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An investigation of the breadth of neutralizing antibody response in cats naturally infected with feline immunodeficiency virus

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Neutralizing antibodies (NAbs) are believed to comprise an essential component of the protective immune response induced by vaccines against feline immunodeficiency virus (FIV) and human immunodeficiency virus (HIV) infections. However, relatively little is known about the role of NAbs in controlling FIV infection and subsequent disease progression. Here, we present studies where we examined the neutralization of HIV-luciferase pseudotypes bearing homologous and heterologous FIV envelope proteins (n=278) by sequential plasma samples collected at 6 month intervals from naturally infected cats (n=38) over a period of 18 months. We evaluated the breadth of the NAb response against non-recombinant homologous and heterologous clade A and clade B viral variants, as well as recombinants, and assessed the results, testing for evidence of an association between the potency of the NAb response and the duration of infection, $CD4^+$ T lymphocyte numbers, health status and survival times of the infected cats. Neutralization profiles varied significantly between FIV-infected cats and strong autologous neutralization, assessed using luciferase-based *in vitro* assays, did not correlate with the clinical outcome. No association was observed between strong NAb responses and either improved health status or increased survival time of infected animals, implying that other protective mechanisms were likely to be involved. Similarly, no correlation was observed between the development of autologous NAbs and the duration of infection. Furthermore, cross-neutralizing antibodies were evident in only a small proportion (13 %) of cats.

INTRODUCTION

Neutralizing antibodies (NAbs) are elicited in response to feline immunodeficiency virus (FIV) and human immunodeficiency virus (HIV) infections, and are believed to be an essential component of the protective immune responses required for successful vaccination against lentiviruses (Kwong *et al.*, 2012). However, relatively little is known about the role of humoral immunity in controlling lentiviral infections and subsequent disease progression, particularly for FIV infection (Piantadosi *et al.*, 2009, Gray *et al.*, 2011; Hosie *et al.*, 2011), in spite of there being a vaccine available commercially that protects cats against FIV infection (Pu *et al.*, 2005).

In HIV infection, NAbs specifically target epitopes on SU and TM, including receptor- and co-receptor-binding sites (Binley *et al.*, 2008). However, their efficacy is subject to significant challenges. The viral envelope (Env) protein contains host glycans that shield neutralization epitopes on Env proteins, often rendering them inaccessible to NAbs (Myers & Lenroot, 1992). Furthermore, HIV and FIV Env proteins may display significant length polymorphisms (Kraase *et al.*, 2010; Euler & Schuitemaker, 2012) that may result in conformational changes, concealing neutralization epitopes (Hoxie, 2010).

Antibodies recognizing HIV-1 Env appear ~2 weeks after infection, but lack neutralizing activity (Tomaras & Haynes, 2009). Autologous, highly strain-specific, polyclonal NAbs appear within 3 months after infection, exert selection pressure and lead to the emergence of escape mutants (Moore *et al.*, 2008; Li *et al.*, 2009). It has been documented in HIV-1 infection that autologous NAbs have little or no protective effect on disease progression (Bunnik *et al.*, 2008; Mahalanabis *et al.*, 2009; van Gils *et al.*, 2010; Gray *et al.*, 2011), largely as a result of the rapid emergence of escape mutants (Bunnik *et al.*, 2010; van Gils *et al.*, 2010). The emergence of neutralization escape mutants

Four supplementary tables are available with the online Supplementary Material.

with altered glycosylation patterns has been demonstrated both in HIV (Burton *et al.*, 2005; van Gils *et al.*, 2010) and FIV (Samman *et al.*, 2010) infections. Neutralization escape, accompanied by the subsequent evolution of the antibody response, occurs during the course of infection in response to the evolving viral Env, until the eventual exhaustion of the immune system (Euler & Schuitemaker, 2012). This explains why NAbs from a specific time point can neutralize viruses isolated from earlier time points, but fail to neutralize contemporaneous viral variants (Mascola & Montefiori, 2010; Overbaugh & Morris, 2012).

It has been suggested that NAbs appear too late following infection with HIV-1 to be effective in controlling disease progression (Richman *et al.*, 2003; Moore *et al.*, 2009; Rong *et al.*, 2009). However, NAbs have been shown to have a potential role in controlling simian immunodeficiency virus (SIV)–HIV (SHIV) infection of macaques depleted of cytotoxic T lymphocytes (Rasmussen *et al.*, 2002). Furthermore, pre-exposure passive transfer of broadly neutralizing mAbs conferred protection against SIV and SHIV-1 in the rhesus macaque model (Veazey *et al.*, 2003; Ferrantelli *et al.*, 2004; Hessell *et al.*, 2009), providing evidence that NAbs do indeed play a protective role and are likely an essential component of a protective vaccine response (Hoxie, 2010).

HIV infection, in the majority of patients, leads to the robust production of antibodies that often possess the ability to neutralize autologous but not heterologous viral variants (Zolla-Pazner *et al.*, 2004). Broadly cross-reactive NAbs (Cr-NAbs) are relatively rare; elicited in some individuals, Cr-NAbs neutralize not only autologous viral variants, but also neutralize other viral subtypes (Walker *et al.*, 2011). Several broadly neutralizing mAbs have been isolated (including b12, 2G12, 2F5 and 4E10), their binding epitopes have been characterized (Muster *et al.*, 1993; Burton *et al.*, 1994; Gorny *et al.*, 1994; Trkola *et al.*, 1996; Zwick *et al.*, 2001) and protective roles in animal models have been demonstrated (Mascola *et al.*, 1999; Mascola *et al.*, 2000; Binley *et al.*, 2004). Recent evidence suggests that Cr-NAbs are more common than previously estimated, arising in approximately one-third of HIV-1-infected individuals (Stamatatos *et al.*, 2009; Bonsignori *et al.*, 2011; Medina-Ramírez *et al.*, 2011; Mikell *et al.*, 2011; Walker *et al.*, 2011; Euler & Schuitemaker, 2012). However, neutralization breadth does not develop until ~3 years post-infection (Gray *et al.*, 2011; Mikell *et al.*, 2011). It remains unknown why, and by which mechanism, such antibodies develop in some individuals or why the broadly neutralizing response is significantly delayed in response to infection (Gray *et al.*, 2011). Furthermore, it is unclear whether neutralization cross-reactivity can be attributed to a single, highly potent antibody or a combination of antibodies acting in synergy (Scheid *et al.*, 2009).

The strength and breadth of the NAb response was greater in HIV progressors compared with aviraemic or long-term non-progressors (Doria-Rose *et al.*, 2009). Studies of elite controllers revealed that individuals who controlled viral replication, such that their viraemia was below detectable levels, had the lowest levels of NAbs (Lambotte *et al.*, 2009; Pereyra *et al.*, 2009). Consistent with this observation, the breadth of Cr-NAbs was positively correlated with higher plasma viral loads, lower $CD4^+$ T lymphocyte counts and disease progression (Piantadosi *et al.*, 2009; Sather *et al.*, 2009; van Gils *et al.*, 2009; Euler *et al.*, 2010). These results suggested that the development of Cr-NAbs is influenced by strong antigenic stimulation (Gray *et al.*, 2011). However, individuals who did not develop Cr-NAbs might have failed to do so as a result of insufficient antigenic stimulation and possibly non-specific hypergammaglobulinaemia (Euler & Schuitemaker, 2012). Despite the breadth and potency of Cr-NAbs *in vitro*, such antibodies do not appear to influence HIV-1 disease progression; rather, their incremental development is associated with increased viral loads and declining numbers of $CD4^+$ T lymphocytes (Piantadosi *et al.*, 2009; van Gils *et al.*, 2009; Euler *et al.*, 2010; Gray *et al.*, 2011).

Little is known about the role of NAbs in controlling natural FIV infection and subsequent disease progression (Hosie *et al.*, 2011), although NAbs appear to be involved in vaccine-induced protective immunity (Hosie & Flynn, 1996; Pu *et al.*, 2001). What is the relationship between the duration of infection, health status, survival time and the NAb response in FIV-infected cats? Can a strong NAb response delay disease progression? Is there evidence for broadly Cr-NAbs in plasma samples from naturally infected cats? Although the gold standard neutralization assay utilizes primary PBMCs as target cells, as well as uncloned primary field isolates, the reproducibility of such systems is limited by the variability in susceptibility of PBMCs to infection, as reviewed previously (Hosie *et al.*, 2011). Therefore, in this study we utilized a pseudotype-based neutralization assay, similar to the assay systems that have been shown to be robust and highly reproducible for measuring HIV neutralization, to measure NAb responses in cats naturally infected with FIV in order to investigate the role of neutralization.

RESULTS

FIV-infected cats display variable neutralization patterns

Plasma samples from 38 cats displayed variable autologous and heterologous neutralization patterns, ranging from strong, through moderate to no neutralization (Table S1, available in the online Supplementary Material). Plasma samples from 16 cats (16/38, 42.1 %) strongly neutralized pseudotypes bearing autologous Env variants. This pattern was observed in eight (8/16, 50 %) of the cats that remained alive for the duration of the study and eight (8/16, 50 %) of the cats that died during the study. Six cats (6/38, 15.8 %) displayed moderate neutralization of pseudotypes bearing autologous Env variants; all but one of these cats remained alive during the observation period.

Plasma samples from 16 cats (16/38, 42.1 %) failed to neutralize pseudotypes bearing autologous Env variants; nine of these cats (9/16, 56 %) remained alive, whereas seven (7/16, 44 %) of the cats with no detectable NAbs died during the study period. Only five cats (5/38, 13 %) demonstrated strong heterologous neutralization of at least one pseudotype; all of those cats remained alive during the study.

Autologous NAbs and duration of infection

We investigated whether the development of autologous neutralization was positively correlated with the age of the cats and the duration of infection. Fig. 1 illustrates the relationship between the potency of autologous NAb response and the duration of infection for cats from the entire study group. The median duration of infection for cats with strong, moderate and absent autologous NAb responses was 3.1 (1.1–6.3), 2.9 (1.5–5.4) and 3.8 (range 0.8–8.8) years, respectively. No statistically significant differences between the groups were observed.

NAb responses in cats infected with recombinant and non-recombinant viruses

We hypothesized that cats infected with recombinant *env* viruses (n=14) would have more potent autologous and heterologous NAb responses than cats infected with non-recombinant *env* viruses (n=24). However, no statistically significant differences were observed between the strength of autologous neutralization in cats infected with recombinant compared with non-recombinant viruses (Fig. 2).

Next, we asked whether cross-reactive NAb responses were more common in cats infected with recombinant *env* viruses compared with cats infected with non-recombinant *env* viruses. There was no statistically significant difference between the two groups; of five cats that demonstrated heterologous neutralization (against at least one pseudotype), three were infected with non-recombinant viruses, whilst two were infected with recombinant viruses.

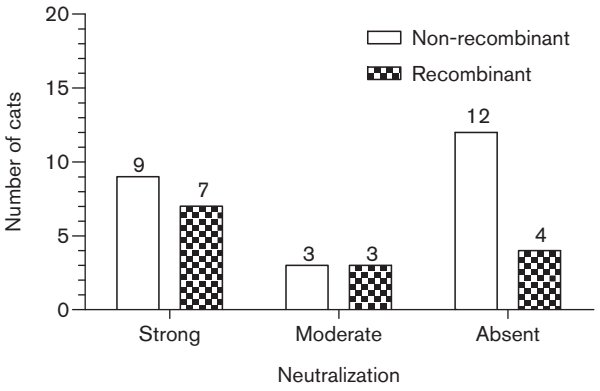

Fig. 2. Autologous neutralization responses in cats infected with non-recombinant (n=24) and recombinant (n=14) *env* viruses. Entire *env* sequences from the study group (n=355), together with reference full-length *env* sequences obtained from GenBank (n=19), were subjected to rigorous fivefold recombination testing as described previously (Bęczkowski *et al.*, 2014).

NAb response and health status of infected animals

We examined the data for an association between the presence of autologous NAbs and the health status of infected cats (Fig. 3). Health status was assessed by a board-certified feline medicine specialist, but was nevertheless subjective, and so we also examined the data to test for an association between autologous NAbs and declining $CD4^+$

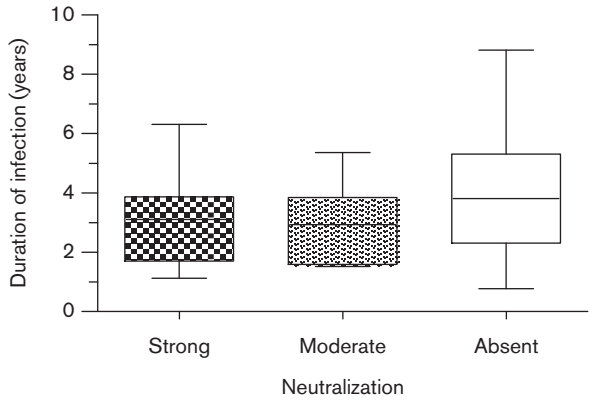

Fig. 1. Relationship between the duration of infection and the development of autologous NAbs. Duration of infection for cats with strong (n=16), moderate (n=6) and absent (n=16) autologous neutralization responses is shown from left to right (median 3.1, 2.9 and 3.8 years, respectively).

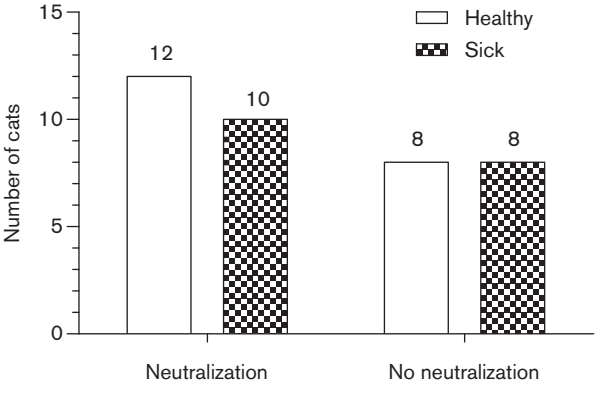

Fig. 3. NAb responses according to health status. Within the group of cats expressing strong and moderate autologous NAb responses (n=22), there were 12 healthy and 10 sick cats. Within the group with no autologous neutralization (n=16), there were eight healthy and eight sick cats.

Table 1. $CD4^+$ T lymphocyte counts for each time point (A, B, C and D) unless a cat was deceased (X) or a sample was not available (NA)

$\Delta CD4^+$ in the final column represents the difference between the first (A) and the last available sampling. All but two cats from the Memphis cohort (M) displayed a progressive decline in $CD4^+$ T lymphocyte numbers over the 18-month observation period. Five cats from the Chicago cohort (P) (5/14) displayed increasing $CD4^+$ T lymphocyte numbers over the 12 month observation period.

Cat	$CD4^+$ count ($\times 10^3$ cells μl^{-1})				$\Delta CD4^+$ ($\times 10^3$ cells μl^{-1})	Cat	$CD4^+$ count ($\times 10^3$ cells μl^{-1})				$\Delta CD4^+$ ($\times 10^3$ cells μl^{-1})
	A	B	C	D			A	B	C	D	
M2	1.74	0.36	0.76	0.62	−1.12	M5	0.56	0.15	0.16	X	−0.40
M29	1.48	0.62	0.93	0.62	−0.86	M50	1.38	1.32	X	X	−0.07
M1	0.87	0.36	0.34	0.26	−0.61	M33	0.20	X	X	X	NA
M15	0.87	1.03	1.09	0.47	−0.40	M3	0.33	X	X	X	NA
M8	0.55	0.21	0.2	0.15	−0.40	M44	1.24	X	X	X	NA
M49	0.41	0.31	0.27	0.04	−0.38	P4	0.09	0.34	0.33	NA	0.24
M28	1.23	0.89	1.77	0.90	−0.34	P14	0.97	1.16	1.09	NA	0.12
M14	0.45	0.29	0.15	0.14	−0.30	P8	0.50	0.86	0.60	NA	0.10
M25	0.36	0.39	0.68	0.09	−0.27	P6	0.48	0.35	0.57	NA	0.09
M20	1.50	0.81	NA	1.25	−0.25	P7	0.40	0.26	0.40	NA	0
M47	0.29	0.14	0.14	0.10	−0.19	P11	0.45	0.35	0.45	NA	0
M32	0.38	0.32	0.35	0.21	−0.17	P13	0.46	0.28	0.27	NA	−0.18
M30	0.13	0.15	0.26	0.10	−0.04	P17	0.49	0.55	0.28	NA	−0.21
M46	0.15	0.19	0.18	0.18	0.03	P9	0.63	0.35	0.18	NA	−0.45
M11	0.98	0.14	0.48	X	−0.50	P5	0.79	0.72	0.30	NA	−0.49
M16	0.35	0.37	0.36	X	0.01	P2	0.40	0.36	X	NA	−0.03
M26	0.54	0.14	0.13	X	−0.41	P21	NA	0.93	0.57	NA	−0.36
M31	0.80	0.88	0.26	X	−0.55	P22	NA	1.55	0.79	NA	−0.76
M41	0.34	0.35	0.12	X	−0.22	P18	NA	0.73	0.75	NA	0.02

T lymphocyte numbers. As demonstrated in Table 1, all but two of the cats within the Memphis cohort (n=24) demonstrated a progressive decline in $CD4^+$ T lymphocytes. The median $\Delta CD4^+$ over the 18 month observation period was –340 cells μl^{-1} (ranging from −1120 to +30 cells μl^{-1}). In contrast, within the Chicago cohort (n=14), seven cats displayed progressive declines in $CD4^+$ T lymphocyte numbers, five displayed increased numbers, whilst two cats maintained their $CD4^+$ T lymphocyte numbers over a period of 12 months (Table 1). The median $\Delta CD4^+$ was calculated as −15 cells μl^{-1} (ranging from –760 to +240 cells μl^{-1}).

We then asked whether a strong autologous NAb response might protect cats against a progressive decline in $CD4^+$ T lymphocytes. We compared three groups of cats: those with absent (n=15), moderate (n=5) or strong (n=15) NAb responses for which $\Delta CD4^+$ values were available (n=35). $\Delta CD4^+$ values were not available for three cats (3/38; M3, M33 and M44) as these cats died prior to the second blood sampling (Table 1). As demonstrated in Fig. 4, there was no evidence that cats with strong NAb responses were less likely to display progressively declining $CD4^+$ T lymphocyte numbers (median $\Delta CD4$=–270 cells μl^{-1}); a similar range of $\Delta CD4^+$ values was observed within the group of cats which failed to mount autologous NAb responses (median $\Delta CD4$=–250 cells μl^{-1}) and there were no statistically significant differences between the two groups. Three animals with moderate levels of NAbs maintained their $CD4^+$ T lymphocyte numbers, whilst two animals showed declining $CD4^+$ T lymphocyte numbers.

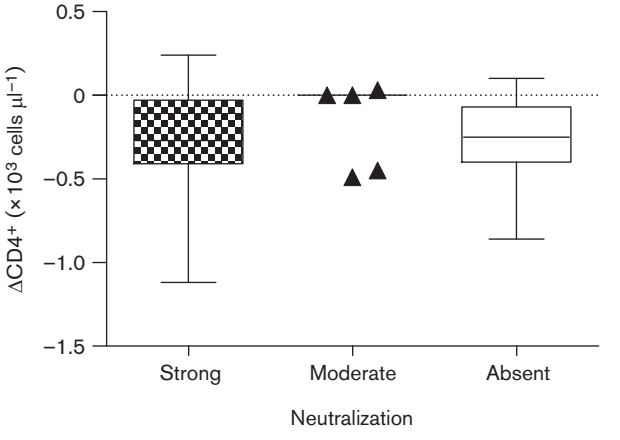

Fig. 4. Changes in $CD4^+$ lymphocyte count ($\times 10^3$ cells μl^{-1}) over the course of infection in cats with strong (n=15; median −0.27, range −1.12 to +0.24), moderate (n=5; median 0.0, range −0.49 to +0.03) or absent (n=15; median −0.25, range −0.86 to +0.10) NAb responses. $\Delta CD4^+$ values were not available for three cats (Table 1).

NAb response and survival time of infected animals

We examined the relationship between the autologous neutralization responses and survival times of infected cats since the estimated time of infection. Kaplan–Meyer survival curves were constructed for three groups of cats expressing strong (n=16), moderate (n=6) or no (n=16) autologous neutralization (Fig. 5).

The estimated median survival time for cats with strong autologous NAb responses was 2840 (range 1061–2840) days and for the group with no NAbs was 2476 (range 1384–3387) days. There was insufficient data within the moderate neutralization group to estimate median survival. A comparison of the Kaplan–Meyer curves [log-rank (Mantel–Cox) test] revealed that survival times were not significantly different amongst the three groups (P=0.48). Further testing (log-rank test for trend) revealed no significant trend between the three survival curves (P=0.36).

NAbs in the terminal stages of disease

Finally, we asked whether the NAb response was preserved in the terminal stages of disease, when the immune system was weakened. There were 13 cats in the study group with $CD4^+$ T lymphocyte counts <200 cells μl^{-1} recorded at the final sampling (Table 1), indicative of terminal infection (by analogy to HIV infection). Plasma samples from six (6/13; 46 %) of these cats did not neutralize pseudotypes bearing homologous Env proteins, whilst plasma samples from seven (7/13; 54 %) of the cats neutralized pseudotypes bearing autologous Env proteins, in spite of the cats being assumed to be in the terminal stage of disease.

DISCUSSION

We demonstrated that cats naturally infected with FIV have variable NAb responses against pseudotypes bearing autologous and heterologous Env proteins. When neutralization assays were conducted against pseudotypes bearing Env proteins from autologous and heterologous viruses, no correlation was evident between either the health status or the survival time following infection and the strength of the NAb response. Similar neutralization profiles were observed for plasma samples tested from healthy and unhealthy cats as well as cats that survived or died during the study period. Such findings argue against a role for NAbs in controlling disease progression. Furthermore, our results demonstrate that FIV-infected cats, regardless of the strength of the NAb response induced, showed progressive declines in $CD4^+$ T lymphocyte numbers; NAb responses, even when potent, did not appear to protect against the loss of $CD4^+$ T lymphocytes. A similar trend has been reported for HIV-1 infection, where the presence of autologous, Cr-NAbs was not associated with a prolonged AIDS-free, asymptomatic period (Schmitz *et al.*, 2003; Piantadosi *et al.*, 2009; van Gils *et al.*, 2010; Euler *et al.*, 2010). Several studies have suggested that neutralization breadth and potency depend on the duration of infection (Moog *et al.*, 1997; Deeks *et al.*, 2006; Sather *et al.*, 2009). However, the results presented here do not support this proposal; rather, no association was observed between the duration of infection and the potency of autologous neutralization.

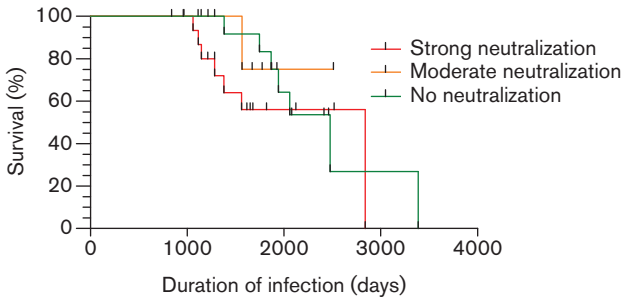

Fig. 5. Kaplan–Meyer survival curves for cats with strong (n=16) (red), moderate (n=6) (orange) or weak/absent (n=16) (green) autologous neutralization.

Almost half of the cats examined displayed strong autologous NAb responses; 24 % of the cats showed a steady increase in neutralization potency during the course of infection. A similar increase in the strength of autologous NAb response has been reported for HIV-1 infection (Arendrup *et al.*, 1992; Geffin *et al.*, 2003; Richman *et al.*, 2003). Although NAbs failed to protect against contemporaneous viruses, it was suggested that such antibodies might exert selection pressure on the emergence of viral variants of lower fitness, e.g. with decreased replicative capacity, and thus might indirectly delay HIV-1-associated disease progression (Friedrich *et al.*, 2004; Leslie *et al.*, 2004). However, in this study we found no evidence of any correlation between the strength of NAb response and survival time in cats naturally infected with FIV.

The remaining half of the cats that were examined did not develop NAbs. As a high level of antigenic stimulation is crucial for the development of broad and potent NAb responses (Rodriguez *et al.*, 2007; Doria-Rose *et al.*, 2009; Sather *et al.*, 2009), it is possible that the immune systems of those cats that did not neutralize pseudotypes bearing autologous Env proteins had not been exposed to sufficient antigenic stimulation following infection to induce NAbs. This is most likely the case with cat M1, which acquired the virus vertically (Bęczkowski, 2013) and failed to mount a NAb response. Given that a high viral load and high viral diversity following infection influence the development of potent and broad NAbs (Piantadosi *et al.*, 2009; Sather *et al.*, 2009; Euler *et al.*, 2010; Gray *et al.*, 2011), it is tempting to speculate that those cats in our study group which failed to develop NAb responses may have had relatively low viral load set points compared to the cats which developed NAbs. Unfortunately, viral load set point

data following the postulated transmission events were not available to test this hypothesis.

Furthermore, the non-specific $CD4^+$ T lymphocyte-dependent polyclonal hypergammaglobulinaemia that arises as an initial response to FIV infection (Recher *et al.*, 2004; Lang *et al.*, 2007) might also contribute to the lack of effective neutralization observed in this group. It is possible that high numbers of $CD4^+$ T lymphocytes at the time of virus acquisition might be responsible for a non-specific, overwhelming hypergammaglobulinaemia and subsequent failure of NAb responses to develop (Euler *et al.*, 2010; Gray *et al.*, 2011). This scenario could also explain why only a small fraction of cats in our study demonstrated cross-neutralization of the heterologous GL-8 and B2542 pseudotypes, regardless of whether they were infected with recombinant or non-recombinant viruses.

A study examining the breadth of neutralization in a similar number of HIV-1-infected individuals (n=40) revealed that 17.5 % of patients developed broadly NAbs (Gray *et al.*, 2011). Other studies reported higher numbers (up to 30 %) of individuals with broadly NAbs (Doria-Rose *et al.*, 2009; Gray *et al.*, 2009; Piantadosi *et al.*, 2009; Sather *et al.*, 2009; Euler *et al.*, 2010). In contrast, only 13 % of plasma samples from our study group displayed cross-reactivity. This may suggest that Cr-NAbs are rarer in FIV-infected cats than in HIV-1-infected individuals. However, a limitation of this study was that plasma samples were tested for neutralization against only two reference pseudotypes bearing heterologous FIV Env proteins; it is possible that testing a greater number of pseudotypes bearing Env proteins from more strains of FIV might have revealed a higher prevalence of Cr-NAbs. In studies of HIV, it has been suggested that neutralization breadth develops slowly over a period of 2–4 years post-seroconversion (Gray *et al.*, 2011; Mikell *et al.*, 2011). Given the duration of infection in our study group, it would be predicted, by analogy, that more cats would have developed Cr-NAbs, but this scenario was not supported by the data presented here.

Finally, differences in neutralization profiles might have been related to different kinetics of viral replication between the various strains of FIV infecting the cats. It is possible that more virulent, and more replication competent, viral strains are more likely to induce effective humoral responses compared with isolates of lower replicative capacity.

The results presented here demonstrate that humoral immunity was preserved in cats that subsequently developed AIDS, consistent with the observation that the rate of viral evolution slows during the terminal stage of disease (Bęczkowski, 2013). Thus, autologous antibodies elicited during the earlier stages of infection remain capable of neutralization, owing to the relatively high genetic stability of the virus terminally (Bęczkowski, 2013). However, such NAbs, although capable of neutralization *in vitro* and despite being preserved in terminal disease, failed to prevent disease progression.

Pseudotypes bearing 1–18 Env variants from each time point were used to assess sensitivity to neutralization by autologous plasma. It is difficult to assess how representative the cloned Env proteins were compared to the pool of Env variants within the cats. Nevertheless, the alternative approach of testing a single 'representative' clonal Env variant would have led to an underestimation of viral diversity within the host. The sensitivities to neutralization amongst pseudotypes bearing Env variants isolated from each cat tended to be similar; these data suggest that, where changes were observed, these were likely a true indication of the range of neutralization sensitivity and resistance amongst the pool of variants in individual cats.

Any assay system used to assess NAb responses *in vitro* will be limited in its representation of *in vivo* neutralization. Nevertheless, the indicator cell line transduced with CD134 that was selected for use in this study displayed a pattern of susceptibility to infection consistent with that of the MYA-1 cell line, an IL-2-dependent, $CD4^+$ feline T cell line expressing CD134 and CXCR4 that has been utilized previously in neutralization assays (Hosie *et al.*, 2011). This suggests that the pattern of CD134 and CXCR4 expression on the indicator cell line recapitulates the cell surface phenotype of the IL-2-dependent primary T cell line used formerly in neutralization assays.

We demonstrated that humoral immunity did not significantly alter the clinical course of natural FIV infection and, although this study was limited by the size of the cohort examined, and thus the statistical power obtained, it provides the basis for future studies. One explanation for this apparent lack of correlation is that any factor promoting a strong NAb response may negatively influence other immune responses, e.g. leading to the exhaustion of polyfunctional $CD4^+$ and $CD8^+$ T lymphocytes (Harari *et al.*, 2004; Betts *et al.*, 2006; Streeck *et al.*, 2008). In light of recent evidence from studies with HIV-1, it is plausible that cell-mediated immunity, as well as host genetic factors, are more likely to influence the clinical course of lentiviral infection than NAbs (Huang *et al.*, 2012; Nomura & Matano, 2012). It will be intriguing to evaluate the performance of FIV vaccination in the field by measuring the development of NAbs in vaccinated cats, in order to determine whether NAbs are protective in the face of natural challenge.

METHODS

Cats and plasma samples. Forty-four cats from Memphis, TN, USA (n=27) and Chicago, IL, USA (n=17) were enrolled in the study on the basis of a history of FIV infection, regardless of breed, sex, age and health status (Bęczkowski, 2013). Twenty-seven of the FIV-positive cats enrolled were housed together in a large multi-cat household in Memphis. The remaining 17 FIV-positive cats had been previously adopted from a large metropolitan adoption-guarantee shelter (PAWS Chicago) and lived in single-cat households in Chicago, except for seven cats: two cats (P7 and P4) cohabited in a two-cat household, one cat (P13) lived in a two-cat household with a FIV-negative cat, one cat (P9) was housed at PAWS Chicago for the

first 11 weeks of the study and then was adopted into a house with an FIV-positive cat not enrolled in the study, and three cats (P2, P15 and P21) were housed at PAWS Chicago in a room containing up to three FIV-positive cats before they were each adopted into single-cat households at 2, 14 and 58 weeks after enrolment, respectively. The FIV status of each cat was confirmed by virus isolation (Hosie *et al.*, 2009). All cats were feline leukemia virus antigen-negative at enrolment. Four blood samples (denoted A, B, C and D, related to each collection time point) were obtained from each cat at 6 month intervals over an 18 month period, unless the cat had died during the interim period. During the study, one of 17 (5.9 %) cats from the Chicago cohort and 17 of 27 (63 %) FIV-positive cats from the Memphis cohort died. Analysis of $CD4^+$ and $CD8^+$ T lymphocyte subsets (Table 1) and post-mortem findings (Bęczkowski, 2013) suggested that, in the majority of cases, FIV infection played a role in the observed morbidity and mortality. Detailed recording of signallement, clinical history, physical examination data and body weight, and flow cytometry analysis of $CD4^+$ and $CD8^+$ lymphocyte subsets were performed at the time of each sampling (Bęczkowski, 2013), and are summarized in Table S2.

The study and its aims were reviewed and approved by the University of Glasgow Ethics Committee and the Purdue Animal Care and Use Committee. Cat owners provided written informed consent for their participation in the study.

Amplification and cloning of WT FIV *env* genes. Full-length FIV *env* genes (~2500 bp) were amplified from whole-blood samples using a two-step nested PCR protocol. First-round PCRs were performed using Phusion Blood Direct II Polymerase (Thermo Fisher Scientific) followed by direct nucleic acid sequence determination. The nucleic acid sequence of the first-round PCR product informed primer design for the second-round PCR, which was performed using Roche High Fidelity Master (Roche); strain-specific primers for second-round PCR incorporated restriction sites to facilitate subcloning into the expression vector for pseudotyping (Table S3). In addition, reference *env* genes from clade A (GL-8) and clade B (B2542) were cloned into the eukaryotic expression vector VR1012 (Hartikka *et al.*, 1996) and transformed into *Escherichia coli* MAX Efficiency DH5α Competent Cells (Invitrogen). Next, VR1012 plasmids expressing FIV *env* genes were transiently co-transfected with the HIV pNL4-3-Luc-E^-R^-luc plasmid (an *env*-deleted HIV provirus containing the luciferase reporter gene) (Connor *et al.*, 1995) into HEK 293T cells (Graham *et al.*, 1977), using Superfect Transfection Reagent (Invitrogen). Following 72 h incubation in six-well culture clusters (Corning), culture fluids containing pseudoviruses were harvested, centrifuged at 1000 r.p.m. (~200 ***g***) for 5 min, passed through 0.45 μm filters and stored at −80 °C until required.

In this way, pseudotypes (n=278) were prepared, bearing naturally occurring (n=276) and reference (n=2) FIV Env proteins on an HIV backbone; the single-round, replication-competent pseudoviruses were used to assess the neutralization properties of test plasma samples. Pseudotypes were prepared bearing Env proteins from 38 cats (38/44, 86.4 %); it was not possible to produce viable pseudoviruses bearing Env proteins from five cats (5/44, 11.4 %; largely because of premature stop codons occurring in the *env* sequences) and plasma samples from one cat were not available for testing.

Neutralization assay. Plasma samples from 38 cats were tested for NAbs using HIV(FIV)luc pseudotypes. Plasma samples were heat inactivated at 56 °C for 30 min in order to inactivate complement and diluted 10-fold from a starting dilution of 1 : 10 in complete RPMI 1640 medium (Invitrogen). For 1 h at 37 °C, 25 μl of each plasma dilution (1 : 10, 1 : 100 and 1 : 1000) were incubated in triplicate with 25 μl HIV(FIV)luc pseudotype [luciferase activity on CLL-CD134 cells (Willett *et al.*, 2006) of $\sim 5 \times 10^7$ c.p.m.] before 5×10^4 CLL-CD134 cells were added in 50 μl and cultured in CulturPlate-96 assay plates (Perkin Elmer) for 72 h. Next, luciferase activity was quantified following the addition of 100 μl Steadylite HTS (Perkin Elmer) substrate and single-photon counting was conducted using a MicroBeta luminometer (Perkin Elmer).

The neutralization activity of the tested plasma samples is presented as 'fold neutralization'. Fold neutralization was calculated by dividing the mean luciferase counts of control wells containing no plasma (NP luc) by the mean luciferase counts for wells containing 1 : 10 plasma dilutions (P luc). Fold neutralization may be compared with the percentage neutralization calculated according to:

$$\text{Neutralization (\%)} = \frac{(\text{NP luc} - \text{P luc})}{\text{NP luc}} \times 100$$

Plasma samples were classified as not neutralizing, or weakly, moderately or strongly neutralizing according to the empirical cut-off values shown in Table S4.

Graphs and statistical analyses. Graphs and statistical analyses were performed in Prism version 5.00 (GraphPad Software). Descriptive data were shown as medians and interquartile range (fifth and 95th quartile). Binary data were analysed using Fisher's exact test. Kaplan–Meier curves were compared using the Mantel–Cox 'log-rank' test and tested with the log-rank test for trends. Significance was set at $P<0.05$. For clarity, values for fold neutralization at 1 : 10 plasma dilutions are shown in Table S1.

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