 containing all the possible antibodies from one animal – or from several animals, if desired –
250 this library can be screened against different therapeutic targets by the process of biopanning
251 (Barbas et al., 1991). Since the library can be constructed from the target species, instead of
252 mice, the antibodies it generates will be fully human (or canine, for example). Mice modified
253 to contain a 'human' immune system are also used as an alternative for the production of
254 fully human mAbs, but such a technology is not available for production of antibodies for
255 domestic animals (Shultz et al., 2012). The aforementioned technologies associated with
256 antibody production are illustrated in Fig. 4.
257
258 Although full speciation of antibodies has become the industry standard (Modjtahedi
259 et al., 2012), more recently opinions have emerged questioning the real benefits of
260 engineering antibodies beyond chimerisation. As stated previously, chimerisation is
261 responsible for the main reduction of immunogenicity of the mAbs. Indeed, in many cases
262 chimerisation is enough to prevent anti-mAb formation. Full speciation also has the

263 disadvantages that it is a lengthy and labour-intensive laboratory process, delaying product
264 launch, and in many cases leads to lower antibody affinity, since it interferes with the
265 antibody variable regions (Kettleborough et al., 1991). New speciation techniques have been
266 developed to try and circumvent these problems; one such example is the "PETization
267 platform", developed by a veterinary company (Gearing et al., 2013). However, full
268 speciation does not guarantee that a mAb will not raise a host response, as has been seen with
269 some fully humanised mAbs (Clark, 2000; Singh et al., 2012; van Meer et al., 2013;
270 Waldmann, 2014).
271
272 In veterinary medicine, the first speciated mAbs are now attaining the necessary legal
273 approvals and are entering the market (Acharya et al., 2015), and it will be interesting to
274 analyse the responses of large cohorts of animals to such caninised antibodies, in special with
275 regards to side effects.
276
277 **Potential side effects of mAb therapy**
278 The side-effects of mAb therapy can arise as a consequence of a host reaction against
279 the antibody or of the direct activity of the mAb. The host reactions against mAbs can be
280 either acute (infusion reactions or anaphilactoid) or dependent on the formation of anti-mAbs.
281 Acute reactions include the cytokine release syndrome (CRS) and systemic inflammatory
282 response syndrome (SIRS). A number of counter-measures are recommended, such as
283 hydration, premedication and gradual increase in the rate of infusion. Anaphylactic and
284 'anaphylactoid' reactions occur more frequently with some antibodies than with others,
285 probably due to the generation of IgE by the host. Adverse reactions can also be derived from
286 the formation of anti-mAb antibodies after administration. These tend to appear and increase
287 with subsequent doses, when the immune response has matured to secondary and adaptive

288 reactions. In these cases, high titres of anti-mAb IgGs can be found in the circulation of the
289 patient. Monitoring anti-mAb responses relies on specialized techniques, not commonly
290 available to the veterinary clinician; it depends on the concomitant development of the mAb
291 with its coupled monitoring tests. Anti-mAb responses can be monitored, among other
292 possibilities, by specific enzyme-linked immune sorbent assays (ELISAs), such as anti-
293 idiotype ELISA (for an explanation on anti-idiotypes, see Fig. 5 below and associated
294 discussion) or antigen capture methods (Shankar et al., 2007; Renaudineau et al., 2009).
295
296 Anti-mAb reactions may cause CRS and influenza-like symptoms in humans
297 (McLaughlin et al., 1998; Hansel et al., 2010). In dogs, the murine mAb 231 caused clinical
298 signs such as oedema and fever, but the occurrence of these signs did not impede subsequent
299 administration of the treatment; however, the dogs were preventively treated with
300 antihistamine, corticosteroids and the rate of infusion was reduced (Jeglum, 1996). It must be
301 noted that side effect management and mAb treatment withdrawal should be considered on a
302 case-by-case basis (Melosky et al., 2009). These host reactions tend to be more frequent
303 when non-speciated (humanised or caninised) formulations are used, demonstrating the
304 relevance of speciation (Adams and Weiner, 2005). The speciation process of antibodies,
305 from chimerisation to full speciation, followed by fully human (or canine, feline) mAbs,
306 reduces but does not exclude the probability of formation of strong host adverse reactions
307 against the antibody. Moderate and mild host reactions may continue to occur, nonetheless
308 (Hwang and Foote, 2005).
309
310 Reactions against fully speciated mAbs (canine against canine antibodies, for
311 instance) occur due to the inherent variability of antibodies. The antigen-binding region
312 (named the complementarity-determining region, or CDR) is by definition always structurally

313 novel to the immune system itself – that means that antibodies are always immunogenic,
314 regardless of how speciated the constant region of the mAb is. Although it is possible to
315 change a few (1-2) amino acids in the CDR of a mAb to reduce its immunogenicity, these
316 alterations always risk reducing the functionality of the antibody, since its very binding
317 region is being altered (Harding et al., 2010). The immunogenic property of the CDRs create
318 the phenomenon of the idiotype cascade presented in Fig. 5 (Herlyn et al., 1986). The main
319 importance of full speciation seems to be to reduce "marked" anti-antibody responses. When
320 reactions do occur against speciated mAbs, these tend to be "tolerable" or "negligible"
321 (Hwang and Foote, 2005).
322
323 The most common signs that follow the rapid infusion of rituximab, a human chimeric
324 antibody, are vomiting and nausea, rash and hypotension. The prophylactic use of
325 antipyretics and antihistamines is widespread to prevent these effects (Lang et al., 2011).
326 However, major acute reactions such as CRS can occur. Similar effects are also known to
327 occur after the administration of other chimeric mAbs such as Cetuximab (an anti-EGFR
328 mAb). These strong adverse reactions are less common for fully human mAbs, such as
329 Panitumumab (also anti-EGFR), but signs, such as skin rashes, still occur widely with the use
330 of this antibody (Hansel et al., 2010). Nevertheless, it has been highlighted that the most
331 common biological consequence of anti-mAb responses tends to be loss of efficacy, which is
332 deleterious but not a safety concern (Shankar et al., 2007).
333
334 The second type of possible reactions is dependent on antibody activity. One possible
335 effect of mAbs is the formation of Tumour Lysis Syndrome (TLS), where the release of
336 intracellular contents from the tumour after being targeted by treatment leads to metabolic
337 disorders, such as hyperkalaemia, hyperphosphatemia and hypocalcaemia (Jeha, 2001;

338 Gilbert and Wright, 2015). Undesired antibody activity has led to life threatening situations in
339 the famous case of the CD28 superagonist antibody TGN1412, used with the goal of
340 stimulating immune responses. This mAb had been trialled in human cells *in vitro*, in mice
341 and in primates before reaching a human phase I clinical trial. However, in humans the mAb
342 induced a severe cytokine storm (CRS): within minutes of administration patients had
343 headaches, nausea, vomiting, pyrexia, a systemic inflammatory response with hypotension.
344 This was followed by severe respiratory deterioration and several other signs of metabolic
345 failure. It was later found that these effects were not predicted by pre-clinical tests due to
346 immune differences between primates and mice compared humans – CD4 from macaques
347 lose CD28 expression at the effector memory level, while mice induce strong regulatory T
348 lymphocyte responses when administered with the superagonist. Additionally, the mere detail
349 of cell density in cell culture assays seems to have affected the results so that the cytokine
350 storm was also not predicted from these tests (Suntharalingam et al., 2006; Hünig, 2012).
351
352 Other side-effects related to antibody function may derive from off-targets. None of
353 the currently approved anti-cancer mAbs target a tumour-specific molecule. The targets are
354 also expressed in normal tissues, albeit often at different concentrations. Adverse reactions
355 therefore may originate from the off-targeting of the mAbs to molecules associated with
356 physiological functions, but these events are rarely life threatening or debilitating
357 (Modjtahedi et al., 2012).
358
359 When an antibody is used to block the function of leukocytic cancers, the possibility
360 of side effects related to suppression of normal immune cells exists. Alemtuzumab, used for
361 lymphoid malignancies, targets CD52, which also exists on the surface of normal circulating
362 lymphocytes, leading to lymphocytopoenia for up to one year after treatment is discontinued.

363 One of the possible consequences is the appearance of opportunistic infections. Rituximab
364 targets CD20 on the surface of B cells, but secondary infection is normally only a problem in
365 patients that were already immunosuppressed or taking immunosuppressive drugs
366 concomitantly. Nevertheless, transient decline in leukocytes can occur after use of Rituximab
367 and caution is recommended when administering multiple courses of the antibody.
368 Additionally, reactivation of latent hepatitis B infections has been recorded in patients
369 receiving Rituximab (Sharma et al., 2007; Kelesidis et al., 2011; Uettwiller et al., 2011).
370
371 *Benefits of immunosuppression*
372 Several factors influence the formation of anti-antibody responses by the patient
373 receiving a mAb, such as the number of injections of the antibody, its immunogenicity and
374 the immunocompetence of the recipient (Kuus-Reichel et al., 1994). Reducing the immune
375 response against the mAb is therefore an intuitive procedure that could make possible the use
376 of extraneous antibodies or reduce the responses against antibodies. Indeed, it is believed that
377 the lack of a negative reaction to some antibodies is due to the immunosuppressed status of
378 the patients, either by the use of chemotherapy or because of the cancer activity itself
379 (Glennie and Johnson, 2000). Suppression of human anti-mouse (HAMA) responses has been
380 attempted with the administration of cyclosporine and other compounds. The use of
381 cyclosporine was able to reduce the formation of anti-mAb responses and increase the
382 amount of useful doses of mAbs that could be administered, but could not completely
383 suppress HAMA in all the individuals tested or for an indefinite amount of administered
384 doses (Ledermann et al., 1988; Weiden et al., 1994; Dhingra et al., 1995). CTLA-4 inhibits T
385 lymphocyte activation by affecting the CD28-B7 co-stimulatory signal. Healthy beagle dogs
386 simultaneously receiving an antibody fraction and the recombinant human CTLA-4 had
387 better antibody responses. While dogs treated with the CTLA-4 had no side-effects after 5

388 doses of human antibody fractions, dogs that did not receive CTLA-4 simultaneously with the
389 antibody showed hypersensitivity responses after 4 doses (pale mucous membranes, tremors,
390 nasal discharge, and other signs). Also, the treatment seemed to reduce anti-antibody
391 formation, allowing antibody plasma levels to be higher for longer periods (Siegall et al.,
392 1997). However, another study could not reproduce these results. The addition of CTLA-4
393 did not inhibit the formation of anti- antibodies in this second study (Henry et al., 2005).
394
395 **Hurdles and opportunities**
396 The hurdles and opportunities in the study and production of new antibodies for
397 veterinary use are discussed below and are summarised in Table 3.
398
399 The immunogenicity of therapeutic molecules, including monoclonal antibodies,
400 affects the efficacy and safety of the product (Chirino et al., 2004). For the veterinary field
401 this still represents the greatest barrier for mAb usage in the clinical setting, although much
402 still needs to be studied about the possible use of non-speciated mAbs. Clinical use of
403 antibodies will benefit from the production of species-specific molecules (Gearing et al.,
404 2013).
405
406 Cost represents an important concern for monoclonal therapies in humans. In
407 veterinary medicine, this issue is even more relevant. The need for high cumulative doses and
408 the fact that cancer is a chronic disease add to the fact that current methods for antibody
409 production in mammalian cells are intrinsically expensive. Novel technologies such as
410 transgenic animals and use of yeast, bacteria and insect culture systems are probable paths to
411 be followed in the future for the production of antibodies (Daniell et al., 2001; Farid, 2007).
412 This highlights the importance of better understanding the possibilities of using single-dose

413 non-speciated mAbs, which are substantially cheaper to produce, since the price of
414 production will likely hinder the market accessibility of the veterinary biologics industry.
415
416 Another limiting factor is the ability of the antibody to penetrate into the tumour. This
417 accounts for one of the reasons why many mAbs that are promising during the developmental
418 stage are not so successful clinically. Clearance from circulation, blood vessel density,
419 vascular permeability and binding site barrier are some of the factors that influence mAb
420 delivery (Lobo et al., 2004; Niu et al., 2009). Solutions include the use of antibody fragments
421 such as scFv or Fab which exhibit better pharmacokinetics for penetration into the cancerous
422 tissue and also possess high binding specificity (Batra et al., 2002). Direct intratumoural
423 antibody administration and the administration of microencapsulated whole antibody-
424 producing cells or other *in situ* antibody producing technologies are possible means to
425 circumvent these difficulties (Pelegrin et al., 1998; Azemar et al., 2003; Orive et al., 2003).
426 Imaging techniques such as positron emission tomography (PET) offer high sensitivity which
427 is required to monitor distribution of the drug throughout the body, to study pharmacokinetics
428 and pharmacodynamics and also provide the information on optimal timing and antibody
429 dosage (Rudin et al., 2005). These techniques will be central in the establishment of better
430 mAb tumour penetration.
431
432 Reduced efficiency of monoclonal antibodies during human clinical trials when
433 compared to mouse trials is of major concern (Stein, 1997), and this might also be an issue
434 when mAbs are to be used in veterinary medicine. Dogs represent a good model to study
435 cancer on in respect to possible future applications in human patients. The genetic diversity
436 of dogs is comparable to that of humans, and is larger than that of most rodent models; their
437 cancer-associated genes show great similarities; cancers are naturally occurring, and appear

438 in animals with intact immune systems; similar factors influence the appearance of cancer in
439 dogs and humans, such as age and environmental exposure to carcinogens. Indeed, clinical
440 studies in dogs have shown that they serve as good predictors of both clinical toxicity and
441 response to therapy (Paoloni and Khanna, 2008; London et al., 2009; Richards and Suter,
442 2015). It would be in the interests of both veterinary medicine and of human medicine that
443 dogs became an established intermediate in the studies of monoclonal antibodies.
444 Experiments using human or murine mAbs in animals need to consider the potential benefits
445 of the idiotype responses and the possibility of using mAbs in short dosing protocols. Quick
446 chimerisation of antibodies and the study of cheaper expression systems would facilitate this
447 process. It is very likely that once an antibody is fully arranged to be tested in dogs, the
448 relevance of the results for human clinical trials would be improved when compared to mouse
449 studies.
450
451 **Conclusions**
452 The current trend of biological therapeutics is now reaching veterinary medicine.
453 While there are still many challenges to overcome in order to achieve commercial viability
454 and wide accessibility of monoclonal antibodies for animal use, there are many opportunities
455 in this field, as can be seen by the renewed interest of many research laboratories and private
456 companies. Testing and using monoclonals clinically in veterinary medicine will be of benefit
457 for both the animal patients and the development and improvement of human therapeutics.
458
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Conflict of Interest

The authors declare no conflicts of interest.

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887 **Table 1**

888 Molecular mechanisms of action of monoclonal antibodies in cancer.

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Classes	Mechanisms	Mediators	mAbs	Effects
		Direct effects		
Blockade of signalling	Neutralisation of mediators, cytokines, blockade of receptors, induction of apoptosis	Apoptosis mediators	Daclizumab Trastuzumab (other mechanisms also involved) Cetuximab Panitumumab Nivolumab Ipilimumab [nearly all commercially available antibodies for cancer affect signalling to some extent]	Inhibition of proliferation and apoptosis
Induction of signalling	Protein tyrosine phosphorylation, upregulation of Myc	p38 Map kinase, protein kinase C, Myc	Rituximab	Regulation of cell growth
Conjugated antibodies	Induction of cell death and inhibition of proliferation	Drugs, toxin fragments, radioisotopes	Tositumomab Ibritumomab tiuxetan	Direct cell death
		Immune Mediated Effects		
ADCC[a]	Interaction of mAb with FcγR bearing cells (NK, macrophages, neutrophils) with mAbs of IgG2a and IgG3 (in dogs receiving mouse mAb) or IgG1 and IgG3 (in humans)*	[NK cells] IFNγ, cytotoxic granules (perforin, granulysin, granzymes), [Macrophages] ROS, proteases	Rituximab Trastuzumab (other mechanisms also involved) Alemtuzumab	Inhibition of cell proliferation, anti-angiogenesis, cell lysis. The antigen presentation following cell death may induce a host

				response.
CDC	Classical activation of complement system	IgG1 and IgG3 Fc region (in humans)[b]; IgG2 region (in mice) and complement cascade (initiating with C1q)	Alemtuzumab Rituximab	Lysis of cells by the complement through the creation of pore on cell membrane. Enhancement of ADCC.
Immunomodulation **Checkpoint inhibitors**	Blockade of immune inhibitory receptors; approximation of immune cells to its target (trifunctional antibodies); activation of cells and induction of immune memory	CTLA-4, PD-1, CD40, CD3, Fcγ receptors (mouse IgG2a and rat IgG2b in combination, for humans)[c]	Catumaxomab; Ipilimumab	Induction of T mediated lysis, ADCC, phagocytosis; reduction of tumour-induced immune anergy

[a]ADCC, antibody-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; IFN, interferon; FcγR, Fc gamma receptor; ROS, reactive oxygen species; NK, natural killer cells.

[b]Shuffled (engineered) isotypes of IgG1 and IgG3 in humans show a strong enhancement in the ADCC and CDC capacity (Kubota et al., 2009)

[c]Shuffled isotypes of mouse IgG2a and rat IgG2b show increased activation of human FcγR.

References: (Rosales et al., 1988; Helfand et al., 1994; Pedersen et al., 2002; Iannello and Ahmad, 2005; Seimetz et al., 2010; Eissler et al., 2012; Golay and Introna, 2012; Anderson and Modiano, 2015) .

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909 **Table 2**

910 Potential targets in domestic animal species for monoclonal antibodies in oncology.

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Target (name of mAb)	Condition targeted	Potential to be used in animals	Outcome in dogs (cats)	Hurdles	References
CD20	B-cell lymphoma	Dog PBMC stained with Rituximab. CD20 occurs in canine B-cell lymphomas. New antibodies developed for dogs (AT-004, 6C8, 1E4) mAb 6C8 had specific binding to canine B-cells mAb 1E4 (Elanco) has 'rituximab-like' properties and depletes B-cells; chimeric version maintains function	Rituximab does not bind peripheral blood dog cells. mAbs AT-004 reached the market [Aratana/Novartis], with poor outcomes.	Difference in aa[a] where Rituximab binds. Low affinity of AT-004 for its target	(Jubala et al., 2005; Binder et al., 2006; Impellizeri et al., 2006; Acharya et al., 2015; Aratana Therapeutics Inc, 2015; Ito et al., 2015; Rue et al., 2015)
CD52	T-cell lymphoma	mAb received USDA approval for therapeutic use in dogs (AT-005)	Canine mAbs reached the market, but with poor responses [Aratana]	Low affinity of the antibody for its target	(Acharya et al., 2015; Aratana Therapeutics Inc, 2015)
CD47	B-cell lymphoma	Innate anti-tumor immune response such as phagocytosis increased against canine lymphoma by anti-CD47 mAb	mAb treatment increased canine lymphoma phagocytosis *in vitro*	None described	(Anderson and Modiano, 2015)(Anderson et al., 2016)
α4β1 receptor (LLP2A)	NHL	Biodistribution of LLP2A studied in dog as model for human	Tumour affected tissues strongly targeted (preferentially B cells)	None described	(A L Zwingenberger et al., 2012; Allison L Zwingenberger et al., 2012)
Lymphoma [target not defined] (mAb 231)	Lymphoma	FDA approved for dog lymphoma. Binds 73% of tested lymphomas	Increased complete remission in dogs combined with VCAA*	Discontinued due to lack of demand. No longer USDA licensed	(Steplewski et al., 1987; Jeglum, 1996, 2009; USDA, 2016)
VEGF (Bevacizumab)	Various cancers	VEGF correlation with outcome ambiguous in dogs and cats. Molecular structure of VEGF/R similar between dog and man. Bavacizumab inhibited canine osteosarcoma and	Delayed tumour growth and blood vessel formation in mice grafted with canine osteosarcoma	Humanized mAb and cost of therapy	(Scheidegger et al., 1999; Millanta et al., 2002, 2006; Restucci et al., 2002; Coomer, 2008; Michishita et al., 2013; Scharf et

		hemangiopericytoma xenografts in mice.			al., 2013)
Lewis^y (BR96)	Breast and lung carcinomas	33% of dog tumour samples tested express Le^y (several carcinomas)	Partial response in 25% of dogs tested. Stable disease in 33%. Dogs negative for Le^y had progressive disease.	75% cases had anti-mAb reactions not preventable by immunosupression. Anti-mAb reactions curtailed clinical benefits	(Friedman et al., 1993; Henry et al., 2005)
HER2/neu (trastuzumab)	Breast cancer overexpressing HER2/neu	Dog tumours expressing HER2/neu have been identified, but prognostic value is uncertain. Canine and human receptors are similar; dogs have a similar antigenic phenotype to humans. Prognostic value and tumour behaviour are more similar to humans in cats	Human monoclonal antibodies are able to bind dog antigens.	In cats no adaptation is possible from trastuzumab, since human and feline receptors differ at the EC* region	(Mottolese et al., 1994; Demaria et al., 2005; Valabrega et al., 2007; Gama et al., 2008; Gianni et al., 2010; Singer et al., 2012)(Cannon, 2015)
CEA*	Tumours of colon; other epitelial tumours	CEA ligand and receptor aa* are 99% conserved in dogs compared to humans.	CEA receptor is expressed in canine mammary carcinoma cell lines	The CEA ligand is not differentially expressed in cancer in dogs	(Watine et al., 2001; Sturgeon et al., 2008; Weichselbaumer et al., 2011; Campos et al., 2012; Grunnet and Sorensen, 2012)
EGFR (cetuximab)	Colorectal; head and neck cancer	In canine mammary tumours, EGFR has been associated with malignancy and poor prognosis. Human and canine receptors are similar in the epitopes for cetuximab. A caninised version of Cetuximab was developed	Cetuximab is tumoristatic to canine mammary carcinoma cell lines. Caninised cetuximab (can225IgG) maintained function	Cetuximab is a human chimeric antibody. Caninised version is in trial phase.	(Gama et al., 2009; Shiomitsu et al., 2009; Selvarajah et al., 2012; Singer et al., 2012, 2014; Terragni et al., 2014; Acharya et al., 2015; Queiroga et al., 2015)
Human leucocyte antigen-DR [MHC II] (Murine version: L243; humanised: IMMU-114)	Lymphoma	L243 and IMMU-114 bind human lymphocytes and malignant cells in nodal tissue	Transient disease stabilisation. Murine led to fever, vomiting and short mAb half-life. Humanised had lymphocytopenia and half-life of 2h	Author recommended the canine chimerisation of mAb	(Stein et al., 2011)
Prostatic tumour antigens	Prostate cancer and human colon	Met5 binds to the EC region of human and canine Met; dog prostate express PSMA mRNA	Met5 binds to canine prostate cancer metastatic cell line	None described	(Anidjar et al., 2001; Ross et al., 2003; Hay et al.,

(Met5; PSM-P12; CC49)	cancer	and 50% of cancers have the antigen (but not benign tissue)	GN4; PSM-P12 binds to a prostate cell line (DPC-1); CC49 was successful in imaging canine metastases		2005; Lewis et al., 2005; Lai et al., 2008)
PD-1	Several tumour types	Several canine tumours express PD-1, and some also express its ligand. Intra-tumoural lymphocytes in canine melanoma also express PD-1. Anti-PD-1 increases IFNγ production	PD-1 being researched by Nexvet/Zenoaq	None described	(Maekawa et al., 2014, 2016; Nexvet, 2016)

[a]aa = aminoacids; CEA = carcinoembryonic antigen; EC = extracellular; EGFR = epidermal growth factor receptor; PSMA = prostate specific membrane antigen; VCAA = vincristine, cyclophosphamide, L-asparaginase and doxorubicin; VEGF/R = Vascular endothelial growth factor/receptor;

918 **Table 3**

919 Hurdles and opportunities in monoclonal antibody therapies in veterinary medicine.

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Hurdles	Opportunities
Immunogenicity of human and mouse mAbs in dogs	Caninised and chimeric mAbs
Cost of production of conventional mAbs, with cumulative doses	Alternative mAb production systems and the use of non-speciated single-dose mAbs. Possible expression systems: Insect and yeast cells, transgenic plants and animals. Bacterial production of scFv
Difficult tumour penetration	*In vivo* production of the antibody by the host, through viral transduction or direct DNA injection; use of scFv antibody fragments
Weak results *in vivo* following promising results in mice	Use of naturally occurring cancers in animals as models to study the therapies

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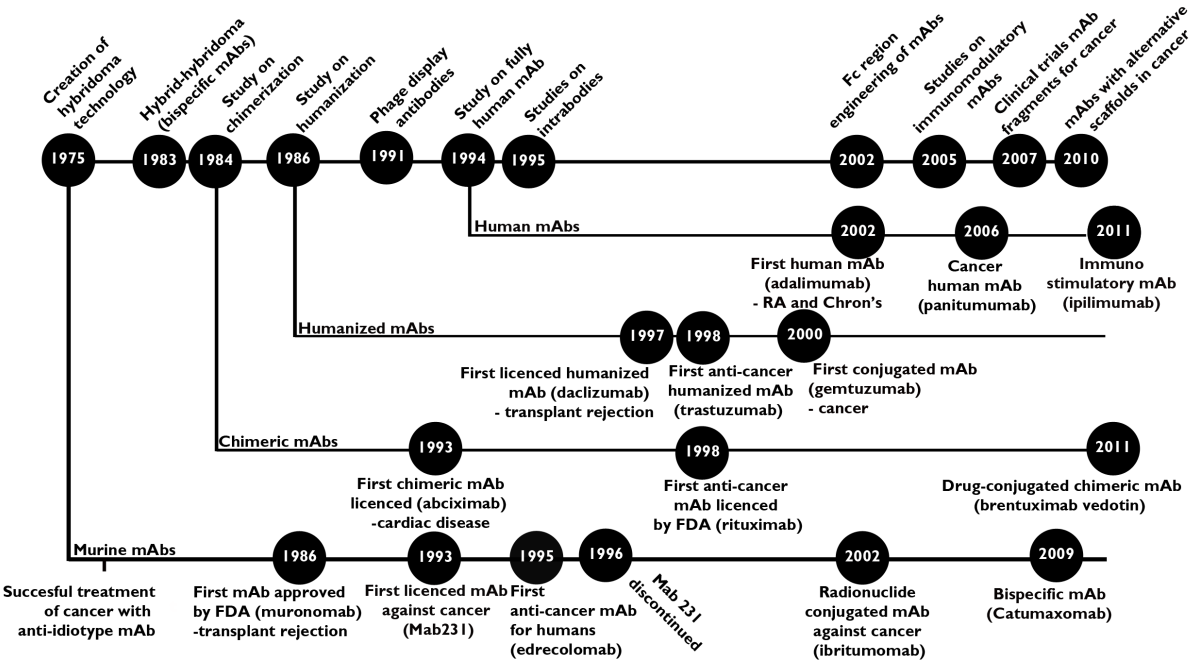
924 **Figure legends**



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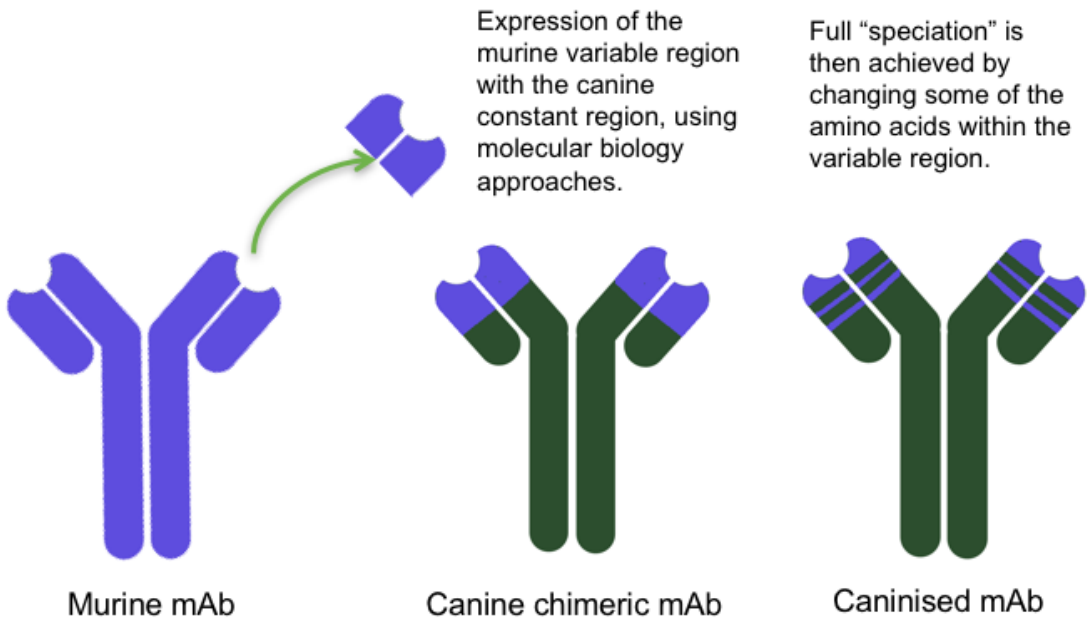
926 Fig. 1. History of monoclonal antibodies and its clinical uses. The top horizontal line presents
927 the main technological breakthroughs and the year in which they occurred (or the year around
928 which significant research was done on that subject). The parallel lower lines represent the
929 mAbs used clinically categorised by their structural classes with regard to humanisation. The
930 mAbs cited represent a milestone in the use of the technology; other dates of interest are also
931 shown. RA = rheumatoid arthritis; References: Carter et al., 2001; Lobato and Rabbitts, 2001;
932 Fong and Small, 2008; Reichert, 2012; Kellerman and Green, 2002; Enever et al., 2009.



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934 Fig. 2. The most common methods of antibody production and the resulting classes of
935 antibodies (Jakobovits et al., 2007).

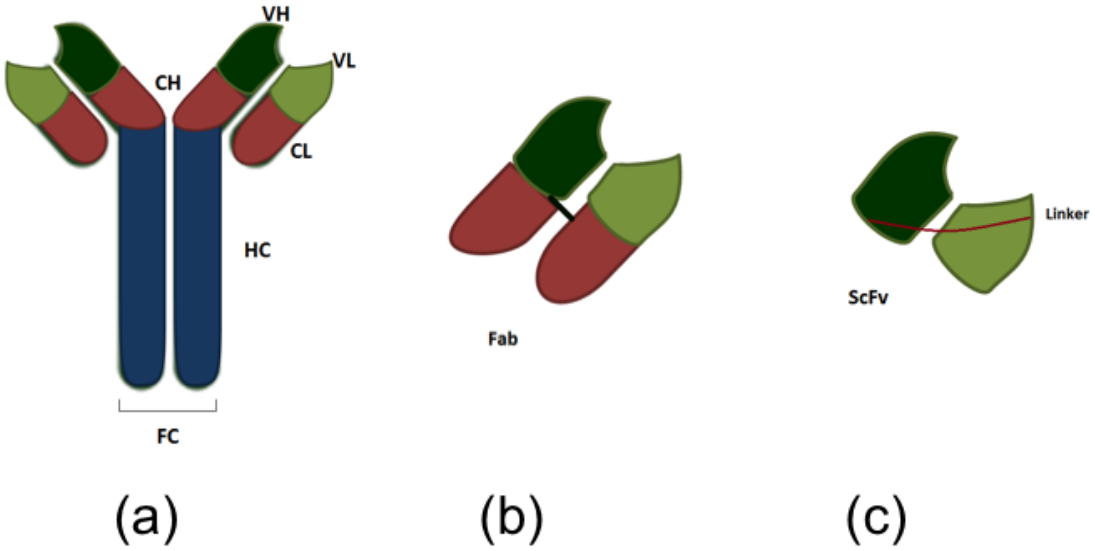

Fig. 3. Representation of different formats of the antibody molecule. (a) Full length monoclonal antibody showing heavy as well as light chain comprised of variable and constant regions, also the hinge region and the Fc portion which interacts with cell surface receptors; (b) Fab molecule which contains no Fc portion of the antibody, thus containing variable as well as constant regions from heavy and light chains; and (c) scFv which contains only variable domains of the heavy and light chains for antigen binding.

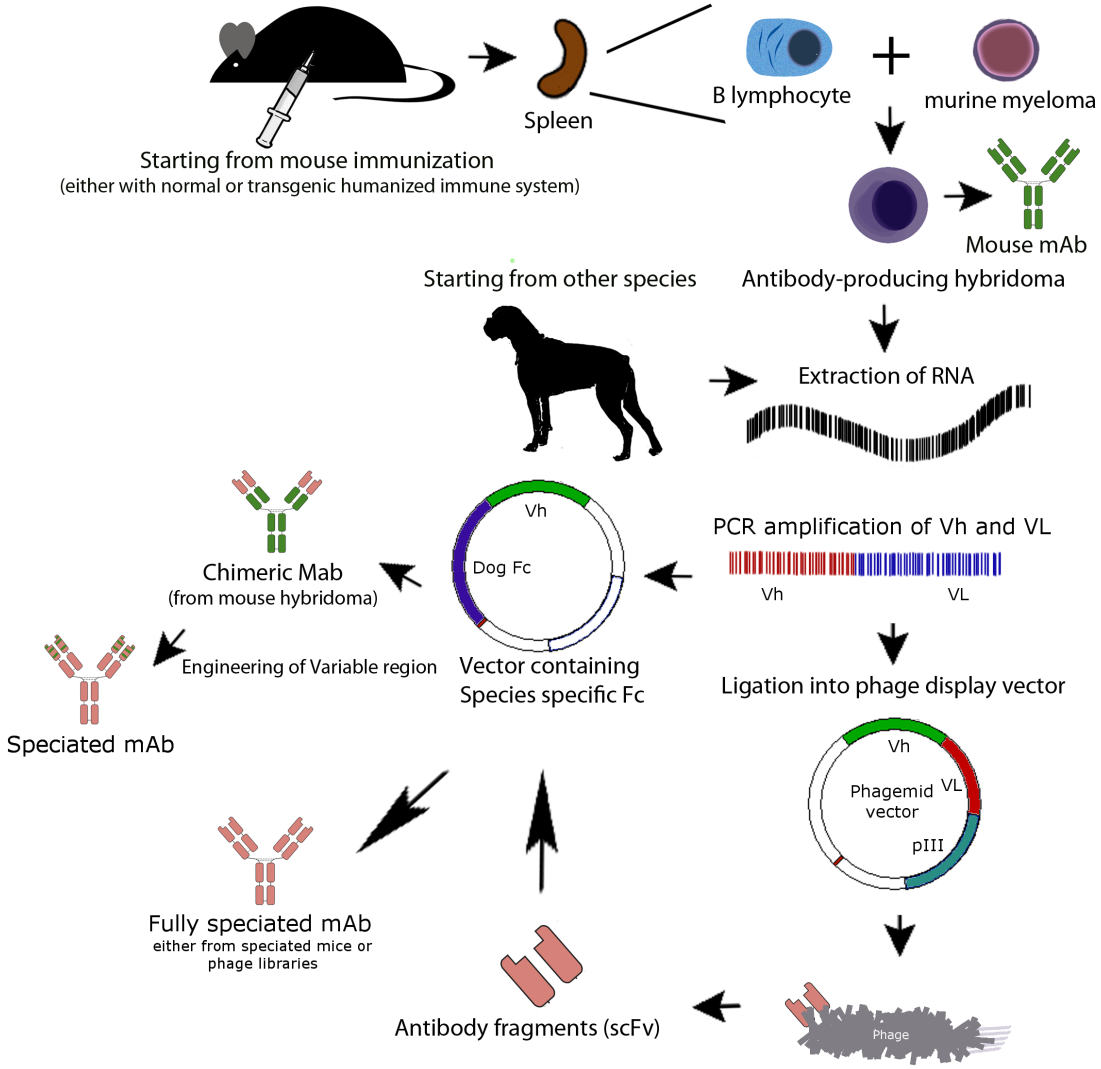

Fig. 4. Techniques for producing antibodies of different speciation levels. Antibodies can be derived either from: 1) mice or other laboratory animals immunized with the antigen of

interest; unless the mouse was altered genetically to express the immune system from another species (such as the XenoMouse), antibodies derived from laboratory species will be at most 'speciated' (humanized, for instance); 2) directly from the species of interest, in which case phage display can be used to select the antibody of interest from the vast repertoire available in any animal. In this case, the antibodies that are derived are 'humanized', or 'caninised', etc.

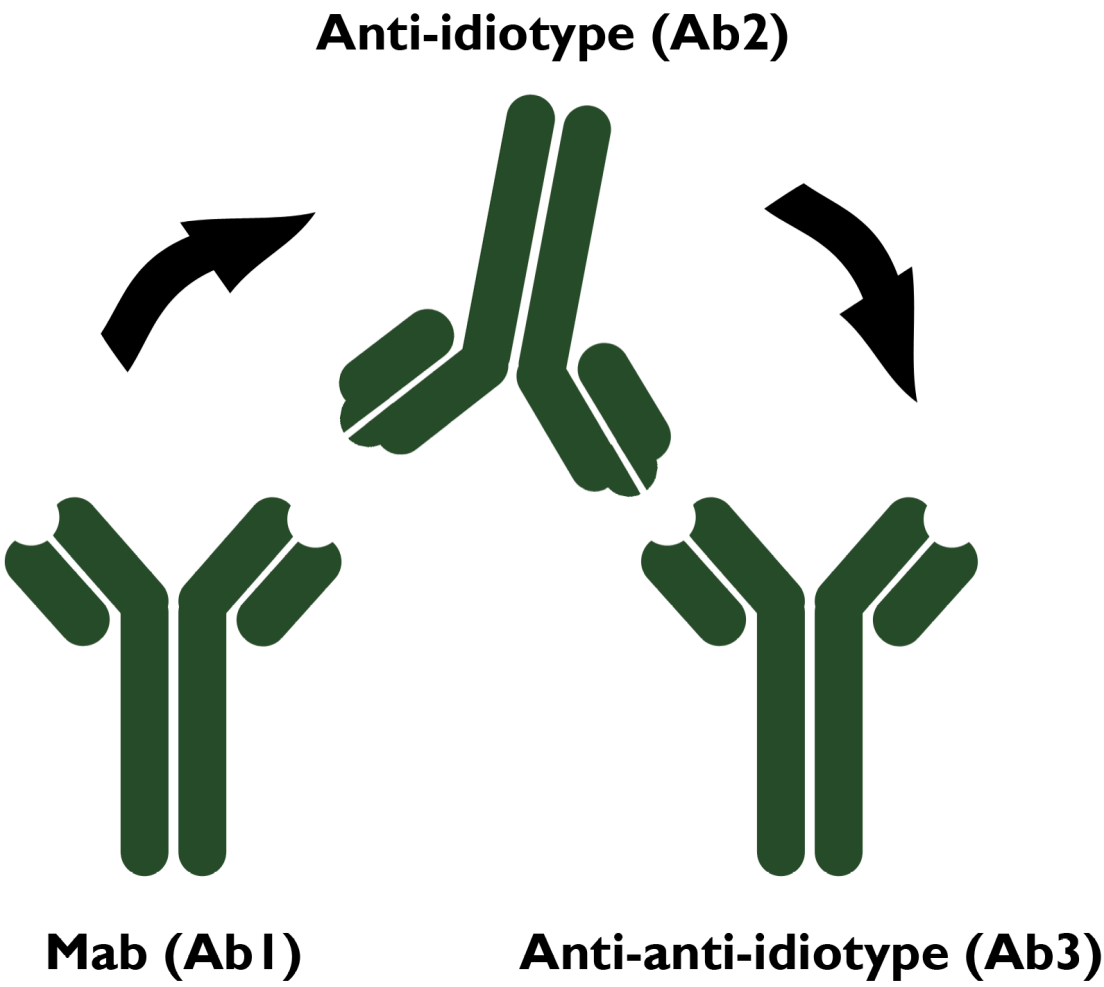

Fig. 5. The idiotype cascade. The mAb itself is an antigen, and the patient receiving it will raise an immune response against all the foreign parts of the mAb. Ab2 are created by the host as a response against the antigen-binding region of the mAb (Ab1). Ab3 is then raised recognizing the antigen-binding region of Ab2. Therefore, the antigen-binding region of Ab3 is identical to that of the therapeutic mAb (Ab1).