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The generation of domainspeccific monoclonal anti-CD4 antibodies as novel candidate HIV therapies

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2016-03-11
http://hdl.handle.net/10539/20069

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LIST OF CORRECTIONS

I would like to thank the examiners for taking time during their busy schedule to read my dissertation. I appreciate the constructive comments and corrections made, which undoubtedly served to the betterment of the dissertation. As requested, below are the corrections and alterations made to the MSc.

Examination report 1:

i. Pg 15, 1.1.1. Paragraph 1, line 6-7: "Encouragingly, we are witnessing the stabilisation of the prevalence of global infected population" was deleted in order to avoid the impression that prevalence does not increase due to less people dying of HIV/AIDS.

ii. Pg 15, 1.1.1. Paragraph 1, last line: "…the leading infectious cause of death" was altered to "…the leading cause of death by an infectious agent worldwide". WHO reference was added.

iii. Pg 15, 1.1.1. Paragraph 3, line 4-6: Added suggested reference "For example,, following introduction of ARVs in the public health system in KwaZulu-Natal (South Africa) in 2003, the adult life expectancy of the population cohort under observation (101000 individuals) was increased from 49.2 to 60.5 years by 2011 (Bor et al., 2013)"

iv. Pg 17, 1.1.2.1, line 7-8: Added suggested reference "In fact, population studies in Kenya have shown that regular malaria infections results in repeated increases in viral loads in HIV-infected patients, which is likely to contribute to the spread of HIV (Abu-Raddad et al., 2006)"

v. Pg 20, 1.1.2.4.: Expanded the description of viral latency to one paragraph, as follows:

"After integration of viral DNA into the host cell genome, HIV is capable of entering the so called 'latent'state , a state of viral dormancy during which active viral replication is interrupted (Siliciano and Greene, 2011) Following immune activation by antigen-presenting cells, a small number of activated CD4+ T-cells revert to the resting (Go) state, and persist as long-lived memory cells. HIV contained within memory cells in the form of stably integrated DNA escapes canonical humoral and cell-based immune surveillance mechanisms, and these provide an important

reservoir that sustains the emergence of viral quasi-species (Chun et al., 1997, Chun et al., 1995, Spina et al., 1997)".

vi. Pg 20,1.1.3.1: As asked by the examiner, suggested references were included in section 1.1.3.3.1, on pg 24, lines 15-19 as follows:

"For instance, by tracking the changes in the genome of HIV during early infection in patients, it was observed that low neutralising antibody titres allowed for selection and escape of HIV resistant strains (Bar et al., 2012). Similarly, neutralising antibodies produced by HIV-infected patients drove the emergence of resistant strains for as long as 10 years after of initial infection (Chaillon et al., 2012)."

vii. Pg 25, sections 1.1.3.3.2 and 1.1.3.3.3, as per examiner's recommendation were combined. Additional suggested references were added.

"Another important mechanism of immune evasion involves the inaccessibility of neutralising epitopes on Env (Johnson and Desrosiers, 2002). Several such epitopes remain buried at the core of the viral envelope and are only exposed transiently during the course of viral entry as a result of induced conformational changes. Whilst antibodies are produced in HIV-infected patients, these generally target the exposed variable regions, rather than the conserved, recessed regions on gp120, leading to very restricted strain-specific neutralisation (Burton et al., 2012a). Furthermore, the heavily glycosylated variable loops of gp120, often blamed for concealing the more conserved regions (Poignard et al., 2001, Johnson and Desrosiers, 2002), are therefore a reason for the inability to develop efficient bNAbs. It is estimated that 50 % of the gp120-gp41 molecular weight is attributed to the associated carbohydrates. In effect, the viral envelope is coated with a dense "glycan shield" that blocks antibody interaction with underlying epitopes (Wei et al., 2003). It is interesting to note that the CD4-binding site of gp120 lacks oligosaccharides (Kwong et al., 1998). It is however protected by the shielding variable loops, which in addition, are glycosylated. Artificially altering the positions or removal of the oligosaccharides of the V1 or V3 loop dramatically increased the sensitivity of the virus to antibody neutralisation (Back et al., 1994, Overbaugh and Rudensey, 1992, Koch et al., 2003). Certain antibodies such as 2G12, however, can actually recognise and bind to the carbohydrates on gp120 (Calarese et al., 2003). More recently, the PGT group of antibodies could bind directly to the glycans and many within this group are capable of potent, cross-clade neutralisation (Pejchal et al., 2011). Nevertheless, it has been shown that the glycan layer is not static, but changes throughout the course of viral infection (Overbaugh and Rudensey, 1992, Moore et al., 2012, Wei et al., 2003). To illustrate this, a study demonstrated that within 6 months of infection, a shift in the position of a critical glycan molecule on gp120 resulted in HIV resistance to PGT128 (Moore et al., 2012)."

viii. For maintaining the flow of the text, the last lines of 1.1.3.3.2 of pg 24, was deleted:
"Studies involving X5, a CD4-induced (CD4i) binding monoclonal antibody showed that only the Fab fragments as opposed to the entire antibody were capable of inhibiting viral infection (Labrijn et al., 2003). It is therefore suggested that when gp120 is engaged with CD4, the gap between the viral and host cell membranes is insufficient to enable the binding of a whole CD4i antibody to its epitope."

ix. Pg 25, 1.1.3.3.4., is now 1.1.3.3.3 on pg 26

x. Pg 26, 1.1.3.3.5., is now 1.1.3.3.4 on pg 26-27

xi. Pg 26-27, 1.1.3.3.4 was expanded and suggested references were included, as follows:

"With increasing usage of high-throughput antibody screening and sequencing approaches, a number of novel bNAbs have been identified in HIV-infected individuals (Walker et al., 2009, Zhu et al., 2013, Johnson et al., 2009). However, these mature bNAbs only come to existence after extensive somatic hypermutation in some of the chronically infected HIV individuals (Haynes et al., 2012, Verkoczy et al., 2011). Understanding the development of the antibodies and recognising the immunogen precursor that elicited such response would be of immense help in rationale vaccine design (Simek et al., 2009, Sather et al., 2009, Wu et al., 2011, Doria-Rose et al., 2010). However, directing guided evolution of the antibody remains a complex task due to the number of variables that affect somatic hyper-mutation evolution (Gray et al., 2009, Piantadosi et al., 2009, Sather et al., 2009). For instance, following an analysis of over a hundred patient sera against several clinical features, it was shown that neutralisation breadth correlated only with viraemia, and 20% of these sera could be qualified as bNABs (Doria-Rose et al., 2010). Elsewhere, it is indicated that additionally, the generation of potent bNABs is influenced by length of time since the patient was infected and the binding avidity of the early antibodies against viral envelope (Sather et al., 2009). Moreover, it has also been suggested the development of bNABs has to be triggered during early infection (Piantadosi et al., 2009). Taken together, a number of studies suggest that the generation of bNABs in a patient begins from an early unmutated germline antibody with strong affinity to CD4-binding site of viral envelope that goes through a lengthy and complex maturation process, whilst being continuously exposed to heterogeneous viral antigens (Zhou et al., 2010, Xiao et al., 2009, Wu et al., 2010, Wu et al., 2011, Kwong and Mascola, 2012, Liao et al., 2013). Recently, the evolution and structure determination of mature CD4-binding site CH103 antibody, which could neutralise about 55% of HIV-1 strains, provides critical information on the pathway for maturation and the structural characteristics that bNABs require (Liao et al., 2013).In the future, with more studies, it might be possible to indentify the exact requirements for the development of potent bNABs against HIV (Burton et al., 2012b)"

xii. Pg 31, 2.1.2: References, which described presence of CD4 on various cells, were added. An extra paragraph indicating the role of CD4 on non-T-cells was added as follows:

"Although canonically located on CD4+ T-cells (Maddon et al., 1985), the CD4 receptor is also present on dendritic cells (Patterson et al., 1995), macrophages (Lee et al., 1999) and natural killer cells (Bernstein et al., 2006). The role of CD4 on non-T-cells remains largely unknown. However, recent evidence indicates that CD4 on non-T-cells is involved in roles other than formation of the 'immunological synapse'. . For instance, following ligation of CD4 on activated natural killer cells, CD4 has been shown to mediate the migration of the cells towards CD4-specific chemotactic factor IL-16, and induce cytokine expression (Bernstein et al., 2006)."

xiii. Pg 31, 2.1.2: Since reference for CD4 on T-reg was deemed a bit arcane, and to enhance flow of text, line 2 –3, "Interestingly, CD4 receptors are also located on CD4+CD25+ regulatory T-cells (T-regs) (Beissert et al., 2006)", was deleted.

xiv. Pg 31, 2.1.2: Reference for review on the role of CD4 T-cell activation was added. The following last sentence was added to the paragraph:
"For an extensive description on the role of CD4 T-cell in antigen recognition, the review by Merwe and Davis should be consulted (van der Merwe and Davis, 2003)."

xv. Pg 39 - 40, 2.1.6.3: For better clarity, the section was re-written as follows:

"Reduced, monomeric forms of CD4 have been shown to be the preferred state of the receptor for HIV entry (Matthias et al., 2010). The predominant explanation for this observation is that CD4 occurs in a conformation that enhances viral entry. Although, the detailed intricacies of these conformational changes remain elusive, there is strong evidence that they arise from redox alterations in the disulphide bond of domain 2 of CD4 (Matthias et al., 2002, Cerutti et al., 2014).The importance of the state of the disulphide bond of CD4 for gp120 binding was most recently demonstrated in our laboratory using recombinant two-domain CD4 fragment 2 (Cerutti et al., 2014)."

xvi. Pg - 48, 2.2.7.4: As requested by the examiner, the protocol for the protein refolding method was referenced. In addition, a brief description of protein refolding, along with references for extensive reviews, was include as follows:

"The 2dCD4 re-folding protocol, previously developed in our laboratory to facilitate formation of canonical disulphide bonds in proteins expressed in E coli, involves slow dialysis in glutathione-containing buffersand (Cerutti et al., 2014, Cerutti et al., 2010). Briefly, the insoluble proteins were extracted from inclusion bodies by solubilisation with strongly denaturing (8M urea) buffer. The extensive re-folding protocol allows the gradual removal of solubilisation buffer and its replacement with PBS at physiological pH of 7.4. The slow removal of denaturant, at cold temperature, is important to promote the renaturation and correct refolding of the protein into its native conformation, and to limit precipitation. Sucrose and glycine act as stabilisers,

and enhance the refolding process, whilst also limiting precipitation (Tsumoto et al., 2003). Additionally, with the inclusion of glutathione and glutathione disulphide in the refolding buffer, an appropriate redox system is setup to encourage correct formation of disulphide bonds (Singh and Panda, 2005). The following references should be consulted for extensive reviews on protein refolding (Sorensen et al., 2003, Middelberg, 2002, Tsumoto et al., 2003, Singh and Panda, 2005)."

xvii. Pg 51; 2.2.7.6, line 2: "binding affinity" was altered to "binding ability"

xviii. Pg 52; 2.3.1.1, last lines: Reference was added to "Additionally, the substitution of isoleucine to proline at position 76 could also enhance the stability of D1 by firmly "kinking" the backbone of the polypeptide against the overall domain (MacArthur and Thornton, 1991)".

xix. Pg 52; 2.3.1.1; with reference to examiner's comment of section 2.3.2.3, a table references of previous studies which involved the expression of individual domains of D1 and D2 has been provided in the appendix C.f. Accordingly, the following sentence was added "References from previous authors who have successfully expressed of D1 and D2, and how were they expressed, is provided in Appendix C.f." at the end of the 1st paragraph of 2.3.1.1 on pg 52

xx. With reference to examiner's comment of section 2.3.2.3, an extra paragraph was in 2.4 on pg 68.

"Previous studies have described the use of the *E. coli* expression system for the expression and purification of mutant D1 as insoluble inclusion bodies (Sharma et al., 2005, Saha et al., 2011). Although the use of commercially available recombinant *E.coli* expressed was mentioned (Matthias et al. 2010), the eleborate details for its expression and purification were not described. Whilst the use of mammalian cell culture for recombinant protein expression provides the ideal microenvironment for protein folding and includes post-translation modification, it remains a costly and tedious task. Our laboratory demonstrated that mutant D1(m1.1) and D2 could be produced in high yields from inclusion bodies, and subsequently purified following solubilisation and extensive refolding process. Aside from the disulphide bonds, domain 1 of CD4 does not include any other post-translation modification (glycosylation, lipidation…). With the established in-house GSH-GSSG containing refolding protocol, and as indicated by SDS-PAGE gels, correct disulphide pairing could be confirmed in D1(m1.1) and D2. Furthermore, with the CD- and binding studies that were conducted in this study, the maintenance structural and functional integrity of the recombinant protein is suggested.

xxi. Pg 54, 2.3.4.1, line 4: References describing mt310 as a D1-specific antibody were added.

xxii. Pg 61, 2.3.4.1, line 4-6: the statement was changed from "Later in Chapter 2, we however demonstrate that D2 maintains functional properties in that it is recognised

by supernatants from hybridoma cell lines generated following immunization of mice with a native 2dCD4" to "Later in Chapter 2, we however provide evidence that D2 - recognisable by supernatants from hybridoma cell lines generated following immunization of mice with a native 2dCD4 – has native structure".

xxiii. Pg 61, 2.3.4.1, line 4: With regards to above, "…functionality of D2…" was altered to "…structural intergrity of D2…"

xxiv. Pg 63-64, 2.3.4.3: "Gp120 binding affinity" was changed to "Gp120 binding ability of CD4 variants"

xxv. Pg 64, 2.3.4.3, Figure 14: Graph title was changed from "Gp120 binding affinity of CD4 variants" was changed to "Gp120 binding to CD4 variants"

xxvi. Pg 64, 2.3.4.3, Figure 14, Legend: "Gp120 binding affinity to CD4 variants" was changed to "Gp120 binding to CD4 variants"

xxvii. Pg 69, 2.4, last paragraph: The paragraph was re-written with appropriate reference. The term "isoform" was replaced with "conformation"

"The next phase of this study involved the generation of a panel of novel anti-CD4 monoclonal antibodies as novel candidate anti-HIV therapies. We also attempted to verify whether such antibodies bound preferably to specific CD4 conformations. Recently, an antibody directed against HER3 (Human Epidermal Growth factor Receptor 3), a critical receptor that when overexpressed leads to oncogenesis, could lock HER3 into an inactive conformation (Garner et al., 2013). In the same analogy, we sought to verify whether certain anti-CD4 antibodies may mediate inhibitory effects by antagonizing specific conformations of CD4 that are required for viral entry."

xxviii. Pg 75, 3.1.2.3, line 2: the term "immunological synapse" was replaced with "interaction"

xxix. Pg 75, 3.1.2.3, figure 17, Legend: "…the cornerstone of the immunological synapse…" was deleted.

xxx. Pg 78, 3.1.3.1, line 3: The strong statement was changed to a suggestion, which is more appropriate based on given evidence. The term "signifies" was changed to "could suggest"

xxxi. Pg 85, 3.2.1, line 5-6: "These 40 antibody-containing supernatants samples were selected by the manufacturer based on thethe strengths of their reactivities with 2dCD4, a number defined per contract prior to immunzation " was added in order to specify that supernatants contained antibodies against 2dCD4, and how supernatants were selected.

xxxii. Pg 86, 3.2.2.1, line 4: term “dilute” was deleted.

xxxiii. Pg 91, 3.3.1.2, line 12-13: For clarity, “Differences in binding abilities of CD4-Mabs 21, 22, 25, and 26 were confirmed as artefacts after further analysis” was deleted.

xxxiv. Pg 92, 3.3.1.2, figure 21, legend: Accordingly, “Supernatants 21, 22, 25 and 26, showing differences in their binding affinity towards 2dCD4-D2A were later confirmed as an artifact” was deleted

xxxv. Pg 93, 3.3.1.2, figure 23, was renamed figure 22.

xxxvi. Pg 95, 3.3.1.4, figure 25, was renamed figure 24.

xxxvii. Pg 95, 3.3.1.4: It could be argued that all the D2-specific antibodies showed significant (if not absolute) reductions in their abilities to bind a 2dCD4 in which the disulfides were ablated (Figure 21), which surely supports the suggestion that the D2 protein has at least some of the secondary (perhaps tertiary) structural elements formed. Hence we qualified this notion by stating that the ‘D2 folds into a structure that reconstitutes **at least** some of the native Ig-fold-like CD4 secondary structure’

xxxviii. Pg 97, 3.3.2.1: Calculation of TCID was added in Appendix F on pg 149.

xxxix. Pg 105, 3.4, paragraph 3, line 2: “Furthermore, all of these were able to inhibit gp120 binding and viral infection” was changed to “Furthermore, ight of these were able to inhibit viral infection as demonstrated from the pseudoviral assay”

xl. With regards to the examiner’s comment relating to “CD4 binding site is formed differently in gp120 as compared to gp140 or other trimer mimics”, a paragraph covering the suggestion was added in the results section 3.3.2.3 on pg 101.

“Effectively, all 40 supernantants could inhibit gp120 binding to CD4 in the ELISA. However only 12 of the CD4 supernatants showed significant ability to prevent viral infection and viral infection to varying degrees. A plausible explanation for this observation could be due the unknown, but certainly varying concentration of antibodies present in the supernatants. Supernatants having lower MAb concentration, whilst being able to inhibit gp120 binding to CD4 in the ELISA, could be insufficient to saturate CD4 molecules present on TZMBls in order cause observable reduction in the infection during the viral assay. Another explanation could be due to the occurrence of gp120-gp41 (gp140) trimers in the viral assay (Ozel et al., 1988), instead of recombinant monomeric gp120 which was present in the ELISA. As compared to monomeric gp120, native trimeric gp140 could bind to CD4 in such a way that it is not sterically hindered by the anti-CD4 – thus allowing viral entry and

infection. It is also possible that mammalian expressed CD4 is functionally different to that CD4 expressed from bacteria when it concerns viral binding and entry. For instance, mammalian CD4 has glycosylated residues in domains 3 and 4 (Spellman et al., 1991), and these could reduce binding affinity of MAbs to the CD4 molecule by imposing steric constrains. Interestingly, CD4MAb-20 enhanced viral infection and therefore, it would be interesting to further characterise the antibody responsible for such observation."

Examination report 2:

i. Pg 3, Key words: "Pseudoviral assay" was changed to "Pseudovirion Assay"
ii. Pg 16, 1st paragraph, lines 8 and 11: "viremia" was changed to "viraemia"
iii. Pg 17, line 3: "Karposi Sarcoma" was altered to "Karposi's Sarcoma"
iv. Pg 25, 1.1.3.3.4, line 5; now on pg 26, 1.1.3.3.3, line 5: "suggest" was altered to "suggests"
v. Pg 26, 1.1.4, line 9; now on pg 27, 1.1.4, line 9: "…generated of a panel…" was altered to "generated a panel of…"
vi. Pg 31, line 2; now on pg 33, line 2: "Figure 1" was altered to "Figure 2"
vii. Pg 31, Figure 2: Reference for figure 2 was added.
viii. Pg 45, 2.2.7.1, line 2; now on pg 47, 2.2.7.1, line 2: "E.coli" was altered to "*E.coli*"
ix. Pg 78, line 6; now pg 80, line 6: "…properties the murine…" was altered to "…properties to the murine…"
x. Pg 80, Legend, Fig. 19; now on pg 82: "…From Freeman et al.,2010" was altered to "from Freeman et al., 2010)
xi. Pg 89, Legend, Fig. 20; now on pg 91: "…570 readout" was altered to "570nm readout"
xii. Pg 101, Legend, Fig. 28; now on pg 103: "T-test" was altered to "t-test"
xiii. Pg 102, 1st sentence; now on pg 104, 1st sentence: "…generation a panel…" was altered to "…generation of a panel…"
xiv. Pg 102, 2nd paragraph, line 3; now on pg 104, 2nd paragraph, line 3: "E.coli" was altered to "*E.coli*"
xv. Pg 102, 2nd paragraph, line 5; now on pg 104, 2nd paragraph, line 5: "…and presence of heavily…" was altered to "…and presence of a heavily…"
xvi. Pg 106, 2nd paragraph, line 2; now on pg 108, 2nd paragraph, line 2: "…structurally and functionally intact" was altered to "were structurally and functionally intact"
xvii. Pg 106, 2nd paragraph, line 9; now on pg 108, 2nd paragraph, line 9: "potencies the MAbs" was altered to "potencies of the MAbs"
xviii. Pg 106, 2nd paragraph, line 11; now on pg 108, 2nd paragraph, line 11: "began" was changed to "begun"

xix. Restriction analysis gel for section 2.2.6 was added in Appendix C, f. on pg 142. Accordingly, added: “(NB: For restriction analysis figure, please see appendix C.f.)” at the end of section 2.2.6 on pg 46)