genome is unknown, a homology search showed that a *Mafa-MICH3* gene (AY032639) was homologous to *Mafa-ULBP4/RAET1E* because the nucleotide sequences of *Mafa-ULBP4*1.1* showed a 96% homology to *Mafa-MICH3*. Similarly, nucleotide sequences of a chimpanzee gene, *Patr-MICH3* (AY032638), showed a 94% homology to the rhesus *ULBP4/RAET1E*. These findings strongly suggest that *MICH3* in the crab-eating macaque and chimpanzee is orthologous to *ULBP4/RAET1E* in the human and rhesus macaque.

In conclusion, we revealed a large diversity of *ULBP4/RAET1E* in two related species of the Old World monkey. Because there were extremely large polymorphisms in the extracellular domain of the ULBP4/RAET1E molecule in the Old World monkey, which was larger than that in the human, the functional impact of the polymorphisms and its significance in the evolution of primates should be investigated in future studies.

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Review Article

Mycobacterium bovis Bacille Calmette-Guérin as a Vaccine Vector for Global Infectious Disease Control

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Mycobacterium bovis bacille Calmette-Guérin (BCG) is the only available vaccine for tuberculosis (TB). Although this vaccine is effective in controlling infantile TB, BCG-induced protective effects against pulmonary diseases in adults have not been clearly demonstrated. Recombinant BCG (rBCG) technology has been extensively applied to obtain more potent immunogenicity of this vaccine, and several candidate TB vaccines have currently reached human clinical trials. On the other hand, recent progress in the improvement of the BCG vector, such as the codon optimization strategy and combination with viral vector boost, allows us to utilize this bacterium in HIV vaccine development. In this paper, we review recent progress in rBCG-based vaccine studies that may have implications in the development of novel vaccines for controlling global infectious diseases in the near future.

1. Introduction

Mycobacterium bovis bacille Calmette-Guérin (BCG) is the only licensed vaccine that has substantially helped controlling tuberculosis (TB) for more than 80 years. This vaccine affords ~80% protection against TB meningitis and miliary TB in infants and young children [1]. However, the BCG-induced protective effects against pulmonary diseases over all ages are variable; the escalation of the worldwide TB epidemic is evidence that the vaccine does not work well to prevent pulmonary TB [2]. Recently, studies on the advanced molecular biology and genomics of mycobacteria have revealed that the BCG genome has various mutations and deletions compared with the original virulent strain of *Mycobacterium tuberculosis* and *M. bovis* [3]. Interestingly, there are substantial differences in the genomic DNA even among BCG substrains [4, 5] that can cause biological differences in the population of BCG vaccines.

Since a host-vector system in mycobacteria was developed in 1987 [6], recombinant BCG (rBCG) technology has been extensively applied in the development of vaccines against a variety of infectious diseases, including bacterial, viral, and parasitic infections in addition to TB [7, 8]. BCG is attractive as a vaccine vector because of its extensive safety record in humans, heat stability, low production cost, induction of long-lasting type 1 helper T cell (Th1) immunity, $CD8^+$ T-cell triggering, adjuvant activity, usability in newborns and its mucosal immune induction by oral administration. Taking the current situation of serious epidemics of emerging and reemerging diseases mainly in developing African and Asian countries into account, a new global vaccine should be affordable in such areas. Therefore, the low price and heat stability of BCG-based vaccines would be desirable. In this paper, we review various efforts to develop novel BCG vector-based vaccines mainly for controlling TB and HIV/AIDS.

2. Immunological Properties of BCG Vector

The immune responses induced by BCG are outlined in Figure 1. The most characteristic response to BCG is the induction of innate (nonspecific) immunity by cell wall components through toll-like receptors (TLRs) 2 and 4 on dendritic cells and macrophages [9]. After phagocytosis,

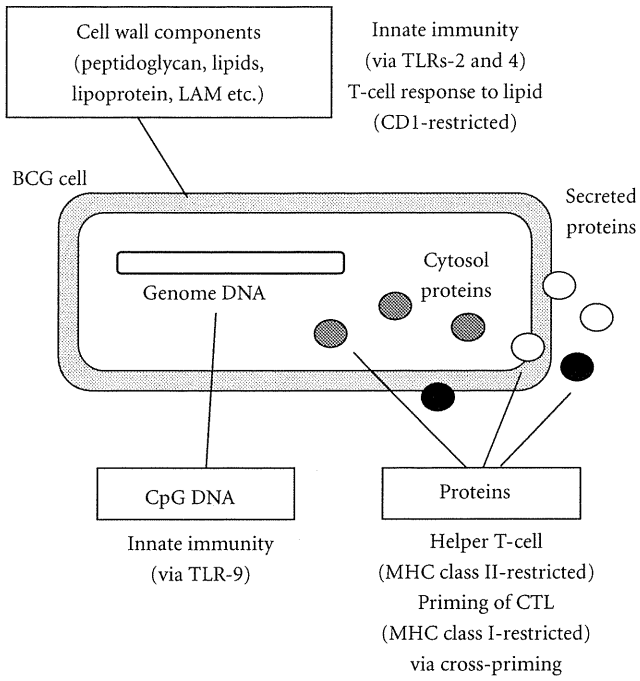

FIGURE 1: Outline of immune responses by BCG. Both innate immunity via TLRs and antigen-specific immunity via MHC- or CD1-restricted antigen presentation to T cells are induced by various BCG cell components.

BCG is degraded by lysosomal enzymes, and the processed antigen can be presented to the host immune system via various pathways. DNA fragments containing the CpG motif may activate innate immunity via the TLR9 route [10]. Lipids such as mycolic acid presented by CD1 stimulate CD1-restricted $CD8^+$ T cells [11]. Protein antigens, such as antigen 85 complex produced by BCG, induce Th1 response through presentation by major histocompatibility complex (MHC) class II. This pathway is the major route of BCG-induced responses and is indispensable for protective immunity against *M. tuberculosis* infection via protective cytokine interferon (IFN)-γ production. On the other hand, the processing and presentation of protein antigens via the MHC class I pathway are also elicited in the BCG-infected antigen presenting cell (APC). As reported by Goonetilleke et al. [12], immunizing BCG-sensitized animals with recombinant vaccinia virus MVA expressing antigen 85A greatly enhances the MHC class I-restricted CTL response against antigen 85A, indicating that BCG priming could be a novel type of prime-boost vaccine. This immunological feature of BCG vector allows its application in vaccines against chronic viral infectious diseases such as HIV/AIDS. In addition, the strong Th1 induction by BCG would be favorable to aid the maturation and maintenance of CTL [13]. Thus, the BCG vector is expected to induce effective cell-mediated immunity against a targeted antigen.

3. TB Vaccine

3.1. Background of the Global TB Epidemic. TB kills 1.7 million people worldwide each year; someone dies from TB every 19 seconds [14]. Although the TB treatment protocol was established a long time ago, the recent increase of multidrug-resistant *M. tuberculosis* infection has generated a serious situation. New vaccines are urgently needed to eliminate TB as a public health threat and should be a major global public health priority. TB is a disease that is spread from person to person through the air. Furthermore, the terrible synergy between TB and HIV makes this disease even more dangerous, especially in sub-Saharan African countries. For instance, according to the World Health Organization's (WHO) Global TB report 2010 [14], South Africa had nearly 400,000 new TB cases in 2009 with an incidence rate of an estimated 806 cases per 100,000; TB is one of the leading causes of death in both adults and children of this country. The case fatality rate has increased from 3% in 1993 to 24.3% in 2007. A major reason for the increased fatality rate is South Africa's concurrent HIV epidemic. The prevalence of HIV infection in South Africa in 2009 was approximately 7%, which has been decreasing as a result of various efforts toward prevention. TB is a common opportunistic infection among people living with HIV, and 60% of new TB cases occurred in persons who were also infected with HIV in 2009 [14]. We can observe similar critical situations in the countries surrounding South Africa. Regarding the vaccination, such situation has raised concerns about the safety of using BCG vaccine in HIV-infected infants because between 10 and 30% of pregnant women are HIV infected in many sub-Saharan African countries.

3.2. Current Efforts toward New TB Vaccine Development. The global plan to stop TB 2011–2015 report [15] offers 7 objectives as follows: (i) to maintain a robust TB vaccine pipeline by supporting research and discovery, (ii) to conduct research to identify correlates of protection and preclinical studies to assess new TB vaccine candidates, (iii) to ensure the availability of vaccine production capacity by expanding manufacturing facilities for TB vaccines, (iv) to build capacity for large-scale clinical trials (phases II and III) of TB vaccine candidates at field sites in TB-endemic countries, (v) to conduct phase I, II, and III clinical trials of TB vaccine candidates, (vi) to develop delivery, regulatory, and access strategies for new TB vaccines, (vii) to build support for TB vaccine development and uptake through advocacy, communications, and resource mobilization. All these objectives are important to realize new TB vaccine development.

The main goal of vaccine development in the Global Plan to Stop TB 2006–2015 is for 2 vaccines to be in proof-of-concept trials by 2010 and that 1 new and safe vaccine is available by 2015. As of 2009, 12 TB vaccine candidates had entered clinical trials. Of these, 9 are still being tested (Table 1) : 5 are in phase I clinical trials, 2 are in phase II trials, and 2 are in phase IIb proof-of-concept trials [15]. One vaccine has produced estimates of safety and effectiveness in a targeted HIV-infected population. At least 6 TB vaccine candidates are in preclinical development, and at least 21 additional next-generation candidates are in the vaccine discovery phase [15]. As mentioned earlier, the current BCG vaccine has limited and variable effectiveness against TB.

Table 1: Summary of candidate TB vaccines in clinical trials 2009. Nine candidate preventive TB vaccines are currently in clinical phases.

Status	Products	Product description	Sponsor
Phase IIb	MVA85A/AERAS-485	Vaccinia virus MVA	OETC/AERAS
Phase IIb	AERAS-402/Crucell Ad35	rBCG/adenovirus 35	Crucell/AERAS
Phase II	Hybrid-I + IC31	Ag85B/ESAT6 + adjuvant	SSI/TBVI
Phase II	M72	Fusion protein + adjuvant	GSK/AERAS
Phase I	AdAg85A	adenovirus 5/Ag85A	McMaster Univ.
Phase I	VPM 1002	rBCG/listeriolysin::ΔureC	Max Planck/TBVI
Phase I	Hyvac 4/AERAS-404	Fusion protein + adjuvant	SSI/Sanofi/AERAS
Phase I	RUTI	Fragmented Mtb cell	Archivel Farma
Phase I	Hybrid-I + CAF01	Ag85B/ESAT6 + adjuvant	SSI

Abbreviations in the sponsors: AERAS, AERAS Global TB Vaccine Foundation; GSK, GlaxoSmithKline; OETC, The Oxford-Emergent Tuberculosis Consortium Ltd.; SSI, Staten Serum Institute; TBVI, Tuberculosis Vaccine Initiative.

Therefore, the first choice of strategy may be improving BCG by using recombinant DNA technology even though it may imply safety issue of vaccination in HIV-infected individuals. Overproduction against a protective antigen of TB in BCG (rBCG30) exhibited enhanced immunogenicity in humans [16]. Moreover, the expression of the listeriolysin gene in BCG (rBCG/*hly*$^+$::Δ*ureC*) is proven to be more potent in the induction of TB-specific cellular immune responses [17]. Another strategy for improving BCG vaccines is boosting BCG immunity with protein [18, 19] or viral vector vaccine such as modified vaccinia virus Ankara (MVA) strain [20] and adenovirus type 35 [21]. BCG-prime and recombinant MVA-antigen 85A boost regimen [22] exhibited efficient immune responses in humans and have entered the first phase IIb trial in newborns. Furthermore, a combination of such strategies in which 3 major antigens are overproduced and the perfringolysin gene is incorporated into BCG and boosted with a recombinant adenovirus vaccine has been developed [23]. However, it is unknown whether such strategies are relevant for developing vaccines that are effective against adult pulmonary TB. It is necessary to test whether these candidate vaccines effectively induce mucosal immunity and protect against lung disease.

4. HIV/AIDS Vaccine

4.1. Background of the Global HIV Epidemic. In 2009, there were an estimated 2.6 million people who became newly infected with HIV. This is more than 21% less than the estimated 3.2 million who became infected in 1997, the year in which annual new infections peaked. In 33 countries, the incidence of HIV has decreased by more than 25% between 2001 and 2009; 22 of these countries are in sub-Saharan Africa. This trend reflects a combination of factors including the impact of HIV prevention efforts and the natural course of HIV epidemics [24].

Although highly activated antiretroviral therapy apparently contributes to control HIV replication in infected individuals [25], several problems remain to be resolved. These problems include: (i) the following viral load recovers soon after the interruption of treatment; (ii) chronic toxicities cause abnormalities in lipid metabolism and mitochondria; (iii) drug-resistant viruses increase during long period of treatment; (iv) long-term treatment carries a risk of carcinogenesis [26]; (v) expensive drugs are still difficult to access in developing countries. Even in developed countries, the high cost of antiretroviral drugs produces a sense of impending crisis in public health policy [27]. In such circumstances, although the rate of new infections with HIV-1 is gradually decreasing, an effective preventive vaccine is still urgently needed to stem further spread of the virus [28]. Even though considerable recent progress has been made in the development of an HIV vaccine [29, 30], the immune correlate of viral protection is not fully elucidated due to the complicated interaction of viral, immunological, and genetic factors [31, 32]. Since it is known that some populations of HIV-1-infected people do not present disease progression when HIV-1 replication is regulated by host immunity [33, 34], targeted vaccine immunogens are designed to closely mimic the long-lasting protective immunity induced in the long-term human survivors of natural infection [35, 36]. Due to safety issues, a live-attenuated HIV vaccine is not practical. This inevitably led the trend of HIV vaccine development to component- and vector-based vaccines.

4.2. Current Trends in HIV/AIDS Vaccine Research. The first large-scale efficacy trial of an HIV/AIDS vaccine was conducted by a US company, Vaxgen Co., in which a genetically engineered surface envelope (Env) glycoprotein, gp120, vaccine was tested in humans. Although the vaccine was targeted toward inducing effective virus-neutralizing antibodies, the phase III efficacy trial revealed its ineffectiveness [37, 38]. The failure of the gp120 vaccine changed the trend of HIV/AIDS vaccine research from an antibody-targeted strategy to a cell-mediated immunity-targeted strategy. Because HIV-1 causes chronic infection due to its cell-associated features, cellular immunity especially virus-specific cytotoxic T lymphocyte (CTL) should be a more important arm of the host immune system. Indeed, immune deficiency virus-specific cell-mediated immunity has been suggested to effectively control viral replication during the natural course of viral infections [39–41]. Based on these findings, various vaccine modalities, including live viral vectors and DNA vaccines, have been used to elicit strong CTL and Th1 type

responses in nonhuman primate models. Although single-vaccine delivery systems sometimes exhibit insufficient immune responses, boosting with viral vector vaccines such as vaccinia virus [40, 41], adenovirus [42, 43], and Sendai virus [44] in DNA-primed individuals strongly amplified CTL responses and resulted in the effective control of simian immunodeficiency virus (SIV) replication. Among such viral vectors, adenovirus type 5 (Ad5) had the strongest CTL enhancement effect, and the DNA-prime and recombinant Ad5 boost vaccine strategy is recognized as the most promising. However, in 2007, Merck Co. reported that a recombinant Ad5 vaccine expressing HIV-1 Gag, Pol, and Nef antigens did not demonstrate any protective efficacy in a phase IIB clinical trial [45]. Surprisingly, the vaccinated group exhibited a significantly higher HIV-1 infection rate than the placebo group [45], suggesting that the recombinant Ad5 immunization may have some unknown effect in enhancing HIV-1 infection. Thus, we were aware that T-cell vaccine approaches may involve certain risks and limitations; this paradigm appears to have reached an impasse.

In September 2009, there was ground-breaking news that the RV144 large-scale efficacy trial in Thailand demonstrated a partial effect of reducing HIV-1 infection rate in the recipients of ALVAC (canarypox)/gp120 prime-boost vaccine [46]. Although the results demonstrated limited effects, they demonstrated the possibility of preventing HIV infection with the active immunization for the first time. Furthermore, although there was no apparent correlation between protection and virus-specific cellular immune response or neutralizing antibody levels in the vaccinees, more detailed analyses of the total host responses are expected in the future. Taking the vaccine formulation with the gp120 protein boost into account, some antibody-mediated reactions may be involved in this partial protection. On the other hand, a new T-cell-targeted vaccine also demonstrated protective efficacy in a macaque study in the same year. A rhesus cytomegalovirus-vectored vaccine expressing SIV Gag, Rev-Tat-Nef, and Env persistently infected rhesus macaques, primed, and maintained robust SIV-specific $CD4^+$ and $CD8^+$ effector memory T-cell responses in the absence of neutralizing antibodies [47]. The report suggests that T cell vaccines may have greater potential than previously estimated. Although the importance of broadly neutralizing antibody production would not change despite tremendous difficulties, cellular immunity-targeted candidate vaccines should be also clinically tested for proofs of concept.

4.3. BCG-Vectored HIV Vaccine. The most practical advantage of the BCG vector is its high safety. In addition to being effective at inducing protective immunity, an HIV-1 vaccine regimen must be shown to be safe, affordable, and compatible with other vaccines before it can be considered promising [39]. In this respect, vectors that have already been used in humans without serious complications and with low cost should be utilized for HIV vaccines. BCG is a unique live vaccine vector because of its easy antigen delivery to the professional APC to be presented to T cells. Therefore, this bacterium is expected to be an important vector for HIV vaccine development.

At the early stage of rBCG research in the 1990s, Aldovini and Young [48] demonstrated immunogenicity of rBCG against genetically engineered HIV-1 antigens in mice. We independently worked on an rBCG-vectored anti-HIV vaccine simultaneously. First, we demonstrated effective cellular immune induction against SIV Gag antigen by the rBCG vector in rhesus macaques [49, 50]. Furthermore, we cloned an extracellular α antigen (antigen 85B) gene from both BCG [51] and *Mycobacterium kansasii* [52], and established a foreign antigen secretion system in mycobacteria [53]. Based on this system, we extensively evaluated several rBCG constructs for candidate HIV vaccines and reported that an rBCG-HIV vaccine could induce protective humoral immune responses in guinea pigs [54]. These studies suggest that rBCG-based vaccines are feasible as AIDS vaccines. However, the CTL activity did not reach protective levels with a single injection of rBCG-HIV vaccine in the macaque model. To overcome the low immunogenicity of the rBCG vaccine in CTL induction, we utilized various strategies for enhancing the immune potential of the BCG vector.

4.4. Prime-Boost Regimen for Enhancing Immune Responses. The first strategy by which we tried to improve the potential of the rBCG-HIV vaccine was the use of a safe recombinant viral vector for a booster vaccine. With respect to safety, traditional live vaccines, which have been administered safely to both the healthy and the HIV-infected individuals, may be the vectors of choice for HIV-1 vaccines. To fully take advantage of the benefits of such traditional vaccines in the development of anti-HIV vaccines, we studied BCG Tokyo 172 strain and the replication-deficient vaccinia vaccine strain DIs [55, 56] both of which have been shown to be nonpathogenic when inoculated into immune-deficient animals as live recombinant vaccine vehicles [57]. The vaccinia virus DIs have been tested clinically as a smallpox vaccine in Japanese infants and proved to be quite safe. We chose this highly attenuated virus as a booster vaccine vector and constructed recombinant DIs (rDIs) expressing the HIV *gag* [58] or SIV *gag-pol* gene [59]. Both rDIs constructs were found to be effective in eliciting HIV- or SIV-Gag-specific immunity in mice. When they were administered as a booster antigen after priming with an SIV-DNA vaccine, the cellular immunity to SIV Gag was greatly enhanced [59]. In brief, we tested a new combination regimen: priming with rBCG-SIV Gag followed by boosting with rDIs-SIV Gag.

In the macaque study, we found that BCG/DIs vaccination induced a long-lasting and effective cellular immunity that was able to control a highly pathogenic virus SHIV C2/1 [60], after mucosal challenge [61]. A possible mechanism of effective Gag-specific cell-mediated immunity is shown in Figure 2. The strong Th1 response induced by the BCG vector may contribute to eliciting the Gag-specific CTL response. How these immune inductions are correlated with protective efficacy requires further investigation. In this study, the BCG/DIs vaccination developed high levels of cellular immunity in the macaques that were protected against the loss of $CD4^+$ T lymphocytes with reduced viral RNA levels after virus challenge. Furthermore, the BCG/DIs group showed no evidence of clinical diseases or mortality

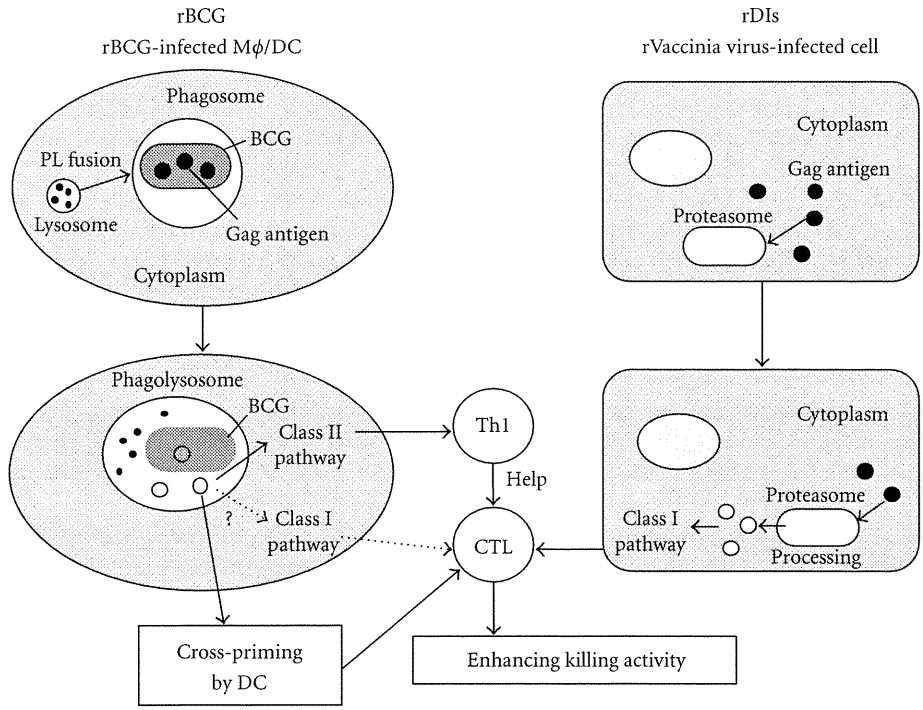

FIGURE 2: A possible mechanism of effective Gag-specific cell-mediated immunity induction with the rBCG/rDIs prime-boost vaccine. Abbreviations: DC, dendritic cell; Mϕ, macrophage; PL, phagosome-lysozome; Th1, type 1 helper T cell; CTL, cytotoxic T lymphocyte.

after viral challenge during the 1-year observation period [61]. These results suggest that the BCG/DIs prime-boost regimen might be a potential candidate for an effective and safe anti-HIV vaccine. Recent studies in macaques subjected to BCG/Ad5 [62] and BCG/MVA [63] regimens strongly support the effectiveness of the BCG vector. In the latter study, a hemolysin-expressing BCG strain, which was devised for more efficient antigen presentation to the CTL precursor, elicited a robust and broad range of HIV-1 specific T-cell responses along with recruitment of multiple T-cell clonotypes into the memory pool.

4.5. Codon Optimization Strategy. The major issue with BCG vehicle vaccines is the low expression level of the foreign antigen gene in BCG cells. In general, sufficient levels of foreign antigen-specific immune responses are obtained with high doses of rBCG between 10- and 100-fold greater than that needed for a practical dose against TB in humans [54]. This is considered the main limitation for the clinical use of rBCG-based vaccines. To address this substantial issue, we applied a codon optimization strategy for foreign genes in the rBCG system to increase its expression level. The aims of the study were to increase the immunogenicity of the foreign antigen, decrease inoculation dosages as small as the conventional BCG vaccine against TB, avoid adverse reactions, prevent possible association with Th2-type immune responses, and ward off the exacerbation of retroviral infections.

First, we determined the in vitro effects of codon optimization of the HIV gene in rBCG. Although the effect of codon optimization in mammalian cells is well documented [64–66], its effect in rBCG vehicle had never been fully elucidated. We targeted the HIV-1 *gag p24* gene as a model antigen to clarify the effect of codon optimization in the rBCG system. A specially designed synthetic p24 gene consisting of mycobacterial-preferred codons resulted in an increase in their GC content from 43.4% to 67.4%. Furthermore, codon-optimized rBCG was generated without any detectable changes in its characters including the growth rate. This rBCG exhibited a dramatic increase in Gag p24 antigen production approximately 40-fold greater than the non-optimized rBCG. Moreover, we successfully obtained data regarding the enhancement of immune responses in codon-optimized rBCG-immunized mice [67]. Inoculation of mice with a single low dose of the codon-optimized bacteria elicited effective cellular immunity. In the ELISPOT assay, the number of Gag-specific IFN-γ spot-forming cells elicited by codon-optimized rBCG was significantly greater than that elicited by non-optimized recombinants [67]. These cellular immune responses would decrease if the $CD8^+$ T cells were depleted. The results also suggest that effective MHC-class I-restricted CTL responses are inducible by vaccination with codon-optimized rBCG. Furthermore, Gag-specific lymphocyte proliferative responses were also detected in the codon-optimized rBCG-immunized mice [67].

We also applied this strategy to an SIV Gag construct and successfully generated an rBCG harboring the codon-optimized SIV *gag* gene with an expression 10-fold greater than that of the native *gag* gene. In the macaque study, compared with a native *gag* gene construct, a low-dose (10^6 bacilli) injection of this construct induced optimal priming of Gag-specific $CD4^+$ and $CD8^+$ T cells and prolonged the maintenance of memory T-cell response after vaccinia DIs

boost [68]. These results imply that the quality of the priming vaccine is a critical factor for inducing a desirable immune response against immunodeficiency viruses. Thus, the codon optimization strategy should generally be applied to other foreign genes in rBCG-based vaccine development.

5. Vaccine for Other Infectious Diseases

There were various candidate rBCG vaccines targeting infectious diseases other than TB or HIV. Stover et al. [69] reported that the rBCG system would be useful in Lyme disease vaccine development; the vaccine incorporated with the surface protein of *Borrelia burgdorferi* first reached clinical phase I trials. However, the vaccine was rejected due to its low antibody production response [70]. Two groups [71, 72] applied rBCG in malaria vaccine development and demonstrated efficacy in a mouse model. Malaria is recognized as one of the three major infectious diseases as well as TB and AIDS. Although there is a long history of malaria vaccine development, we have not seen any licensed vaccine. The strategy to induce cellular immunity against conserved antigens using BCG vector could be effective to overcome substantial difficulties in producing vaccine due to antigenic diversity and unique life cycle of this parasite. In addition, BCG vector was tested for vaccine discovery against some viral diseases. A rBCG expressing the measles virus nucleoprotein demonstrated protection against measles virus pneumonia in macaques [73]. Furthermore, we demonstrated that a rBCG with a single hepatitis C virus (HCV) NS5 CTL epitope into antigen 85B induced HCV-specific CTL response in mice [74]. HCV is recognized as one of the major infectious pathogens of which the global infection rate is ~3%. Although the priority for preventive HCV vaccine development has become lower because of the remarkable progress in the treatment, BCG vector of targeting CTL induction may have implication for therapeutic vaccine against this disease. All these candidates at the early stage of rBCG study could not proceed to further development stages at those times. The rBCG-based vaccine development for these diseases should be reconsidered because the advanced technology that enhances the potential of BCG vectors has become currently available.

6. Conclusion and Future Perspective

As described in Section 3, several rBCG-based candidate vaccines are currently being evaluated for the development of TB vaccines. Such human trials would provide a greater insight into the paradigm of immune correlation in *M. tuberculosis* infection. In addition, the application of the codon optimization strategy enables us to utilize this bacterial vector as a primer of a heterologous prime-boost regimen for a preventive HIV vaccine. These results could suggest that the BCG vector is possible divalent vaccine controlling both TB and HIV/AIDS with a single construct; such study may help resolve the serious public health problem in the sub-Saharan African countries in which both diseases are highly prevalent [14].

Another potential outcome is the utility of the BCG vector for infant vaccines. One of the largest advantages of rBCG vaccines is their applicability to newborns. Because BCG as a TB vaccine is integrated into the expanded program on immunization in many countries, we have the earliest chance to immunize newborns with BCG within 3 months of birth before they are exposed to a variety of infectious pathogens. Substituting the current BCG with a novel rBCG vaccine possessing protective antigens against pathogens that cause serious diseases in infants, such as severe diarrhea and respiratory diseases, could be effective in developing countries. Such vaccine concepts should be also tested in appropriate animal models before they are tested in humans. Thus, after much trial and error in the last 2 decades, rBCG-based vaccines may contribute to the control of global infectious diseases in the near future.

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Research paper

Flow cytometry based identification of simian immunodeficiency virus Env-specific B lymphocytes

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ABSTRACT

SIV infection of macaques is the most widely employed model for preclinical AIDS vaccine and pathogenesis research. In macaques, high-titer virus-specific antibodies are induced by infection, and antibody responses can drive evolution of viral escape variants. However, neutralizing antibodies (Nabs) induced in response to SIVmac239 and SIVmac251 infection or immunization are generally undetectable or of low titer, and the identification and cloning of potent Nabs from SIVmac-infected macaques remains elusive. Based on recent advances in labeling HIV-specific B lymphocytes [1–3], we have generated recombinant, secreted, soluble SIVmac envelope (Env) proteins (gp120 and gp140) for detection and quantification of SIVmac Env-specific B lymphocytes. In contrast to HIV-1, we found that soluble SIVmac239 gp140 retains the ability to form stable oligomers without the necessity for introducing additional, stabilizing modifications. Soluble oligomeric gp140 reacted with rhesus anti-SIV Env-specific monoclonal antibodies (MAbs), and was used to deplete Env-specific antibodies with SIV neutralization capability from plasma taken from a rhesus macaque immunized with live attenuated SIVmac239Δnef. Soluble gp120 and gp140 bound to SIV-specific immortalized B cells, and to SIV Env-specific B lymphocytes in peripheral blood of immunized animals. These reagents will be useful for analyzing development of Env-specific B cell responses in preclinical studies using SIV-infected or vaccinated rhesus macaques.

1. Introduction

The development of an effective HIV vaccine has so far remained elusive, a situation that is exacerbated by the limitations of current animal models. Despite over two decades of research, there is still no convenient, small animal model that faithfully recapitulates the persistent infection and pathogenesis of HIV-1 infection in humans (Sato and Johnson, 2007; Okada et al., 2009; Keppler et al., 2001; Keppler et al., 2002; Browning et al., 1997). Consequently, the SIV/macaque model is critical for preclinical evaluation of AIDS vaccine candidates and for pathogenesis studies.

Numerous assays are currently available for investigation of the role of different T cell subsets in the immune response to HIV/SIV infection or vaccination. In contrast, the evaluation of humoral immune responses has largely been restricted to titration of virus antigen-specific binding antibodies or neutralizing antibodies from serum of infected individuals. In SIV-infected macaques, initial reports were limited to the

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description of total B cell populations in the periphery (Dykhuizen et al., 1998; Mattapallil et al., 2004; Steger et al., 1998). The phenotypic identification of different B (naïve and memory) cell subsets during the course of SIV infection is more recent (Kuhrt et al., 2010). To our knowledge, specific phenotypic identification of SIV envelope (Env)-binding B cells has not been reported. For HIV-1-infected individuals, the use of engineered trimeric HIV gp140 bearing a biotinylation target sequence (Avitag) has permitted the identification of HIV Env-binding B cells by flow cytometry (Doria-Rose et al., 2009; Scheid et al., 2009a, b). This reagent also made it possible to clone HIV-Env-specific immunoglobulin genes directly from sorted B cells (Scheid et al., 2009a). Because of the central role of the SIV-infected macaque as a model for studying AIDS pathogenesis and exploring vaccine strategies, we developed similar recombinant, soluble SIVmac239 gp120 and gp140 proteins for flow cytometry based identification of SIV Env-specific B Lymphocytes (Doria-Rose et al., 2009; Scheid et al., 2009a,b).

2. Materials and Methods

2.1. Plasmids

SIVmac239 Env sequences have previously been optimized for expression in mammalian cells (Rosati et al., 2005). In order to generate soluble gp120 and gp140 proteins for efficient biotinylation and detection by flow cytometry, both proteins were modified to contain a protein tag commonly known as Avitag (LNDIFEAQKIEWHE) that can be biotinylated with high efficiency using biotin ligase; once biotinylated, the Avitag can serve as a high affinity substrate for streptavidin-based reagents. (Scheid et al., 2009b). To generate the tagged proteins, gene sequences corresponding to SIVmac239 Env gp120 and gp140 were amplified by PCR and the Avitag was introduced as part of the reverse primer. The same Forward primer EcoRI-SIVmac239envF-BF (5′-AGCGAATTCATGGGATGTCTTGGGAATCAGCTGCTTATCGCCATC-3′) containing an EcoRI restriction site was used for both gp120 and gp140. Reverse primers gp120_avitag_XhoI-R-BF (5′-GGCCTCGAGTCACTCGTGCCACTCGATCTTCTGGGCCTCGAAGATGTCGTTCAGCCGCTTGTTCCGCGACGTCCCCCCGGTCGT-3′) and gp140_avitag_XhoI-R-BF (5′-GGCCTCGAGTCACTCGTGCCACTCGATCTTCTGGGCCTCGAAGATGTCGTTCAGCGACGCCAGGTCGAACCAGTTGCCGAACAC-3′) containing a sequence corresponding to the Avitag signal and an XhoI restriction site were used for gp120 and gp140, respectively. The PCR amplification product was clone into pCDNA3.1(+) via EcoRI and XhoI restriction sites.

A non-modified gp120 protein was produced as a control for immunoblotting experiments. For that purpose, a stop codon was created after the amino-acid position 525 by quick change mutation (Strategene, La Jolla, CA) using primers oli082 (5′-CGTCGCGGAACAAGCGGTGATCATTCGTCCTGGGGTTCC-3′) and oli083 (5′-GGAACCCCAGGACGAATGATCACCGCTTGTTCCGCGACG-3′).

2.2. Protein Production and Biotinylation

Protein expression plasmids were used to transfect 293T cells using the Transfectin reagent, according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA). Culture supernatants (serum-free medium) were harvested twice, at 2 and 5 days post-transfection, and pooled. Pooled supernatants were centrifuged 2095 RCF for 5 min to remove cell debris. The supernatants were further clarified using a 0.45 μm syringe filter (Millipore, Billerica, MA). Protein was purified using *Galanthus nivalis* Lectin-Agarose (Sigma, St-Louis, MO). Biotinylation was performed with biotin ligase according to the manufacturer's suggestions (Avidity, Denver, Co). Biotinylation efficiency was evaluated by reference to a commercial, fully-biotinylated protein, BIS-300 (Avidity, Denver, Co). Proteins were quantified by determining adsorbance at 280 nm using a spectrophotometer (Nanodrop Technologies Inc, Wilmington, DE).

2.3. Glutaraldehyde (GA) Cross-Linking

Culture supernatant from transfected cells was harvested 48 h post-transfection and centrifuged 2095 rcf for 5 min to pellet cell debris. Supernatant was further cleared using a 0.45 μm syringe filter (Millipore, Billerica, MA). 200 μl of filtered supernatant were mixed with gluteraldehyde (GA) at different concentrations (0, 1, 2.5, 5 and 10 mM). The mixture was then incubated for 5 minutes at room temperature and the reaction was quenched with 0.1 M Tris-HCl pH 7.4. Treated protein was then analyzed by denaturing SDS-PAGE on an 8% gel (BioRad, Hercules, CA).

2.4. Protein Antigenicity

Prior to biotinylation, antigenicity of Avitag bearing gp120 and gp140 was determined by immunoblotting. Purified and cross-linked proteins were denatured by addition of equal volume of 2x concentrated sample buffer containing SDS and ß-mercaptoethanol (Sigma, St-Louis, MO) and boiling at 99 °C for 5 min. Denatured proteins were separated by SDS-PAGE on an 8 or 12% gel (BioRad, Hercules, CA). Staining with Coomassie blue was performed to verify equal loading of purified proteins. The presence of Avitag sequence on the non-biotinylated proteins was tested using anti-Avitag monoclonal antibody (Avidity, Denver, Co.) while reactivity with SIV Env-specific antibodies was tested using monoclonal antibody 3.11H (Robinson et al., 1998) and pooled plasma from SIV-infected macaques.

The antigenicity of none or biotinylated gp120 and gp140 was analyzed by ELISA using plasma of SIV-infected macaque (Mm376-04) and SIV gp120-specific macaque MAbs obtained from Dr James Robinson (Tulane University Medical School, New Orleans, Lo). The binding sites of these MAbs have been more or less characterized and they have been assigned to different competition groups (Robinson et al., 1998; Cole et al., 2001; Johnson et al., 2003). MAb 3.11H is V3-loop specific and belongs to the competition group IV while MAb 1.10A assigned to group VII has a less defined binding site but encompasses the V4 loop. MAb 3.4E and 1.9C respectively assigned to group V and VI have an undefined binding sites but seems to exclude the variable loops of gp120. ELISA was performed as previously described (Hammonds et al., 2005). ELISA plates were coated at 50 ng of antigen per well. HRP-conjugated anti-human IgGs (Santa Cruz Biotechnology, Santa Cruz, CA) was used for detection with TMB solution (BioRad, Hercules, CA).

2.5. Binding to Immortalized, SIV-specific Rhesus B Cell Lines

Rhesus macaque B cell lines producing anti-SIV gp120 MAb 3.11H and MAb 3.4E were obtained from Dr James Robinson (Tulane University Medical School, New Orleans, Lo).

Rhesus macaque B cell lines were stained with biotinylated 10 μg/ml of gp120 or gp140 for 30 min at 4 °C in 100 μl of staining buffer corresponding to phosphate buffer saline PBS (Invitrogen, Carlsbad, CA) containing 0.1% bovine serum albumin (Sigma, St-Louis, MO). Cells were then incubated with streptavidin-APC (BD Biosciences, San Jose, CA). Data were acquired on a FACS Calibur (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (TreeStar, Cupertino, CA).

2.6. Plasma Adsorption and Elution of Anti-gp120 and -gp140 Antibodies

Adsorption and elution of plasma anti-HIV-1 gp120 or anti-membrane proximal external region (MPER) antibodies have previously been described (Li et al., 2007; Gray et al., 2009). Purified gp120, gp140 or control BSA (BioRad, Hercules, CA) were coated to Dynabeads MyOne Tosylactivated at the ratio of 40 μg protein per mg of beads according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA).

To verify integrity of gp120 and gp140, protein-coated beads were first stained with MAb 3.11H or pooled plasmas from SIV-infected animals and then FITC-conjugated anti-human IgGs (BD Biosciences, San Jose, CA). Data were acquired on a FACS calibur (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (TreeStar, Cupertino, CA).

For antibody depletion, 50 μl of plasma from a rhesus macaque (Mm 372–04) previously immunized with SIV-mac239Δnef was diluted with 150 μl of RPMI (Invitrogen, Carlsbad, CA). Diluted plasma was separately added in 2 ml Eppendorf tubes to 1 mg of beads previously coated with 40 μg of corresponding proteins. Tubes were incubated for 1 h at room temperature with rotation and the beads where collected using a magnet. A second round of adsorption was performed with fresh beads (1 mg, 40 μg of proteins). The efficiency of the adsorption was tested by ELISA, using plates coated with gp120 or gp140.

gp120- and gp140-binding antibodies were eluted by addition of 200 μl of 0.1 M glycine to beads pellets. Beads were then retained with a magnet and supernatants (eluates) were collected and brought to neutral pH by addition of 7.5 μl of 1.5 M Tris-HCl pH 8.5. The antigenic specificity of eluted IgGs was evaluated by ELISA, using plates coated with gp120 or gp140. The IgG content of eluates was determined using an IgG quantification ELISA Kit following recommendations from the manufacturer (Columbia Bio LLC, Elmhurst, NY). A cut-off value was set at 2× mean background values.

2.7. Virus Neutralization Assay

To test interaction of the Env proteins with neutralizing antibodies, plasma was depleted by treatment with g120- and gp140-coated beads and tested for virus neutralization using the CEMx174-SEAP indicator cell line as previously described (Johnson et al., 2003). Untreated plasma, plasma treated with "beads-only," and plasma treated with BSA-coated beads were all included as controls. Neutralization assays were performed using the SIVmac316 (neutralization sensitive) and SIVmac239 (neutralization resistant) isolates as test viruses.

2.8. Detection of gp120- and gp140-specific B Cells in Peripheral Blood

Mononuclear cells were prepared from peripheral blood using Lymphocyte Separation Medium (MP Biomedicals LLC, Irvine, CA) following the manufacturer's instructions. $CD3^+$ cells were depleted using anti-human CD3 clone SP34-2 (BD Biosciences, San Jose, CA) and Pan Mouse IgG Dynabeads (Invitrogen, Carlsbad, CA) in order to enrich for B cells. $CD3^+$ cell depletion resulted in depletion of $CD4^+$ T cells, which gp120 and gp140 could bind through the CD4 receptor. Enriched B cell fractions were stained as previously described (Doria-Rose et al., 2009). At first, cells were stained with non-conjugated purified anti-CD4 antibody (NIH Nonhuman Primate Reagent Resource, Boston, MA) at the ratio of 10 μg/ml at 4 °C in order to prevent gp120 and gp140 binding to potentially remaining CD4 positive cells. After 15 min of incubation, cells were stained with 10 μg/ml of biotinylated gp120 or gp140 for 30 min at 4 °C. Cells were then incubated with streptavidin-V450 and anti-human antibodies IgG-PE-Cy5, CD3-V500 and CD20-APC-H7 (BD Biosciences, San Jose, CA). Data were acquired (800 000 events) using an LSRII Flow Cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software (TreeStar, Cupertino, CA).

2.9. Animals

Mm 376–04 is a rhesus macaque that was immunized with SIVmac239Δnef. The animal had an ongoing but limited replication of the vaccine strain SIVmac239Δnef with viral loads of ~1000 ceq/ml. Two months after immunization, SIVmac239Δnef viral load was equal to 1200 ceq/ml. Mm 376–04 was sterilely protected following intravenous challenge with wild type SIVmac239, 5 weeks after SIVmac239Δnef-immunization (wild type SIVmac239 viral load always below 60 ceq/ml).

Mm 376–02 is a rhesus macaque that was immunized with SIVmac239Δnef and had persistent low-level replication of the vaccine strain of 60–300 ceq/ml. Mm 376–02 was sterilely protected following vaginal challenge with SIV-mac251 at 20 weeks after SIVmac239Δnef-vaccination (SIV-mac251 viral load always below 10 ceq/ml).

Naïve animals Mm 400–08 and 9–010 are SIV-negative.

All 4 animals were utilized in experiments aiming to detect gp120- and gp140-binding B cells. Only Mm 376–04 was involved in plasma antibody adsorption experiments.

3. Results

3.1. Protein Antigenicity/Recognition

To identify SIV Env-specific B cells in the peripheral blood of SIV-infected animals, we engineered recombinant, soluble SIVmac239 Env proteins bearing a high affinity biotinylation target sequence, or Avitag (Avidity, Denver, Co) at the C-terminus. Soluble gp120 encompasses the entire coding region of the SIVmac239 surface subunit (SU or gp120), whereas soluble gp140 includes all of gp120 and the

extracellular portion of the transmembrane subunit (TM or gp41). The Avitag sequence was placed at the C-terminus of gp120 and gp140; thus, in the case of gp140, processing of the protein into the SU and TM subunits results in only the TM subunit (the truncated gp41) bearing the Avitag. Proteins were generated by transient transfection of 293T cells and purified using *G. nivalis* Lectin-Agarose (Sigma, St-Louis, MO).

The purified non-biotinylated, soluble gp120 and gp140 proteins were denatured (99 °C, 5 min) and separated on a 12% SDS-PAGE gel in reducing conditions, along with untagged soluble gp120 as a control (Fig. 1a). We confirmed expression of the proteins by coomassie staining and by immunoblotting using Env-specific and Avitag specific antibodies. As expected, all three proteins were detected with the SIV V3 loop-specific MAb 3.11H (Fig. 1b, left panel) and pooled plasma from SIV-infected rhesus macaques (Fig. 1b, center panel). In contrast, only the Avitag-bearing proteins were detected with an Avitag-specific MAb (Fig. 1b, right panel). With both SIV + plasma and the anti-Avitag MAb a band of smaller size (~ 25 Kda) corresponding to the truncated, gp41-derived subunit of gp140 was detected; importantly, the intensity of the 25kD band is similar whether probing with SIV + plasma or the anti-Avitag MAb (Fig. 1b, center and right panels). The combined results confirmed that purified soluble SIV gp140 is comprised of two subunits, gp120 and the ectodomain region of gp41. The 2 subunits were separated under denaturing/reducing conditions as shown by the detection of gp41 ectodomain (Fig. 1, center and right panels). In the same lanes, the higher molecular weight fragment is a mixture of gp140 and gp120. Probing with SIV^+ plasma detected both gp140 and gp120 (Fig. 1, center panel) generating a strong band. In contrast, because the Avitag sequence is at the C-terminus of the TM subunit, the anti-Avitag antibody detected only gp140 but not gp120 (Fig. 1, right panel); this result also reveals that the gp140 preparation contains both cleaved and uncleaved gp140 proteins.

We next anaylzed the oligomeric structure of soluble SIV gp140 by glutaraldehyde cross-linking. In the absence of glutaraldehyde, gp140 migrates as two bands on an 8% SDS gel under denaturing/reducing conditions (Fig. 2). In the presence of increasing concentrations of glutaraldehyde, the gp140 bands shift into a slower migrating band corresponding to oligomeric gp140. In contrast, glutaraldehyde cross-linking did not result in oligomerization of gp120, even at the highest concentration tested. On the 8% SDS gel the Avi-tagged gp120 ran slightly above the 160 kD marker. The dramatic shift in size between gp140 in the absence and presence of glutaraldehyde suggests that the

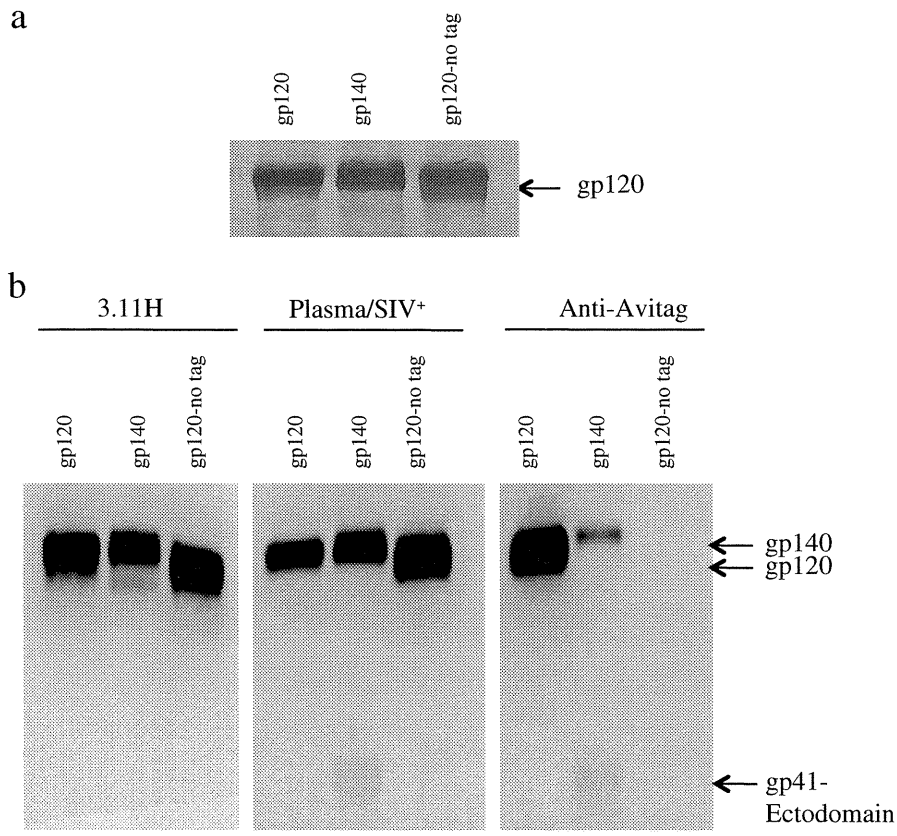

Fig. 1. Recognition of Avitag-bearing gp120 and gp140 by immunoblotting. SIV gp120 and gp140 specificities were detected with monoclonal antibody (MAb) 3.11H (V3 loop-specific) and pooled plasma from SIV-infected macaques. Avitag specificity was determined with anti-Avitag MAb. Purified proteins were separated by SDS-PAGE. (a) Coomassie stained gel showing Avitag-bearing gp120 and gp140 and untagged control gp120. (b) immunoblotting with MAb 3.11H (left panel), pooled plasma from SIV-infected macaques (center panel) and anti-Avitag MAb (right panel).

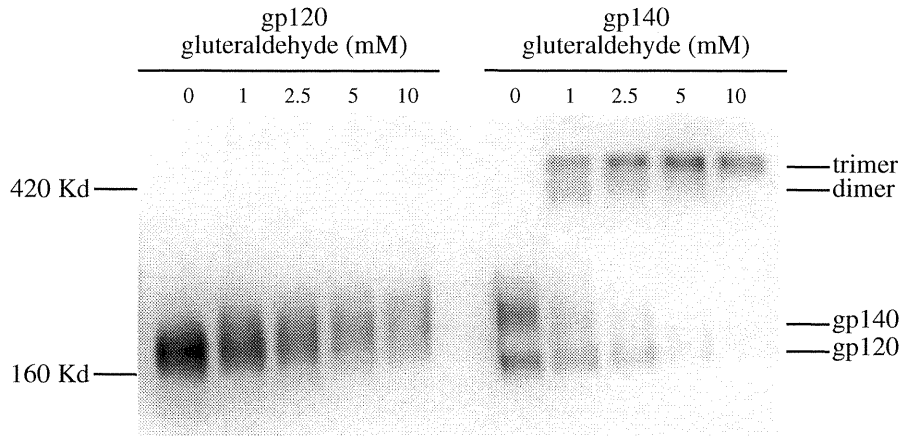

Fig. 2. Oligomerization of soluble SIV gp140. Gluteraldehyde (GA) cross-linking was used to determine the oligomeric structure of soluble gp140 on a 8% SDS gel and in reducing conditions following transfection of 293T cells with Avitag-bearing gp120 or gp140 expression vectors. Culture supernatant was harvested 48 h post-transfection. gp140 supernatant was cross-linked with different concentrations (mM) of GA. The reaction was stopped after 5 min by addition of 0.1 M Tris–HCl. Samples were loaded along with gp120 supernatant as control.

slowest migrating gp140 oligomer is most likely a trimer. An additional, faint band estimated to be a gp140 dimer was also observed, although this disappeared with increasing concentrations of glutaraldehyde (and was undetectable at the highest concentration tested).

Following the analysis of the oligomeric structure of gp140, purified Avi-tagged gp120 and gp140 were biotinylated using biotin ligase. Efficacy of biotiylation was verified by ELISA by comparison to a reference protein Bis-300 (Supplementary Fig. 1). The integrity of biotinylated gp120 and gp140 proteins was analyzed by ELISA using plasma from an SIV^+ macaque and SIV-gp120 specific rhesus macaque MAbs. For SIV^+ plasma, binding to gp120 and gp140 was identical to corresponding biotinylated forms (Fig. 3). These data suggest that biotinylation did not grossly alter the antigenic structure of these proteins. Even though the results were not as striking as for polyclonal plasma, MAbs presented a similar trend (with the exception of MAbs 3.4E and 1.10A where biotinylation improved antibody binding to gp140). For all MAbs and SIV + plasma, binding to gp120 was always superior to binding to gp140. These results are consistent with the assumption that oligomeric gp140 is structurally constrained and some surfaces are less accessible to binding antibodies.

In order to demonstrate the ability of biotinylated gp120 and gp140 to bind SIV Env-specific antibody producing cells, we used two immortalized rhesus macaque B cell lines (BLCL) that produce MAb 3.11H and MAb 3.4E (Robinson et al., 1998; Cole et al., 2001; Johnson et al., 2003). Both proteins readily stained these SIV-specific BLCLs, whereas neither of the biotinylated proteins (gp120 and gp140) bound to BLCL generated from naïve (SIV-negative) rhesus macaques (Fig. 3, left panel). Binding of gp120 and gp140 to BLCL-3.11H was superior to BLCL-3.4E consistent with the ELISA results (Fig. 3). In addition, gp120 binding was also superior to gp140, similar to the result obtained by ELISA (Fig. 3). Because the Avitag is located at the C-terminus of the gp41 ectodomain, and binding to 3.11H and 3.4E occurs through the gp120 subunit, the gp120 subunit and the gp41 ectodomain must have remained associated throughout the assay.

These results indicate that soluble SIVmac239 gp140 can form an oligomeric structure. The oligomeric structure of soluble SIVmac gp140 has been previously demonstrated with the Env protein of CP-MAC, a lab-adapted variant of SIVmacBK28 (LaBranche et al., 1995; Edinger et al., 2000). These authors showed that introduction of a premature stop codon in the transmembrane (TM) domain of gp41 resulted in a highly stable gp120/gp41 association and the modification of the cleavage site between gp120 and gp41 was not necessary. Our results with soluble SIVmac239 gp140 are in accordance with these previous findings. In contrast, the association between HIV-1 gp120 and gp41 is apparently more labile, and retention of stable oligomeric structures typically requires experimental modifications, such as elimination of the gp120-gp41 cleavage site, introduction of cysteine residues into the gp41 N36 coiled coil, and inclusion of a heterologous trimerization motif (Binley et al., 2000; Yang et al., 2000a,b; Farzan et al., 1998). The minimal alteration of SIVmac239 gp140 is desirable in order to retain the original antigenicity of the trimeric Env complex.

3.2. Plasma Antibody Adsorption and Elution

In order to further assess the structural integrity of soluble SIV gp120 and gp140, the proteins were tested for ability to adsorb SIV Env-specific antibodies in plasma from an animal immunized with SIVmac239Δnef (Mm 376–04). To do this, soluble gp120 and gp140 were coated to Dynabeads. Both gp120 and gp140 coated beads reacted with MAb 3.11H (V3 loop-specific) and pooled SIV^+ plasmas (Fig. 4a).

gp120 and gp140 coated Dynabeads were then used to adsorb SIV Env-specific antibodies from the plasma. The efficiency of plasma adsorption was evaluated by ELISA (Fig. 4b). gp120 and gp140 adsorptions resulted in reduction of SIV Env-specific antibodies in plasma in comparison to controls (controls included untreated plasma, plasma treated with beads-only, and plasma treated with BSA-coated beads controls) (Fig. 4). Both gp120 and gp140 adsorption resulted in similar levels of depletion (Fig. 4b, left panel). BSA-coated

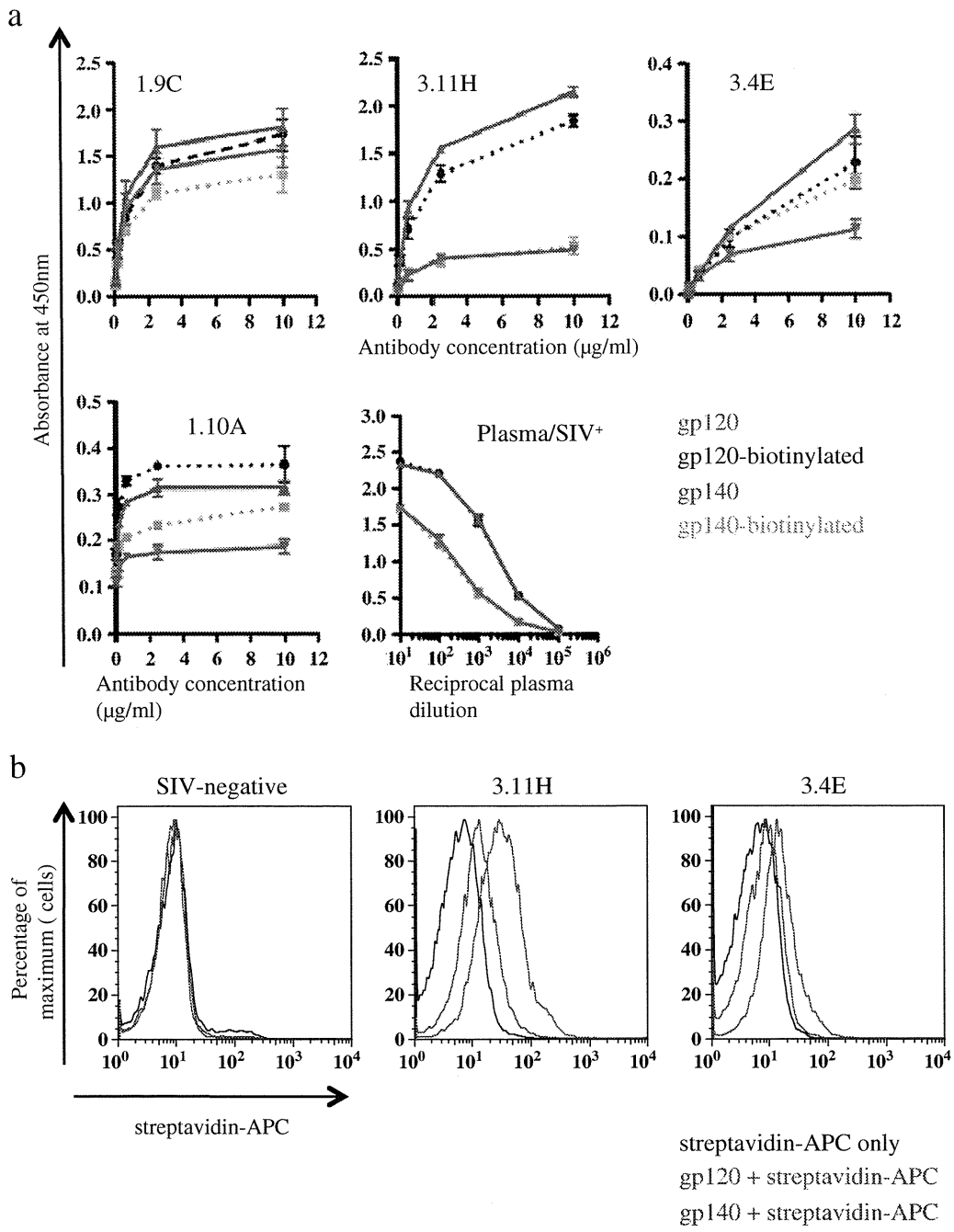

Fig. 3. Recognition of biotinylated gp120 and gp140 by ELISA and binding to macaque B cell lines. (a) SIV gp120 and gp140 were detected with monoclonal antibodies (MAbs) 3.11H, 3.4E, 1.9C and 1.10A (competition group IV, V, VI and VII, respectively) and plasmas (Plasma/SIV^{+}) from sterilely protected SIVmac239Δnef immunized macaque (Mm376-04) followed by HRP-conjugated anti-human IgGs. ELISA plates were coated with biotinylated gp120 and gp140 or non-biotinylated gp120 and gp140. OD, optical density. Background values were obtained using control wells (no antigen). Bars represent OD value obtained after background subtraction. Error bars are based on standard deviation of OD values for triplicate wells for each antigen. (b) Anti-SIV ENV gp120-directed monoclonal antibody (MAb) producing B cell lines (3.11H and 3.4E) were stained with biotinylated gp120 and gp140 or buffer only. Cells were washed and further stained with APC-conjugated streptavidin. The control B cell line was developed from a naïve (SIV-negative) macaque.

beads, beads-only and untreated plasma resulted in little or no depletion. We concluded that both SIV gp120 and SIV gp140 bind anti-Env-IgGs. Oligomeric SIV gp140 most likely encompasses the same specificities as SIV gp120 with regard to the gp120 component.

Plasma anti-gp140 IgG adsorption was more efficient with gp140-coated beads than gp120-coated beads (Fig. 4b, right panel). BSA-coated beads and beads-only had a similar level of plasma anti-gp120 IgG content compared to untreated plasma (Fig. 4b, right panel). We conclude that oligomeric SIV gp140

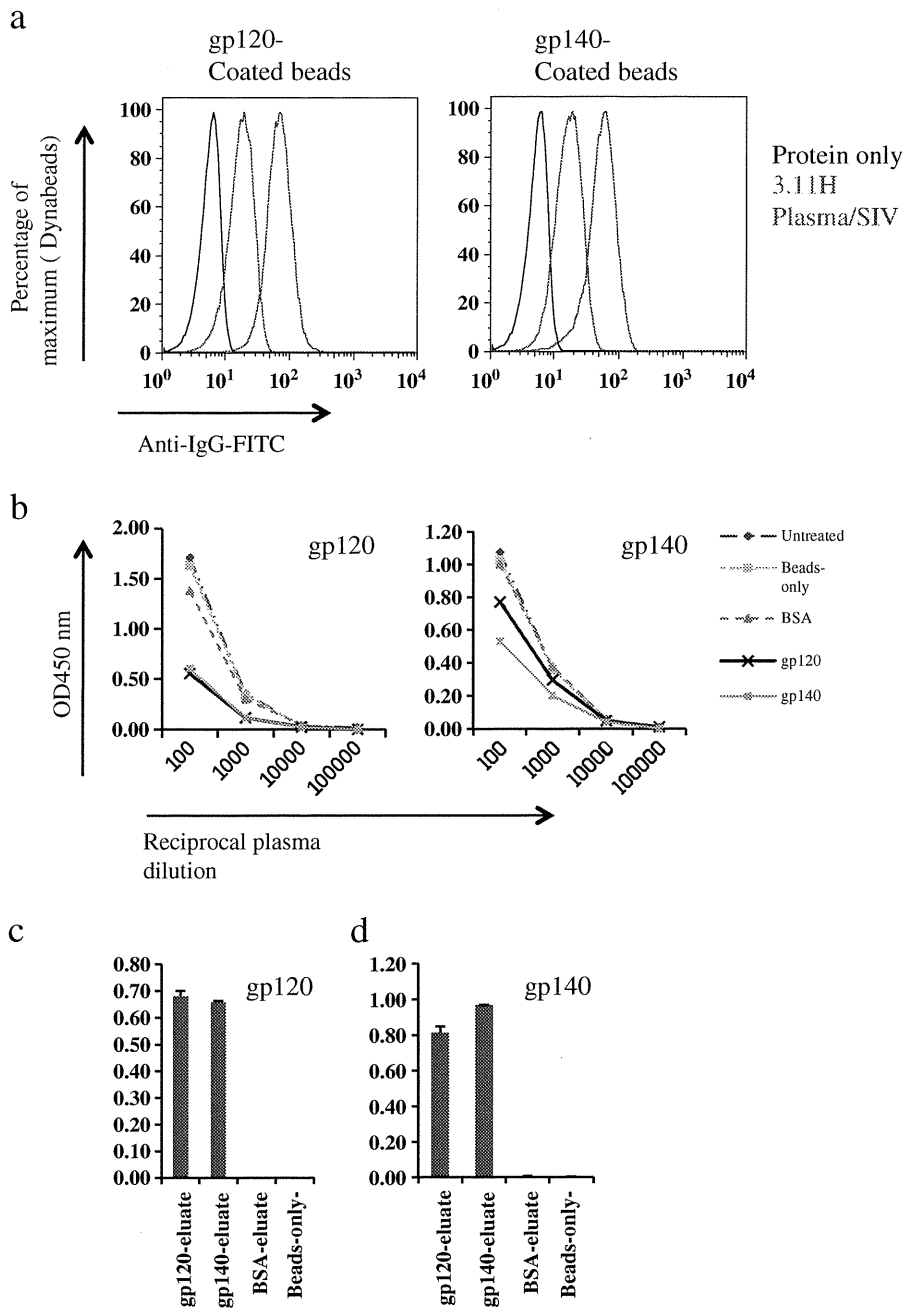

Fig. 4. Plasma antibody adsorption and elution. (a) Recognition of gp120 and gp140 coated Dynabeads. Coated beads were stained with monoclonal antibody (MAb) 3.11H (V3 loop-specific) and pooled plasmas from SIV-infected monkey (Plasma/SIV^{+}) or buffer only. Antibody binding to gp120 and gp140 was revealed by flow cytometry using an anti-human FITC-conjugated IgG. (b) Anti-gp120 and anti-gp140 antibody adsorption from plasma from SIVmac239Δnef-immunized macaque (Mm 376–04). Antibodies were adsorbed with gp120-coated beads, gp140-coated beads, BSA-coated beads or beads-only or left untreated. Resulting plasmas were then assayed by ELISA for binding to gp120 (left panel) or gp140 (right panel). (c) and (d) Bead-eluate content in anti-SIV ENV-directed antibodies. gp120 and gp140 binding IgGs were eluted with glycine. Eluates were then brought to neutral with Tris-HCl and tested using ELISA plates coated with gp120 and gp140. OD, optical density.

possesses additional specificities to those of SIV gp120 subunit, possibly due to epitopes in the ectodomain of gp41 or epitopes unique to the oligomeric structure.

In order to confirm that Env-specific IgGs in the plasma from an animal immunized with SIV239Δnef bound to gp120- and gp140-coated beads, the beads were treated with a low pH buffer to elute any bound IgG and the eluate was tested for the presence of Env-specific binding antibodies by ELISA. Similar antibody levels were obtained for the gp120-eluate (247 ng/ml) and the gp140-eluate (247 ng/ml). Eluates from control BSA-coated beads and the beads-only control were below the cut-off value. An equal volume of each

eluate (50 µl) was assayed using ELISA plates coated with gp120 and gp140. Similar gp120-specific IgG content was detected in the eluates from gp120-coated beads and gp140-coated beads (Fig. 4c). A similar level of gp120- and gp140-specific IgG content was observed in the eluate from both gp120- or gp140-coated beads (Fig. 4d). Neither gp120- nor gp140-specific IgGs were detected in beads-only eluate or BSA eluate. These results demonstrate that purified soluble SIV gp120 and oligomeric SIV gp140 can bind SIV Env-specific antibodies from a macaque immunized with SIVmac239Δnef.

3.3. Depletion of Neutralizing Activity from SIV+ Plasma with gp120 and gp140

Finally, we sought to analyze the functionality of depleted anti-Env IgGs by performing virus neutralization assays. The SIV strain SIVmac316, like many laboratory strains of HIV-1, is sensitive to antibody-mediated neutralization when treated with SIVmac+ sera or anti-SIV Env antibodies (Johnson et al., 2003). Pre-depletion of plasma from macaque Mm376-04 with gp140 resulted in a significant decrease in 50% neutralizing titer (from 1:16,384 for the untreated control to 1:265 for the gp140 depleted control)(Fig. 5b, left panel). SIVmac239 is generally resistant to neutralization by autologous plasma, and consequently we found that only the lowest plasma dilution (1:16) achieved close to 50% neutralization compared to controls. Nevertheless, depletion with gp120 and gp140 reduced neutralization at the 1:16 dilution from 50% to 80% (gp120) and 100% (gp140) (Fig. 5b, right panel). No detectable difference was observed for neutralization of SIVmac239 using plasma from control samples (untreated, beads-only and BSA-coated beads) (Fig. 5b). Together, these results confirm that the SIV gp140 protein is recognized and bound by antibodies that also recognize native envelope spikes as they exist on the surface of infectious virions.

3.4. Detection of SIV gp120- and gp140-Specific B Cells in Peripheral Blood

We next asked whether biotinylated gp120 and gp140 proteins could be used to identify SIV Env-specific B lymphocytes in peripheral blood using flow cytometry. The assay was performed using PBMC from SIVmac239Δnef-immunized macaques (Mm 376–02 and Mm 376–04) and a naïve (SIV-negative) control. To do this, CD3-positive cells were first depleted in order to enrich for B lymphocytes. Additionally, to prevent binding to residual CD4+ T-cells via the Env receptor binding domain, cells were treated with a CD4 blocking antibody prior to staining. While HIV gp140-binding $CD19^{+}IgG^{+}$ memory B cells have previously been reported (Scheid et al., 2009a,b), the anti-human CD19 (Non-Human Primate Reagent Resources, Boston, MA) is less efficient for staining rhesus B cells even when used at high concentrations (data not presented). Preliminary studies using a number of surface markers specific for B-cells showed that in general CD19, CD21 and CD22 were detected on the surface of $CD20^{+}$ cells, while a significant number of $CD20^{+}$ cells were negative for CD19, CD21 and CD22 (data not presented). Therefore, in this study we chose CD20 as B-cell

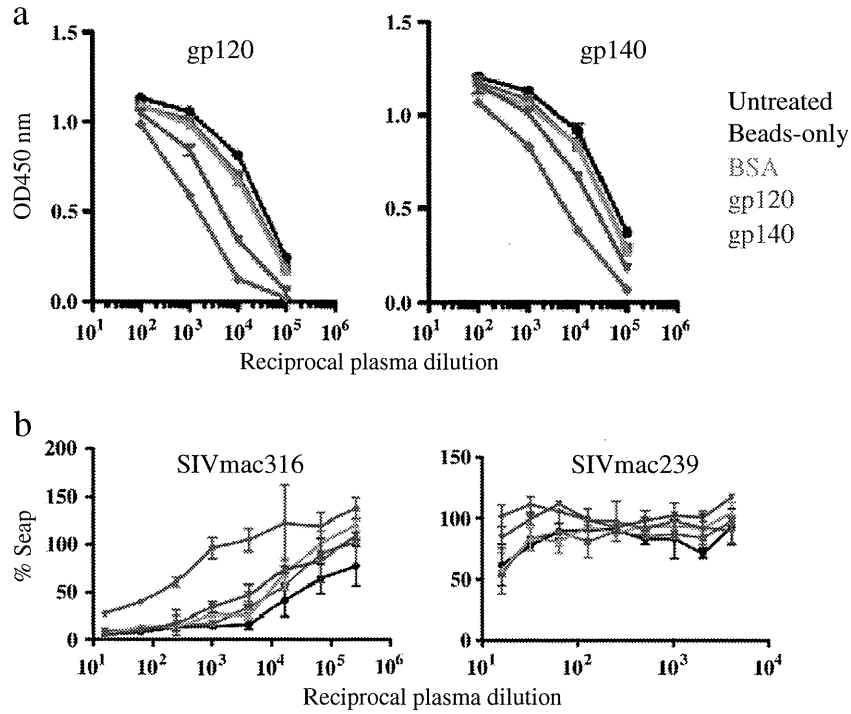

Fig. 5. Plasma antibody adsorption and virus neutralization assay. (a) Anti-gp120 and anti-gp140 antibody adsorption from plasma from SIVmac239Δnef-immunized macaque (Mm 376–04). Antibodies were adsorbed with gp120-coated beads, gp140-coated beads, BSA-coated beads or beads-only or left untreated. Resulting plasmas were then assayed by ELISA for binding to gp120 (left panel) or gp140 (right panel). OD, optical density. (b) anti-gp120 and -gp140 IgG depleted plasma were assayed for neutralization of SIVmac239 and SICmac316.

marker using an anti-human CD20 antibody (BD Biosciences, San Jose, CA). In human, one advantage of using anti-CD19 over anti-CD20 antibody can be the detection of plamablasts/early plasma cells. However, our preliminary studies in rhesus macaques failed to detect $CD19^{+}CD20^{-}$ plamablasts/early plasma cells. Our lab and others are actively pursuing alternative protocols for phenotypic identification of plasmablasts/early plasma cells in macaques.

SIV Env-specific (gp120- and gp140-binding) $CD20^{+}$ IgG^{+} B cells were detected in both animals (Mm 376–02 and Mm 376–

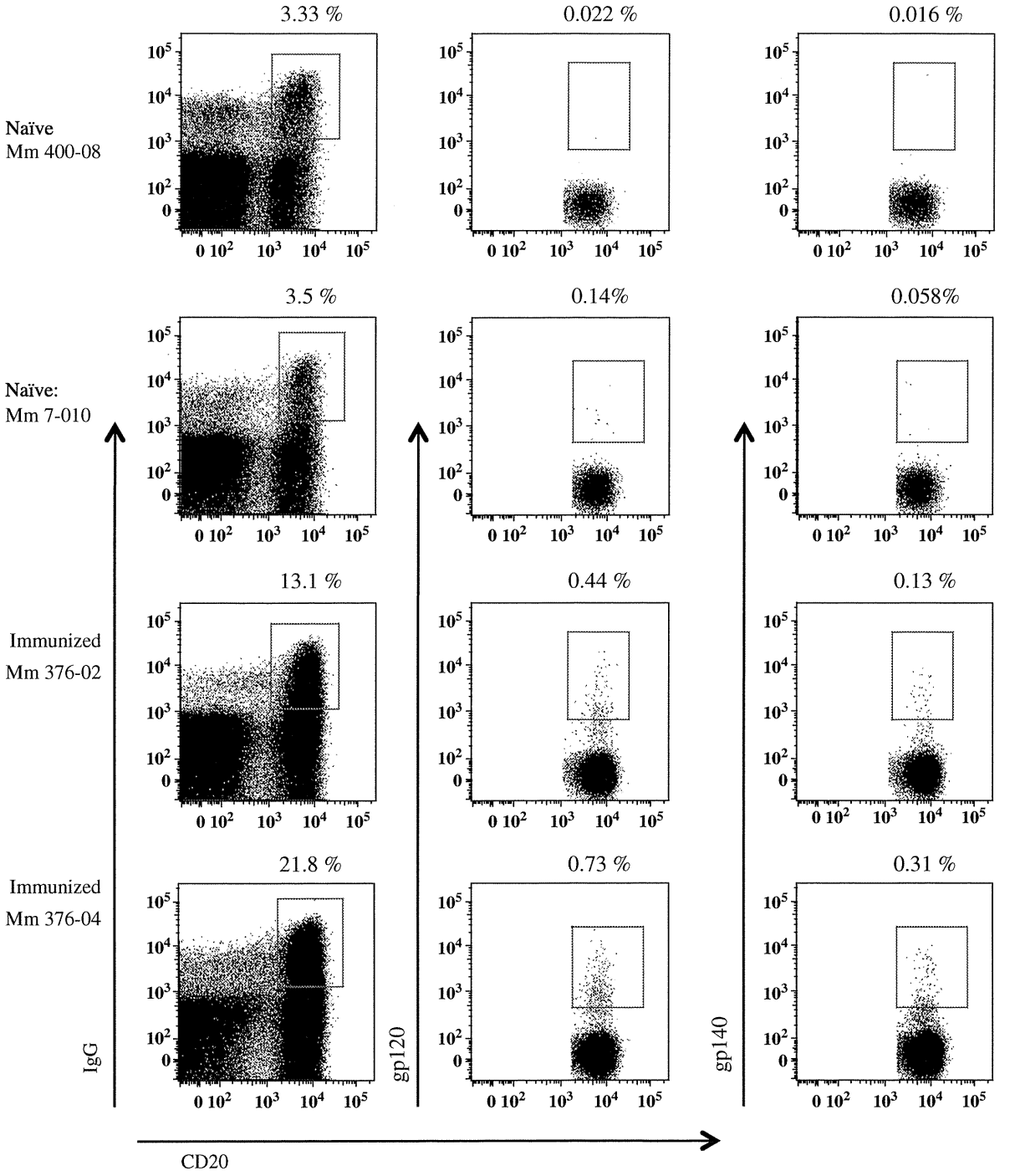

Fig. 6. Identification of gp120- and gp140-binding B cells. Gating was performed on CD3-negative lymphocytes from SIVmac239-immunized macaques (Mm 376–02 and Mm 376–04) and naïve (SIV-negative) controls. The plots on the left show the gating on $CD20^{+}IgG^{+}$ B cells. The plots on the center show the gating on gp120-binding $CD20^{+}IgG^{+}$ B cells. The plots on the right show the gating on gp140-binding $CD20^{+}IgG^{+}$ B cells. Numbers indicate the frequency of $CD20^{+}IgG^{+}$ B cells (left) and the frequency of gp120-binding $CD20^{+}IgG^{+}$ B cells (center) and the frequency of gp140-binding $CD20^{+}IgG^{+}$ B cells (right).