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(54) **Title**: B-CELL STIMULATING FUSION PROTEINS OF AN ANTIGEN WITH BAFF OR APRIL

(57) **Abstract**: The invention relates to the fields of molecular biology, medicine, virology and vaccine development. Because the different forms of the presently available vaccines all have their specific drawbacks, there is a need for alternative vaccine strategies. The current invention provides means and methods for such alternative vaccine strategies. It provides a fusion protein of an antigen (e.g. HIV gp120 or gp160) with the extracellular domain of APRIL or BAFF, or with GMCSF or IL-21, as well as therapeutic applications of said fusion protein in vaccination.

# B-CELL STIMULATING FUSION PROTEINS OF A AN ANTIGEN WITH BAFF OR APRIL

The invention relates to the fields of molecular biology, medicine, virology and vaccine development.

Vaccination is the administration of antigenic material (the vaccine) to produce immunity to a disease. Vaccines can prevent or ameliorate the effects of infection by many pathogens and are generally considered to be the most effective means of preventing infectious diseases. The material administered can for instance be live but weakened forms of pathogens (such as for instance bacteria or viruses), killed or inactivated forms of these pathogens, or purified material such as proteins.

Disadvantages of live, but weakened forms of pathogens are that these pathogens are still able to replicate and can in some cases manifest as a full-blown possibly life-threatening infection, especially in immunocompromised hosts. These forms of pathogens also have the possibility to mutate and become more virulent through such mutations. Killed or inactivated forms, if properly inactivated, do not have these disadvantages, but they have other disadvantages. Since a killed or inactivated pathogen cannot multiply, a large number of pathogens is required to stimulate immunity. Further, periodic boosters must be given to maintain immunity, only humoral immunity can be induced and since the vaccine must be injected (and not for instance orally taken), it is costly to administer.

Subunit vaccines are protein based vaccines that contain purified antigens rather than whole organisms; an example is the *Bordetella pertussis* antigens included in the acellular DPT vaccine. The acellular DPT vaccine comprises diphtheria and tetanus toxins and selected antigens of the pertussis

pathogen. Subunit vaccines are not infectious, so they can safely be given to immunocompromised individuals, and they are less likely to induce unfavorable immune reactions that may cause side effects. Disadvantages of subunit vaccines are that the antigens may not retain their native conformation, so that antibodies produced against the subunit may not recognize the same protein on the pathogen surface. In general it takes at least two inoculations with a protein based vaccine to confer adequate protection. Another disadvantage is that isolated protein does not stimulate the immune system as well as a whole organism vaccine, because various components of live-attenuated and whole-inactivated vaccines provide co stimulatory signals.

In general, the effectiveness of subunit vaccines is increased by giving them in combination with adjuvant. Adjuvant slows antigen release for a more sustained immune stimulation, binds toll-like receptors on macrophages and dendritic cells to stimulate production of inflammatory cytokines, and activates APC to express B7. Alum (aluminum salts) is a common adjuvant used in human vaccines; it aggregates proteins to make them easier for phagocytes to engulf. Pertussis toxin, one of the components of the acellular DPT, acts as an adjuvant in that vaccine. Some bacterial components used as adjuvant in animals but which cause too much inflammation to be safe in humans are whole Mycobacterium tuberculosis, muramyl dipeptide from Mycobacterial cell walls, and bacterial DNA. One drawback of adjuvant used in human subunit vaccines is that such adjuvant induces a broad nonspecific immune response rather than a specific immune response restricted to the administered antigen.

Because the different forms of the presently available vaccines all have their specific drawbacks, there is a need for alternative vaccine strategies.

The current invention provides means and methods for such alternative vaccine strategies.

In a first embodiment, the invention provides a fusion protein comprising an antigen and a ligand capable of inducing, enhancing or sustaining a B cell immune response, wherein said ligand is selected from the group consisting of a compound comprising at least the extracellular domain of a proliferation inducing ligand (APRIL), a compound comprising at least the extracellular domain of a B-cell activating factor (BAFF), a compound comprising an amino acid sequence having at least 80% sequence identity with at least the extracellular domain of APRIL, and a compound comprising an amino acid sequence having at least 80% sequence identity with at least the extracellular domain of BAFF (Cerutti 2008, Kimberley 2009, Mackay 2009)(Figures 1 and 38). In a more preferred embodiment said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably at least 98%, most preferably 100%. In a preferred embodiment, said ligand is selected from the group consisting of a compound comprising at least the extracellular domain of a proliferation inducing ligand (APRIL), a compound comprising an amino acid sequence having at least 80% sequence identity with at least the extracellular domain of APRIL. In a more preferred embodiment said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably at least 98%, most preferably 100%

As used herein, a compound comprising at least the extracellular domain of a proliferation inducing ligand (APRIL) or comprising an amino acid sequence having at least 80% sequence identity with the extracellular domain of APRIL will be referred to as “APRIL” or an “APRIL-like compound or protein”. Consequently, a compound comprising at least the extracellular domain of a B-cell activating factor (BAFF) or comprising an amino acid sequence having at least 80% sequence identity with at least the extracellular domain of BAFF will be referred to as “BAFF” or a “BAFF-like compound or protein”. Also

within this definition of an APRIL-like and a BAFF-like compound said sequence identity is preferably at least 85%, more preferably at least 90%, more preferably at least 95%, more preferably at least 98%, most preferably 100%. De terms "APRIL" and "BAFF" are used whenever said amino acid sequence has 100% sequence identity with at least the extracellular domain of APRIL and BAFF, respectively. As used herein, de terms APRIL and BAFF include any orthologue thereof. The terms "APRIL" and "BAFF" thus includes sequences with 100 % sequence identity with at least the extracellular domain of APRIL or BAFF of any species, preferably with at least the extracellular domain of human APRIL or human BAFF. The antigen can be any antigen known in the art, such as proteins or peptides, DNA, RNA, or chemicals, such as a hapten. It is preferred, however, that the antigen is an immunogenic part of a microorganism, preferably of a pathogenic microorganism, such as a bacterium, fungus, yeast, parasite or virus. It is preferred that the antigen resembles its native conformation when present in a fusion protein of the invention in order to closely mimic the antigen as present on the microorganism.

The term "% sequence identity" is defined herein as the percentage of nucleotides in a nucleic acid sequence that is identical with the nucleotides in a nucleic aid sequence of interest, after aligning the sequences and optionally introducing gaps, if necessary, to achieve the maximum percent sequence identity. Methods and computer programs for alignments are well known in the art. As used herein, the terms "nucleic acid sequence" and "nucleotides" also encompass non-natural molecules based on and/or derived from nucleic acid sequences, such as for instance artificially modified nucleic acid sequences, peptide nucleic acids, as well as nucleic acid sequences comprising at least one modified nucleotide and/or non-natural nucleotide such as for instance inosine, LNA, Morpholino, and 2'-O-methyl RNA.

The TNF family ligands BAFF (also called BLyS) and APRIL regulate lymphocyte survival and activation (Cerutti 2008, Kimberley 2009, Mackay 2009). BAFF can bind to three receptors, BAFF-R, TACI and BCMA, whereas APRIL can interact with TACI, BCMA and proteoglycans. BAFF and APRIL contribute amongst other to B-cell and plasma-cell survival, CD154 (CD40L)-independent antibody isotype switching, germinal center maintenance, T cell-dependent and T cell-independent antibody responses, and T cell co-stimulation. Human BAFF is a 285-amino acid long peptide glycoprotein which undergoes glycosylation at residue 124. It is expressed as transmembrane protein on various cell types including monocytes, dendritic cells and bone marrow stromal cells.
Human APRIL is a 250-amino acid long peptide glycoprotein. It is expressed as transmembrane protein on various cell types including B cells, dendritic cells, monocytes, macrophages, epithelial cells, T cells and osteoclasts. Importantly, both BAFF and APRIL are expressed as membrane forms as well as secreted forms.

A fusion protein of the present invention comprises APRIL or BAFF or an APRIL-like or a BAFF-like compound as defined above. A fusion protein of the present invention thus comprises at least the extracellular domain of APRIL or BAFF or comprises an amino acid sequence having at least 80% sequence identity with said extracellular domain. The extracellular domain of human BAFF consists of the amino acids 183 – 323, wherein the numbers correspond to the numbers used in Figure 12. For human APRIL, the extracellular domain consists of the amino acids 115 – 250, wherein the numbers correspond to the numbers used in Figure 12. A skilled person is capable of determining the corresponding domain in APRIL or BAFF of other species. A fusion protein comprising APRIL or BAFF or an APRIL-like or BAFF-like compound is superior in eliciting an immune reaction to an antigen than fusion proteins comprising no co-stimulatory molecule or some other co-stimulatory molecule,

for instance CD40L. In a preferred embodiment, a fusion protein according to the invention is provided, wherein said antigen comprises a protein derived from a pathogen, preferably a virus protein, or an immunogenic part thereof.

Normally BAFF and APRIL act on their receptor counterpart in the form of a trimer. This can be either a heterotrimer or a homotrimer. Heterotrimers comprise for instance 1 APRIL protein and 2 BAFF proteins or for instance 2 APRIL proteins and 1 BAFF protein.
In another preferred embodiment, therefore, a fusion protein according to the invention is provided, further comprising a multimerizing polypeptide, preferably a trimerizing polypeptide. A trimerizing polypeptide, for instance an isoleucine zipper peptide, enables the formation of trimeric complexes comprising preferably three fusion proteins according to the invention.

As already mentioned, a fusion protein of the invention preferably comprises a virus protein or an immunogenic part thereof. One example of a virus for which vaccine development is hampered because of many of the problems discussed previously is human immunodeficiency virus (HIV). The classical vaccination approaches that have been successful in the control of various viral diseases by priming the adaptive immunity to recognize viral envelope proteins have failed for instance in the case of HIV. Reasons for the failure may be that the epitopes of the HIV viral envelope glycoprotein complex (Env) are too variable (due to HIV's high mutation rate) and that the functionally important epitopes of the HIV Env are masked by glycosylation, trimerisation and receptor-induced conformational changes, making it difficult to induce neutralizing antibodies to the virus.

Protein-based vaccines to HIV have failed to elicit protective immune responses. We improved the immunogenicity of HIV envelope glycoprotein (Env) based protein vaccines by fusion of an Env antigen to APRIL(-like) or

BAFF(-like) proteins which are co-stimulatory molecules that target the Env antigen directly to B cells and at the same time activate these cells. The present invention provides the surprising insight that APRIL and BAFF are superior to CD40L. Unexpectedly, CD40L is less efficient in inducing an immune response than APRIL and BAFF. This is a surprising finding, because one would expect CD40L to be efficient in inducing an immune response, as CD40L is known to target dendritic cells. Dendritic cells are very efficient antigen presenting cells and one would thus expect that CD40L would be very efficient in inducing an immune response. Use of CD40L as an adjuvant is therefore frequently suggested in the literature, for instance in virus vaccines or for tumor therapy. The present invention shows, however, that direct targeting of antigens to B cells via APRIL(-like) and BAFF(-like) proteins, is superior for subunit vaccines for HIV and other microbial diseases. The invention furthermore provides the insight that especially APRIL(-like) proteins are superior for subunit vaccines for several viruses, because APRIL preferably induces an IgA response, which is beneficial for protection against viruses that enter via the mucosal route, such as HIV, Ebola and Influenza. As IgA is an immunoglobulin present on mucosa, it is especially useful to induce an IgA response for strengthening the first line of defense against a virus that uses the mucosa for entry. Non-limiting examples of such viruses are HIV, Ebola and Influenza virus.

Although live-attenuated SIV/HIV vaccines have consistently elicited protective immune responses in monkey models and are thus also superior to subunit or protein-based vaccines, the use of live-attenuated HIV vaccines is considered to be unsafe for human use (Reynolds 2008). Recombinant viral vectors such as adenovirus that express HIV-1 proteins continue to be evaluated despite recent setbacks, but they have so far not elicited neutralizing antibody (NAb) responses efficiently (Liu 2009). Mucosal immunity against HIV-1 has also proven hard to elicit by any vaccine approach, a substantial problem considering that the virus is sexually transmitted (Shattock 2008).

Inducing high titers of broadly active NAbs is a major goal of many HIV vaccine approaches that has not yet been achieved. The most common approaches are based around protein subunit immunogens that mimic the native viral envelope glycoprotein complex (Env), which is the only target for NAbs. Unfortunately, most anti-Env antibodies are unable to neutralize primary HIV-1 isolates. Vaccines based on monomeric gp120 proteins failed to confer protection in efficacy trials (Flynn 2005, Pitisuttithum 2006). The difficulty in inducing NAbs is in part rooted in the structure of the Env complex, which has evolved multiple defenses that limit the induction and binding of such antibodies. Thus, various structural devices shield otherwise vulnerable conserved neutralization epitopes such as the receptor binding sites (Burton 2004, Eggink 2007, Kwong 2009), and highly immunogenic but non-neutralizing epitopes exposed on non-functional forms of Env serve as immune decoys (Parren 1997).

As already said above, one limitation to subunit protein vaccines in general (or DNA plasmid vaccines that encode such proteins) is their poor immunogenicity compared to live-attenuated or inactivated viral vaccines. Moreover, the HIV-1 Env proteins are particularly poor immunogens. Thus, the anti-Env titers in current studies with vaccinated individuals are relatively low compared to those raised against other protein antigens, and the anti-Env antibodies have an unusually short half-life of 30-60 days (Gilbert 2005). Other factors such as the magnitude and duration of the antibody response, affinity maturation and the induction of B cell memory are also relevant to the design of an effective B-cell vaccine against HIV-1. The poor performance of Env-based vaccines in these areas is rooted in the structure of the Env complex and how the latter interacts with the immune system. By providing additional stimulatory signals to B cells it is possible not only to increase the extent and duration of antibody production, but also improve their quality, probably because the increase in B cell stimulation promotes antibody affinity maturation (Delgado 2009). A few attempts to conjugate HIV-1 Env

immunogens to co-stimulatory molecules to improve antibody responses have been made, but with limited success (Bower 2004, Koch 2005). Another approach to the problem, using model antigens, showed that antigen targeting to dendritic cells (DC) via lectins such as DC-SIGN, DEC205, DCIR2 or Clec12A can augment antigen-specific immune responses (Bonifaz 2004, Dudziak 2007, Yang 2008, Lahoud 2009).
DC are thought to be critical in orchestrating efficient antibody and T cell responses and targeting vaccines to DC is thought to be the most efficient way to enhance the immunogenicity of vaccines (Steinman&Banchereau 2007). This is a reason why others have used CD40L as an adjuvant in vaccine development. In contrast to these approaches that aim at targeting vaccines to DC, the present invention uses APRIL(-like) and BAFF(-like) compounds which do not target dendritic cells. Instead, an antigen is directly targeted to B cells. Although dendritic cells, which play a central role in immunity are not targeted, antigenic constructs according to the invention appear to provide better immune responses as compared to currently known vaccines.

The invention shows for the first time that a fusion protein according to the invention is preferred for the development of an immunogenic composition for HIV. Of course, fusion proteins according to the invention are also useful for the development of an immunogenic composition for other pathogens, such as for instance Ebola virus and influenza virus.

In a preferred embodiment, therefore, a fusion protein according to the invention is provided, wherein said antigen has at least 80% sequence identity with a virus protein of HIV, influenza virus, or Ebola virus, or with an immunogenic part of a protein of any of these viruses. In a more preferred embodiment said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably least 98%, most preferably 100%.

In a more preferred embodiment, the invention provides a fusion protein according to the invention, wherein said virus protein is a gp120 protein or a gp160 protein of HIV, a hemagglutinin protein of influenza or a glycoprotein of Ebola.

An immunogenic part of a protein is defined herein as a part of a protein which is capable of eliciting an immune response in a human individual and/or a non-human animal. Preferably said immunogenic part is capable of eliciting the same immune response in kind, albeit not necessarily in amount, as said protein. The immune response elicited by said immunogenic part is preferably directed to the native (whole) protein as it is present in vivo, for instance on the surface of a pathogen. An immunogenic part of a protein preferably comprises one or more epitopes of said protein. An epitope of a protein is defined as a part of said protein, at least about 5 amino acids in length, capable of eliciting a specific antibody and/or immune cell capable of specifically binding said epitope. Two different kinds of epitopes exist: linear epitopes and conformational epitopes. A linear epitope comprises a stretch of consecutive amino acids. A conformational epitope is formed by several stretches of consecutive amino acids that are folded in position and together form an epitope in a properly folded protein. An immunogenic part of the invention is capable of comprising either one, or both, of said kinds of epitopes. An immunogenic part of a protein comprises at least 5 amino acid residues. Preferably said immunogenic part comprises at least 10, more preferably at least 15, more preferably at least 25 and most preferably at least 30 amino acids. Said immunogenic part preferably comprises at most about 500 amino acid residues, more preferably at most 250 amino acid residues, depending on the kind of protein from which said immunogenic part is derived.

The invention preferably makes use of APRIL or BAFF or BAFF-like or APRIL-like compounds because the invention shows that a fusion protein according to the invention comprising a BAFF(-like) protein or an APRIL(-like) protein is superior in inducing an immune response to an antigen as compared to a fusion protein comprising CD40L. APRIL and BAFF are important in inducing IgA secretion from mucosal B cells (Cerutti 2008, Xu 2008) and are therefore also superior in improving mucosal immunity to an antigen as compared to CD40L. Examples of pathogens that are preferably targeted at mucosal sites are HIV, hepatitis virus, influenza virus, and salmonella bacteria.

In a preferred embodiment, a fusion protein according to the invention is provided, wherein said inducing, enhancing or sustaining a B cell immune response involves immunoglobulin class switching, preferably a class switch to an immunoglobulin of class A (IgA). In more preferred embodiment, a fusion protein according to the invention is provided, wherein said ligand is an APRIL or APRIL-like protein.

In a working example, the invention shows that soluble trimeric gp140-APRIL/BAFF/CD40L fusion proteins can be expressed and trimerize efficiently, and bind CD4 and anti-Env Nabs demonstrating that the proteins are well folded. In another working example the invention shows that Env-APRIL and Env-BAFF induce higher titers of Env-specific antibodies in rabbits than Env alone or than Env fused to CD40L. Importantly, the virus neutralizing antibody responses were also improved, in particular with Env-APRIL. The invention further shows that rabbits primed with Env-APRIL induced an enhanced memory response upon recall with Env protein (without co-stimulatory molecule) and an Env-specific T cell response was enhanced when mice were immunized with Env-APRIL or Env-BAFF as compared to Env alone or Env-CD40L. Further, the examples show that Env-CD40L was

less effective than the APRIL or BAFF containing fusion proteins in eliciting B- or T-cell responses.

Although an antigen can be directly linked to an APRIL(-like) protein or a BAFF(-like) protein of the invention, it is preferred that the antigen is linked via a linker. Such linker for instance enables the correct folding of the BAFF(-like) or APRIL(-like) protein and of the antigen, independently from one another. The linker preferably supplies enough freedom for the two protein domains to not interact with each other and to not disturb the correct folding of the domains. Said linker preferably comprises an amino acid stretch with a preferred length of between 1 and 20 amino acids, preferably between 4 and 13 amino acids, most preferred with a length of 11 amino acids.
Preferably, said linker is a glycine-rich linker, preferably comprising an amino acid sequence selected from the group consisting of GGGX, GGGXGGG, GGGGXGGGGXGGG, GGGGXGGGGXGGGGXGGG, GGXGGG, and GGGGXGGGGXG, wherein X is an amino acid selected from the group consisting of Arginine (R), Threonine (T) and Serine (S).

In a preferred embodiment, therefore, a fusion protein according to the invention is provided, wherein said antigen is indirectly linked to said APRIL(like) or BAFF(-like) protein via a linker comprising an amino acid sequence with a length of between 1 and 20 amino acids, preferably between 4 and 13 amino acids, most preferably 11 amino acids. In a preferred embodiment, said linker is a glycine-rich linker, preferably comprising an amino acid sequence selected from the group consisting of GGGX, GGGXGGG, GGGGXGGGGXGGG, GGGGXGGGGXGGGGXGGG, GGXGGG, and GGGGXGGGGXG, wherein X is an amino acid selected from the group consisting of Arginine (R), Threonine (T) and Serine (S).

A fusion protein comprising an APRIL-like compound or a BAFF-like compound of the invention linked directly or indirectly via a linker, to an antigen in one embodiment results in a monomer.

However, a multimer is preferred, because, as said before, APRIL and BAFF exert their function as a trimer. Also, for instance the Env protein of HIV forms trimeric complexes on the surface of a HIV particle. In order to produce a trimeric complex of fusion proteins according to the invention, a multimerizing polypeptide is preferably introduced in said fusion proteins. Said multimerizing polypeptide is preferably inserted in a fusion protein according to the invention between a BAFF(-like) or APRIL(-like) protein and an antigen. Multimerization of multiple fusion proteins according to the invention, however, can result in suboptimal conformation of the BAFF(-like) or APRIL(-like) protein or the antigen when the polypeptide is coupled directly to the compound or the antigen. It is therefore preferred that a linker is introduced between the BAFF(-like) or APRIL(-like) protein and the multimerizing polypeptide. It is also preferred to introduce a linker between the multimerizing polypeptide and the antigen.
Such linker enables the different parts forming a trimeric complex to adapt a conformation that is not restricted by the multimerizing polypeptide. As shown schematically in figure YYY, a complex according to the invention preferably enables the parts that for instance form a BAFF-like or APRIL-like trimer to have some degrees of freedom in order to form a conformation resembling a native conformation. As said above, said linker also enables the correct folding of the BAFF(-like) or APRIL(-like) protein and of the antigen, independently from one another within each monomeric fusion protein. In one preferred embodiment, a fusion protein according to the invention thus comprises an antigen, linked via a first linker to a multimerizing peptide, said multimerizing peptide being linked via a second linker to an APRIL-like or BAFF-like compound. Preferably said first and said second linker have, independently

from one another, a length of between 1 and 20 amino acids, preferably between 4 and 13 amino acids, more preferred with a length of 6 or 11 amino acids. It is most preferred that said first linker has a length of 6 amino acids and said second linker has a length of 11 amino acids. Preferably, said first and/or said second linker is a glycine-rich linker comprising an amino acid sequence selected from the group consisting of GGGX, GGGXGGG, GGGGXGGGGXGGG, GGGGXGGGGXGGGGXGGG, GGXGGG, and GGGGXGGGGXG, wherein X is an amino acid selected from the group consisting of Arginine (R), Threonine (T) and Serine (S).

. Preferably said first linker comprises an amino acid sequence consisting of GGRGGG and said second linker comprises an amino acid sequence consisting of GGGGTGGGGTG. The combination of these two linkers in a fusion protein according to invention enables both, the correct folding of the BAFF(-like) or APRIL(-like) protein and of the antigen, independently from one another within each monomeric fusion protein, and the trimeric complex adapting a conformation that is not restricted by the multimerizing polypeptide.

As the invention provides a fusion protein useful for vaccine development, the invention also provides a nucleic acid molecule encoding a fusion protein according to the invention. Such a nucleic acid molecule can for instance be inserted into a vector or a virus and be used for expressing said fusion protein using a suitable expression system.

As already said above, BAFF and APRIL generally act as trimeric complexes. A trimeric complex of a fusion protein according to the invention is thus preferably used. The invention thus provides an oligomeric complex comprising at least 1, preferably at least 2, more preferably at least 3 fusion protein(s) according to the invention.

In a preferred embodiment, an oligomeric complex according to the invention is provided, comprising a multitude of trimeric fusion proteins according to the

invention. Such polytrimeric constructs form, as the name implies, a multitude of trimers.

Although BAFF and APRIL function when they are trimeric, optimal activation is achieved when they are multimeric (i.e. a multitude of trimers). Multimeric fusion constructs are therefore preferably created. As an additional advantage increased multimerization also contributes to enhanced Env-BCR cross-linking and enhanced B cell activation. Thus, when HIV-Env is used as an antigen, a multimer is preferably used.

Of course, other proteins or for instance particles can be used to form polytrimeric constructs. The inventors show in working examples that this concept works for instance with fusion proteins of the invention immobilized on nanoparticles when the C-terminal trimerization domain is fused to a C-terminal Histidine tag that enables immobilization on a Ni-NTA coated nanoparticle. In addition it is shown that fusion proteins of the invention can be immobilized on nanoparticles when the C-terminal trimerization domain is fused to the Fc part of immunoglobulin G, resulting in hexameric molecules, via binding of the Fc domain to protein A/G on nanoparticles.

Methods for producing a fusion protein according to the invention are also provided. This is for instance done by expressing a nucleic acid sequence encoding a fusion protein according to the invention.

The invention therefore further provides a method for producing a fusion protein according to the invention, said method comprising generating/providing a nucleic acid molecule encoding a fusion protein according to the invention and expressing said nucleic acid molecule in a suitable expression system. This is for instance done by generating a nucleic acid molecule comprising a first nucleic acid sequence encoding an antigen and a second nucleic acid sequence encoding an amino acid sequence with at least 80% sequence identity with at least the extracellular domain of APRIL and/or BAFF. In a preferred embodiment, said nucleic acid molecule further comprises, between said first and said second nucleic acid sequence, a nucleic

sequence encoding a polypeptide, which is a linker. In another preferred embodiment said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably least 98%, most preferably 100%.

As already said before, a complex of multiple fusion proteins according to the invention is preferred. Therefore, in another preferred embodiment, said nucleic acid molecule further comprises a third nucleic acid sequence encoding a multimerizing polypeptide. In a more preferred embodiment, said nucleic acid molecule further comprises, between said first and said second nucleic acid sequence another nucleic acid sequence encoding a polypeptide, which is a first linker, and between said second and said third nucleotide sequence yet another nucleotide sequence encoding yet another polypeptide, which is a second linker. Said first and said second linker can have different lengths and/or different amino acid sequences, but they can also be identical. In a more preferred embodiment, said first and said second linker comprise an amino acid sequence having, independently from one another, a preferred length of between 1 and 20 amino acids, preferably between 4 and 13 amino acids, more preferred with a length of 6 or 11 amino acids. It is most preferred that said first linker has a length of 6 amino acids and said second linker has a length of 11 amino acids. As already outlined above, such linker is preferred because it enables the correct folding of the BAFF(-like) or APRIL(-like) protein and of the antigen, independently from one another, and it enables the different parts forming a trimeric complex to adapt a conformation that is not restricted by the multimerizing polypeptide. In a more preferred embodiment said first and/or said second linker comprise amino acid sequence selected from the group consisting of GGGX, GGGXGGG, GGGGXGGGGXGGG, GGGGXGGGGXGGGGXGGG, GGXGGG, and GGGGXGGGGXG, wherein X is an amino acid selected from the group consisting of Arginine (R), Threonine (T) and Serine (S).

. In a most preferred embodiment, said first linker comprises a polypeptide having the amino acid sequence GGRGGG and said second linker comprises a polypeptide having the amino acid sequence GGGGTGGGGTG.

In a preferred embodiment, said allowing expression of said fusion protein from said nucleic acid molecule comprises expression of said fusion protein from said nucleic acid molecule in a virus.

Now that the invention provides fusion proteins, methods, complexes and nucleic acid molecules according to the invention, in yet another embodiment, the invention provides a virus comprising a fusion protein according to the invention or obtainable by a method according to the invention. A virus comprising a complex and/or a nucleic acid sequence according to the invention is also provided. Such a virus is especially useful for preparing an immunogenic composition as such a virus is still capable of reproduction. An advantage of a reproducing virus is that less virions are needed for one vaccine dose, as the virus is able to replicate further in the host. Furthermore, such a virus comprises an APRIL(-like) or BAFF(-like) protein which are very efficient in inducing, sustaining or improving an immune response. In a preferred embodiment, a virus comprising an APRIL(-like) protein is provided, which is very efficient in inducing, sustaining or improving an IgA response. For an immunogenic composition of the invention, a fusion protein, a complex, and/or a nucleic acid according to the invention can also be used. In yet another embodiment therefore, the invention provides an immunogenic composition comprising a fusion protein according to the invention or a fusion protein obtainable by a method according to the invention, and/or comprising a complex, a nucleic acid, and/or a virus according to the invention. An immunogenic composition according to the invention is especially useful for developing a vaccine for use in preventing, treating and/or diminishing an infection with a pathogen. In a preferred embodiment therefore, an immunogenic composition according to the invention comprises a vaccine. Said

vaccine preferably comprises a suitable adjuvant such as for instance Specol or a double oil emulsion.

In one embodiment, an immunogenic composition is provided which comprises at least one fusion protein according to the invention. Preferably, said composition comprises a complex of at least three fusion proteins according to the invention. In another preferred embodiment, however, an immunogenic composition is provided which comprises a virus, which virus comprises at least one fusion protein according to the invention. As stated above, an immunogenic composition comprising a virus according to the invention is especially useful because such virus is preferably capable of replicating to some extent in the host. Said fusion protein is preferably at least in part present on the surface of said virus, so that said fusion protein is exposed to an immune response of an animal and/or human. Said virus preferably comprises an attenuated virus, so that the virus's capability of spreading upon administration to a subject is diminished as compared to a wild-type virus. It is useful to have some spreading in the host in order to increase antigen exposure, but a virus according to the invention preferably does not manifest itself as a full-blown infection. In one embodiment, said virus is an attenuated HIV virus which is especially useful for an AIDS vaccine. Use of a live attenuated HIV vaccine has however generally been avoided by vaccine developers, because of the fear that the attenuated vaccine strain could revert over time to a virulent and pathogenic phenotype, raising serious safety concerns. It is therefore that preferably another kind of viral vector is used, for instance a viral vector based on a virus such as, but not limited to, Sindis virus, Semliki (like) Forest virus, canarypox virus, chicken pox virus, or Vaccina virus.

An immunogenic composition according to the invention is especially useful for developing a vaccine for use in at least in part preventing, treating and/or diminishing a virus infection, especially an HIV infection. In a preferred embodiment therefore, an immunogenic composition according to the invention

comprises a vaccine. An immunogenic composition according to the invention for use in at least in part preventing, treating and/or diminishing an infection, preferably a virus infection, more preferably an infection selected from the group consisting of HIV infection, influenza virus infection and Ebola virus infection is also provided. Also provided is a fusion protein according to the invention or obtainable by a method according to the invention, and/or a complex, a nucleic acid molecule, a virus, and/or an immunogenic composition according to the invention, for use as a medicament and/or prophylactic agent. An immunogenic composition according to the invention is preferably used for inducing or enhancing an immune response specific for a virus, preferably HIV, influenza virus or Ebola virus. A fusion protein of the invention, or a nucleic acid molecule encoding said fusion protein, or a complex or a virus comprising said fusion protein is especially useful for the purpose of inducing or enhancing an immune response specific for a virus, preferably HIV, influenza virus or Ebola virus. Fusion proteins according to the invention allow an immune system of an animal and/or human to recognize at least one part, called epitope, of a virus protein, such as for instance of the HIV envelope glycoprotein or of a hemagglutinin protein of influenza or of a glycoprotein of Ebola, that is shielded in a wild-type virus without the modifications of the invention. A fusion protein of the invention is especially useful for this purpose, because it comprises an antigen and a BAFF(-like) compound or an APRIL(-like) compound, which are very efficient in inducing, sustaining and/or improving an immune response to said antigen. Many viruses enter the human body via the mucosa. It is known that especially IgA plays an important role in mucosal immunological first line of defense. In a preferred embodiment, the invention thus provides a fusion protein for use in inducing an immune response to an antigen, wherein said immune response comprises an IgA respons. In a more preferred embodiment, said antigen is a virus antigen. The immune response thus generated by a fusion protein of the invention allows for the induction of antibodies, preferably IgA antibodies, directed

against said epitope. In one embodiment, said epitope comprises at least part of a conserved epitope of the gp120 protein and/or at least part of a conserved epitope of the gp160 protein and/or at least part of a conserved epitope of the hemagglutinin protein of influenza and/or at least part of a conserved epitope of the glycoprotein of Ebola. Preferably said epitope comprises the receptor binding site of the gp120 protein or of the hemagglutinin protein of influenza or of the glycoprotein of Ebola. Antibodies directed against at least part of a conserved epitope will, since the epitope is conserved, bind several strains of a pathogen. If said epitope is for instance present on a receptor binding-site, an antibody directed towards said epitope will at least partially inhibit a function of the protein, for instance binding of the receptor binding-site of an Env protein to the CD4 receptor on T-cells, thereby for instance inhibiting entry of the virus into said T cell.

In another preferred embodiment, however, said epitope comprises another epitope such as for instance a conformational mannose epitope in gp120, or a membrane proximal region in gp41. Antibodies that are capable of inhibiting function of a pathogen are called neutralizing antibodies, because they are able to neutralize the function of a protein from said pathogen such that the pathogen is deficient in its capability of performing at least one function. For instance the Env protein enables a HIV virus to enter a T cell and spread infection. A neutralizing antibody binding an Env protein will thus prevent a HIV virus entering a T cell, or at least slow down the process of entering a T cell. With deficient is meant herein that the pathogen has a diminished capability of performing at least one of its functions, for example a HIV virus is slowed down in entering a T cell or the virus is completely unable to enter the T cell. It is especially useful if said pathogen is deficient in such a way that the host is able to combat said pathogen and spread of infection is prevented, halted or slowed down.

In a preferred embodiment therefore, the invention provides a fusion protein according to the invention or a fusion protein obtainable by a method

according to the invention, and/or a complex, a nucleic acid molecule, a virus, and/or an immunogenic composition according to the invention for use in inducing, enhancing and/or sustaining an immune response specific for a virus, preferably HIV, influenza virus or Ebola virus. In a more preferred embodiment, said immune response comprises production of an antibody, preferably a neutralizing antibody.

In an even more preferred embodiment, said antibody is specific for HIV, influenza virus or Ebola virus. As said before, it is preferred that an antibody is specific for a conserved amino acid sequence of the envelope glycoprotein complex (Env) of HIV, or specific for a conserved amino acid sequence of the hemagglutinin protein of influenza virus, or specific for a conserved amino acid sequence of a glycoprotein of Ebola virus because an antibody to a conserved epitope is capable of binding several strains. More preferred, said antibody is specific for a CD4 receptor binding-site of HIV.

As a fusion protein, complex, nucleic acid molecule, virus or composition according to the invention is especially useful for inducing, enhancing, and/or improving an immune response against a virus, in yet another embodiment, the invention provides the use of a fusion protein according to the invention or obtainable by a method according to the invention, and/or use of a complex, a nucleic acid molecule, a virus, and/or an immunogenic composition according to the invention for the preparation of a medicament or prophylactic agent for inducing or enhancing an immune response specific for a virus, preferably a human immunodeficiency virus (HIV), influenza virus or Ebola virus.

The invention thus provides means and methods for inducing an antibody response to an antigen, preferably a virus protein, more preferably a virus protein of HIV, influenza virus or Ebola virus. The means and methods according to the invention are especially useful for inducing an improved antibody response because APRIL(-like) or BAFF(-like) proteins are used in said means and methods. Preferably APRIL(-like) proteins are used in said

means and methods. The present invention shows that APRIL(-like) or BAFF(-like) proteins are superior in improving an antibody response when compared to the antigen alone or even when compared to the antigen coupled to another co-stimulatory molecule, for instance CD40L. The use of antibodies to an antigen is thought to be especially useful in at least in part preventing or treating infection when a subject is thought to be very recently infected or if an individual is at risk of getting infected. Use of antibodies for such a purpose is known under the term "passive vaccination". Passive vaccination is used under certain circumstances, sometimes in combination with active vaccination. The passive component of such a combination quickly counteracts the pathogen which has or may have entered the body, whereas the active vaccine counteracts subsequent infections. This concept is also especially useful in the present invention. For instance, a person involved in a so called "prick or puncture accident" in a hospital setting may be first administered a passive vaccine, containing antibodies obtained using a fusion protein according to the invention specific for the pathogen. Preferably said antibodies are neutralizing antibodies. Optionally, such an individual may thereafter be actively immunized with a fusion protein, a complex, a nucleic acid, a virus, and/or an immunogenic composition according to the invention, for instance if said individual still suffers or is at risk of suffering from an infection with the pathogen. In such a case, the passive immunization will counteract penetrated pathogens, for instance virus particles, thereby counteracting spread of infection within the host, whereas a subsequent active immunization will induce antibodies that counteract pathogens that were not neutralized by the passive vaccine, but also any future infections with the same or a similar pathogen. In another situation, it may be preferred to only use passive immunization, for instance if the host is immunocompromised and active immunization is either impossible or dangerous to the host. In another situation, only active immunization maybe preferred, for instance if it is

unclear if and when an infection may occur and when it is useful to induce (protective) immunity in the host as a preventive measure.
By employing forced virus evolution to select for improved Env deletion variants the inventors previously obtained functionally improved Env variants lacking at least part of the V1/V2 domain. Functionally improved variants were obtained wherein for instance at least part of the V1/V2 domain of HIV-Env was deleted and/or wherein amino acids within the V1/V2 domain of HIV-Env had been exchanged. This is described in more detail in PCT/NL2009/050609, which is incorporated herein by reference. Compensatory changes in gp120, but also in gp41 were identified that improve folding and secretion of stable loop-deleted Env trimers and that benefit the generation of recombinant Env trimers for vaccine and structural studies. The present invention thus also encompasses a fusion protein according to the invention, wherein at least part of the V1/V2 domain of HIV-Env is deleted and/or wherein amino acids within the V1/V2 domain of HIV-Env have been exchanged. Such fusion protein has at least two advantages. First, the deletions and/or amino acid exchanges within the V1 and/or V2 loop improve the folding and secretion of the Env trimers. Second, because of the inclusion of at least one APRIL(-like) or BAFF(-like) compound, such fusion protein is very well capable of inducing, sustaining and/or improving an immune response to said improved folded Env trimer. For the amino acid numbering reference is made to the alignment of gp120 of different strains as depicted in Figure 40, whereas the variant numbers refer to the structural variants as depicted in Figure 39.

In respect of the above, the invention thus provides in a preferred embodiment, a fusion protein and/or a method and/or a complex according to the invention, wherein said fusion protein comprises a polypeptide with a length of at least 300 amino acids with at least 80% sequence identity to a gp120 protein or gp160 protein of HIV with a deletion of at least 5 amino acids, preferably at least 10, more preferably at least 20, more preferably at least 40, most

preferably at least 60 amino acids in the region corresponding to amino acid positions 120-204 of HXB2, wherein the amino acid positions are indicated in Figure 40. For other isolates, the corresponding gp120 region is determined by conventional alignment with at least one sequence of Figure 40. Preferably, said deletion comprises at least 10, more preferably at least 20, even more preferably at least 40, most preferably at least 60 amino acids in the region comprising, or corresponding to said amino acid positions 120-204. In a more preferred embodiment said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably least 98%, most preferably 100%. In another preferred embodiment, said polypeptide has a length of at least 350, more preferred at least 375, more preferred at least 400, more preferred at least 425, more preferred at least 450, most preferred at least 475 amino acids. As already stated above, such deletions improve the secretion and/or folding of Env timers. Furthermore, it is possible, as the inventors have shown in a working example, to insert other amino acid sequences at the site of deletion.

There are advantages to inserting an amino acid sequence at the site of deletion in a gp120 or gp160 protein of HIV. In a working example, the inventors have shown for instance that by deleting part of the V1 and V2 loop and replacing the deleted part by an amino acid sequence encoding a GMCSF protein, an Env-specific immune response is induced that is superior to that elicited by a protein without the GMCSF inserted. The invention thus provides proof of principle that replacing at least part of the V1 and/or V2 loop and replacing the deleted part by an amino acid sequence coding for a co-stimulatory molecule, such as a cytokine, improves an immune response towards the Env protein of HIV.

In one embodiment, therefore, the invention provides a fusion protein comprising an antigen and a polypeptide comprising an amino acid sequence having at least 80% sequence identity with a cytokine capable of inducing or

sustaining a B cell immune response. In a preferred embodiment, said sequence identities are, independently from one another at least 85%, preferably at least 90%, more preferably at least 95%, more preferably at least 98%, most preferably 100%.

The polypeptide comprising an amino acid sequence having at least 80%, preferably at least 85%, preferably at least 90%, more preferably at least 95%, more preferably at least 98%, most preferably 100% sequence identity with a cytokine are preferably linked to the antigen directly at the C-terminal or N-terminal end of the antigen sequence. It is also possible to use linkers or spacers for coupling the cytokine sequence to the antigen sequence.

In a preferred embodiment, the polypeptide is not coupled to the C-terminal or N-terminal amino acid, but instead inserted in the antigen sequence, such that it is flanked by two amino acid sequences of the antigenic sequence. It is preferred that a part of the antigen sequence is deleted and that the cytokine sequence is inserted at this deletion site. In a preferred embodiment, the antigen is a HIV antigen. In a most preferred embodiment, the invention provides a fusion protein according to the invention, wherein said antigen comprises an amino acid sequence with a length of at least 300 amino acids with at least 80% sequence identity to a gp120 protein and/or a gp160 protein of HIV envelope glycoprotein complex (Env), wherein at least 5 amino acids of the V1 loop and/or at least 5 amino acids of the V2 loop of said gp120 molecule are absent, and wherein at the deletion site in V1 and/or at the deletion site in V2, said polypeptide is inserted. In a preferred embodiment, said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably at least 98%, most preferably 100%.

In a preferred embodiment, a fusion protein according to the invention is provided, wherein said cytokine is GMCSF or IL-21.

In one embodiment, the invention thus provides a fusion protein comprising an amino acid sequence with a length of at least 300 amino acids with at least

80% sequence identity to a gp120 protein or a gp160 protein of HIV envelope glycoprotein complex (Env), wherein at least 5 amino acids of the V1 loop and/or at least 5 amino acids of the V2 loop of said gp120 molecule are absent, and wherein at the deletion site in V1 and/or at the deletion site in V2, a polypeptide is inserted with at least 80% sequence identity with a co-stimulatory molecule. Said co-stimulatory molecule is preferably selected from the group consisting of GMCSF, IL2, IL4, IL-5, IL-10, IFNγ, IL12, IL17, IL-21, IL-23 and TSLP. In a particularly preferred embodiment, said co-stimulatory molecule is a cytokine selected from GMCSF and IL-21. In another preferred embodiment said sequence identities are, independently from one another at least 85%, preferably at least 90%, more preferably at least 95%, more preferably at least 98%, most preferably 100%. In another preferred embodiment, said polypeptide has a length of at least 350, more preferred at least 375, more preferred at least 400, more preferred at least 425, more preferred at least 450, most preferred at least 475 amino acids.

In a preferred embodiment, said fusion protein also comprises an APRIL(-like) or BAFF(-like) protein, preferably an APRIL(-like) protein. This is, however, not necessary since the present inventors have demonstrated that the introduction of a co-stimulatory molecule into the V1 loop and/or V2 loop also results in an improved immune response in the absence of APRIL or BAFF.

A method for production of a fusion protein according the invention is also provided, said method comprising

a) generating or providing a nucleic acid molecule comprising a nucleotide sequence encoding:

an amino acid sequence with a length of at least 300 amino acids with at least 80% sequence identity to a gp120 protein or a gp160 protein of HIV envelope glycoprotein complex (Env), wherein at least 5 amino acids of the V1 loop and/or at least 5 amino acids of the V2 loop are deleted, and wherein an amino acid sequence with at least 80% sequence identity with a co-stimulatory molecule is inserted at the site of deletion in V1 and/or V2; and

b) allowing expression of said amino acid sequence from said nucleic acid in a suitable expression system.

In a more preferred embodiment said sequence identities are, independently from one another at least 85%, preferably at least 90%, more preferably at least 95%, more preferably at least 98%, most preferably 100%. Said co-stimulatory molecule is preferably selected from the group consisting of GMCSF, IL2, IL4, IL-5, IL-10, IFNγ, IL12, IL17, IL-21, IL-23 and TSLP. In a particularly preferred embodiment, said co-stimulatory molecule is GMCSF. In another more preferred embodiment, said polypeptide has a length of at least 350, more preferred at least 375, more preferred at least 400, more preferred at least 425, more preferred at least 450, most preferred at least 475 amino acids. A fusion protein of the invention is useful for inducing, enhancing and/or improving an immune reaction to a gp120 and/or gp160 protein of HIV, especially when a fusion protein according to the invention is used that comprises both an APRIL(-like) protein and/or a BAFF(-like) protein, and a co-stimulatory molecule at a site of deletion in the V1 and/or V2 loop of the Env protein. Such a fusion protein enhances an immune response to the Env protein both through the APRIL(-like) or (BAFF-like) protein and through the co-stimulatory molecule present within the Env protein sequence. Combinations are preferred that activate immune cells, in particular B cells, synergistically.

In a preferred embodiment, the invention thus provides a fusion protein comprising an amino acid sequence with a length of at least 300 amino acids with at least 80% sequence identity to a gp120 protein or a gp160 protein of HIV envelope glycoprotein complex (Env), wherein at least 5 amino acids of the V1 loop and at least 5 amino acids of the V2 loop of said gp120 molecule are absent, and wherein at the deletion site in V1 or at the deletion site in V2, a polypeptide is inserted with at least 80% sequence identity with a co-stimulatory molecule, preferably selected from the group consisting of GMCSF,

IL2, IL4, IL-5, IL-10, IFNγ, IL12, IL17, IL-21, IL-23 and TSLP, wherein said fusion protein further comprises a compound selected from the group consisting of a compound comprising at least the extracellular domain of APRIL, and/or BAFF, and compounds having at least 80% sequence identity with at least the extracellular domain of APRIL and/or BAFF. In a more preferred embodiment said sequence identities are, independently from one another at least 85%, preferably at least 90%, more preferably at least 95%, more preferably least 98%, most preferably 100%. In another more preferred embodiment, said polypeptide has a length of at least 350, more preferred at least 375, more preferred at least 400, more preferred at least 425, more preferred at least 450, most preferred at least 475 amino acids. Such fusion proteins induce a vigorous immune response against the Env protein because, as said before, it comprises multiple features that improve said immune response.

Using forced virus evolution experiments the inventors frequently observed deletions in the region corresponding to amino acid positions 127-195 of HXB2, which led to functionally improved Env variants.

In another preferred embodiment, therefore, the invention provides a fusion protein and/or a method and/or a complex according to the invention, wherein said fusion protein comprises a polypeptide with a length of at least 300 amino acids with 80% sequence identity to a gp120 protein of HIV with a deletion of at least 5 amino acids, preferably at least 10, more preferably at least 20, more preferably at least 40, more preferably at least 60, even more preferably at least 67, most preferably 69 amino acids in the region corresponding to amino acid positions 127-195 of HIV-virus reference strain HXB2, wherein the amino acid positions are indicated in Figure 40. In a more preferred embodiment said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably least 98%, most preferably 100%. In another more preferred embodiment, said polypeptide has

a length of at least 350, more preferred at least 375, more preferred at least 400, more preferred at least 425, more preferred at least 450, most preferred at least 475 amino acids. As said above, HXB2 is the HIV-virus reference strain to which amino acid numbering is generally adhered to. This by no means implies that the invention is limited to HXB2. It is for instance preferred to use an amino acid sequence of subtype C of HIV in a fusion protein according to the invention for use in Africa and subtype B of HIV in a fusion protein according to the invention for use in the United States and Europe, as these are predominant strains in the respective areas.

Even more frequently, the inventors observed functionally improved Env variants in their forced virus evolution experiments that had deletions in the region corresponding to amino acid positions 142 to 148 and/or deletions in the region corresponding to amino acid positions 168 to 203 of HXB2.

In yet another preferred embodiment, therefore, a fusion protein and/or a method and/or a complex according to the invention is provided, wherein said fusion protein comprises a polypeptide with a length of at least 300 amino acids with at least 80% sequence identity to a gp120 protein of HIV with a deletion of at least 5 amino acids in the region corresponding to amino acid positions 142 to 148 of HXB2 and/or a deletion of at least 5 amino acids, preferably at least 10, more preferably at least 20, more preferably at least 30, most preferably 36 amino acids in the region corresponding to amino acid positions 168 to 203 of HXB2, wherein the amino acid positions are indicated in Figure 40. In a more preferred embodiment said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably least 98%, most preferably 100%. In another more preferred embodiment, said polypeptide has a length of at least 350, more preferred at least 375, more preferred at least 400, more preferred at least 425, more preferred at least 450, most preferred at least 475 amino acids.

Most frequently, the inventors observed deletions in the region corresponding to amino acid positions 133-155 of HXB2 and deletions in the region corresponding to amino acid positions 159-194 of HXB2. In an even more preferred embodiment, the invention therefore provides a fusion protein and/or a method and/or a complex according to the invention, wherein said fusion protein comprises a polypeptide with a length of at least 300 amino acids with at least 80% sequence identity to a gp120 protein of HIV with a deletion of at least 5 amino acids, preferably at least 10, more preferably at least 20, more preferably 23, in the region corresponding to amino acid positions 133-155 of HXB2 and/or a deletion of at least 5 amino acids, preferably at least 10, more preferably at least 20, even more preferably at least 30, most preferably 36, in the region corresponding to amino acid positions 159-194 of HXB2, wherein the amino acid positions are indicated in Figure 40. In a more preferred embodiment said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably least 98%, most preferably 100%. In another more preferred embodiment, said polypeptide has a length of at least 350, more preferred at least 375, more preferred at least 400, more preferred at least 425, more preferred at least 450, most preferred at least 475 amino acids.

The inventors have found that it is especially useful to delete one or more of the disulfide bonds in the gp120 molecule involved in protein folding. Especially deletion of the disulfide bridges formed between cysteines at positions corresponding to position 126 and 196 and between cysteines at positions corresponding to position 131 and position 157 of HXB2 result in improved folding and/or secretion of a fusion protein according to the invention. In one preferred embodiment therefore, the invention provides a fusion protein and/or a method and/or a complex according to the invention, wherein said fusion protein comprises a polypeptide with a length of at least 300 amino

acids with at least 80% sequence identity to a gp120 protein of HIV and wherein at least one cysteine at a position corresponding to position 126 and/or position 196 and/or position 131 and/or position 157 of HXB2 is exchanged for another amino acid, preferably for another non-hydrophobic amino acid(s), more preferably for alanine, wherein the amino acid positions are indicated in Figure 40. In a more preferred embodiment said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably least 98%, most preferably 100%. In another more preferred embodiment, said polypeptide has a length of at least 350, more preferred at least 375, more preferred at least 400, more preferred at least 425, more preferred at least 450, most preferred at least 475 amino acids.

In yet another embodiment, at least two cysteines of gp120 are replaced. A fusion protein useful in the invention is obtained for example by substituting the cysteines at positions 126 and at position 196 of gp120 for another non-hydrophobic amino acid, for instance alanine. Another fusion protein useful in the invention is obtained for example by substituting cysteines at position 131 and 196 for another non-hydrophobic amino acid, for instance alanine. Therefore, in yet another preferred embodiment, a fusion protein and/or a method and/or a complex according to the invention is provided, wherein said fusion protein comprises a polypeptide with a length of at least 300 amino acids with at least 80% sequence identity to a gp120 protein of HIV and wherein at least two cysteines at a position corresponding to position 126 and to position 196, or to position 131 and to position 196 of HXB2 are exchanged into another amino acid, preferably into another non-hydrophobic amino acid, more preferably into alanine, wherein the amino acid positions are indicated in Figure 40. In a more preferred embodiment said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably least 98%, most preferably 100%. In another more preferred embodiment, said polypeptide has a length of at least 350, more preferred at least 375, more

preferred at least 400, more preferred at least 425, more preferred at least 450, most preferred at least 475 amino acids. It is of course also possible to delete or modify at least one cysteine at a position corresponding to position 126 and/or position 196 and/or position 131 and/or position 157 of HXB2 in order to delete at least one disulfide bond in the gp120 molecule.

N-linked carbohydrates are frequently lost upon optimization of a fusion protein useful in the invention, namely at positions corresponding to positions 156, 234, 295, 301, 339 and 625 of HXB2. This implicates that loss of these N-linked carbohydrates improves folding and/or secretion of a fusion protein of the invention.

In a preferred embodiment therefore, a fusion protein and/or a method and/or a complex according to the invention is provided, wherein said fusion protein comprises polypeptide with a length of at least 300 amino acids with at least 80% sequence identity to a gp120 protein of HIV with at least one amino acid exchange comprising loss of a glycosylation site at a position corresponding to amino acid position 156, 234, 295, 301, 339 and/or 625 of HXB2, wherein the amino acid positions are indicated in Figure 40. In a more preferred embodiment, said at least one amino acid exchange comprises loss of a glycosylation site corresponding to amino acid position 156 of HXB2. As said before, the numbering of the amino acid positions is shown in Figure 40. For other HIV isolates, the corresponding amino acid positions are determined by alignment with at least one sequence of Figure 40. As used herein, a reference to an amino acid position therefore also encompasses the corresponding amino acid position in a different HIV strain. In a more preferred embodiment said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably least 98%, most preferably 100%. In another more preferred embodiment, said polypeptide has a length of at least 350, more preferred at least 375, more preferred at least 400, more preferred at least 425, more preferred at least 450, most preferred at least 475 amino acids.

With the term "loss of a glycosylation site" is meant a situation in which said glycosylation site is no longer capable of being glycosylated and/or in which said glycosylation site is no longer present. Inhibiting glycosylation is achieved in various ways. It is for instance possible to delete, substitute and/or insert amino acids near said glycosylation site, such that glycosylation is no longer possible or at least inhibited. A person skilled in the art is aware of the fact that N-glycosylation sites comprise in general a consensus sequence consisting of Asp-Xaa-Ser or Asp-Xaa-Thr, wherein Xaa can be any natural amino acid (except proline) or functional equivalent thereof, for instance a spacer that introduces a similar space between the first aspartate and the third amino acid (serine or threonine) in said above consensus amino acid sequence. Said loss of a glycosylation site can thus be achieved by deleting or exchanging asparagine and/or serine or threonine in said consensus sequence. Further said loss can be achieved by deletion of said natural amino acid or functional equivalent thereof in said consensus sequence, or by insertion of an amino acid and/or functional equivalent thereof in said consensus sequence such, that the new sequence no longer comprises a consensus sequence for glycosylation, and/or exchanging Xaa in said consensus sequence with a proline, leading to inhibition and/or abrogation of glycosylation at said site.

In a preferred embodiment therefore, a fusion protein and/or a method and/or a complex according to the invention is provided, wherein said loss of a glycosylation site comprises a mutation in the N-glycosylation consensus sequence Asp-Xaa-Ser or Asp-Xaa-Thr, wherein Xaa is any natural amino acid except proline, such that the resulting sequence no longer comprises said consensus sequence.

As said before, the loss of a glycosylation site is especially useful in a fusion protein of the invention. On the other hand, a glycosylation site can also be beneficial for improving folding and/or secretion of a fusion protein, a complex, and/or a method according to the invention. This is especially true for

the asparagine on position 197. Some of the HIV strains do not possess a glycosylation site at this position, such as strain WT JR-FL, others do. It has been observed that retaining or introducing a glycosylation site on or near position 197 is especially useful, for instance for correct folding of the Env protein. In one preferred embodiment therefore, a fusion protein and/or a method and/or a complex according to the invention is provided, wherein said fusion protein comprises a polypeptide with a length of at least 300 amino acids with at least 80% sequence identity to a gp120 protein of HIV with at least one amino acid exchange comprising retaining or introducing a glycosylation site at a position corresponding to amino acid position 197 of HXB2, preferably retaining or introducing an asparagine at a position corresponding to amino acid position 197 of HXB2 and a serine/threonine at a position corresponding to amino acid position 199 of HXB2, wherein the amino acid positions are indicated in Figure 40. In a more preferred embodiment said sequence identity is at least 85%, preferably at least 90%, more preferably at least 95%, more preferably least 98%, most preferably 100%. In another more preferred embodiment, said polypeptide has a length of at least 350, more preferred at least 375, more preferred at least 400, more preferred at least 425, more preferred at least 450, most preferred at least 475 amino acids.

All the above mentioned gp120 fusion proteins with deletions and/or mutations in the V1 and/or V2 loop are particularly well capable of eliciting a HIV-specific immune reaction. Furthermore, a virus comprising these gp120 fusion proteins is still capable of infecting host cells, so that production of these viruses is possible.

In yet another embodiment, the invention provides a non-human animal which has been provided with a fusion protein and/or a complex and/or a nucleic acid molecule and/or a virus and/or an immunogenic composition according to the invention. Such a non-human animal preferably mounts a

specific antibody response, wherein said antibody is preferably capable of specifically binding and neutralizing a wild-type pathogen, such as for instance HIV. Said antibodies are preferably harvested from said animal. As said before, antibodies that are capable of specifically binding and neutralizing a wild-type pathogen are especially useful for passive immunization. These antibodies are, when given immediately after an infection, capable of instantly binding and neutralizing said pathogen, thereby inhibiting or at least decreasing the amount of spreading within the infected individual or animal. In another embodiment, therefore, the invention provides an isolated or recombinant antibody and/or functional equivalent thereof, capable of specifically binding to a fusion protein and/or a complex according to the invention. Most preferably, said antibody and/or functional equivalent thereof is capable of specifically binding a pathogen, preferably a virus, more preferably HIV, an influenza virus or an Ebola virus. In a particularly preferred embodiment, said antibody or functional equivalent is capable of neutralizing said pathogen. Preferably said antibody and/or functional equivalent is for use as a medicament. In another preferred embodiment, said antibody and/or functional equivalent is for use in preventing, treating and/or diminishing an infection with a pathogen, preferably a virus infection and/or for the preparation of a medicament for preventing, treating and/or diminishing an infection with a pathogen, preferably a virus infection. In a preferred embodiment, said virus infection is an HIV, influenza virus or Ebola virus infection. In another preferred embodiment, said antibody is a neutralizing antibody.

The invention also provides a method for obtaining an antibody capable of specifically binding a fusion protein and/or a complex and/or a nucleic acid molecule and/or a virus according to the invention, the method comprising

providing a non-human animal with a fusion protein and/or a complex and/or a nucleic acid molecule and/or a virus and/or an immunogenic composition according to the invention; and

obtaining an antibody capable of specifically binding said fusion protein, complex, nucleic acid molecule, and/or virus from said non-human animal.

It is not only possible to obtain binding molecules from a non-human animal, but it is also possible to construct and/or select such a binding molecule *in vitro*. For instance a phage display library is screened. Also provided, therefore is the use of a fusion protein, complex, nucleic acid molecule, and/or virus according to the invention in an *ex vivo* method for producing an antibody, or a functional equivalent of an antibody, which is capable of specifically binding said fusion protein, complex, nucleic acid molecule, and/or virus. The skilled artisan is aware of the different methods for producing an antibody *ex vivo*, such as B-cell hybrodima techniques, antibody phage display technologies and the like.

A functional equivalent of an antibody is defined herein as a part which has at least one same property as said antibody in kind, not necessarily in amount. Said functional equivalent is preferably capable of binding the same antigen as said antibody, albeit not necessarily to the same extent. A functional equivalent of an antibody preferably comprises a single domain antibody, a single chain antibody, a Fab fragment or a $F(ab')_2$ fragment. A functional equivalent also comprises an antibody which has been altered such that at least one property - preferably an antigen-binding property - of the resulting compound is essentially the same in kind, not necessarily in amount. A functional equivalent is provided in many ways, for instance through conservative amino acid substitution, whereby an amino acid residue is substituted by another residue with generally similar properties (size, hydrophobicity, etc), such that the overall functioning is likely not to be seriously affected.

The invention also provides a method for inducing an immune response to an antigen in an individual in need thereof, comprising administering an effective amount of: a fusion protein according to the invention and/or fusion

protein obtainable by a method according to the invention, and/or a nucleic acid molecule according to the invention, and/or a virus according to the invention, and/or an immunogenic composition according to the invention and/or an antibody obtained by a method according to the invention to said individual. Preferably, said individual is first diagnosed with an infection, preferably a virus infection, more preferably a HIV virus infection, an influenza infection or an Ebola virus infection.

The invention further provides a method for treating, diminishing or preventing an infection, preferably a virus infection, more preferably an HIV, influenza virus or Ebola virus infection in an individual in need thereof, comprising administering an effective amount of a fusion protein according to the invention and/or obtainable by a method according to the invention, and/or a nucleic acid, and/or a virus, and/or an immunogenic composition according to the invention and/or an antibody obtained by a method according to the invention to said individual.

The invention is further illustrated by the following non-limiting examples. The examples do not limit the scope of the invention in any way.

FIGURE LEGENDS

**Figure 1**. Concept of trimeric fusion construct of antigen (Ag) and a co-stimulatory molecule such as APRIL. Cartoon (top) and linear (bottom) presentations. White: trimeric antigen; grey: trimerization domain; black: co-stimulatory molecule (example: APRIL) with linkers in between.

**Figure 2.** Design and construction of trimeric gp140-IZ proteins**. (A)** Schematic of the gp140-IZ design. SOSIP.R6 gp140 (amino acids 31-681 encompassing the gp120 and the gp41 ectodomain) contained several modifications that have been previously described: an intermolecular disulfide bond between gp120 and gp41 (A501C T605C)(Binley 2000); a trimer-stabilizing substitution in gp41 (I559P; Sanders 2002), and a hexa-arginine motif to promote gp140 cleavage (R6; Binley 2002). H8: octa-histidine tag; TPA: signal sequence from tissue plasminogen activator. **(B)** Reducing SDS-PAGE and BN-PAGE analysis of SOSIP.R6 gp140 and SOSIP.R6-IZ gp140 (gp140-IZ) proteins derived from transiently transfected 293T cells.

**Figure 3.** Gel filtration analysis of gp140 and gp140-IZ proteins. Concentrated culture supernatants, derived from transiently transfected 293T cells, containing the SOSIP.R6 gp140 or SOSIP.R6-IZ proteins were fractionated on a Superose-6 column, followed by analysis by SDS-PAGE and western blot. The elution of standard proteins is indicated.

**Figure 4.** Design and construction of chimeric gp140-CD40L proteins**. (A)** Schematic of the gp140-CD40L design. The active domain of murine CD40L (amino acids 118-261) was added to the C-terminus of SOSIP gp140, with flexible linkers of varying length (L1-L5) present as indicated. Reducing SDS-PAGE **(B)** analysis of gp140 and gp140-CD40L proteins derived from transiently transfected 293T cells.

**Figure 5**. Design and construction of trimeric gp140-IZ-CD40L proteins. **(A)** Schematic of the gp140-IZ-CD40L design. A GCN4-based isoleucine zipper motif (IZ; amino acid sequence RMKQIEDKIEEILSKIYHIENEIARIKKLIGER; Harbury 1994) was inserted between the gp140 and CD40L moieties, with 11 and 6 amino acid linkers between the gp140 and IZ components, and between the IZ and CD40L components, respectively. Reducing SDS-PAGE **(B)** and BN-PAGE **(C)** analysis of the gp140-L3-CD40L and gp140-IZ-CD40L proteins derived from transiently transfected 293T cells.

**Figure 6.** Linear presentation of trimeric fusion constructs of a stabilized soluble HIV-1 envelope glycoprotein (SOSIP.R6 gp140; in white) with APRIL, BAFF or CD40L (in black) with a trimerization domain (IZ; grey) in between, flanked by flexible linkers. TPA: tissue plasminogen activator derived signal sequence for efficient secretion; His: polyhistidine tag for purification and immobilization. Env from the subtype B JR-FL strain is used throughout this study (although amino acid numbering is based on that of the HXB2 strain).

**Figure 7.** Sequence alignment of human, mouse, macaque and rabbit APRIL (top) and BAFF (bottom) proteins. The extracellular domains used in our studies are underlined.

**Figure 8.** Conformational probing of SOSIP.R6-IZ-APRIL and SOSIP.R6-IZ-BAFF fusion constructs. 293T expressed fusion proteins were immunoprecipitated by polyclonal Ig from infected individuals (HIVIg), a CD4 mimetic (CD4-IgG2), and various MAbs.

**Figure 9.** Rabbit immunization schedule. Rabbits were immunized as described in the material and methods and according to the schedule. Thus, rabbit were primed four times with DNA expressed SOSIP.R6-IZ or SOSIP.R6-

IZ-APRIL/BAFF/CD40L fusion constructs, or gp120 protein (wk0,2,4,8) and all groups were boosted with stabilized Env protein (SOSIP.R6 gp140; wk16) in the saponin-based Quil A adjuvant.

**Figure 10.** Expression of Env fusion constructs using rabbit versions of the co-stimulatory molecules. Reducing SDS-PAGE analysis of SOSIP.R6-IZ gp140 and SOSIP.R6-IZ-rAPRIL/rBAFF/rCD40L proteins derived from transiently transfected 293T cells.

**Figure 11.** Oligomerization of Env fusion constructs using rabbit versions of the co-stimulatory molecules. BN-PAGE analysis of SOSIP.R6-IZ gp140 and SOSIP.R6-IZ-rAPRIL/rBAFF/rCD40L proteins derived from transiently transfected 293T cells.

**Figure 12.** Induction of enhanced gp120-specific antibody titers by Env APRIL in rabbits. Rabbits were immunized with plasmids encoding Env (SOSIP.R6 gp140) or fusion construct using rabbit versions of APRIL, BAFF or CD40L as described in the materials and methods section. The anti-gp120 titers in rabbit sera were measured by ELISA. * $p<0.05$.

**Figure 13.** Induction of enhanced Env trimer-specific antibody titers by Env APRIL in rabbits. The anti-trimer titers in rabbit sera were measured by ELISA similar as done for the anti-gp120 titers. In stead of gp120, Env trimers containing a C-terminal D7324 tag were captured on the ELISA plate. * $p<0.05$.

**Figure 14.** Ratio of trimer and monomer specific antibodies. The ratio of the anti-trimer and anti-gp120 titers were calculated for each rabbit at wk12 and wk18.

**Figure 15.** Enhanced virus neutralization induced by Env-APRIL. Rabbit sera of wk18 were tested in a single cycle neutralization assay against three heterologous viruses (MN, SF162 and BaL). The numbers represent the dilution at which 50% neutralization was achieved. Increased neutralization titers are indicated by increased intensities of grey. The experiments were performed at the NIH reference lab for immune monitoring (David Montefiori, Duke University) and were in accordance with our in-house experiments.

**Figure 16.** Enhanced T cell responses induced by Env-APRIL and Env-BAFF (1). Mice were immunized with plasmids encoding Env (SOSIP.R6 gp140) or fusion construct using mouse versions of APRIL, BAFF or CD40L as described in the materials and methods section. At day the T cell responses in the spleen were analyzed using gp120 as recall antigen. The secretion of IL-2 (top) and IFNg (bottom) was measured. Negative (medium) and positive (anti-CD3) controls were also included (not shown).

**Figure 17.** Enhanced T cell responses induced by Env-APRIL and Env-BAFF (2). Mice were immunized with plasmids encoding Env (SOSIP.R6 gp140) or fusion construct using mouse versions of APRIL, BAFF or CD40L as described in the materials and methods section. At day the T cell responses in the spleen were analyzed using gp120 as recall antigen. The secretion of IL-4 (top) and IL-10 (bottom) was measured. Negative (medium) and positive (anti-CD3) controls were also included (not shown).

**Figure 18**. Concept of trimeric fusion construct of Env and a co-stimulatory molecule such as APRIL at the C-terminus, as well as an co-stimulatory molecule such as GM-CSF replacing the V1V2 domain. Cartoon (top) and linear (bottom) presentations. White: trimeric antigen; grey: trimerization domain; black: co-stimulatory molecule (example: APRIL) with linkers in between; dark grey: co-stimulatory molecule (cytokine such as GM-CSF).

**Figure 19**. Linear representation of soluble and stabilized trimeric Env (top), with a V1V2 deletion (second) and with IL-2, IL-4 or GM-CSF sequences replacing the V1V2 (bottom three).

**Figure 20.** Sequences of gp120 and gp120 variants in which the V1V2 is replaced by a full length cytokine (h(human)IL-2, hIL-4, hGM-CSF, m(mouse)GM-CSF or a truncated cytokine (hGM-CSF 1-3).

**Figure 21.** Expression of Env (SOSIP.R6-IZ gp140) constructs containing co-stimulatory molecules (cytokines: IL-2; IL-4; GM-CSF), or irrelevant molecules (Cherry; mCherry) replacing the V1V2 domain. Reducing SDS-PAGE analysis of proteins derived from transiently transfected 293T cells.

**Figure 22.** Conformational probing Expression of Env (SOSIP.R6-IZ gp140) constructs containing co-stimulatory molecules (cytokines: IL-2; IL-4; GM-CSF), or irrelevant molecules (Cherry; mCherry) replacing the V1V2 domain (1). Proteins were captured using a C-terminal polyhistidine tag onto Ni-NTA plates and the binding of polyclonal Ig from infected individuals (HIVIg), DC-SIGN-Fc, and MAbs 2F5 and 2G12 was assessed.

**Figure 23.** Conformational probing of Env (SOSIP.R6-IZ gp140) constructs containing co-stimulatory molecules (cytokines: IL-2; IL-4; GM-CSF), or irrelevant molecules (Cherry; mCherry) replacing the V1V2 domain (2). Proteins were captured using a C-terminal polyhistidine tag onto Ni-NTA plates and the binding of CD4-IgG2 and MAbs 39F and 17b was assessed.

**Figure 24.** Bioactivity of Env-GM-CSF and Env-IL-4 proteins. The functionality of Env (SOSIP.R6-IZ gp140) constructs containing co-stimulatory molecules (cytokines: IL-2; IL-4; GM-CSF), or irrelevant molecules

(Cherry(1AXX); mCherry) replacing the V1V2 domain was assessed using Tf1 cells. Tf1 cells can only proliferate in the presence of functional GM-CSF or IL-4. Tf1 cells were incubated with recombinant GM-CSF or IL-4 or with transiently expressed Env constructs and proliferation was measured by FACS.

**Figure 25.** Enhanced antibody responses induced by Env-GM-CSF. Mice were immunized with plasmids encoding Env (SOSIP.R6 gp140) or fusion construct using mouse versions of APRIL, BAFF or CD40L with or without GM-CSF as described in the materials and methods section. The anti-gp120 responses were monitored by ELISA. The equally matched groups are separated based on the absence or presence of mouse GM-CSF replacing the V1V2 domain.

**Figure 26.** Expression and oligomerization of Env (SOSIP.R6-IZ gp140) containing a full length GM-CSF (GM-CSF 0) or a truncated GM-CSF (GM-CSF 1) replacing the V1V2 domain. BN-PAGE analysis of proteins derived from transiently transfected 293T cells.

**Figure 27**. Concept of polytrimeric fusion constructs of Env and a co-stimulatory molecule such as APRIL with a multimerizing molecule such as surfactant protein A (SP-A) in between. Cartoon (left) and linear (right) presentations. White: trimeric antigen; grey: trimerization domain; black: co-stimulatory molecule (example: APRIL); dark grey: multimerization molecule (example: SP-A).

**Figure 28.** Sequence alignment of human and mouse surfactant protein A (top) and sequence of a SOSIP.R6-mSP-A-CD40L fusion construct (bottom).

**Figure 29.** Expression and oligomerization of Env (SOSIP.R6-IZ gp140) fused to mouse SP-A followed by CD40L. Reducing SDS-PAGE (top) and BN-PAGE (bottom) analysis of proteins derived from transiently transfected 293T cells.

**Figure 30.** Sequences of the Env-SP-A junction of variants designed to improve polytrimerization of fusion proteins.

**Figure 31.** Expression of Env (SOSIP.R6-IZ gp140) fused to mouse SP-A followed by CD40L with modifications at the Env-SP-A junctions. Reducing SDS-PAGE analysis of proteins derived from transiently transfected 293T cells.

**Figure 32.** Sequences mouse surfactant protein A, C1q, adiponectin, collectin, MBL and human surfactant protein a, all examples of molecules that can be used for polytrimerization of fusion proteins.

**Figure 33**. Concept of multimerization of Env and a co-stimulatory molecules such as APRIL on nanoparticles. Cartoon (left) and linear (right) presentations. White: trimeric antigen; grey: trimerization domain; black: co-stimulatory molecule (example: APRIL); dark grey: polyhistidine tag as an example method for immobilization on nanoparticles. Other tags are also possible such as Fc (for interaction with protein A/G; see below).

**Figure 34**. Efficient immobilization of Env (SOSIP.R6-IZ gp140) trimers on microparticles. Ni-NTA coated microparticles (Talon beads) were incubated with polyhistidine tagged Env (lane 5) and the immobilized Env was analyzed by SDS-PAGE (lane 1). Washes are devoid of Env (lanes 2&3) and low levels of Env are present in supernatant from which Env was depleted (lane 4).

**Figure 35**. Concept of hexameric fusion construct of Env and a co-stimulatory molecule such as GM-CSF replacing the V1V2 domain, fused at the C-terminus to the Fc tail of an antibody. Cartoon (top) and linear (bottom) presentations. White: trimeric antigen; grey: trimerization domain; black: Fc tail; dark grey: co-stimulatory molecule (cytokine such as GM-CSF).

**Figure 36.** Expression of Env (SOSIP.R6-IZ gp140) fused to the Fc tail of human IgG1 and containing a cytokine (IL-2, IL-4 or GM-CSF) replacing the V1V2 domain. Reducing SDS-PAGE analysis of proteins derived from transiently transfected 293T cells.

**Figure 37**. Efficient immobilization of Env (SOSIP.R6-IZ gp140) trimers fused to the Fc domain of human IgG and containing GM-CSF replacing the V1V2 on microparticles. Fc tagged protein are efficiently captured on protein G coated particles (bound lanes).

**Figure 38**. Concept of trimeric fusion constructs of an otherwise monomeric (right) antigen (Ag) and a co-stimulatory molecule such as APRIL. Cartoon (top) and linear (bottom) presentations. White: antigen; grey: trimerization domain; black: co-stimulatory molecule (example: APRIL) with linkers in between.

**Figure 39.** Design of loop deletion variants. **A.** Schematic representation of the V1/V2 deletion variants used in this study. Note that the designation of disulfide bonds is based on studies with the wild-type protein. We do not know whether the designated disulfide bonds do in fact form in these variants. This is particularly questionable in mutants 5 and 6 where one or two *wt* cysteine pairs cannot be formed. In variant 5 an alternative and hypothetical disulfide bond between 126 and 131 is drawn. In variant 6 the native C131-C157 bond is drawn and C126 is left unpaired. **B.** Assumed 3D models of selected ΔV1/V2

variants. The upper panel provides perspectives on gp120 as seen from CD4 (left) and the co-receptor (right; rotated over the y-axis by 90°). The rectangle in the upper right panel encloses the V1/V2-stem and the bridging sheet. The lower panels represent details of this area for the variants 1, 2 and 8 and an overlay of these variants. The four ß-strands that compose the bridging sheet and the local disulfide bonds are indicated. The LAI gp120 core and variant cores were modeled by SWISS-MODEL (swissmodel.expasy.org//SWISS-MODEL.html) using the HXB2 core (pdb accession code 1G9M,) and drawn using Viewerlite (Accelrys Inc.). The overlay in the lower right panel was prepared with Deepview/SWISS pdb Viewer (www.expasy.org/spdbv/) and rendered in Viewerlite. **C**. Schematic representation of the V3 deletion variants. **D.** Rearrangement of the V1/V2-stem in variant 6. The starting situation is in Fig. 39A. Note that the drawn disulfide bond between residues C131 and C157 is purely speculative. However, in the *wt* protein these cysteines do form a disulfide bond. Left panel: hypothetical situation after the first substitution (C131Y) with a new non-native disulfide bond between C126 and C157, resulting in restoration of the V1 to its full length and formation of a pseudo-V2. Right panel: removal of N156 after prolonged culturing. Note that we observe the removal of the glycosylation site at N156 in two independent culture in two different substitutions: N156K (as indicated in the Figure) in culture 6A and S158F in culture 6B. The sequences were derived from sequencing clones at day 38 (6C) and day 99 (6A).

**Figure 40**. Alignment of the amino acid sequences encoding a gp120 of strains JR-CSF, JR-FL, LAI, and HXB2.

**Figure 41.** Env-APRIL activates B cells. Naïve B cells isolated from human blood were activated using IL-4 and IL-10. In addition we supplied CD40L as a control, or Env, Env-APRIL, Env-BAFF or Env-CD40L. **A**. The expression of the cell surface markers HLA-DR, CD40, CD38 and CD86 was measured at

day 5 by FACS and the mean fluorescent intensities are shown. **B.** At day 14 the secretion of IgM, IgG and IgA was measured by ELISA.

**Figure 42.** GP-APRIL and HA-APRIL activate B cells. Naïve B cells isolates from human blood were activated using IL-4 and IL-10. In addition we supplied CD40L as a control, or GP-APRIL, or HA-APRIL. **A.** The expression of the cell surface markers HLA-DR, CD40, CD38 and CD86 was measured at day 5 by FACS and the mean fluorescent intensities are shown. **B.** At day 14 the secretion of IgM, IgG and IgA was measured by ELISA.

**Figure 43.** Env-GMCSF induces enhanced antibody responses in rabbits. Rabbits (6 per group) were immunized at wk0 and wk2 with a plasmid encoding Env or Env-GMCSF. The Env-specific antibody titers were measured at wk4.

**Figure 44.** Chimeric Env-IL21 can be made and expressed. Env-IL21 (human and mouse versions) were expressed in 293T cells and analyzed by SDS-PAGE and western blot analysis.

**Figure 45.** Env-IL21 activates B cells. Naïve B cells isolates from human blood were activated using CD40L. In addition we supplied IL-4 as a control, or Env, Env-IL4 Env-IL21. **A.** The expression of the cell surface markers HLA-DR, CD40, CD38 and CD86 was measured at day 5 by FACS and the mean fluorescent intensities are shown. **B.** At day 14 the secretion of IgM, IgG and IgA was measured by ELISA.

**Figure 46.** Sequence encoding GP antigen of Ebola virus.

**Figure 47.** Sequence encoding HA antigen of Influenza virus.

**Figure 48.** Sequence encoding murine IL-4.

**Figure 49.** Sequence encoding human IL-4.

**Figure 50.** Sequence encoding murine IL-21.

**Figure 51.** Sequence encoding human IL-21.

**Figure 52.** 0900556_Eb-GP1_pMK vector.

**Figure 53.** 0900558_H5_pMK vector.

**Figure 54.** 1029127_mouse_interleukin_4_pMA-T vector.

**Figure 55.** 0900560_IL-4_Human_pMA vector.

**Figure 56.** 1029126_mouse_interleukin_21_pMA-T vector.

**Figure 57.** 1029124_h_Interleukin_21_pMA-T vector.

EXAMPLES

MATERIALS AND METHODS

Plasmid construction

The pPPI4 plasmid (Progenics Pharmaceuticals Inc., Tarrytown, NY) containing a codon-optimized stabilized gp140 gene that is based on the subtype B, R5 isolate JR-FL has been described elsewhere (SOSIP.R6 gp140; Binley 2000, Binley 2002, Sanders 2002). To facilitated subsequent cloning steps, we first introduced a BamH1 site at the C-terminus of pPPI4-SOSIP.R6 gp140. This modification changed the most C-terminal amino acid of the natural gp140 protein (Y681I), and added one more amino acid (682L). These changes did not adversely affect the folding and secretion of SOSIP.R6 gp140 proteins (data not shown).

The gene encoding the functional domain (amino acids 118 to 261) of murine CD40L was amplified from the mouse fibroblast cell line J558 (American Type Culture Collection, Rockville, MD), using the Expand PCR system according to the manufacturer's instructions (Roche, Mannheim, Germany). The PCR was performed with sense and antisense primers (5'mCD40L1BamH1 [5'-CTCATACTCATAGGATCCTCGATCCTCAAATTGCAGC-3'] and 3'mCD40LSfu1 [5'-CTCATACTCATATTCGAATTAGAGTTTGAGTAAGCC-3']). The PCR product was cloned downstream of the gp140 ORF in pPPI4-SOSIP.R6 using BamHI and SfuI, creating pPPI4-SOSIP.R6-L1-CD40L. The plasmids pPPI4-SOSIP.R6-L2-CD40L and pPPI4-SOSIP.R6-L3-CD40L were created by PCR amplification using pPPI4-SOSIP.R6-L1-CD40L as the template and the following 5' primers and, in both cases, 3'mCD40LSfu1: 5'mCD40L2BamH1: [5'-CTCATACTCATAGGATCCTCGGTGGAGGTAGCGATCCTCAA ATTGCAGC-3']; 5'mCD40L3BamH1: [5'-CTCATACTCATAGGATCCTCGGTGGAGGTAGCGGTGGAGG

TGATCCTCAAATTGCAGC-3']. The resulting BamH1-Sfu1 fragments containing the linker sequences and amino acids 118-261 from CD40L were then cloned behind the SOSIP.R6 gp140 sequences.

The pPPI4-SOSIP.R6-L4-CD40L plasmid was generated by PCR amplification, with pPPI4-SOSIP.R6-L3-CD40L as the template and primers 5'mCD40L4BamH1: [5'-CTCATACT CATAGGATCCTCGGCGGTGGCGGTAGCGGTGGTGGAGGTAGC-3'] and 3'mCD40LSfu1. Plasmid pPPI4-SOSIP.R6-L5-CD40L was generated by PCR amplification using pPPI4-SOSIP.R6-L4-CD40L as a template and primers 5'mCD40L5BamH1: [5'-CTCATACTCATAGG ATCCTCGGTGGAGGTGGAAGCGGCGGTGGCGGT-3'] and 3'mCD40LSfu1. These steps created the following spacers between SOSIP.R6 and mCD40L: L1: No spacer; L2: GGGS; L3: GGGSGGG; L4: GGGGSGGGGSGGG; L5 GGGGSGGGGSGGGGSGGG.

To facilitate subsequent cloning steps, the linker region of pPPI4-SOSIP.R6-L5-CD40L between Env and CD40L was further modified to introduce the restriction sites for Asp718, Age1, Not1 and BstE2 (pPPI4-SOSIP.R6-L5MCS-CD40L), creating the 18 amino acid linker sequence GGGGTGGGGTGGGGRGGG (non-silent changes are underlined). The resulting sequence modifications did not adversely affect the secretion of the SOSIP.R6-L5-CD40L fusion protein (data not shown).

A DNA fragment encoding a codon-optimized isoleucine zipper motif (IZ) based on GCN4 (AGAATGAAGCAGATCGAGGACAAGATCGAGGAGATCCTGAGCAAGATCT ACCACA TCGAGAACGAGATCGCCAGAATCAAGAAGCTGATCGGCGAGAGA, which encodes the peptide sequence RMKQIEDKIEEILSKIYHIENEIARIKKLIGER (Harbury 1994)), was annealed using two 5'-sense oligonucleotides, 5'IZ1Age1Bgl2: 5'CCGGTAGAATGAAGCAGATCGAGGA CAAGATCGAGGAGATCCTGAGCAA-3' and 5'IZ2Bgl2Not1: 5'-

GATCTACCACATCGAGAAC GAGATCGCCAGAATCAAGAAGCTGATCGGCGAGAGAGGC-3' and the two antisense oligonucleotides 3'IZ1Age1Bgl2: 5'-GATCTTGCTCAGGATCTCCTCGATCTTGTCCTCGATCT GCTTCATTCTA-3' and 3'IZ2Bgl2Not1: 5'-GGCCGCCTCTCTCGCCGATCAGCTTCTTGATTC TGGCGATCTCGTTCTCGATGTGGTA-3', leading to a double stranded DNA fragment with a 5'AgeI site (single underline), a Bgl2 site (double underlined) and a 3' NotI site (dotted underline). This fragment was cloned into pPPI4-SOSIP.R6-L5MCS-CD40L using AgeI and NotI, leaving a linker of 11 amino acids (GGGGTGGGGTG ) between the SOSIP gp140 and IZ moieties, and a 6-amino acid linker (GGRGGG) between IZ and CD40L. Finally, we added a C-terminal oligo-histidine tag (HHHHHHHHH) using the Quickchange mutagenesis kit (Stratagene, La Jolla, CA) as described above, creating the pPPI4-SOSIP.R6-IZ-CD40L-His construct.

We also created a similar plasmid without the sequences encoding CD40L (pPPI4-SOSIP.R6-IZ), by replacing the NotI-SfuI fragment (CD40L-His) by one containing only the oligo-histidine tag. Codon-optimized genes encoding the extracellular domain of the human and mouse versions of CD40L (amino acids 118 to 261) were synthesized (Mr. gene, Regensburg, Germany) and cloned behind SOSIP.R6-IZ using Not1 and Sfu1. The pPPI4-IZ-CD40L plasmid encoding trimeric CD40L without gp140 was constructed by cutting out the Env-encoding sequences from pPPI4-SOSIP.R6-IZ-hCD40L using Nar1 and Age1, followed by Klenow blunting and self-ligation.

The sequence integrity of all clones was confirmed prior to use. The amino acid numbering of gp140 is based on HXB2 Env. For the rabbit experiments, codon-optimized rabbit based sequences were used for APRIL and BAFF. Like CD40L, only the active domain of APRIL and BAFF was used, corresponding to amino acids 115-250 and 150-289, respectively.

Codon-optimized sequences encoding amino acids 21 to 128 of mouse surfactant protein A (mSP-A) corresponding to the N-terminal region, the

collagen like region and the neck region with AgeI and NotI restriction sites on the 5' and 3'end, respectively, were purchased (Mr Gene). To generate gp140-mSPA-CD40L, the AgeI-NotI fragment was cloned into the gp140-IZ-CD40L construct, replacing IZ and keeping the linkers flanking mSP-A the same those flanking IZ in gp140-IZ-CD40L.

Codon-optimized sequences encoding amino acids 26 to 139 of human granulocyte-macrophage colony-stimulating factor (GM-CSF) were synthesized (Mr Gene) containing a HindIII and BmgBI on either side. The V1V2 domain (amino acids tlnckdvnatnttndsegtmergeikncsfnittsirdevqkeyalfykldvvpidnnntsyrliscdTS) was replaced by GM-CSF (KLTPLCVGSG**STQPWEHVNAIQEARRLLNLSRDTAAEMNETVEVISEMFDLQEPTCLQTRLELYKQGLRGSLTKLKGPLTMMASHYKQHCPPTPETSCATQIITFESFKENLKDFLLVIPFDCW**GSGSC*NTS*) using HindIII and BmgBI on the N and C-terminal side of the V1V2 loop, respectively and underlined in the above sequence. The sequence indicated in bold corresponds to amino acids 26-139 of human GM-CSF, flanked by flexible GSG linkers. SCNTS is based on the JR-FL sequence, but an extra putative N-linked glycosylation site (indicated in italic) was inserted, based on previous data suggesting that glycosylation at this position facilitates correct folding of the Env glycoprotein trimer complex. A similar procedure was followed for the insertion of mouse GM-CSF the amino acids (KLTPLCVGSG**VTRPWKHVEAIKEALNLLDDMPVTLNEEVEVVSNEFSFKKLTCVQTRLKIFEQGLRGNFTKLKGALNMTASYYQTYCPPTPETDCETQVTTYADFIDSLKTFLTDIPFECK**GSGSC*NTS*).

To generate constructs with a C-terminal sequence based on the Fc part of human IgG1 the following codon-optimized amino acid sequence was synthesized (Mr Gene;

GGRSPQPQPKPQPKPEPEGSLQGDQGGGG**EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGV**

**EVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALP APIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSC SVMHEALHNHYTQKSLSLSPGK**-STOP-SfuI). This fragment was cloned at the C-terminus of gp140-IZ, using NotI and SfuI. The locations of both sites are underlined in the sequence above. In bold is indicated the sequence corresponding to amino acids 99 to 330 of the constant region of human IgG1. The introduced Fc-tail effectively replaces the 8X His tag which is usually at this location in our constructs.

To create Ebola GP-APRIL and Influenza HA-April, the env sequence of Env-APRIL were replaced by the sequences encoding GP (Figure 46) and HA (Figure 47), respectively, using Nar1 and BamH1 (see also figures 52 and 53). To create Env-IL4 and Env-IL21, the sequences of Env-GMCSF encoding GMSCF were replaced by the sequences encoding mIL4 (Figure 48), hIL4 (Figure 49), mIL21 (Figure 50), and hIL21 (Figure 51), respectively, using Hind3 and Bmg1 (see also figures 54 – 57).

Rabbit CD40L isolation

Neither the gene sequence nor the mRNA sequence of rabbit CD40L was known. We therefore isolated it ourselves. Peripheral blood mononuclear cells (PBMCs) were isolated from New Zealand White rabbit blood using Ficoll Paque (GE Healthcare, Diegem, Belgium) using the protocol described by (Liu, Hoyt 1996). Briefly, the blood was diluted 3X in Hanks Balanced Salt Solution (HBSS) after which Ficoll Paque was added. This was spun for 30 minutes at 2200 RPM without brake. The serum was then removed and kept apart, followed by removing of the cells from the Ficoll Paque gradient 3X washing with HBSS, of which the first time with homologous serum added. The cells were then stimulated in RPMI 1640 supplemented with 2 mM L-Glutamine, 40 IU/ ml IL-2, 10% FCS, 10 % homologous rabbit serum and 2 ug/ ml PHA at 37 degrees. After three days, RNA was isolated from these cells using the RNeasy

mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions.

Earlier attempts at determining the sequence of rabbit mRNA for CD40L enabled us to determine parts of the 3' sequence. This information was used to design a primer (R1) for first strand synthesis, which was performed using the Thermoscript RT-PCR system (Invitrogen, Breda). Almost complete rabbit CD40L cDNA was then amplified using a forward primer based on mouse CD40L mRNA (F1) and the primer used for first strand synthesis. For this the Expand high fidelity kit (Roche, Almere) was used. The resulting PCR product was then sequenced.

Analysis of a protein BLAST using the blastp algorithm showed that the closest known sequence to the amplified rabbit sequence was an mRNA sequence that was 89 % homologous to human CD40L. If there would have been contamination, blasting analysis would have shown a sequence identity at 100% or very close to it. Blasting of both the amino acid and cDNA sequence into the database with all rabbit sequences showed no significant similarity to any known rabbit sequence. This can be explained by the fact that the rabbit genome identification project is still in process and large pieces of the genome have not yet been puzzled together. This sequence however was incomplete at the 5' and 3' end. Therefore we determined the location and size of the exons in the mouse CD40L mRNA coding sequence and blasted its corresponding putative rabbit sequence, exon by exon into the database with rabbit genome shotgun sequences. Each individual exon could be found within the rabbit sequences and was an exact match with the sequence we identified. The rabbit exons were located in two pieces of shotgun sequence. The spread and distribution was similar to that found in the mouse and human genome. This allowed us to confirm that the sequence found was actual rabbit sequence and to determine the outer 5' and 3' end of the rabbit CD40L mRNA coding sequence. These ends had high homology with its corresponding mouse homologous sequence. An alignment of rabbit, mouse and human CD40L

mRNA sequence can be found in. The sequence of the primers used were ATGATAGAAACATACAGCCAACCTTCC and CAAACACCGAAGCATCCGCTTGC.

Cell culture and transient transfection

293T cells were transiently transfected with Env using linear polyethylenimine as described previously (Kirschner 2006). Briefly, DNA encoding Env (or plasmid DNA for mock transfections) was diluted to 0.1 x the culture volume and mixed with Dulbecco's Modified Eagle's Medium (Invitrogen, Breda, The Netherlands). A volume of 0.15 x the culture volume of a 1 mg/ml solution of linear polyethylenimine (PEI, MW 25,000) was then added and mixed. After incubation for 20 min, the DNA-PEI mix was added to the cells for 4h before replacement with the same culture medium supplemented with 10% fetal bovine serum (FBS) (HyClone, Perbio, Etten-Leur, The Netherlands), penicillin, streptomycin, and MEM non-essential amino acids (0.1 mM, Invitrogen, Breda, The Netherlands). Env-containing supernatants were harvested 48h after transfection.

SDS-PAGE, Blue Native PAGE and Western blotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed according to established protocols using the anti-gp120 V3 loop MAb PA-1 (1:20,000; final concentration, 50 ng/ml; Progenics) (Trkola 1996) and horseradish peroxidase-labeled goat anti-mouse IgG (1:5,000). Luminometric detection of envelope glycoproteins was performed using the western lightning ECL system (PerkinElmer, Groningen, The Netherlands). Blue Native (BN)-PAGE was carried out with minor modifications to the published method (Sanders 2002; Schuelke 2002). Thus, purified protein samples or cell culture supernatants were diluted with an equal volume of a buffer containing 100 mM 4-(N-morpholino) propane sulfonic acid (MOPS), 100 mM Tris-HCl, pH 7.7, 40% glycerol, 0.1% Coomassie blue, just prior to loading

onto a 4 to 12% Bis-Tris NuPAGE gel (Invitrogen). Typically, gel electrophoresis was performed for 2h at 150V (~0.07A) using 50 mM MOPS, 50 mM Tris, pH 7.7 as running buffer.

Immunoprecipitation assays

Supernatants were concentrated 25-fold from 293T cells transiently transfected with gp140-IZ, gp140-IZ-BAFF or gp140-IZ-APRIL constructs and incubated overnight at 4°C with MAbs or CD4-IgG2 in a 500 µl volume containing 100 µl of a 5-fold concentrated RIPA buffer (250 mM Tris-HCl, pH 7.4, 750mM NaCl, 5% NP-40, 12.5 mM sodium deoxycholate, Complete Protease Inhibitor Cocktail (Roche, Mannheim, Germany)). Next, 50 µl of protein G-coated agarose beads (Pierce Inc./Thermo Fisher Scientific, Etten-Leur, The Netherlands) were added for 2h rotation-mixing at 4°C. The beads were washed extensively with ice-cold 1x RIPA buffer containing 0.01% Tween 20. Proteins were eluted by heating the beads at 100°C for 5 min in 50 µl of SDS-PAGE loading buffer supplemented with 100 mM dithiothreitol (DTT). The immunoprecipitated proteins were fractionated on 8% SDS-PAGE gels at 125 V for 2h. Recombinant mouse CD40/TNFRSF5/Fc chimera, anti-mouse CD40L and recombinant human DC-SIGN/CD209/Fc were acquired from R&D Systems (Abingdon, UK). MAbs 2G12, 4E10 and 2F5 were obtained from Hermann Katinger through the NIH AIDS Research and Reference Reagent Program (ARRRRP); HIVIg was obtained through the ARRRP from NABI and NHLBI. MAb b12 was donated by Dennis Burton (The Scripps Research Institute, La Jolla, CA); CD4-IgG2 was a gift from William Olson (Progenics Pharmaceuticals Inc., Tarrytown, NY).

Immunizations (rabbits)

Plasmid DNA was amplified using DH5α cells and isolated using the EndoFree Plasmid Giga Kit (Qiagen, Venlo, The Netherlands). The immunizations were

carried out at Genovac (Freiburg, Germany), under contract. The facilities at Genovac comply with the European Community guidelines for animal housing and in vivo experiments. New Zealand white rabbits were immunized on days 0, 14, 28, 56 with 125 μg of endotoxin-free DNA at the abdominal dermis using gene gun technology. The gp120 protein control group was immunized at the same dates, but instead of plasmid, 30 μg JR-FL gp120 in alum was injected subcutaneously at six sites: twice into the shoulder, abdomen, and hind limb. On day 112, all rabbits were injected with 1 ml PBS containing 30 μg JR-FL SOSIP.R6 protein (Sanders 2002, Beddows 2005) and 60 μg Quil A adjuvant. The injections were performed as follows: 300 μl intradermally (50 μl in each of 6 sites), 400 μl intramuscularly (200 μl into each hind leg) and 300 μl subcutaneously (neck region). Blood samples were obtained on days 0, 14, 28, 42, 56, 84, 112, 126 and the final bleed at day 140. Some animals did not survive until the end of the experiment.

Mice immunizations (gene gun)

Plasmid DNA was amplified using DH5α cells and isolated using the EndoFree Plasmid Giga Kit (Qiagen, Venlo, The Netherlands). The immunizations were carried out at Genovac (Freiburg, Germany), under contract. The facilities at Genovac comply with the European Community guidelines for animal housing and *in vivo* experiments. Out-bred NMRI mice were immunized on days 0, 12, 28 and 42 with 20 μg of endotoxin-free DNA at the abdominal dermis, using gene gun technology. Blood samples were obtained on days 0, 12, 28, 42 and 56 (terminal bleed).

gp120-specific and total immunoglobulin ELISA

Anti-gp120 antibody titers were measured by ELISA essentially as described previously (Sanders 2002). For measuring total serum immunoglobulin levels goat anti-mouse IgG (Jackson ImmunoResearch, Newmarket, UK) was coated onto the wells overnight (10 μg/ml in 0.1 M NaHCO3, pH 8.6; 100 μl/well).

After blocking, serially diluted serum was applied for approximately 2h. Bound mouse IgG was detected with HRP-labeled goat anti-mouse IgG (Jackson Immunoresearch, Suffolk, England; used at 1:5000 (0.2 μg/ml)), followed by luminometric detection.

Ni-NTA trimer ELISA

Supernatants from HEK 293T cells transiently transfected with His-tagged Env proteins were diluted 1:3 in TBS/10% FCS and added for 2h to pre-blocked Ni-NTA HisSorb 96-well plates (Qiagen). After 3 washes using TSM (20 mM Tris, 150 mM NaCl, 1 mM CaCl2, 2 mM MgCl2), various MAbs and polyclonal antibodies diluted in TSM/5% BSA were added for 2h. When appropriate, Env proteins were pre-incubated with sCD4 for 30 min. The wells were then washed 5 times using TSM/0.05% Tween, followed by a 45 min incubation with the corresponding HRP-labeled secondary antibodies in TSM/5% BSA. After 5 washes in TSM/0.05% Tween, luminometric detection using established protocols.

Neutralization assays

The TZM-bl reporter cell line stably expresses high levels of CD4 and HIV-1 co-receptors CCR5 and CXCR4 and contains the luciferase and ß-galactosidase genes under the control of the HIV-1 long-terminal-repeat promoter. The TZM-bl cell line was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health (John C. Kappes, Xiaoyun Wu, and Tranzyme Inc. (Durham, NC)). Single-cycle infection experiments and inhibition experiments using TZM-bl cells were performed as described (Eggink 2009).

T cell functional assays

The numbers of T-cells in spleens of immunized mice were determined using standard cell surface staining protocols and an LSR-II flow cytometer (BD Biosciences). The resulting data were analyzed using FlowJo software. All

antibodies were purchased from BD Biosciences. Surface staining was carried out in buffer (PBS + 10% FBS) for 20 min, followed by 2 washes with the same buffer. Prior to staining for specific cell surface markers, Fc receptors were blocked with an anti-mouse CD16/CD32 antibody (clone 2.4G2). The following labeled antibodies specific for mouse T cell markers were used: TCRß-APC (clone H57-597), CD3 APC (clone 145-2C11), CD4 PCP (clone RM4-5), CD8 PE (clone 53-6.7).

In vitro re-stimulation of T cells ($CD4^+$ and $CD8^+$ combined) in unfractionated splenocyte cultures was carried out by culturing 5 x $10^5$ cells/well with JR-FL gp120 (10 µg/ml) in a final volume of 200 µl/well (RPMI 1640 supplemented with 10% FBS, HEPES, glutamine, sodium pyruvate, penicillin, streptomycin, non-essential amino acids and 2-mercaptoethanol). Positive control wells received an anti-CD3e antibody (2 µg/ml, BD Biosciences, Clone 145-2C11, Cat 553057), negative control wells received media. Supernatants were collected after a 96 h culture at 37°C in 5% CO2 and stored at -80°C till further use. Concentrations of IL-2, IL-4, IL-5, IL-10 and IFNγ in the supernatants were measured by a sandwich ELISA, according to the manufacturer's instructions (OptEIA mouse ELISA kits, BD Biosciences), with the use of a TMB substrate kit (BD Biosciences) to provide a colorimetric endpoint at 405 nm. The assay sensitivity limits were approximately 3 pg/ml for IL-2, 8 pg/ml for IL-4, 16 pg/ml for IL-5 and 30 pg/ml for IL-10 and IFNγ. Cytokine levels were expressed in pg/ml and graphs plotted to show values for each individual mouse.

Tf-1 cell proliferation

TF-1 cells were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% heat inactivated FCS (Hyclone) and 50U/ml GMCSF (Schering-plough). Cells were cultured twice a week. Tf-1 cells were plated in 96-wells plate (Greiner) in 100ul fresh RPMI 1640 medium supplemented with FCS (5.0 * $10^4$ cells/well). Supernatant from 293T cells, transfected with various Env expressing plasmids, was added in 3-fold serial dilutions to Tf-1 cells

(100ul/well). After 3 days of culture at 37°C, cells were harvested and washed twice with PBS supplemented with 2% FCS. Cells were resuspended in 200ul PBS 2%-FCS and the cells were counted in a fixed volume using FACS.

Ni-NTA immobilization

TALON Dynabeads (invitrogen) were washed three times with washing buffer (50 mM Na-phosphate pH 8.0, 300 mM NaCl, 0.01% Tween-20, 0.015 mM imidazole) according to manufacturer's guidelines. Beads were incubated with polyhistidine tagged Env proteins in the supernatant of transfected 293T cells for 2.5 hrs at 4°C, while rotating. Env-bound beads were washed three times in washing buffer.

Protein G immobilization

Fc-tagged Env in the supernatant (800ul) of transiently transfected 293T cells was incubated with protein agarose G beads (20ul) (Pierce biotechnology) for 2 hrs at RT, while rotating. Beads were centrifuged for 4 min at 2.500 rcf and washed three times with IP buffer (150 mM NaCl, 25 mM Tris/HCl pH 6.8) according to manufacture guidelines.

Statistical analyses

One-tailed Mann-Whitney U tests were performed for statistical analysis of immunogenicity experiments.

## CREATION AND CHARACTERIZATION OF TRIMERIC ENV-APRIL/BAFF/CD40L FUSION CONSTRUCTS

### Enhancing SOSIP.R6 gp140 trimer formation

We have previously described modifications that improve the stability of soluble, cleaved gp140 trimers based on the R5 subtype B isolate JR-FL (Binley

2000). The amino-acid sequence of gp120 and the gp41 ectodomain was modified as follows (Figure 2). Briefly, we introduced: (i) a disulfide bond between residues 501 in gp120 and 605 in gp41 (A501C, T605C; Binley00); (ii) a trimer-stabilizing substitution in gp41 (I559P; Sanders 2002); (iii) a sequence-enhanced site for furin cleavage (RRRRRR; Binley 2002). Despite these modifications, the resulting JR-FL SOSIP.R6 gp140 protein is expressed as heterogeneous oligomers, with monomers, dimers and tetramers present as well as the desired trimers. Although trimers based on other HIV-1 sequences (e.g., KNH1144 from subtype A) can be produced more consistently (Beddows 2006), we still wish to learn more about the general influences on gp140 trimer formation. We have therefore assessed the effect of various additional modifications to the JR-FL SOSIP.R6 gp140 sequence (for simplicity, we will use the term gp140, or Env, to describe this particular protein from here on). The addition of heterologous trimerization motifs has been shown to improve gp140 trimer formation (Yang 2000). We therefore introduced a GCN4-based isoleucine zipper (IZ) sequence (Harbury 1994) at the gp140 C-terminus (Figure 2). In addition, we added an octa-histidine (H8) motif immediately C-terminal to the IZ trimerization domain, with flexible 11 and 6 amino acid linkers placed between the gp140 and IZ sequences, and between IZ and the H8 tag, respectively (Figure 2). The optimal linker length was determined in concurrent studies (see below). The resulting gp140-IZ and unmodified gp140 proteins were expressed transiently in 293T cells, but without furin co-transfection, and then analyzed by SDS-PAGE and Blue Native (BN)-PAGE. Both gp140 proteins were efficiently expressed (Figure 2). As expected, the unmodified gp140 was secreted as a mixture in which dimers and trimers predominated (each at ~50% prevalence). The proportion of trimers was markedly greater, however, for the gp140-IZ protein (~90%), presumably because of the impact of the heterologous trimerization domain (Figure 2).

We next studied the gp140 and gp140-IZ proteins using analytical size exclusion chromatography on a Superose-6 column, compared to standard

proteins of defined molecular weight (Figure 3). Analysis of the eluted Env-protein components by SDS-PAGE and western blotting showed that multiple oligomeric gp140 forms were present (Sanders 2002). We previously reported that SOSIP gp140 monomers, dimers and trimers were eluted from a Superdex-200 size exclusion column at positions corresponding to apparent molecular weights of 240, 410 and 520 kDa, respectively. Here, using Superose-6 columns that allow greater resolution at the higher end of the molecular weight range of interest, we observed that most of the SOSIP gp140 protein forms were eluted in volumes corresponding to apparent molecular weights in the range 150-450 kDa, which is consistent with the presence of monomers and dimers. In contrast, the gp140-IZ protein forms were more homogeneous, with a predominant elution peak of ~580 kDa that is consistent with the presence of trimers. Hence, the gel filtration analysis confirms the SDS-PAGE and BN-PAGE studies and shows that the addition of the IZ motif enhances gp140 trimer formation and/or stability.

**Construction of chimeric gp140-CD40L fusion proteins**

The extracellular domain of murine CD40L, consisting of amino acids 118 to 261 and including the CD40 binding site, was fused to the C-terminus of gp140 (Figure 4). To allow the gp140 and the CD40L components to fold independently and the fusion protein to be secreted efficiently, we added flexible linkers between the two elements. Since the optimal linker length could only be established empirically, we compared linkers of 0, 4, 7, 13 and 18 residues (constructs L1-L5; Figure 4). The different gp140-CD40L fusion proteins were expressed transiently in 293T cells and the supernatants analyzed by SDS-PAGE and western blotting (Figure 4). Linkers L2-L4 (4-13 residues) allowed the most efficient secretion of gp140-CD40L; having no linker or a longer linker resulted in lower expression levels (Figure 4). Based on these results, and also cloning considerations, subsequent constructs contained an 11-residue linker between the gp140 and C-terminal components (see below).

**Enhancing gp140-CD40L trimer formation**

Our goal was to create a trimeric gp140-CD40L fusion protein, because the native form of HIV-1 Env is trimeric and CD40L is also optimally functional as either a trimer or a multimer of trimers (Morris 1999, Haswell 2001, Stone 2006). The first gp140-CD40L constructs we evaluated were expressed as a mixture of molecular species, with monomers, dimers and tetramers all present in addition to the desired trimers (Figure 5). The heterogeneity arises because JR-FL gp140 itself is expressed as multiple oligomeric forms (Sanders 2002), and perhaps also because the globular, extracellular domain of CD40L does not form trimers efficiently in the absence of the membrane-spanning domain (Morris 1999). We showed above that the formation of gp140 trimers is improved by the incorporation of a heterologous GCN4 isoleucine zipper (IZ) trimerization domain at the C-terminus, consistent with a previous report (Yang 2000) (Figure 2). Moreover, similar modifications have been shown to improve CD40L trimer formation (Morris 1999). We therefore introduced the IZ motif between gp140 and CD40L, using 11 and 6 amino acid linkers, respectively, at the gp140/IZ and IZ/CD40L junctions (Figure 5). The resulting gp140-IZ-CD40L protein and, for comparison, the gp140-L3-CD40L protein were expressed in 293T cells and analyzed by BN-PAGE. As was observed with gp140 (Figure 5), the gp140-IZ-CD40L protein formed trimers more efficiently (~95% trimeric) than gp140-L3-CD40L, which lacked the IZ motif (~50% trimeric) (Figure 5).

**APRIL and BAFF constructs**

We further explored the possibilities to design constructs with the B cell activating factors APRIL and BAFF as alternatives to CD40L. The constructs are depicted schematically in Figure 6. APRIL and BAFF are more specific for B cells. Therefore the extracellular domain of BAFF and APRIL (AA 183-323

and 115-250, respectively (Figure7)) were fused to the Env-IZ construct in the same way as CD40L was (see "Construction of chimeric gp140-CD40L fusion proteins"). We have made constructs containing the human, mouse and rabbit sequences of BAFF, APRIL and CD40L.

Transient transfection experiments using these constructs, followed by SDS-PAGE and western blotting of the cell supernatant show that the constructs express well (for the rabbit version see Figure 10; similar results obtained for the human and mouse version, not shown). Blue Native PAGE followed by western blotting showed that these constructs also form trimers efficiently, albeit slightly less efficiently than Env-IZ (for the rabbit versions see Figure 11; similar results obtained for the human and mouse version, not shown). We next probed the structure of the fusion constructs with a panel of monoclonal antibodies, including conformational antibodies. In addition we tested a receptor mimetic, CD4-IgG2. Immunoprecipitation experiments show that despite modifications to the C-terminus of Env, the structure of the Env part is intact and well folded, as indicated by the efficient binding of pooled Ig from HIV-infected individuals (HIVIg), b12 (directed to the CD4 binding site), CD4-IgG2 and 2F5 (directed to gp41) (Figure 8). 17b binds to a conformational epitope that is induced by CD4 binding and overlaps with the co-receptor bindingsite. We observed some binding of 17b to Env-BAFF and En-APRIL in the absence of (soluble) CD4 but 17b binding was increased after addition of soluble CD4, indicating that the Env domain of the constructs is able to undergo CD4-induced conformational changes. Combined these data indicate that the constructs are well-folded (Figures 8,10,11).

**Env-APRIL and Env-BAFF elicit enhanced antibody responses in rabbits**

In order to test whether fusion of BAFF or APRIL to Env can provide enhanced immune responses *in vivo*, we designed an immunization experiment in rabbits (Figure 9). The immunizations were carried out at Genovac (Freiburg,

Germany), under contract. New Zealand white rabbits were immunized on days 0, 14, 28, 56 with 125 μg of endotoxin-free DNA at the abdominal dermis using gene gun technology. Group H was immunized at the same dates as group A-G, but instead of DNA 30 μg JR-FL gp120 in alum was injected subcutaneously at six sites: twice into the shoulder, abdomen, and hind limb. On day 112, all rabbits were injected with 1 ml PBS containing 30 μg JR-FL SOSIP protein (reference) and 60 μg Quil A adjuvant. The day 112 protein injections were performed as follows: 300 μl intradermally (50 μl in each of 6 sites), 400 μl intramuscularly (200 μl into each hind leg) and 300 μl subcutaneously (neck region). Blood samples were obtained on days 0, 14, 28, 42, 56, 84, 112, 126 and the final bleed at day 140. One animal in the Env-BAFF group did not survive until the end of the experiment: it died of an unknown cause.

Anti-gp120 antibody titers in the rabbit sera were measured by ELISA. The resulting midpoint IgG anti-gp120 binding titers shown in Figure 12 indicate that Env-APRIL and Env-BAFF induce increased antibody responses compared to the Env DNA construct or the gp120 protein immunization control group. This was significant ($p < 0.05$ according to the one-tailed Mann-Whitney test) at days 42, 126 and 140 for Env-APRIL compared to Env and on days 28, 42, 126 and 140 for Env-APRIL compared to gp120.

Since virus-associated Env is trimeric, we also analyzed the antibody responses against trimeric Env. Anti-trimeric gp140 titers were measured by using a gp140 construct which had the epitope which is recognized by the D7324 antibody (APTKAKRRVVQREKR) attached at its C-terminus. The resulting midpoint anti-trimeric Env IgG binding titers are shown in Figure 13. These data show that at week 18 (day 126), Env-APRIL and Env-BAFF induce the highest IgG anti-trimeric Env binding titers of the tested constructs, even after boosting with trimeric protein. To get a better sense of the quality of the induced immune response we divided the anti-trimer midpoint binding titers by the anti-gp120 midpoint binding titers. The resulting trimer-gp120 ratios

are depicted in figure 14. At week 18 Env-APRIL and Env-BAFF provided the highest trimer-gp120 ratio's of the tested constructs, indicating that a higher percentage of the induced antibodies are directed against trimeric Env.

To rule out that we induced unspecific immune activation by the fusion of co-stimulatory molecules, we measured total IgG levels in the serum. Goat anti-mouse IgG (Jackson ImmunoResearch, Newmarket, UK) was coated overnight (10 μg/ml) in 0.1 M NaHCO3, pH 8.6 (100 μl/well). After blocking, serially diluted serum was applied for approximately 1 hr. Bound mouse IgG was detected with HRP-labeled goat anti-mouse IgG (Jackson Immunoresearch, Suffolk, England); used at 1:5000 dilution followed by luminometric detection. There were no differences in total IgG levels indicating that there was no unspecific immune activation (data not shown).

The analysis of serum IgA and mucosal IgG and IgA responses is in progress. APRIL is important for class-switching to IgA and mucosal B cell response so we expect these responses to be enhanced. Improvement of mucosal responses is of course highly relevant for HIV-1 vaccine design.

**Env-ARPIL induces enhanced neutralizing antibody responses in rabbits**

Env binding antibodies are not necessarily predictive of antiviral activity and therefore we studied the capacity of the rabbit sera to neutralize two different virus strains. SF162 is a neutralization sensitive virus strain. In our experiment is represents a heterologous challenge since the vaccine Env was from the JR-FL strain. We also tested neutralization of JR-FL, the homologous strain, which is notoriously neutralization resistant.

Sera from rabbits taken at week 12 and week 18 were tested in a standard single cycle TZM-bl based neutralization assay. TZM-bl cells express the HIV receptor and co-receptors and further contain a luciferase reporter gene under control of the HIV-1 LTR. Luciferase activity is directly correlated with virus infection. Serially diluted, heat-inactivated serum was mixed with SF162 or

JR-FL pseudovirus and incubated for 1 hr at room temperature, after which the virus-serum mix was added to the TZM-bl cells and incubated for two days at 37°C. The cells were then lysed and luciferase expression was measured. The results revealed that Env-APRIL induced the most potent and most consistent neutralization (not shown). Importantly our in house results were corroborated independently by the NIH reference laboratory for immune monitoring using a panel of different challenge viruses (Figure 15). The neutralization titers are given in Figure 15. The heat maps show that the most consistent neutralization of MN, SF162 and BaL is observed with Env-APRIL. In summary, the data indicate that fusion of Env to co-stimulatory molecules APRIL and BAFF, enhances the humoral immune response in rabbits, leading to higher antibody levels, better memory and more efficient virus neutralization.

**Env-APRIL and Env-BAFF induce improved T cell responses in mice**

Since APRIL acts on B cells it is likely that B cells are directly activated by the fusion construct. To test, however, whether the improved antibody response was accompanied by an improved T helper response. We used gene gun methodology to immunize out-bred NMRI mice with plasmids encoding either Env, Env-CD40L, Env-BAFF or Env-APRIL. The plasmids were administered on days 0, 14, 28 and 42 and the T cells responses in the spleens was measured at day 56 (Figures 16,17), focusing on the secretion of cytokines relevant for B cell responses. Env-specific responses were analyzed using gp120 as a recall antigen, with culture medium and anti-CD3 stimulation serving as negative and positive controls, respectively. The negative and positive controls performed as expected and are not shown.

Figures 16 and 17 show that Env-APRIL and Env-BAFF enhance T cell responses to the gp120 antigen dramatically.

**Env-APRIL activates B cells in vitro**

Env-APRIL was tested for its capability to activate naïve human B cells . Naïve B cells isolated from human blood were stimulated using a cocktail of IL-4, IL-10 and CD40L to provide a baseline activation. In addition mock, Env, Env-APRIL, Env-BAFF or Env-CD40L were supplied. At day 5 the expression of the cell surface markers HLA-DR, CD40, CD38 and CD86 was measured.
The levels of CD86 and CD40 were similar irrespective of the fusion protein, but Env-APRIL induced higher levels of CD38, a plasma cell marker, and this was accompanied by lower expression of HLA-DR (Figure 41A). At day 14 the secretion of IgM, IgG and IgA was measured (Figure 41B). Env-CD40L induced enhanced IgM and IgG secretion by B cells compared to mock and unconjugated Env. Env-APRIL also enhanced secretion of IgM and IgG but not as efficiently as Env-CD40L. In contrast, Env-APRIL potently induced IgA secretion, consistent with the role of APRIL in class-switching to IgA and mucosal antibody responses.

**Ebola GP-APRIL and Influenza HA-APRIL activate B cell in vitro**

To provide evidence that the fusion of APRIL is applicable to other antigens, APRIL was fused to the ebola virus glycoprotein (GP) and the influenza virus hemagglutinin (HA). It was tested whether the fusion constructs are able to activate human B cells. Naïve B cells isolated from human blood were stimulated using a cocktail of IL-4, IL-10 and CD40L to provide a baseline activation. In addition the B cells were incubated with mock, Env-APRIL, GP-APRIL or HA-APRIL. At day 5 the expression of the cell surface markers HLA-DR, CD40, CD80 and CD86 was measured. There were no major changes, except for the enhanced CD38 expression with Env-APRIL as noted above (Figure 42A). At day 14 we measured the secretion if IgM, IgG and IgA (Figure 42B). While Env-APRIL induced a pronounced IgA response in particular, HA-APRIL induced high levels of IgM and IgG in addition to IgA. GP-APRIL also induced secretion of IgM, IgG and IgA but not as efficiently as HA-APRIL.

These data indicate that fusion to APRIL benefits the antibody response to various antigens.

## VARIATIONS AND IMPROVEMENTS

### Combination with co-stimulatory molecules at a different location

Thus far we have described the addition of co-stimulatory molecules at the C-terminus of Env. We choose the C-terminus for the addition of co-stimulatory molecules for mainly two reasons. First, addition to the N-terminus of Env is likely to result in misfolding since the N-terminus is not exposed, but buried in the Env complex. The addition at the exposed C-terminus allows for independent folding of Env and APRIL/BAFF etc. Second, it allows for the addition of trimerization domains or other domains between Env and the co-stimulatory molecule.

We explored the possibility of introducing small co-stimulatory molecules within Env as an alternative to the C-terminus. The possibility of two locations for insertion of co-stimulatory molecules also opens up the possibility of combining co-stimulatory molecules. Our second projected location for the introduction of co-stimulatory motifs is the variable V1V2 domain. Since we previously removed a large number of amino acids (PCT/NL2009/050609), we hypothesized that we could exploit the resulting "gap" and "fill it" with a co-stimulatory molecule. The V1V2 is not required for function and can easily be manipulated to contain heterologous sequences (Law 2007; Pantophlet 2009) and we have extensive experience with modification of the V1V2 domain ourselves (Sanders 2000; Bontjer 2009). We therefore felt confident that we would be able to incorporate heterologous immunostimulatory sequences. We have previously generated functional Env variants that lack the entire V1V2-domain resulting in a 20 kDa reduction in size. Considering the plasticity of Env, in particular in this region, we expected that we could replace the V1V2

domain with cytokine sequences, many of which are of the same size as the V1V2-domains itself. Our initial choice was the cytokine GM-CSF, but to create a more extensive proof of principle we also include IL-2 and IL-4. GM-CSF (size ~14 kDa) recruits and activates DC, but it has pleiotropic effects, also recruiting and activating NK cells, neutrophils and macrophages, which then produce cytokines to create a milieu favorable for the initiation of a potent immune response. GM-CSF is successfully used in a variety of preclinical and clinical settings both for therapeutic use and to augment vaccine responses. Importantly, GM-CSF can enhance anti-HIV responses and has good track record in clinical use.

Based on our studies on our V1V2 deletion studies (Bontjer 2009), we replaced the V1V2 domain (amino acids 128-194) in a stable recombinant and soluble Env trimer construct (JR-FL SOSIP.R6-IZ gp140) by the sequences encoding human IL-2, flanked on both sides by a flexible linker Gly-Ser-Gly (Figures 18,19,20). The constructs were then expressed in 293T cells and analyzed by SDS-PAGE and western blot (Figure 21). The Env-IL-2, Env-IL-4 and Env-GM-CSF constructs were expressed efficiently, indicating that the introduced cytokine sequences did not affect protein folding. The constructs were also recognized efficiently by conformational antibodies and receptor mimics (Figures 22,23).

We next investigated whether the IL-4 and GM-CSF components in Env-IL-4 and Env-GM-CSF were functional. The Tf1 cell line requires IL-4 or GM-CSF for proliferation and when neither is present in the culture medium, they do not proliferate (Figure 24). When recombinant IL-4 or GM-CSF is present in the medium, Tf1 cells proliferate efficiently (Figure 24). We found that the Tf1 cells also proliferated in the presence of Env-IL-4 or Env-GM-CSF, but not in the presence of Env or Env-dV1V2, demonstrating that the IL-4 and GM-CSF components in the chimeric proteins are well-folded, well-exposed and functional (Figure 24). Importantly, mice immunized with Env-GM-CSF developed higher Env-specific antibody levels compared to mice immunized

with Env alone, indicating that GM-CSF replacing the V1V2 domain exerts adjuvant activity in vivo (Figure 25). In another experiment it was shown that Env-rGMCSF enhances the antibody response to Env in rabbits (Figure 43).

It is possible to truncate cytokine sequences inserted in Env. We have already made one truncated GM-CSF construct successfully (GM-CSF 1) in which contains 15 amino acids less GM-CSF sequence compared to the first generation Env-GM-CSF. This construct is expressed efficiently and forms trimers efficiently (Figure 26). As an alternative to rationally designed truncations, the cytokines are placed in the context of full length gp160 and live virus to allow the virus to optimize and accommodate the inserted sequences by virus evolution, similar to what we have done previously with dV1V2 viruses (Bontjer 2009).

Env-IL21 chimera's based on mouse and human IL21 molecules were also efficiently expressed (Figure 44). These chimera's are especially useful since it is intended to target B cells.

It was investigated whether Env-hIL-4 and Env-hIL21 were able to activate human B cells in vitro. Naïve B cells isolated from human blood were stimulated using a cocktail of IL-4, IL-10 and CD40L to provide a baseline activation. In addition, mock, Env, Env-IL4 or Env-IL21 were added. At day 5 the expression of the cell surface markers HLA-DR, CD40, CD38 and CD86 was measured (Figure 45A). Compared to unconjugated Env, both Env-IL4 and Env-IL21 induced modestly increased levels of CD38, a marker for plasma cells. Env-IL4 also induced an increase in CD40 expression. At day 14, the secretion if IgM, IgG and IgA was also measured (Figure 45B). Env-IL4 induced enhanced IgM, IgG and IgA secretion compared to mock or Env alone, but Env-IL21 induced dramatic increases in the secretion of IgM, IgG and IgA. These data first of all confirm that the embedded IL-21 domain is functional, but also show that embedding IL-21 into Env to target and activate B cells is a valid vaccine strategy.

Another location for the introduction of heterologous sequences is the V3 domain. This is a particularly useful site for placing CCR5- and CXCR4-binding chemokines, such as SDF-1.. Importantly, Env binds to CCR5 or CXCR4 via its V3 loop and replacement of the V3 by SDF-1, which is also able to bind to CCR5 or XCXR4 results in a functional Env (Yonezawa 2001), providing evidence that our strategy is feasible.

The generation and development of hyperactive cytokines may of course have many future applications. A plethora of cytokines is used in laboratory studies as well as clinical applications and more active cytokine variants could be very valuable. Our lab has a long-standing expertise with the introduction and optimization of heterologous sequences including complete proteins within an HIV-1 backbone (Zhou 2006).

**Polytrimeric fusion constructs**

BAFF and APRIL function when they are trimeric. However, optimal activation is achieved when they are multimeric (i.e. a multitude of trimers). Therefore, multimeric antigen-APRIL/BAFF fusion constructs are created. As an added advantage increased multimerization also contributes to enhanced Env-BCR cross-linking and enhanced B cell activation.

A first strategy is the addition of the sequences encoding the N-terminal domain, the collagen-like domain and the neck domain from surfactant protein A or similar molecules such as adiponectin, collectin, C1q and MBL (Figures 27-32). Surfactant protein A forms octadecameric 'bouquet-like' structures consisting of 6 trimers (Figure 27). The appropriate connection of Env or another antigen with APRIL/BAFF/ using Surfactant protein A (SPA) should results in octadecameric Env-APRIL/BAFF fusion constructs with optimal APRIL/BAFF activity. As an added advantage the increased valence from 3 to 18 contributes to enhanced Env-BCR cross-linking and enhanced B cell activation. Another strategy to 'multimerize' Env and APRIL/BAFF is their immobilization on nanoparticles (Figure 33). We have already explored

methods to immobilize our Env trimers on synthetic microparticles using a C-terminal His tag. Since the His tag is trimerized on Env trimers, the affinity for Ni-NTA is extremely high. When magnetic Ni-NTA coated microparticles were incubated with His-tagged Env trimers, we found that Env trimers were immobilized on these beads extremely efficiently and stably (Figure 34). Thus, vaccine antigens are improved for instance by immobilizing them in a densely packed array on nanoparticles in combination with immunostimulatory molecules.

A third strategy to enhance the valency of antigens and co-stimulatory molecules is the fusion of Env with a C-terminal trimerization domain to the Fc part of immunoglobulin G. This results in hexameric molecules (Figure 35). Env-Fc constructs were efficiently expressed (Figure 36). In addition, we have shown that it is possible to combine this strategy with the inclusion of cytokines in Env (Figure 36). The Fc tail was functional as we could capture Env-Fc constructs efficiently using protein G (Figure 37).

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## Claims

1 A fusion protein comprising an antigen and a ligand capable of inducing, enhancing or sustaining a B cell immune response, wherein said ligand is selected from the group consisting of a compound comprising at least the extracellular domain of a proliferation inducing ligand (APRIL), and a compound comprising an amino acid sequence having at least 80% sequence identity with at least the extracellular domain of APRIL.

2 A fusion protein comprising an antigen and a polypeptide comprising an amino acid sequence having at least 80% sequence identity with a cytokine capable of inducing or sustaining a B cell immune response.

3 A fusion protein according to claim 2, wherein said antigen comprises an amino acid sequence with a length of at least 300 amino acids with at least 80% sequence identity to a gp120 protein and/or a gp160 protein of HIV envelope glycoprotein complex (Env), wherein at least 5 amino acids of the V1 loop and/or at least 5 amino acids of the V2 loop of said gp120 molecule are absent, and wherein at the deletion site in V1 and/or at the deletion site in V2, said polypeptide is inserted.

4 A fusion protein according to claim 2 or 3, wherein said cytokine is GMCSF or IL-21.

5 A fusion protein according to any one of claim 1, 2 or 4, wherein said antigen comprises a pathogenic protein, preferably a virus protein, or an immunogenic part thereof, said antigen preferably having at least 80% sequence identity with a virus protein of HIV, influenza virus or Ebola virus, or with an immunogenic part thereof.

6 A fusion protein according to any one of claims 1-5, further comprising a multimerizing polypeptide.

7 A fusion protein according to any one of claims 1-6, wherein said antigen is linked to said ligand or said cytokine via at least one linker comprising an amino acid sequence with a length of between 1 and 20 amino acids, preferably between 4 and 13 amino acids, most preferably with a length of 11 amino acids.

8 A nucleic acid molecule encoding a fusion protein according to any one of claims 1-7.

9 A method for producing a fusion protein according to any one of claims 1-7, said method comprising

generating/providing a nucleic acid molecule comprising a nucleotide sequence encoding a fusion protein according to any one of claims 1-7; and

allowing expression of said fusion protein from said nucleic acid molecule in a suitable expression system.

10 An oligomeric complex comprising at least 1, preferably at least 2, more preferably at least 3 fusion protein(s) according to any one of claims 1-7 or obtainable by a method according to claim 9.

11 A fusion protein according to any one of claim 1-7 and/or a nucleic acid molecule according to claim 8 and/or a method according to claim 9 and/or a complex according to claim 10, wherein said fusion protein comprises an amino acid sequence with a length of at least 300 amino acids with at least 80% sequence identity to a gp120 protein of HIV with a deletion of at least 5 amino acids in the region corresponding to amino acid positions 120-204 of reference HIV strain HXB2, wherein the amino acid positions are indicated in Figure 40.

12 A virus comprising a fusion protein according to any one of claims 1-7 or 11, and/or a fusion protein obtainable by a method according to claim 9, and/or a complex according to claim 10 or 11, and/or a nucleic acid sequence according to claim 8 or 11.

13 An immunogenic composition comprising a fusion protein according to any one of claims 1-7 or 11, and/or a fusion protein obtainable by a method according to claim 9, and/or a complex according claim 10 or 11, and/or a nucleic acid sequence according to claim 8 or 11, and/or a virus according to claim 12.

14 A fusion protein according to any one of claims 1-7 or 11, and/or a fusion protein obtainable by a method according to claim 9, and/or a complex according to claim 10 or 11, and/or a nucleic acid sequence according to claim 8 or 11, and/or a virus according to claim 12, and/or an immunogenic composition according to claim 13 for use as a medicament or prophylactic agent.

15 A fusion protein according to any one of claims 1-7 or 11, and/or a fusion protein obtainable by a method according to claim 9, and/or a complex according to claim 10 or 11, and/or a nucleic acid sequence according to claim 8 or 11, and/or a virus according to claim 12, and/or an immunogenic composition according to claim 13 for use in inducing, enhancing and/or sustaining an immune response specific for a pathogen, preferably HIV, influenza virus or Ebola virus.

16 A fusion protein according to claim 15, wherein said immune response comprises an immunoglobulin A (IgA) response.

17 A non-human animal which has been provided with a fusion protein according to any one of claims 1-7 or 11, and/or a fusion protein obtainable by a method according to claim 9, and/or a complex according to claims 10 or 11, and/or a nucleic acid sequence according to claim 8 or 11, and/or a virus according to claim 12, and/or an immunogenic composition according to claim 13.

18 A method for obtaining an antibody capable of specifically binding a fusion protein according to any one of claims 1-7 or 11, and/or a fusion protein obtainable by a method according to claim 9 and/or a complex according to claim 10 or 11, and/or a nucleic acid sequence according to claim 8 or 11, and/or a virus according to claim 12, the method comprising

providing a non-human animal with a fusion protein according to any one of claims 1-7 or 11, and/or a fusion protein obtainable by a method according to claim 9, and/or a complex according to claim 10 or 11, and/or a nucleic acid sequence according to claim 8 or11, and/or a virus according to claim 12, and/or an immunogenic composition according to claim 13; and

obtaining from said non-human animal an antibody capable of specifically binding said fusion protein, complex, nucleic acid, and/or virus.

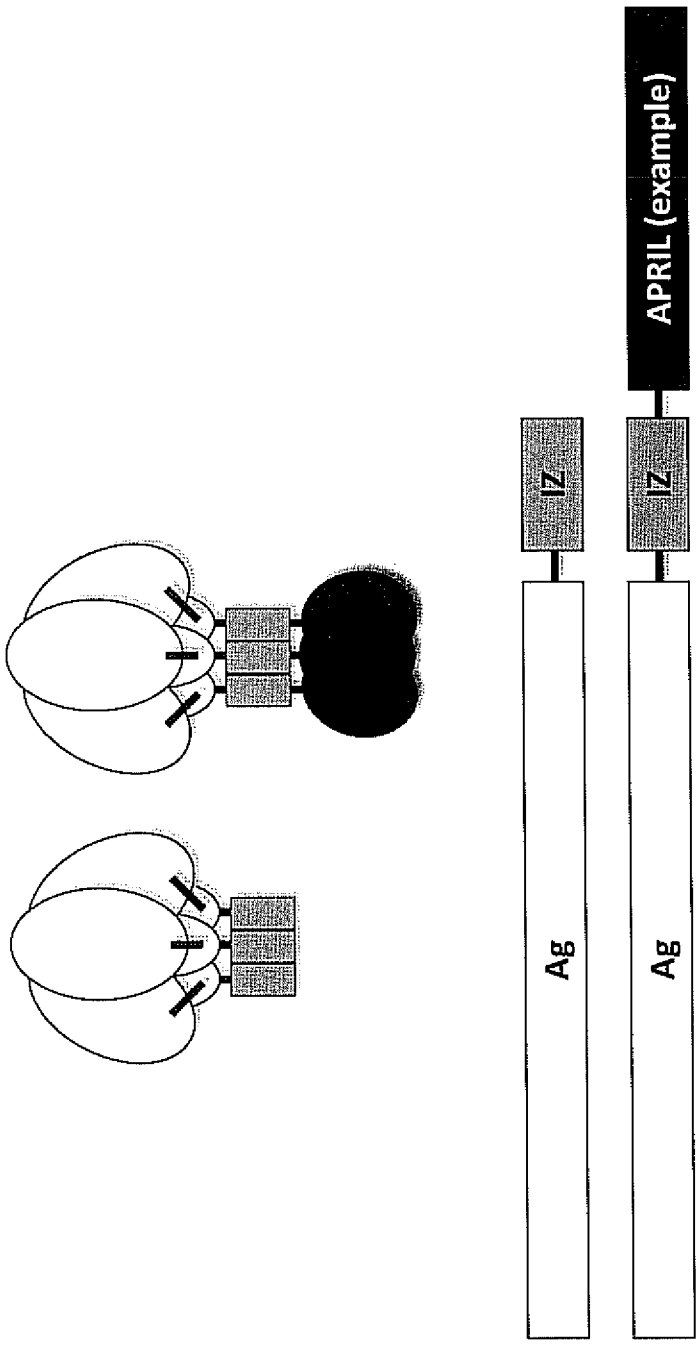



Figure 1

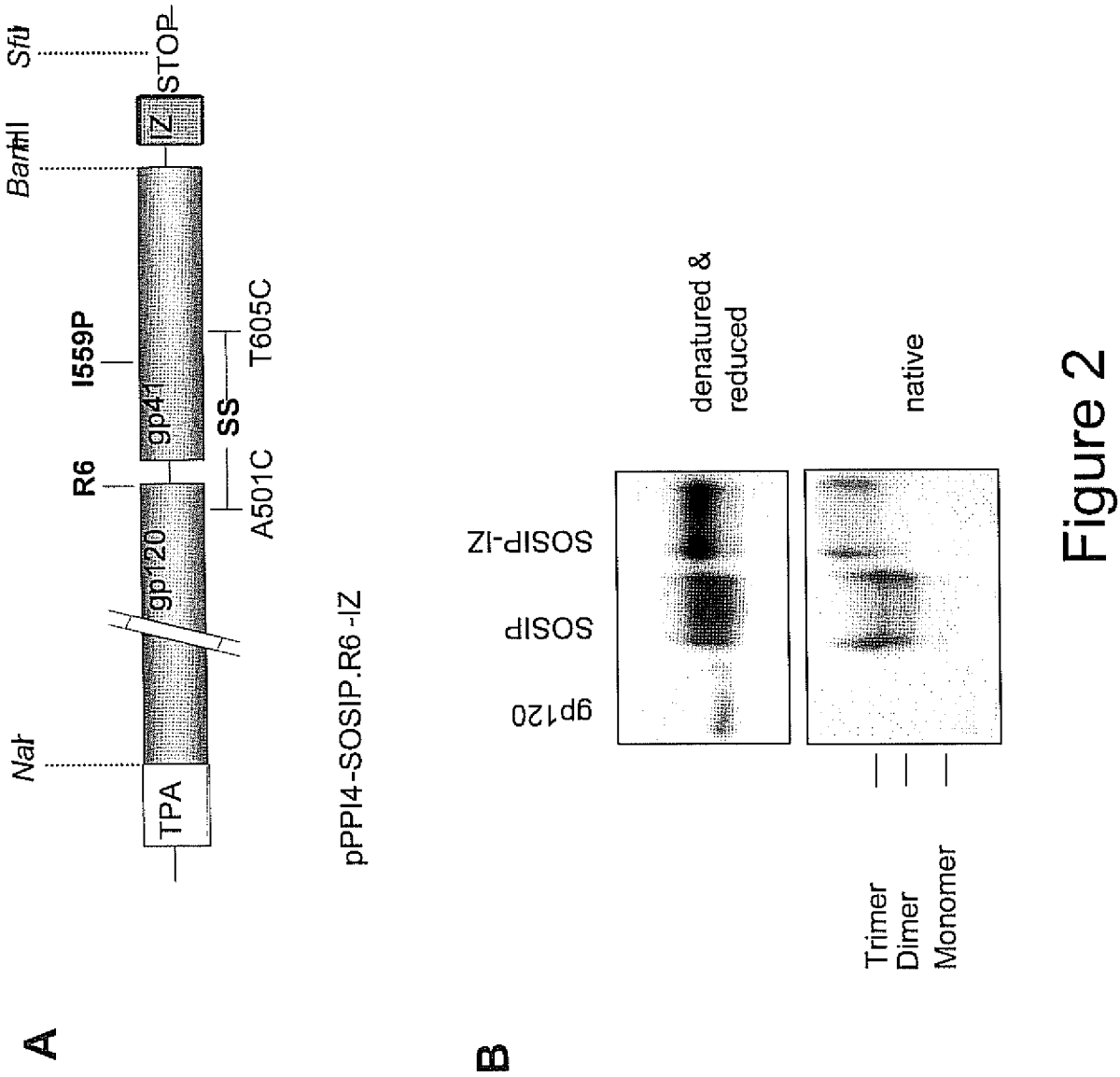

A
NaI
TPA
gp120
R6
gp41
I559P
BamHI
IZ
SfiI
STOP
SS
A501C
T605C
pPPI4-SOSIP.R6 -IZ
B
gp120
SOSIP
SOSIP-IZ
denatured &
reduced
native
Trimer
Dimer
Monomer

Figure 2

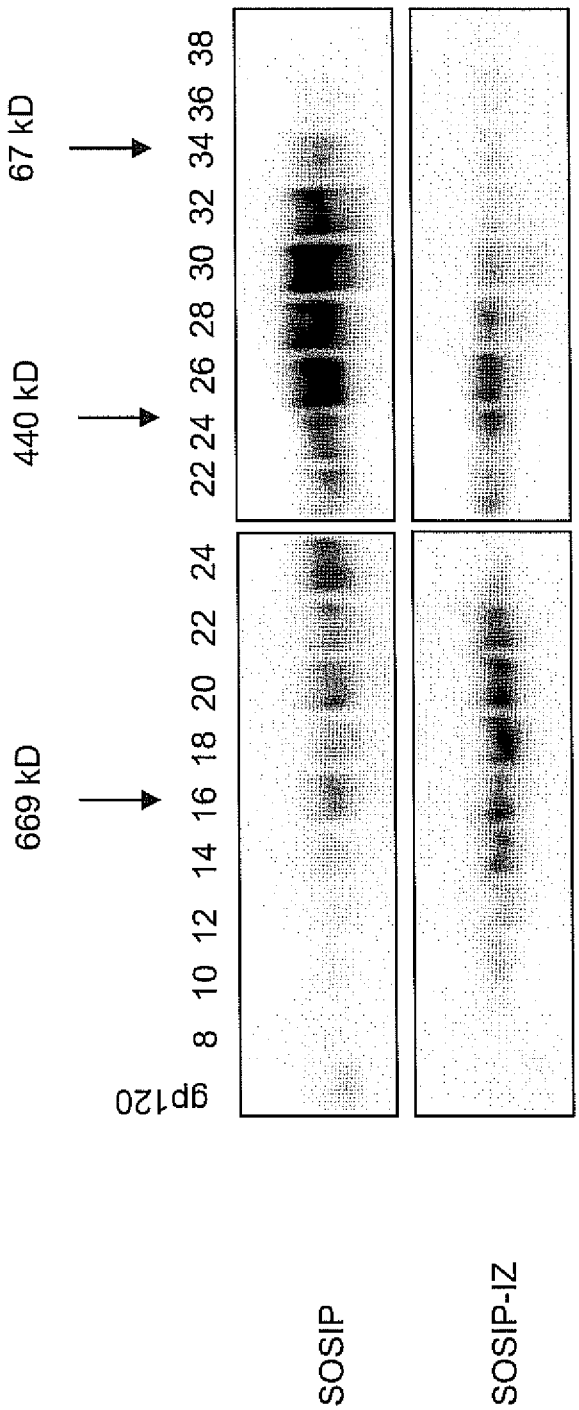
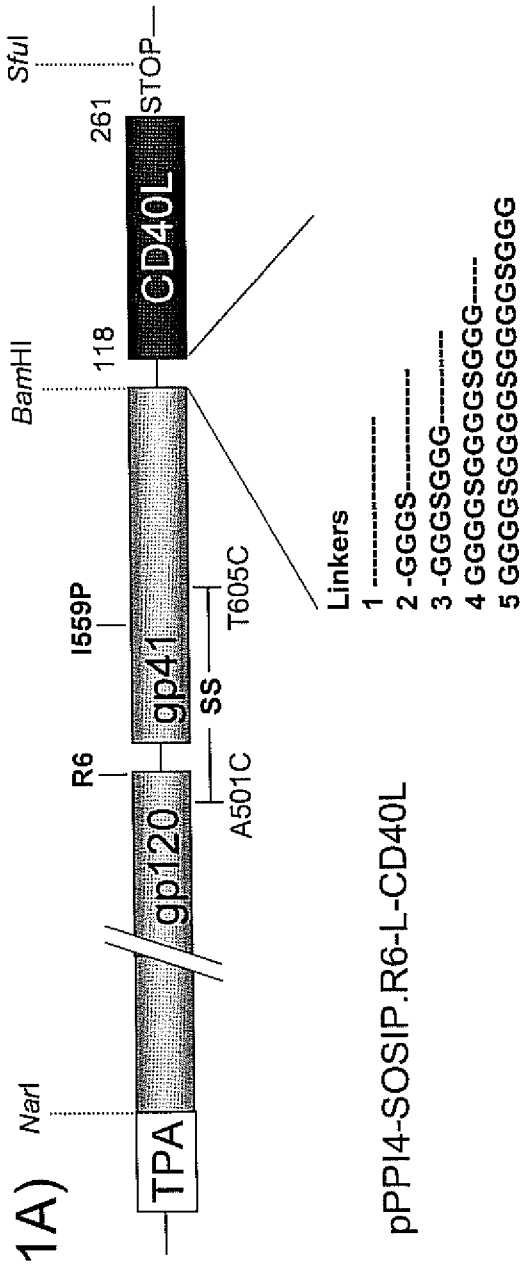

669 kD
440 kD
67 kD
gp120
8 10 12 14 16 18 20 22 24
22 24 26 28 30 32 34 36 38
SOSIP
SOSIP-IZ

Figure 3


1A)
NarI
TPA
gp120
R6
A501C
gp41
SS
I559P
T605C
BamHI
118
CD40L
261
STOP
SfuI
pPPI4-SOSIP.R6-L-CD40L
Linkers
1 ----------
2 -GGGS----------
3 -GGGSGGG----------
4 GGGGSGGGGSGGG----
5 GGGGSGGGGSGGGGSGGG

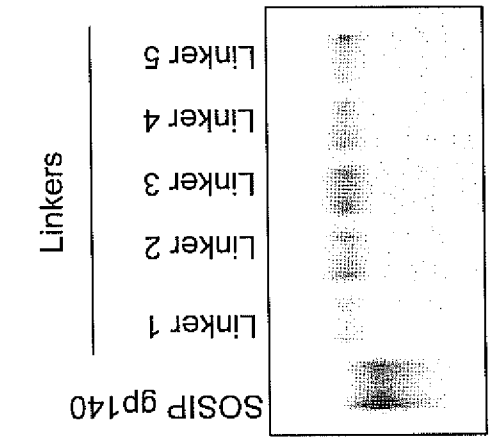

B
Linkers
Linker 1
Linker 2
Linker 3
Linker 4
Linker 5
SOSIP gp140

Figure 4

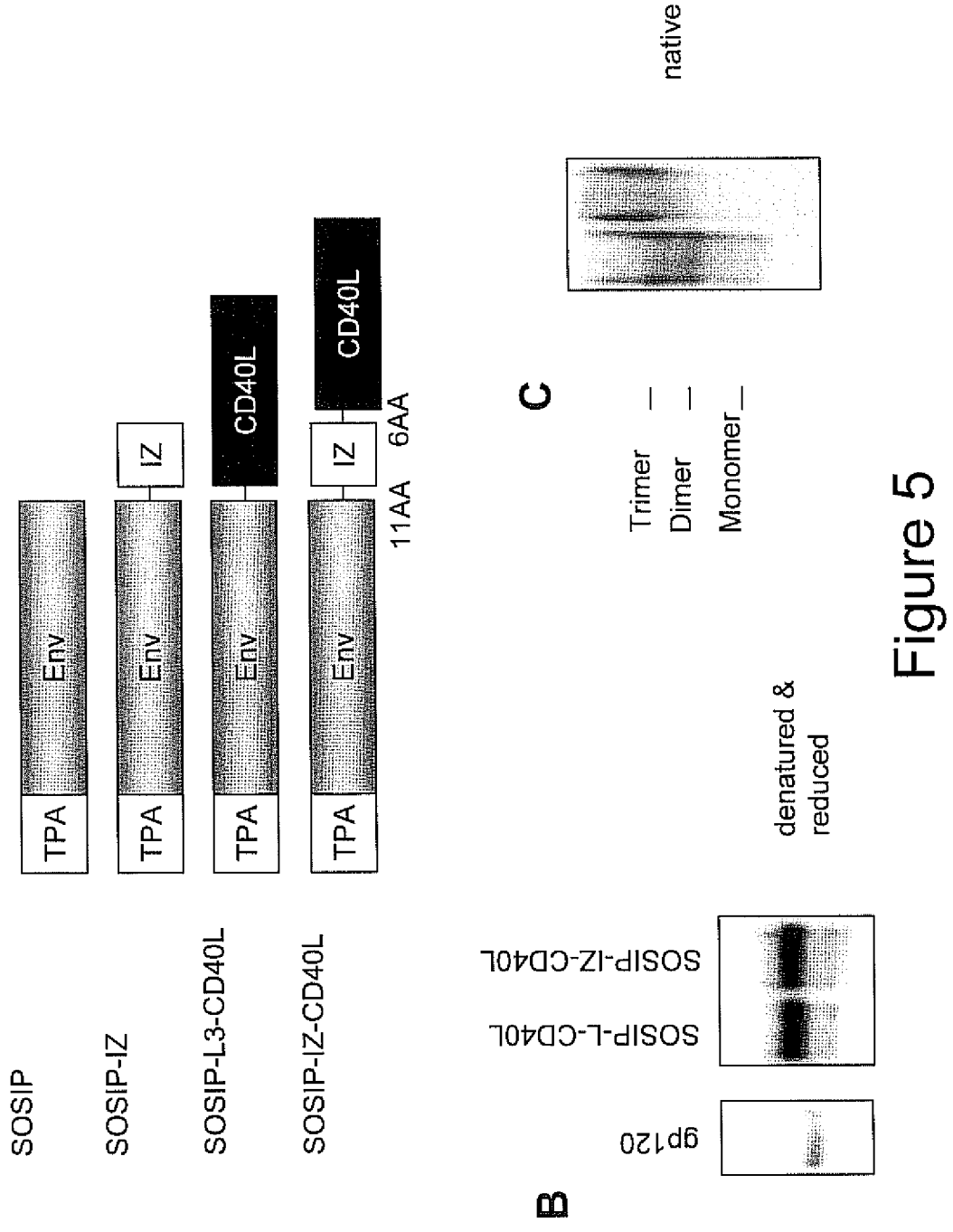

A
SOSIP
SOSIP-IZ
SOSIP-L3-CD40L
SOSIP-IZ-CD40L
TPA
Env
IZ
CD40L
11AA
6AA
B
gp120
SOSIP-L-CD40L
SOSIP-IZ-CD40L
denatured &
reduced
C
native
Trimer
Dimer
Monomer

Figure 5

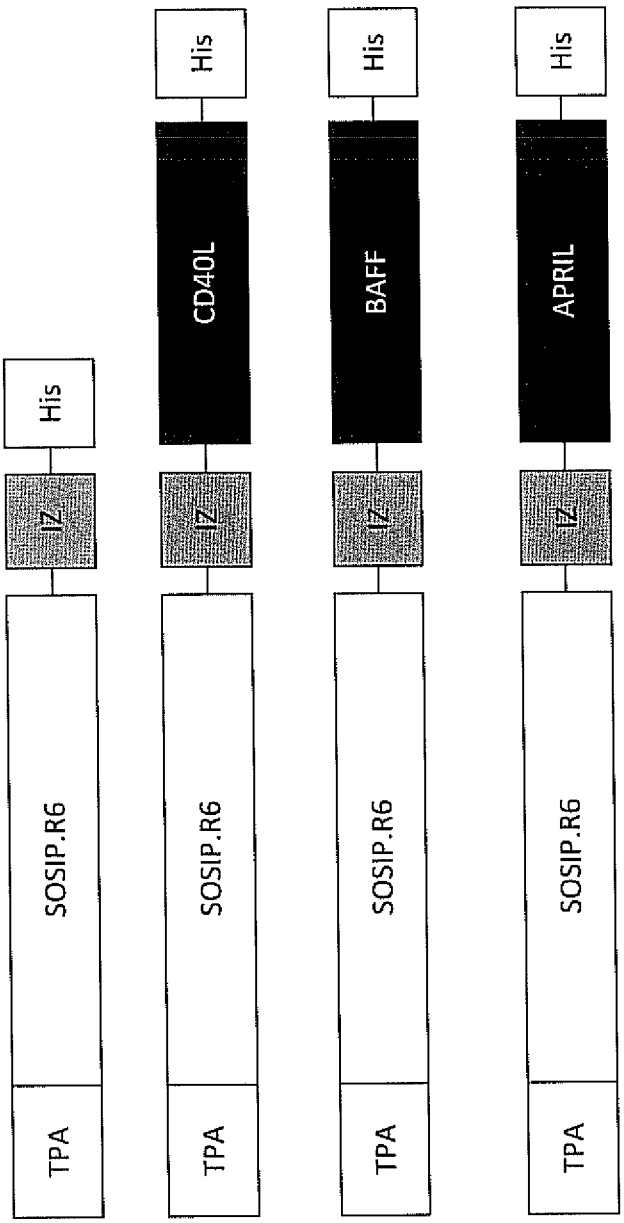

His
IZ
SOSIP.R6
TPA
His
CD40L
IZ
SOSIP.R6
TPA
His
BAFF
IZ
SOSIP.R6
TPA
His
APRIL
IZ
SOSIP.R6
TPA

Figure 6

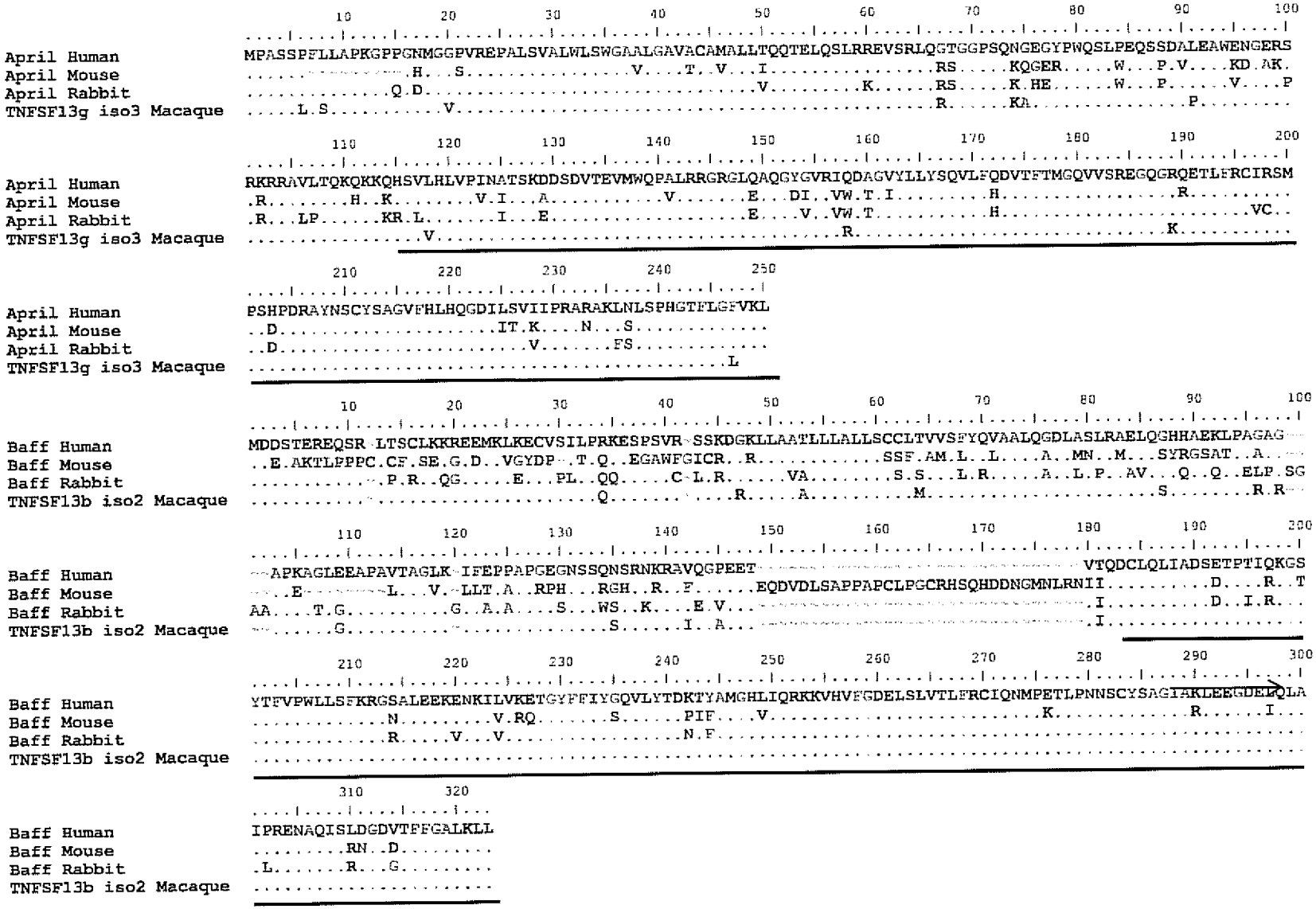

April Human
April Mouse
April Rabbit
TNFSF13g iso3 Macaque
Baff Human
Baff Mouse
Baff Rabbit
TNFSF13b iso2 Macaque

Figure 7

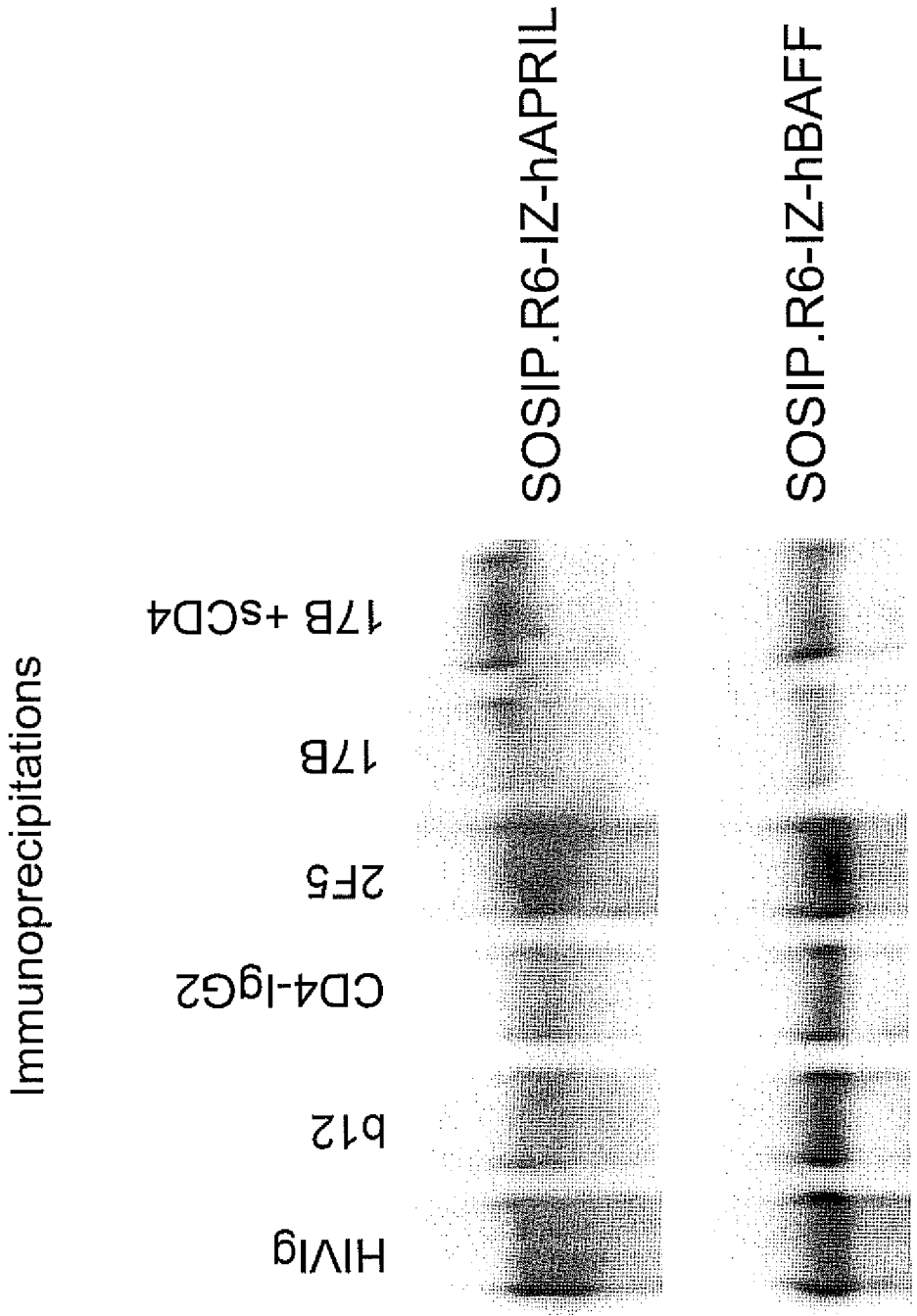
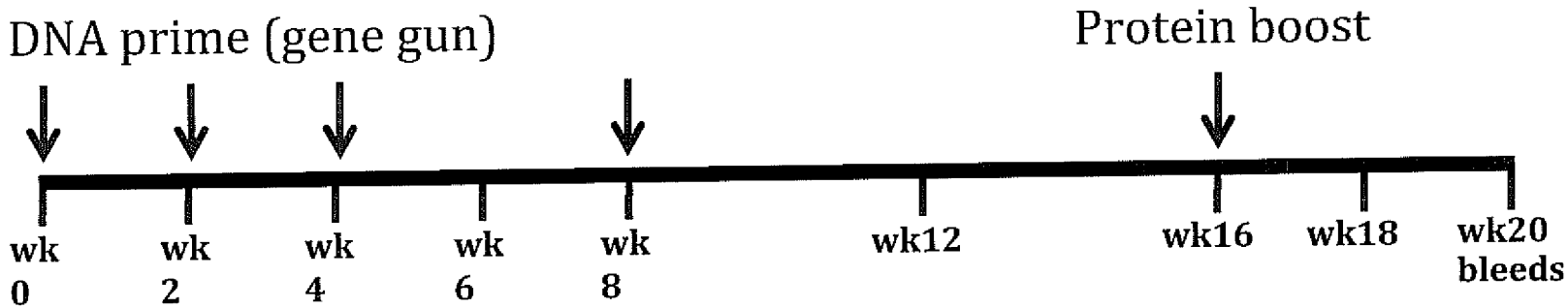

SOSIP.R6-IZ-hAPRIL
SOSIP.R6-IZ-hBAFF
Immunoprecipitations
17B +sCD4
17B
2F5
CD4-IgG2
b12
HIVIg

Figure 8

# Rabbit immunization schedule




| DNA prime | | protein boost |
|---|---|---|
| A. | JR-FL SOSIP.R6-IZ | JR-FL SOSIP.R6 (Progenics) |
| B. | JR-FL SOSIP.R6-IZ-rAPRIL | JR-FL SOSIP.R6 (Progenics) |
| C. | JR-FL SOSIP.R6-IZ-rBAFF | JR-FL SOSIP.R6 (Progenics) |
| D. | JR-FL SOSIP.R6-IZ-rCD40L | JR-FL SOSIP.R6 (Progenics) |
| E. | JR-FL gp120 protein | JR-FL SOSIP.R6 (Progenics) |

4 rabbits per group
DNA prime (gene gun): 125 $\mu$g per injection
Protein boost: 30 $\mu$g SOSIP.R6, 60 $\mu$g QuilA adjuvant in 1 ml PBS

Figure 9

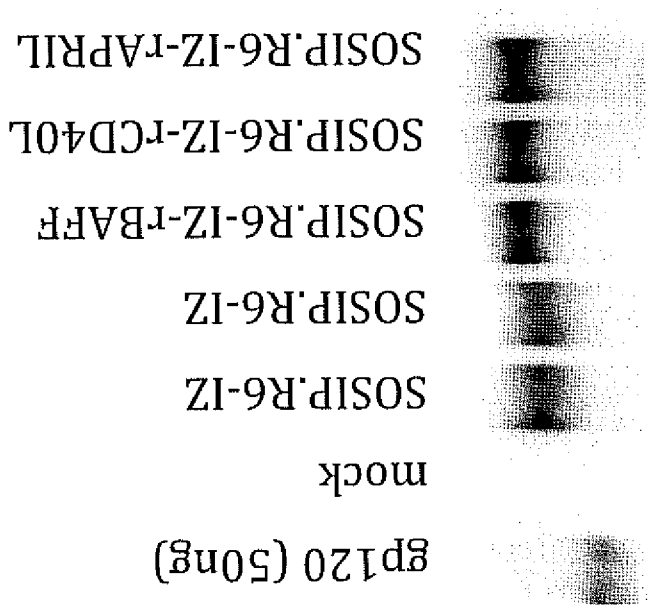

gp120 (50ng)
mock
SOSIP.R6-IZ
SOSIP.R6-IZ
SOSIP.R6-IZ-rBAFF
SOSIP.R6-IZ-rCD40L
SOSIP.R6-IZ-rAPRIL

Figure 10

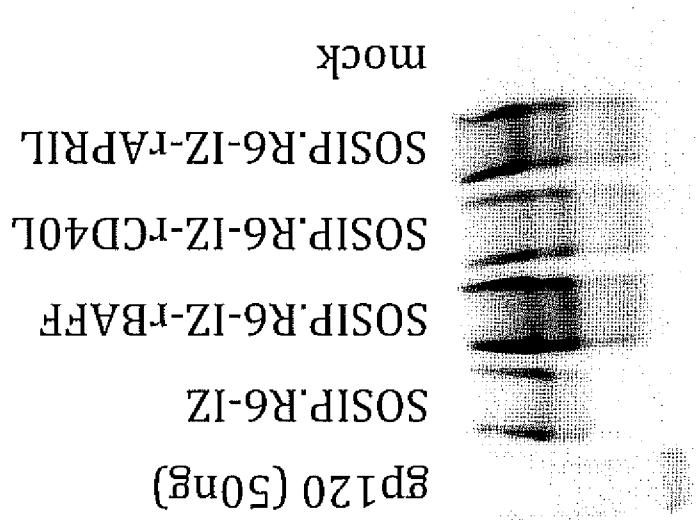

gp120 (50ng)
SOSIP.R6-IZ
SOSIP.R6-IZ-rBAFF
SOSIP.R6-IZ-rCD40L
SOSIP.R6-IZ-rAPRIL
mock

Figure 11

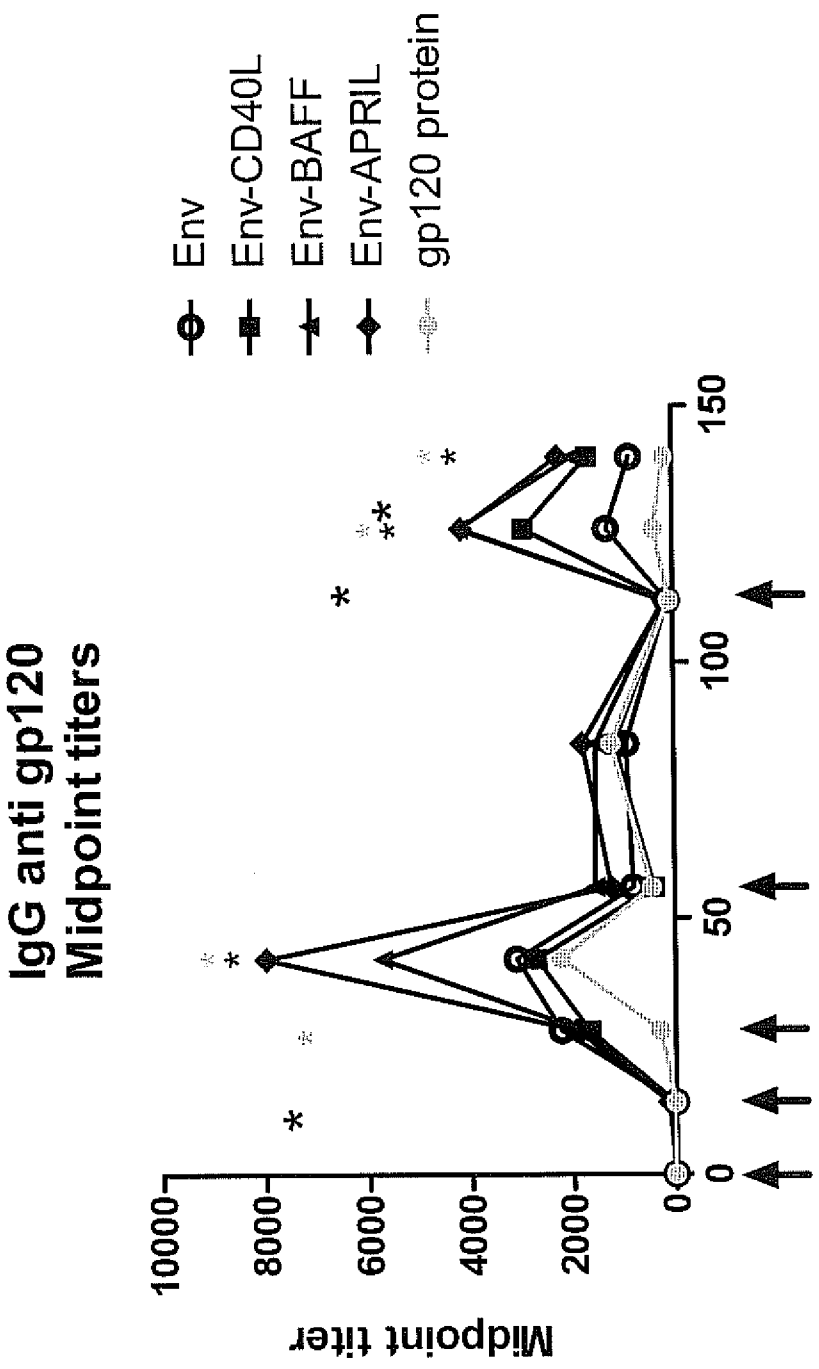

IgG anti gp120
Midpoint titers
Env
Env-CD40L
Env-BAFF
Env-APRIL
gp120 protein
Midpoint titer
10000
8000
6000
4000
2000
0
0
50
100
150
Time (days)

Figure 12

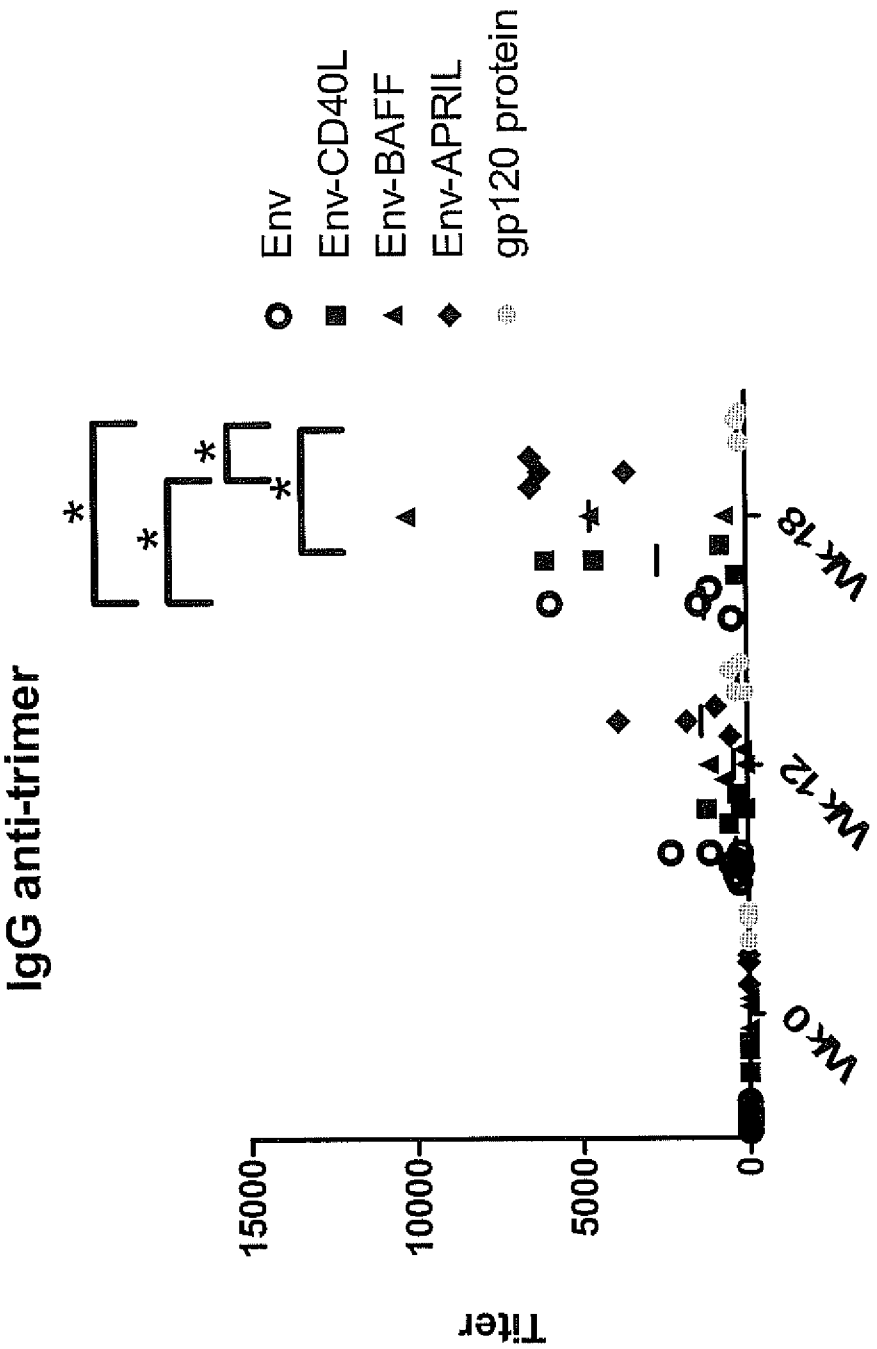

IgG anti-trimer
Titer
15000
10000
5000
0
Wk 0
Wk 12
Wk 18
Env
Env-CD40L
Env-BAFF
Env-APRIL
gp120 protein
*
*
*
*

Figure 13

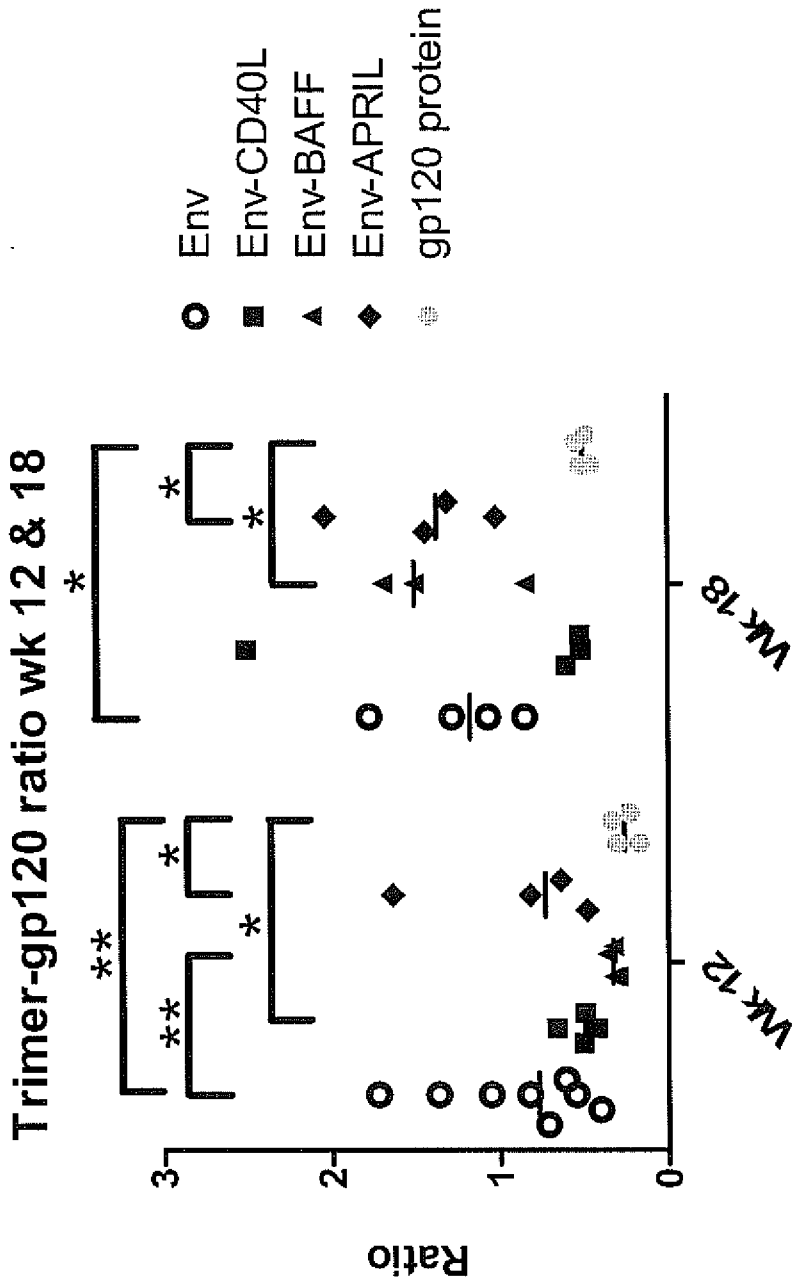

Trimer-gp120 ratio wk 12 & 18
Ratio
0
1
2
3
wk 12
wk 18
Env
Env-CD40L
Env-BAFF
Env-APRIL
gp120 protein
*
**

Figure 14

| Antigen | Animal | Virus | | |
|---|---|---|---|---|
| | | MN | SF162.LS | Bal.26 |
| Env | A289 | 2348 | 2298 | 177 |
| Env | A290 | 111 | 207 | <20 |
| Env | A323 | 519 | 54 | <20 |
| Env | A292 | 65 | 33 | <20 |
| Env-APRIL | B293 | 421 | 2003 | 117 |
| Env-APRIL | B294 | 112 | 176 | 57 |
| Env-APRIL | B295 | 3647 | 2170 | 164 |
| Env-APRIL | B296 | 4195 | 3118 | 191 |
| Env-BAFF | C297 | 6764 | 1589 | 347 |
| Env-BAFF | C299 | 241 | <20 | <20 |
| Env-BAFF | C300 | 53 | 817 | <20 |
| Env-CD40L | D301 | 68 | 2136 | 170 |
| Env-CD40L | D302 | 122 | 281 | 33 |
| Env-CD40L | D303 | <20 | 76 | <20 |
| Env-CD40L | D304 | 1438 | 2076 | 109 |
| gp120 protein | H317 | 39 | 50 | <20 |
| gp120 protein | H318 | <20 | 66 | <20 |
| gp120 protein | H319 | 64 | 250 | <20 |
| gp120 protein | H320 | 23 | <20 | <20 |

Figure 15

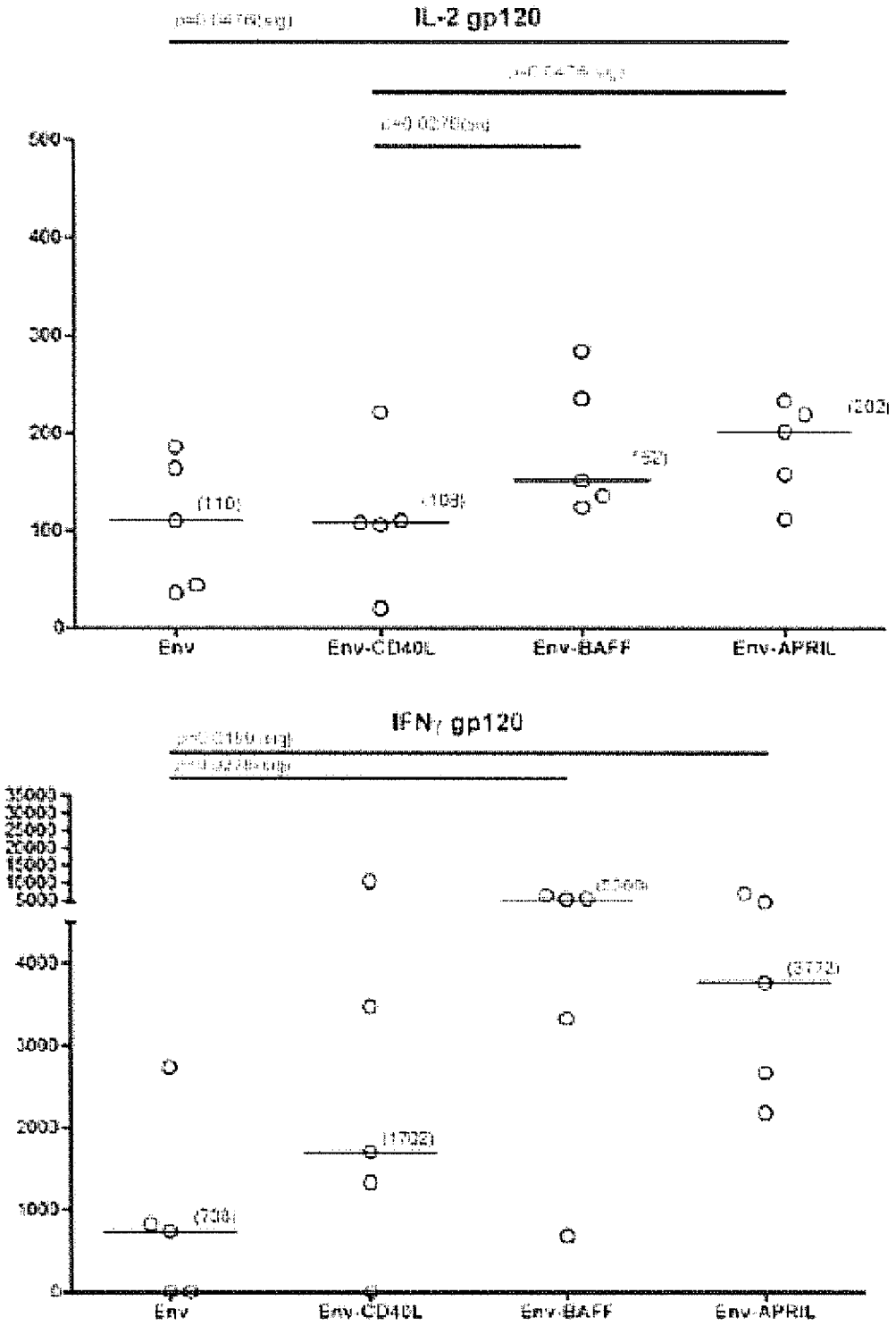

IL-2 gp120
400
300
200
100
0
(110)
(109)
(152)
(202)
Env
Env-CD40L
Env-BAFF
Env-APRIL
IFNγ gp120
4000
3000
2000
1000
0
(738)
(1732)
(3772)
Env
Env-CD40L
Env-BAFF
Env-APRIL

Figure 16

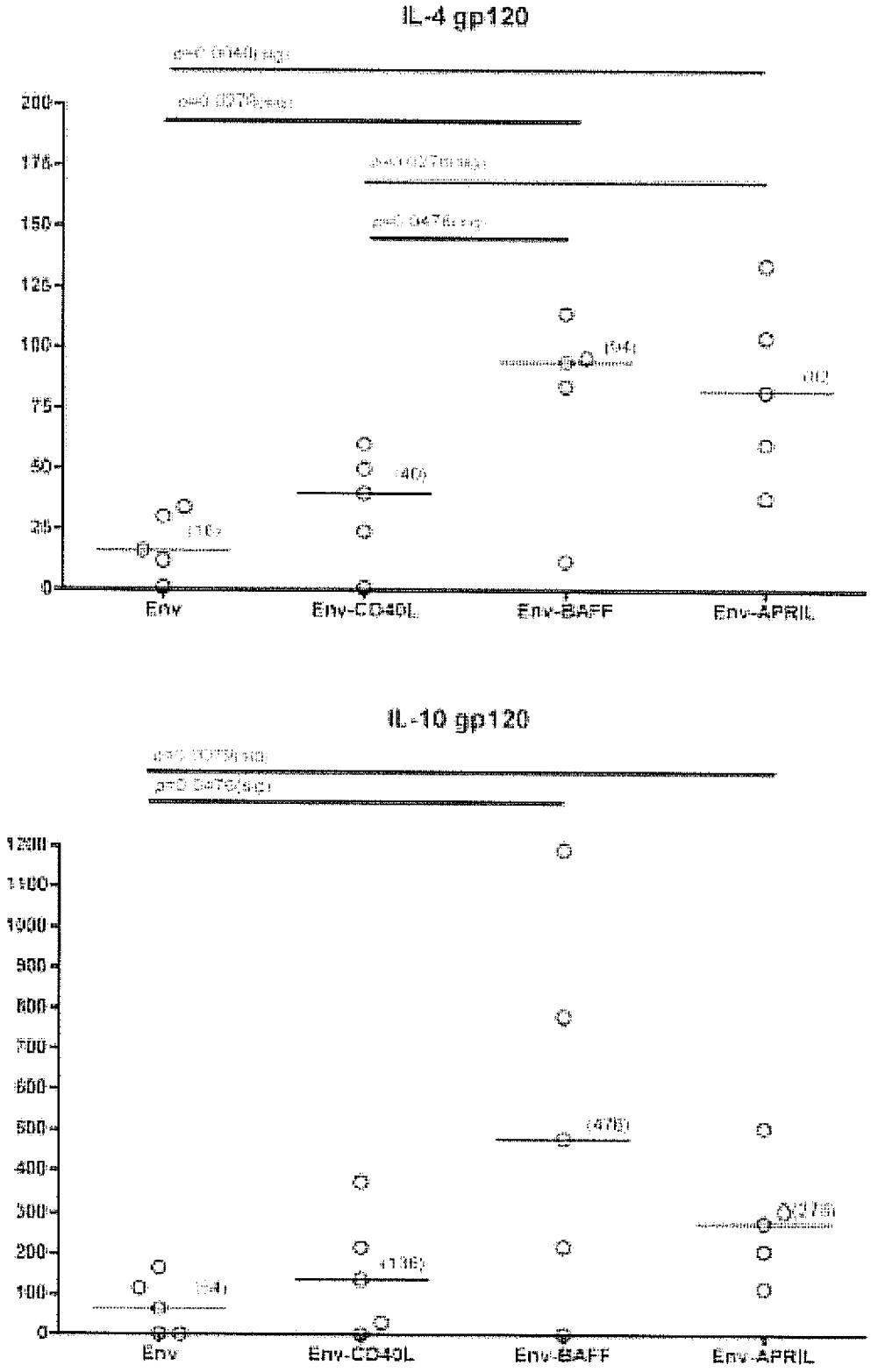

IL-4 gp120
200
175
150
125
100
75
50
25
0
Env
Env-CD40L
Env-BAFF
Env-APRIL
IL-10 gp120
1200
1100
1000
900
800
700
600
500
400
300
200
100
0
Env
Env-CD40L
Env-BAFF
Env-APRIL

Figure 17

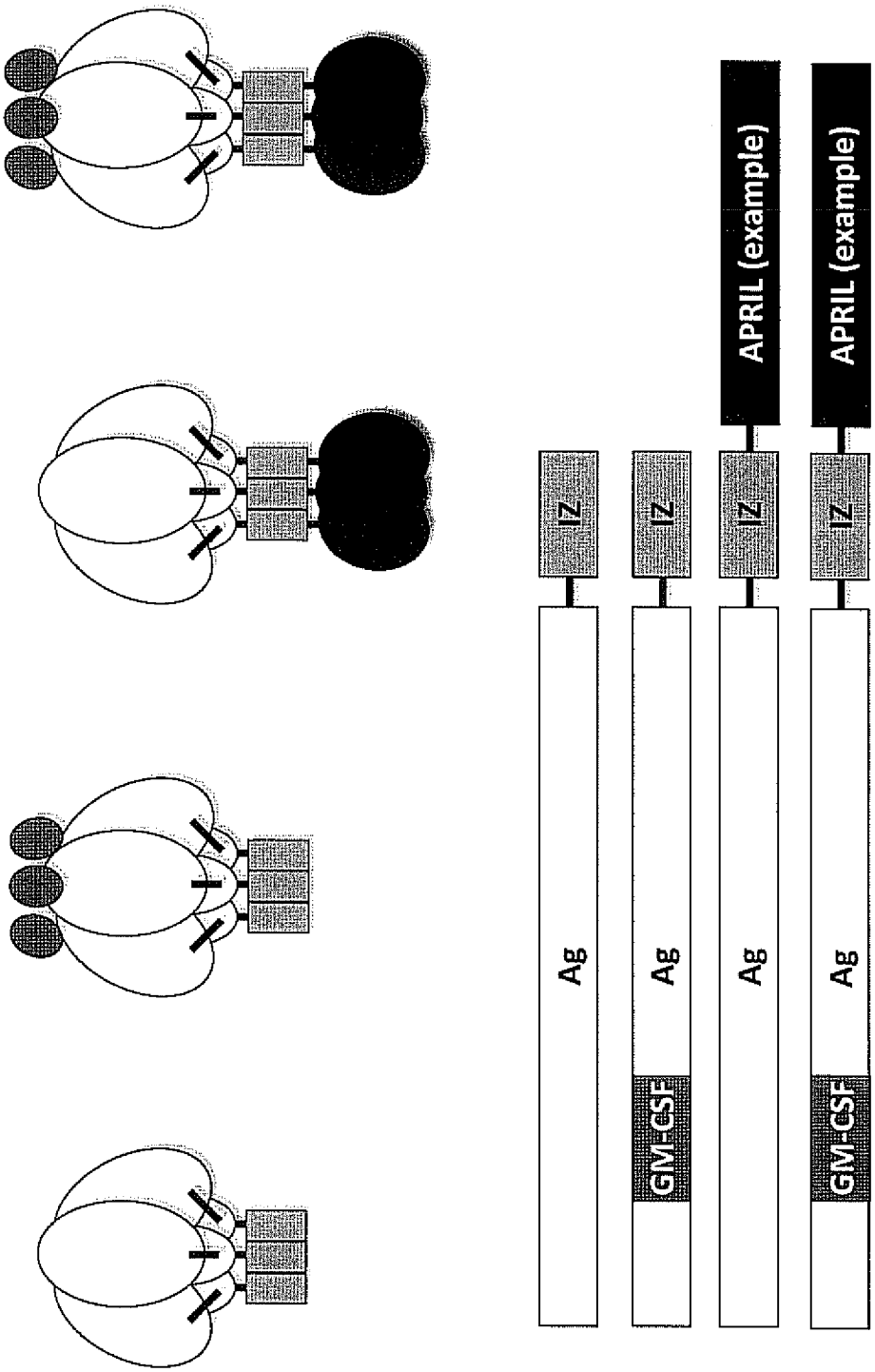

IZ
IZ
IZ
IZ
APRIL (example)
APRIL (example)
Ag
Ag
Ag
Ag
GM-CSF
GM-CSF

Figure 18

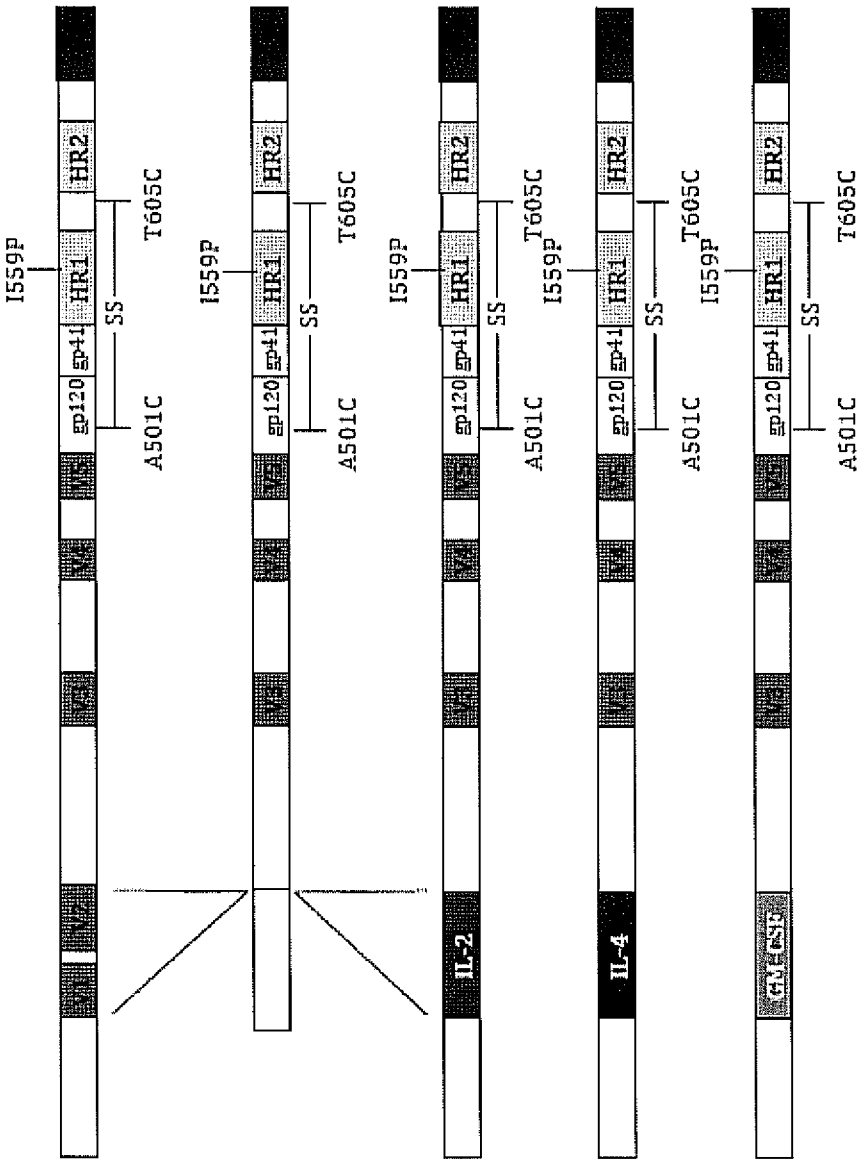

Env (gp140)
Env dV1V2
Env-IL2
Env-IL-4
Env-GM-CSF
I559P
T605C
A501C
SS
gp120
gp41
HR1
HR2
V1
V2
V3
V4
V5
IL-2
IL-4
GM-CSF

Figure 19

Env with IL-2, IL-4 GM-CSF inserts
(only gp120 shown) 65 amino acids of V1V2 removed

gp120
GARVEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLENVTEHFNMWKNNMVEQMQEDIISLWDQSLKPCVKLTPLCVTLNCKDVNATNTTNDSEGTMERGEIKNCSFNITTSIRDEV
QKEYALFYKLDVVPIDNNNTSYRLISCDTSVITQACPKISFEPIPIHYCAPAGFAILKCNDKTFNGKGPCKNVSTVQCTHGIRPVVSTQLLLNGSLAEEEVVIRSDNFTNNAKTIIVQLKESVEINCTRPNNNTRKSIHI
GPGRAFYTTGEIIGDIRQAHCNISRAKWNDTLKQIVIKLREQFENKTIVFNHSSGGDPEIVMHSFNCGGEFFYCNSTQLFNSTWNNNTEGSNNTEGNTITLPCRIKQIINMWQEVGKAMYAPPIRGQIRCSSNITGLLLT
RDGGINENGTEIFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQREKR
gp120 hIL-2: 128 amino acids inserted (plus 6 amino acids linker)
GARVEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLENVTEHFNMWKNNMVEQMQEDIISLWDQSLKPCVKLTPLCVGSGSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTF
KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFAQSIISTLTGSGSCDTSVITQACPKISFEPIPIHYCAPAGFAILKCNDKTFNGKGPCK
NVSTVQCTHGIRPVVSTQLLLNGSLAEEEVVIRSDNFTNNAKTIIVQLKESVEINCTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRAKWNDTLKQIVIKLREQFENKTIVFNHSSGGDPEIVMHSFNCGGEF
FYCNSTQLFNSTWNNNTEGSNNTEGNTITLPCRIKQIINMWQEVGKAMYAPPIRGQIRCSSNITGLLLTRDGGINENGTEIFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQREKR
gp120 hIL-4: 129 amino acids inserted (plus 6 amino acids linker)
GARVEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLENVTEHFNMWKNNMVEQMQEDIISLWDQSLKPCVKLTPLCVGSGHKCDITLQEIIKTLNSLTEQKTLCTELTVTDIFAASK
NTTEKETFCRAATVLRQFYSHHEKDTRCLGATAQQFHRHKQLIRFLKRLDRNLWGLAGLNSCPVKEANQSTLENFLERLKTIMREKYSKCSSGSGSCDTSVITQACPKISFEPIPIHYCAPAGFAILKCNDKTFNGKGPC
KNVSTVQCTHGIRPVVSTQLLLNGSLAEEEVVIRSDNFTNNAKTIIVQLKESVEINCTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRAKWNDTLKQIVIKLREQFENKTIVFNHSSGGDPEIVMHSFNCGGE
FFYCNSTQLFNSTWNNNTEGSNNTEGNTITLPCRIKQIINMWQEVGKAMYAPPIRGQIRCSSNITGLLLTRDGGINENGTEIFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQREKR
gp120 hGM-CSF 0: 114 amino acids inserted (plus 6 amino acids linker)
GARVEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLENVTEHFNMWKNNMVEQMQEDIISLWDQSLKPCVKLTPLCVGSGSTQPWEHVNAIQEARRLLNLSRDTAAEMNETVEVISE
MFDLQEPTCLQTRLELYKQGLRGSLTKLKGPLTMMASHYKQHCPPTPETSCATQIITFESFKENLKDFLLVIPFDCWGSGSCDTSVITQACPKISFEPIPIHYCAPAGFAILKCNDKTFNGKGPCKNVSTVQCTHGIRPV
VSTQLLLNGSLAEEEVVIRSDNFTNNAKTIIVQLKESVEINCTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRAKWNDTLKQIVIKLREQFENKTIVFNHSSGGDPEIVMHSFNCGGEFFYCNSTQLFNSTWN
NNTEGSNNTEGNTITLPCRIKQIINMWQEVGKAMYAPPIRGQIRCSSNITGLLLTRDGGINENGTEIFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQREKR
gp120 mGM-CSF 0: 111 amino acids inserted (plus 6 amino acids linker)
GARVEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLENVTEHFNMWKNNMVEQMQEDIISLWDQSLKPCVKLTPLCVGSGVTRPWKHVEAIKEALNLLDDMPVTLNEEVEVVSNEFS
FKKLTCVQTRLKIFEQGLRGNFTKLKGALNMTASYYQTYCPPTPETDCETQVTTYADFIDSLKTFLTDIPFECKGSGSCDTSVITQACPKISFEPIPIHYCAPAGFAILKCNDKTFNGKGPCKNVSTVQCTHGIRPVVST
QLLLNGSLAEEEVVIRSDNFTNNAKTIIVQLKESVEINCTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRAKWNDTLKQIVIKLREQFENKTIVFNHSSGGDPEIVMHSFNCGGEFFYCNSTQLFNSTWNNNT
EGSNNTEGNTITLPCRIKQIINMWQEVGKAMYAPPIRGQIRCSSNITGLLLTRDGGINENGTEIFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQREKR
gp120 hGM-CSF 1: 96 amino acids inserted (plus 6 amino acids linker)
GARVEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLENVTEHFNMWKNNMVEQMQEDIISLWDQSLKPCVKLTPLCVGSGIQEARRLLNLSRDTAAEMNETVEVISEMFDLQEPTCL
QTRLELYKQGLRGSLTKLKGPLTMMASHYKQHCPPTPETSCATQIITFESFKENLKDFLGSGSCDTSVITQACPKISFEPIPIHYCAPAGFAILKCNDKTFNGKGPCKNVSTVQCTHGIRPVVSTQLLLNGSLAEEEVVI
RSDNFTNNAKTIIVQLKESVEINCTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRAKWNDTLKQIVIKLREQFENKTIVFNHSSGGDPEIVMHSFNCGGEFFYCNSTQLFNSTWNNNTEGSNNTEGNTITLPC
RIKQIINMWQEVGKAMYAPPIRGQIRCSSNITGLLLTRDGGINENGTEIFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQREKR
gp120 hGM-CSF 2: 104 amino acids inserted (plus 6 amino acids linker)
GARVEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLENVTEHFNMWKNNMVEQMQEDIISLWDQSLKPCVKLTPLCVGSGWEHVNAIQEARRLLNLSRDTAAEMNETVEVISEMFDL
QEPTCLQTRLELYKQGLRGSLTKLKGPLTMMASHYKQHCPPTPETSCATQIITFESFKENLKDFLLVGSGSCDTSVITQACPKISFEPIPIHYCAPAGFAILKCNDKTFNGKGPCKNVSTVQCTHGIRPVVSTQLLLNGS
LAEEEVVIRSDNFTNNAKTIIVQLKESVEINCTRPNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRAKWNDTLKQIVIKLREQFENKTIVFNHSSGGDPEIVMHSFNCGGEFFYCNSTQLFNSTWNNNTEGSNNTE
GNTITLPCRIKQIINMWQEVGKAMYAPPIRGQIRCSSNITGLLLTRDGGINENGTEIFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQREKR
gp120 hGM-CSF 3: 70 amino acids inserted (plus 6 amino acids linker)
GARVEKLWVTVYYGVPVWKEATTTLFCASDAKAYDTEVHNVWATHACVPTDPNPQEVVLENVTEHFNMWKNNMVEQMQEDIISLWDQSLKPCVKLTPLCVGSGAAEMNETVEVISEMFDLQEPTCLQTRLELYKQGLRGS
LTKLKGPLTMMASHYKQHCPPTPETSCATQIITGSGSCDTSVITQACPKISFEPIPIHYCAPAGFAILKCNDKTFNGKGPCKNVSTVQCTHGIRPVVSTQLLLNGSLAEEEVVIRSDNFTNNAKTIIVQLKESVEINCTR
PNNNTRKSIHIGPGRAFYTTGEIIGDIRQAHCNISRAKWNDTLKQIVIKLREQFENKTIVFNHSSGGDPEIVMHSFNCGGEFFYCNSTQLFNSTWNNNTEGSNNTEGNTITLPCRIKQIINMWQEVGKAMYAPPIRGQIR
CSSNITGLLLTRDGGINENGTEIFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQREKR

Figure 20

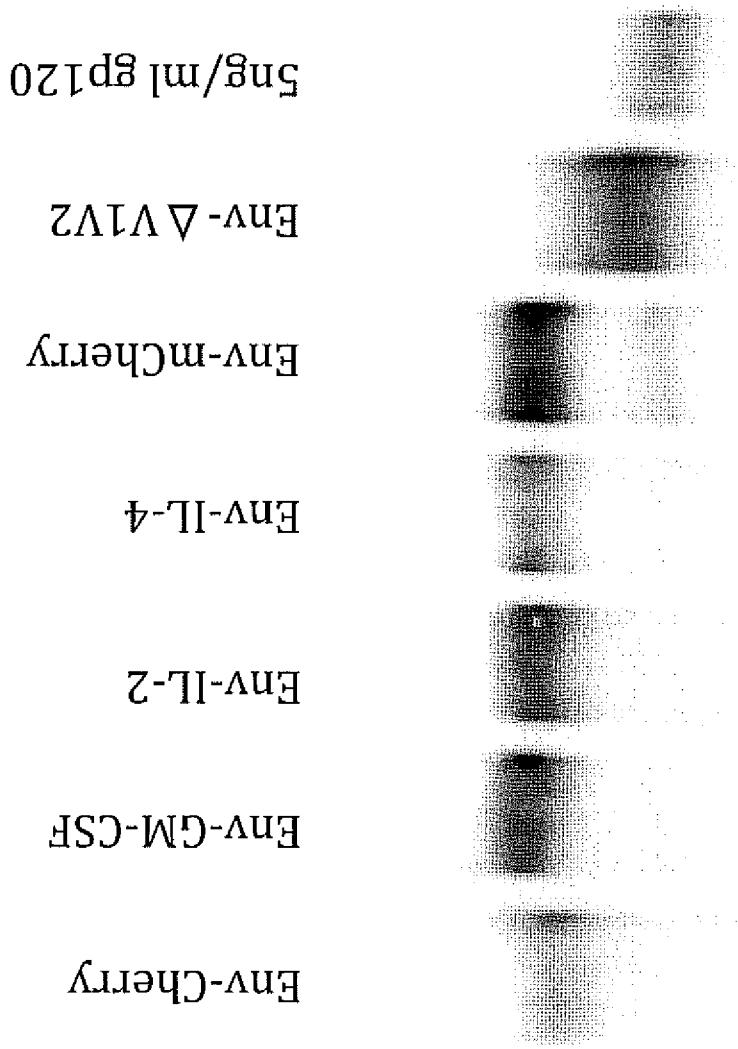

Env-Cherry
Env-GM-CSF
Env-IL-2
Env-IL-4
Env-mCherry
Env-ΔV1V2
5ng/ml gp120

Figure 21

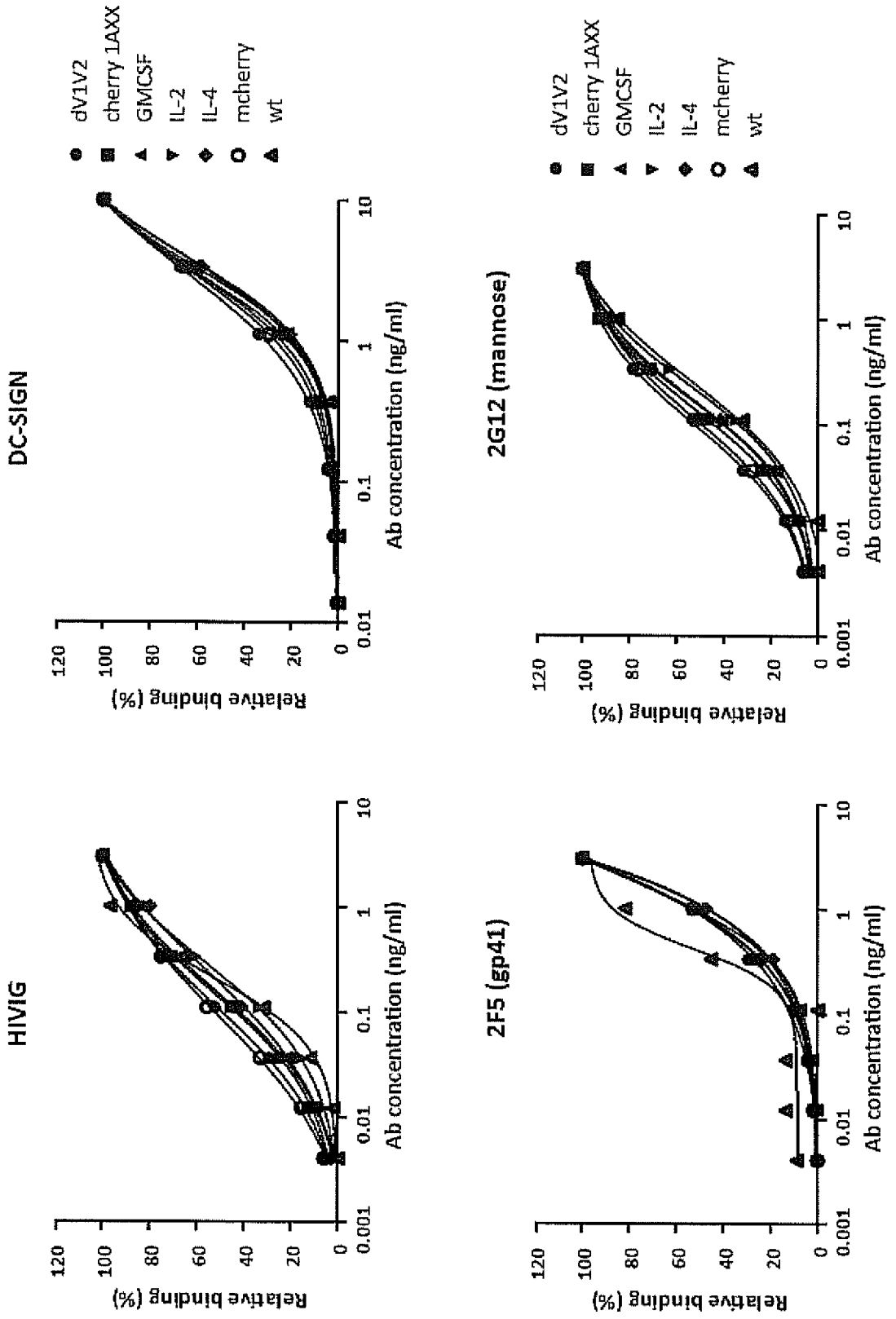

DC-SIGN
2G12 (mannose)
HIVIG
2F5 (gp41)
dV1V2
cherry 1AXX
GMCSF
IL-2
IL-4
mcherry
wt
Relative binding (%)
Ab concentration (ng/ml)
0
20
40
60
80
100
120
0.001
0.01
0.1
1
10

Figure 22

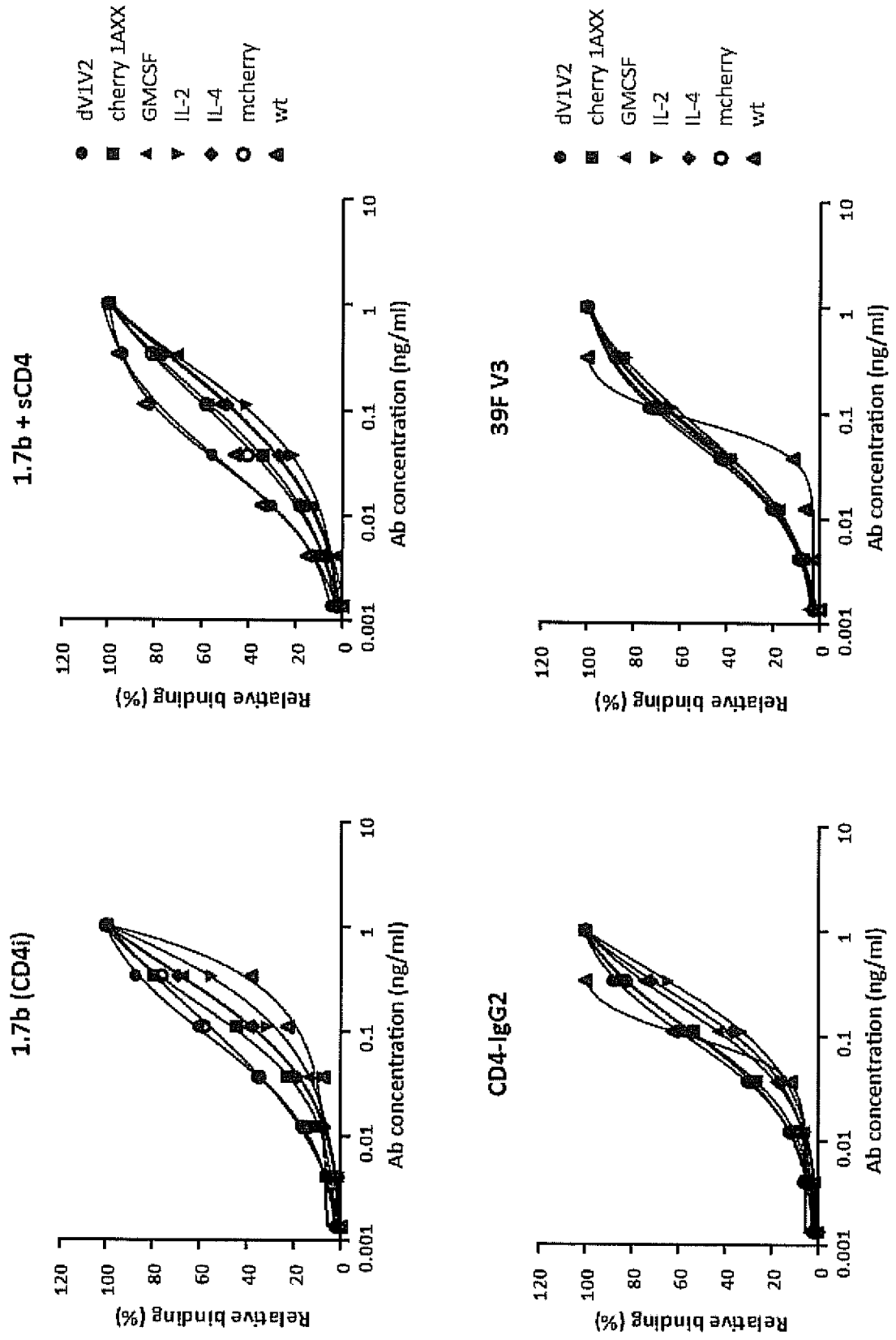

1.7b + sCD4
39F V3
1.7b (CD4i)
CD4-IgG2
dV1V2
cherry 1AXX
GMCSF
IL-2
IL-4
mcherry
wt
Relative binding (%)
Ab concentration (ng/ml)
0
20
40
60
80
100
120
0.001
0.01
0.1
1
10

Figure 23

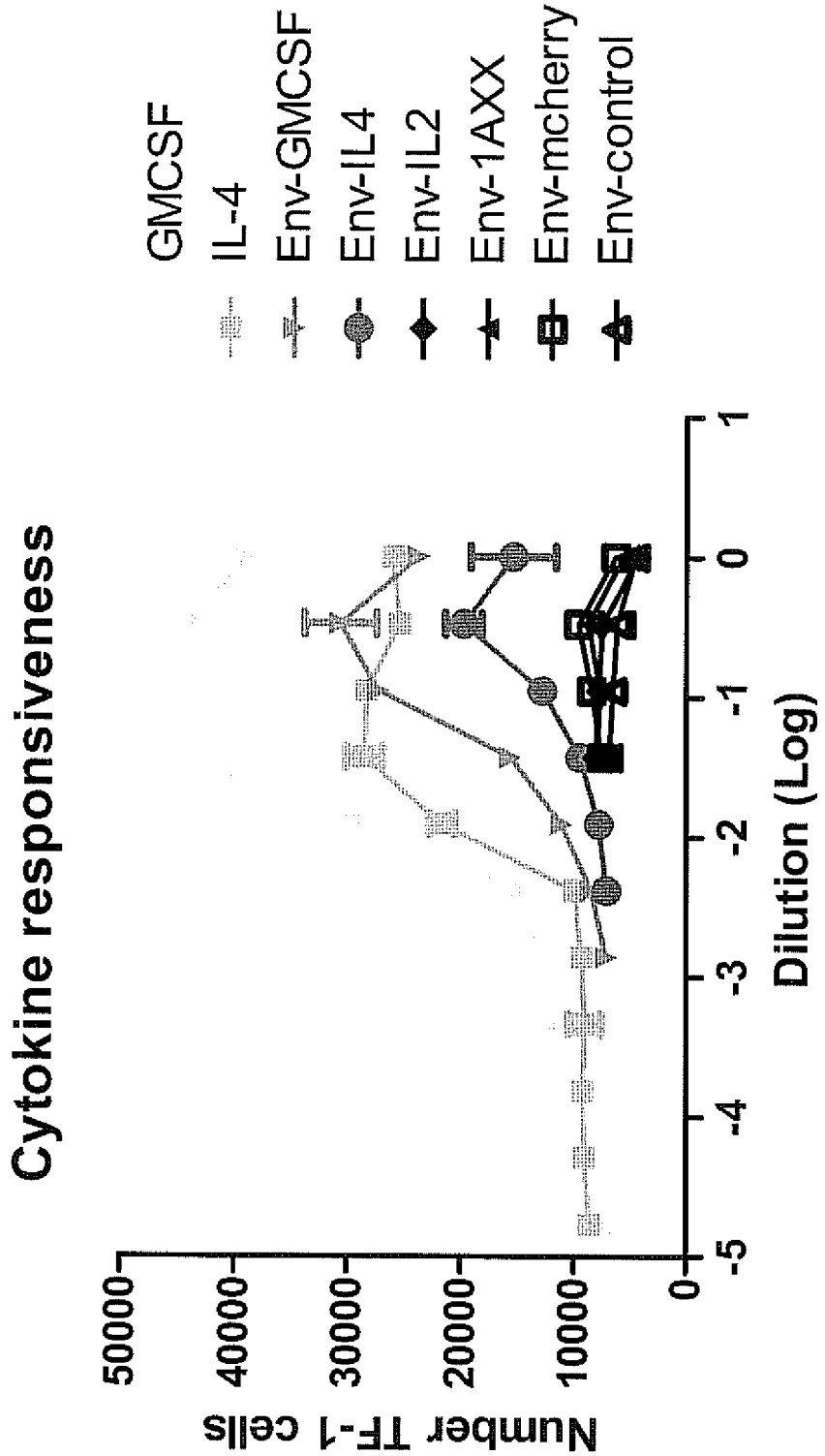

Cytokine responsiveness
GMCSF
IL-4
Env-GMCSF
Env-IL4
Env-IL2
Env-1AXX
Env-mcherry
Env-control
Number TF-1 cells
50000
40000
30000
20000
10000
0
-5
-4
-3
-2
-1
0
1
Dilution (Log)

Figure 24

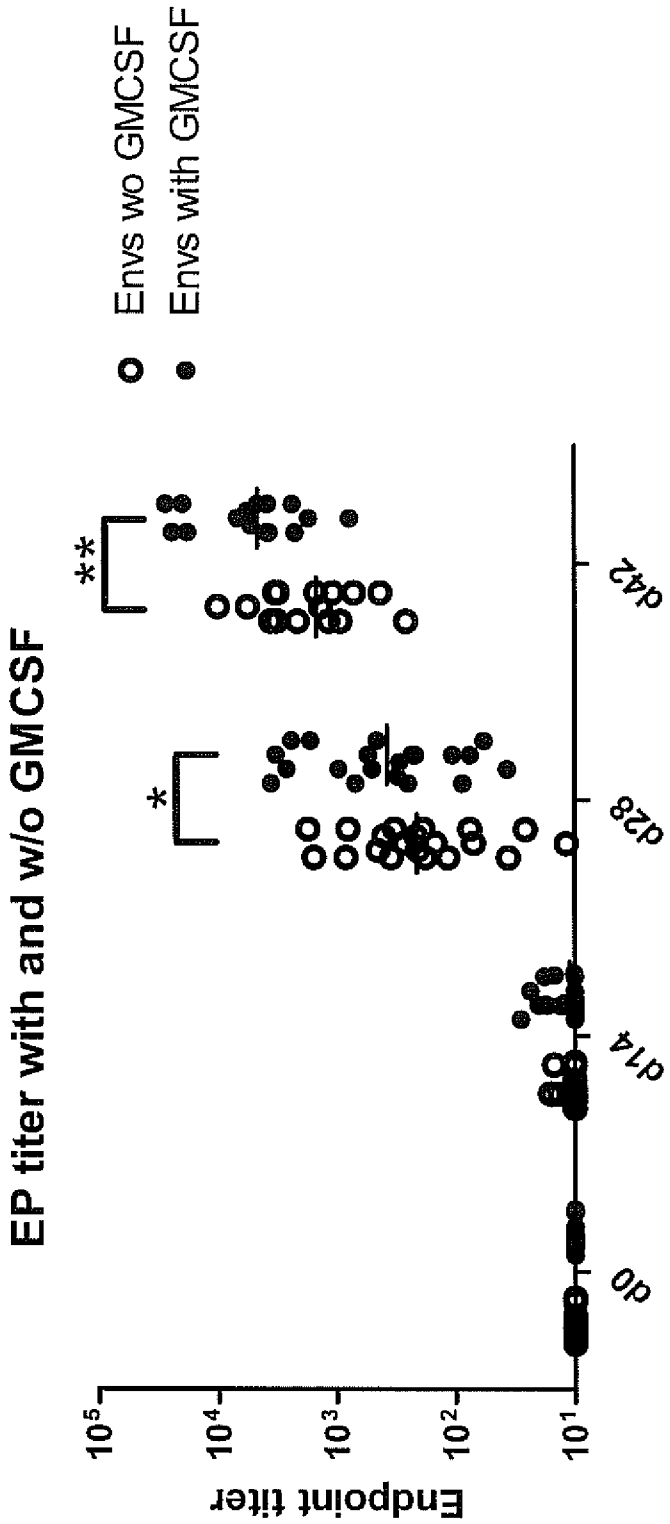

EP titer with and w/o GMCSF
Envs wo GMCSF
Envs with GMCSF
Endpoint titer
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
d0
d14
d28
d42
*
**

Figure 25

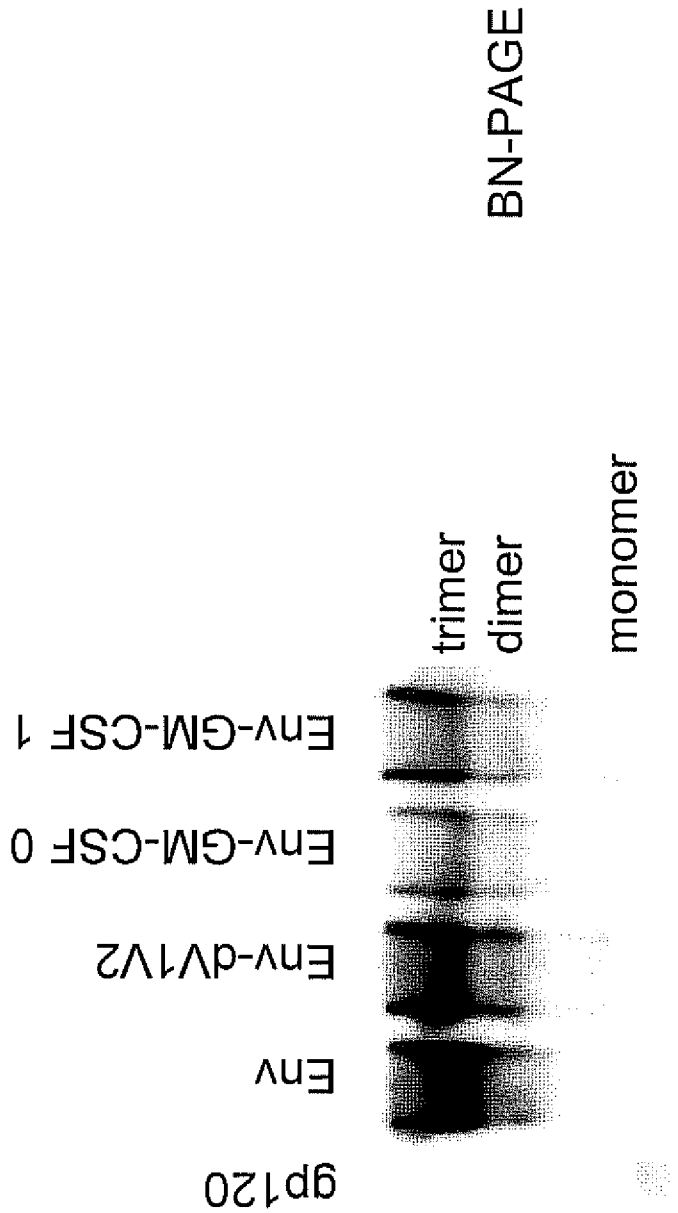

BN-PAGE
trimer
dimer
monomer
Env-GM-CSF 1
Env-GM-CSF 0
Env-dV1V2
Env
gp120

Figure 26

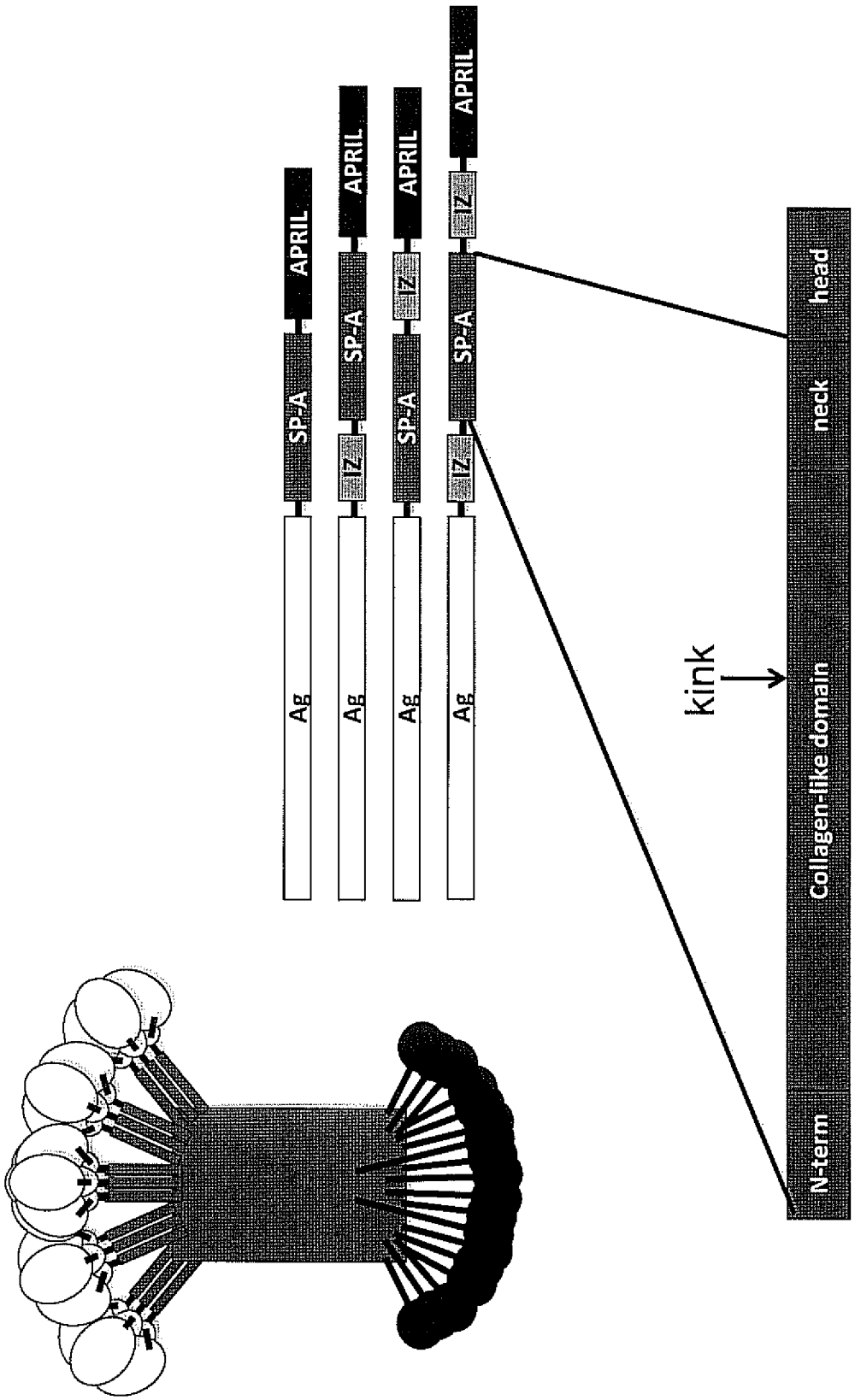



Figure 27

ALIGNMENT MOUSE AND HUMAN SURFACTANT PROTEIN A

```
mSP-A   MSLGSLAFTL FLTVVAGIKC NGTEVCAGSP GIPGTPGNHG LPGRDGRDGI KGDPGPPGPM GPPGGMPGLP GRDGLPGAPG APGEHGDKGE PGERGLPGFP
hSP-A1  MWLCPLALNL ILMAASGAVC EVKDVCVGSP GIPGTPGSHG LPGRDGRDGL KGDPGPPGPM GPPGEMPCPP GNDGLPGAPG IPGECGEKGE PGERGPPGLP
hSP-A2  MWLCPLALTL ILMAASGAAC EVKDVCVGSP GIPGTPGSHG LPGRDGRDGV KGDPGPPGPM GPPGETPCPP GNNGLPGAPG VPGERGEKGE AGERGPPGLP

mSP-A   AYLDEELQTA SYEIKHQILQ TMGVLSLQGS MLSVGDKVFS TNGQSVNFDT IREMCTRAGG HIAAPRNPEE NEAIASITKK YNTYPYLGVI EGQTPGDFHY
hSP-A1  AHLDEELQAT LHDFRHQILQ TRGALSLQGS IMTVGEKVFS SNGQSITFDA IQEACARAGG RIAVPRNPEE NEAIASFVKK YNTYAYVGLT EGPSPGDFRY
hSP-A2  AHLDEELQAT LHDFRHQILQ TRGALSLQGS IMTVGEKVFS SNGQSITFDA IQEACARAGG RIAVPRNPEE NEAIASFVKK YNTYAYVGLT EGPSPGDFRY

mSP-A   LDGASVNYTN WYPGEPRGRG KEKCVEMYTD GKWNDKGCLQ YRLAICEF
hSP-A1  SDGTPVNYTN WYRGEPAGRG KEQCVEMYTD GQWNDRNCLY SRLTI
hSP-A2  SDGTPVNYTN WYRGEPAGRG KEQCVEMYTD GQWNDRNCLY SRLTICEF
```

JR-FL SOSIP.R6-mSPA-CD40L-H9

```
MDAMKRGLCC VLLLCGAVFV SPSQEIHARF RRGAAVEKLW VTVYYGVPVW KEATTTLFCA SDAKAYDTEV HNVWATHACV
PTDPNPQEVV LENVTEHFNM WKNNMVEQMQ EDIISLWDQS LKPCVKLTPL CVTLNCKDVN ATNTTNDSEG TMERGEIKNC
SFNITTSIRD EVQKEYALFY KLDVVPIDNN NTSYRLISCD TSVITQACPK ISFEPIPIHY CAPAGFAILK CNDKTFNGKG
PCKNVSTVQC THGIRPVVST QLLLNGSLAE EEVVIRSDNF TNNAKTIIVQ LKESVEINCT RPNNNTRKSI HIGPGRAFYT
TGEIIGDIRQ AHCNISRAKW NDTLKQIVIK LREQFENKTI VFNHSSGGDP EIVMHSFNCG GEFFYCNSTQ LFNSTWNNNT
EGSNNTEGNT ITLPCRIKQI INMWQEVGKA MYAPPIRGQI RCSSNITGLL LTRDGGINEN GTEIFRPGGG DMRDNWRSEL
YKYKVVKIEP LGVAPTKCKR RVVQRRRRRR AVGIGAVFLG FLGAAGSTMG AASMTLTVQA RLLLSGIVQQ QNNLLRAPEA
QQRMLQLTVW GIKQLQARVL AVERYLGDQQ LLGIWGCSGK LICCTAVPWN ASWSNKSLDR IWNNMTWMEW EREIDNYTSE
IYTLIEESQN QQEKNEQELL ELDKWASLWN WFDITKWLWI LGGGGTGGGG TGNGTEVCAG SPGIPGTPGN HGLPGRDGRD
GIKGDPGPPG PMGPPGGMPG LPGRDGLPGA PGAPGEHGDK GEPGERGLPG FPAYLDEELQ TALYEIKHQI LQTMGVLSLQ
GGRGGGDPQI AAHVVSEANS NAASVLQWAK KGYYTMKSNL VMLENGKQLT VKREGLYYVY TQVTFCSNRE PSSQRPFIVG
LWLKPSSGSE RILLKAANTH SSSQLCEQQS VHLGGVFELQ AGASVFVNVT EASQVIHRVG FSSFGLLKLH HHHHHHHH
```

Figure 28

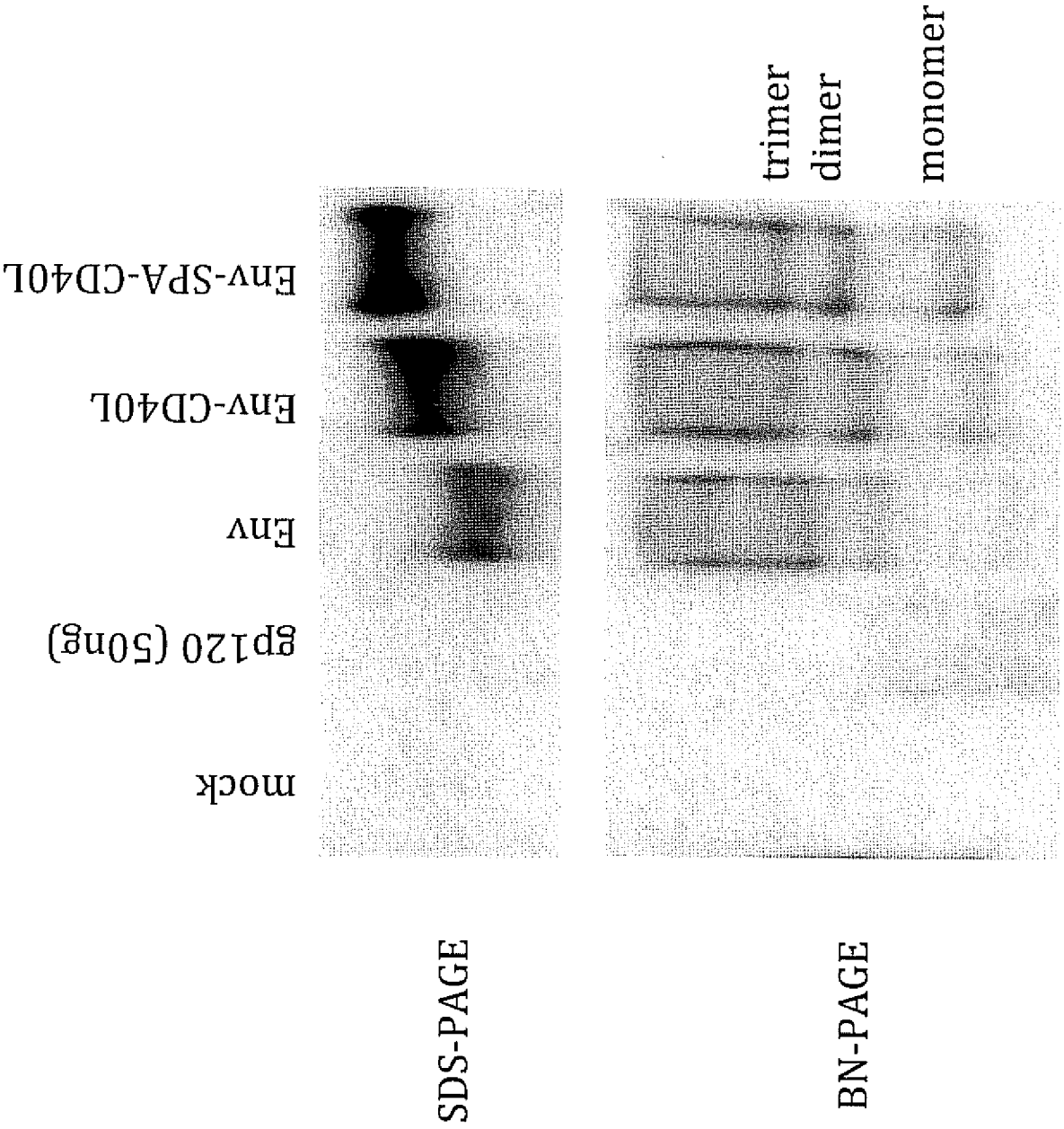

mock
gp120 (50ng)
Env
Env-CD40L
Env-SPA-CD40L
SDS-PAGE
trimer
dimer
monomer
BN-PAGE

Figure 29

```
Improvement gp140-mSPA-mCD40L octadecamer (bouquet)
TEMP    DITKWLWILGGGGTGGGGTG----------NGTEVCAGSPGIP---------GTPGNHGLPGRDGRDGIKGDPGPPGPMGPPGGMPGLPGRD
DWIWY DITK-----GGGGTGGGGTG----------NGTEVCAGSPGIP---------GTPGNHGLPGRDGRDGIKGDPGPPGPMGPPGGMPGLPGRD
1.      DITK-----GGGGTGGGGTG----------NGTEVCAGSPGIP---------GTPGNHGLPGRDGRDGIKGDPGPPGPMGPPGGMPGLPGRD
1A.     DITK-----GGGGTGGGGTG---------KCNGTEVCAGSPGIP---------GTPGNHGLPGRDGRDGIKGDPGPPGPMGPPGGMPGLPGRD
1B.     DITK-----GGGGTGGGGTG--------IKCNGTEVCAGSPGIP---------GTPGNHGLPGRDGRDGIKGDPGPPGPMGPPGGMPGLPGRD
2.      DITK-----GGGGTGGGGTG----------EVKDVCVGSPGIP---------GTPGNHGLPGRDGRDGIKGDPGPPGPMGPPGGMPGLPGRD
2A.     DITK-----GGGGTGGGGTG---------VCEVKDVCVGSPGIP---------GTPGNHGLPGRDGRDGIKGDPGPPGPMGPPGGMPGLPGRD
2B.     DITK-----GGGGTGGGGTG--------AVCEVKDVCVGSPGIP---------GTPGNHGLPGRDGRDGIKGDPGPPGPMGPPGGMPGLPGRD
3.      DITK-----GGGGTGGGGTGSGSQTCEDTLKTCSVIACGSPGIP---------GTPGNHGLPGRDGRDGIKGDPGPPGPMGPPGGMPGLPGRD
4.      DITK-----GGGGTGGGGTG----------NGTEVCAGSPGIP---------GTPGNHGLPGRDGRDGIKGDPGPPGPMGPPGEMPCLPGRD
5.      DITK-----GGGGTGGGGTG----------NGTEVCAGSPGIPGRDGRDGPKGTPGNHGLPGRDGRDGIKGDPGPPGPMGPPGGMPGLPGRD

Make dWIWIL (dWIWY) variant by qc (primers present) or cloning Asp718/agel-notl/sful using P382

2: Human SP-A1 variant
1A,1B,2A,2B: Signal peptide cleavage variants
3: Mouse MBL-A variant
4: Human SP-A1 and SP-A2 variants
5: Enhancing oligomerization collagen-domain

Screening: SDS-PAGE, BN-PAGE
Follow-up: gel filtration, IP

Further options:
- Combinations of 1,2,3 with 4 and/or 5
- Add IZ between gp140 and SP-A (wait for results Rafael for optimal configuration)
- Replace SP-A with adiponectin, C1q, MBP, hSP-A1, hSP-A2 etc.
- Replace natural coiled coil between SP-A and CD40L with IZ
- Add C65 (human SP-A1 variant)
- Replace CD40L with BAFF, APRIL, GITRL?
```

Figure 30

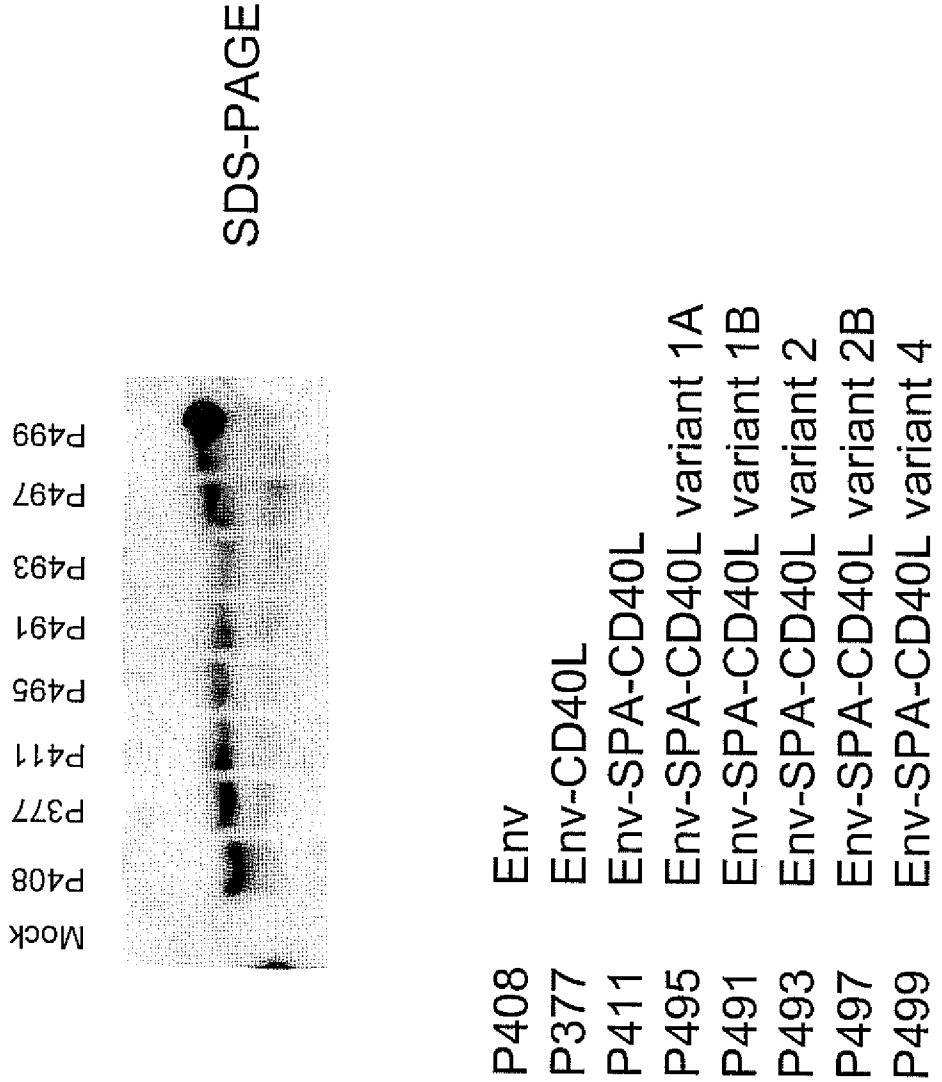

SDS-PAGE
Mock
P408
P377
P411
P495
P491
P493
P497
P499
P408 Env
P377 Env-CD40L
P411 Env-SPA-CD40L
P495 Env-SPA-CD40L variant 1A
P491 Env-SPA-CD40L variant 1B
P493 Env-SPA-CD40L variant 2
P497 Env-SPA-CD40L variant 2B
P499 Env-SPA-CD40L variant 4

Figure 31

```
MOUSE SURFACTANT PROTEIN A
MSLGSLAFTL FLTVVAGIKC NGTEVCAGSP GIPGTPGNHG LPGRDGRDGI KGDPGPPGPM GPPGGMPGLP GRDGLPGAPG APGEHGDKGE PGERGLPGFP
AYLDEELQTA SYEIKHQILQ TMGVLSLQGS MLSVGDKVFS TNGQSVNFDT IREMCTRAGG HIAAPRNPEE NEAIASITKK YNTYPYLGVI EGQTPGDFHY
LDGASVNYTN WYPGEPRGRG KEKCVEMYTD GKWNDKGCLQ YRLAICEF

MOUSE ADIPONECTIN
MLLLQALLFL LILPSHAEDD VTTTEELAPA LVPPPKGTCA GWMAGIPGHP GHNGTPGRDG RDGTPGEKGE KGDAGLLGPK GETGDVGMTG AEGPRGFPGT
PGRKGEPGEA AYVYRSAFSV GLETRVTVPN VPIRFTKIFY NQQNHYDGST GKFYCNIPGL YYFSYHITVY MKDVKVSLFK KDKAVLFTYD QYQEKNVDQA
SGSVLLHLEV GDQVWLQVYG DGDHNGLYAD NVNDSTFTGF LLYHDTN

MOUSE C1Q CHAIN A
METSQGWLVA CVLTMTLVWT VAEDVCRAPN GKDGAPGNPG RPGRPGLKGE RGEPGAAGIR TGIRGFKGDP GESGPPGKPG NVGLPGPSGP LGDSGPQGLK
GVKGNPGNIR DQPRPAFSAI RQNPMTLGNV VIFDKVLTNQ ESPYQNHTGR FICAVPGFYY FNFQVISKWD LCLFIKSSSG GQPRDSLSFS NTNNKGLFQV
LAGGTVLQLR RGDEVWIEKD PAKGRIYQGT EADSIFSGFL IFPSA

MOUSE C1Q CHAIN B
MKTQWGEVWT HLLLLLLGFL HVSWAQSSCT GPPGIPGIPG VPGVPGSDGQ PGTPGIKGEK GLPGLAGDLG EFGEKGDPGI PGTPGKVGPK GPVGPKGTPG
PSGPRGPKGD SGDYGATQKV AFSALRTINS PLRPNQVIRF EKVITNANEN YEPRNGKFTC KVPGLYYFTY HASSRGNLCV NLVRGRDRDS MQKVVTFCDY
AQNTFQVTTG GVVLKLEQEE VVHLQATDKN SLLGIEGANS IFTGFLLFPD MDA

MOUSE C1Q CHAIN C
MVVGPSCQPQ CGLCLLLLFL LALPLRSQAS AGCYGIPGMP GMPGAPGKDG HDGLQGPKGE PGIPAVPGTQ GPKGQKGEPG MPGHRGKNGP RGTSGLPGDP
GPRGPPGEPG VEGRYKQKHQ SVFTVTRQTT QYPEANALVR FNSVVTNPQG HYNPSTGKFT CEVPGLYYFV YYTSHTANLC VHLNLNLARV ASFCDHMFNS
KQVSSGGALL RLQRGDEVWL SVNDYNGMVG IEGSNSVFSG FLLFPD
```

Figure 32

MOUSE MANNOSE-BINDING LECTIN (PROTEIN C)
MSIFTSFLLL CVVTVVYAET LTEGVQNSCP VVTCSSPGLN GFPGKDGRDG AKGEKGEPGQ GLRGLQGPPG KVGPTGPPGN PGLKGAVGPK GDRGDRAEFD
TSEIDSEIAA LRSELRALRN WVLFSLSEKV GKKYFVSSVK KMSLDRVKAL CSEFQGSVAT PRNAEENSAI QKVAKDIAYL GITDVRVEGS FEDLTGNRVR
YTNWNDGEPN NTGDGEDCVV ILGNGKWNDV PCSDSFLAIC EFSD

MOUSE MANNOSE-BINDING LECTIN (PROTEIN A)
MLLLPLLPVL LCVVSVSSSG SQTCEDTLKT CSVIACGRDG RDGPKGEKGE PGQGLRGLQG PPGKLGPPGS VGSPGSPGPK GQKGDHGDNR AIEEKLANME
AEIRILKSKL QLTNKLHAFS MGKKSGKKLF VTNHEKMPFS KVKSLCTELQ GTVAIPRNAE ENKAIQEVAT GIAFLGITDE ATEGQFMYVT GGRLTYSNWK
KDEPNNHGSG EDCVIILDNG LWNDISCQAS FKAVCEFPA

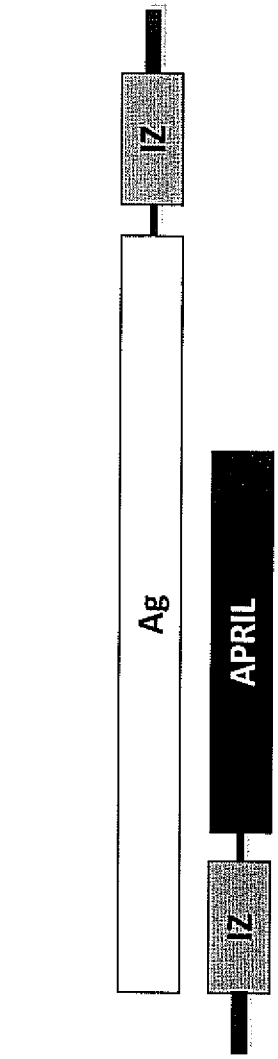
COLLECTIN-L1 [MUS MUSCULUS]
MNGFRVLLRS NLSMLLLLAL LHFQSLGLDV DSRSAAEVCA THTISPGPKG DDGERGDTGE EGKDGKVGRQ GPKGVKGELG DMGAQGNIGK SGPIGKKGDK
GEKGLLGIPG EKGKAGTICD CGRYRKVVGQ LDISVARLKT SMKFIKNVIA GIRETEEKFY YIVQEEKNYR ESLTHCRIRG GMLAMPKDEV VNTLIADYVA
KSGFFRVFIG VNDLEREGQY VFTDNTPLQN YSNWKEEEPS DPSGHEDCVE MLSSGRWNDT ECHLTMYFVC EFVKKKK

HUMAN SURFACTANT PROTEIN A1
MWLCPLALNL ILMAASGAVC EVKDVCVGSP GIPGTPGSHG LPGRDGRDGL KGDPGPPGPM GPPGEMPCPP GNDGLPGAPG IPGECGEKGE PGERGPPGLP
AHLDEELQAT LHDFRHQILQ TRGALSLQGS IMTVGEKVFS SNGQSITFDA IQEACARAGG RIAVPRNPEE NEAIASFVKK YNTYAYVGLT EGPSPGDFRY
SDGTPVNYTN WYRGEPAGRG KEQCVEMYTD GQWNDRNCLY SRLTI

HUMAN SURFACTANT PROTEIN A2
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AHLDEELQAT LHDFRHQILQ TRGALSLQGS IMTVGEKVFS SNGQSITFDA IQEACARAGG RIAVPRNPEE NEAIASFVKK YNTYAYVGLT EGPSPGDFRY
SDGTPVNYTN WYRGEPAGRG KEQCVEMYTD GQWNDRNCLY SRLTICEF



Figure 32, contd.



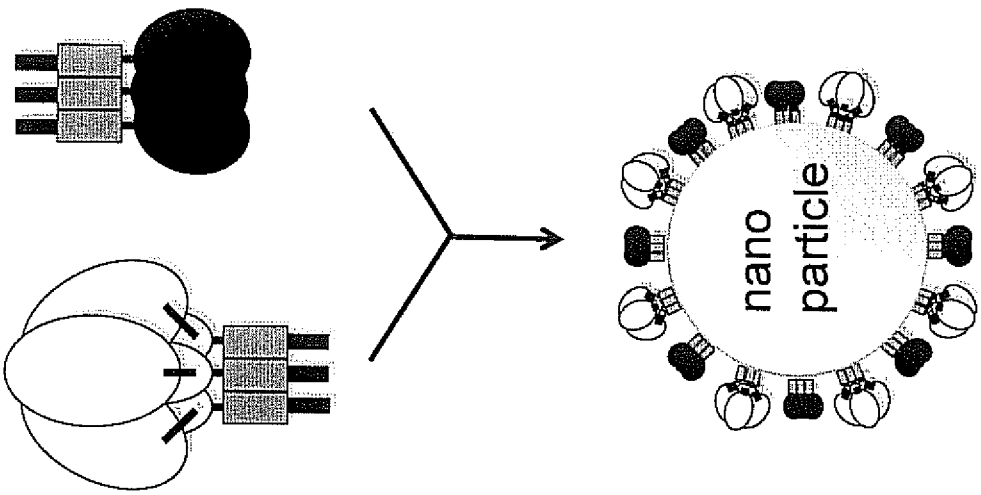
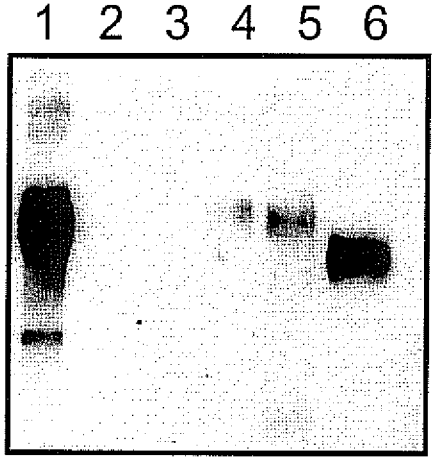




Figure 33




1) Env Beads 15ul (van 30 ul eluate, van 12 ml culture sup)
2) Supernatant Env beads after DNA treatment
3) Supernatant Env beads
4) Medium after Env-bead extraction
5) Medium Env
6) gp120 5 ng/ml

Experimental procedure:

Beads were incubated for 2.5 hrs with Env medium (5) at 4 degrees

Env medium after bead extraction was collected (4)

Beads fraction (1) was washed and wash supernatant was collected (3)

Additional bead fraction was treated for DNA restriction

Beads were collected and supernatant was collected (2)





Figure 34

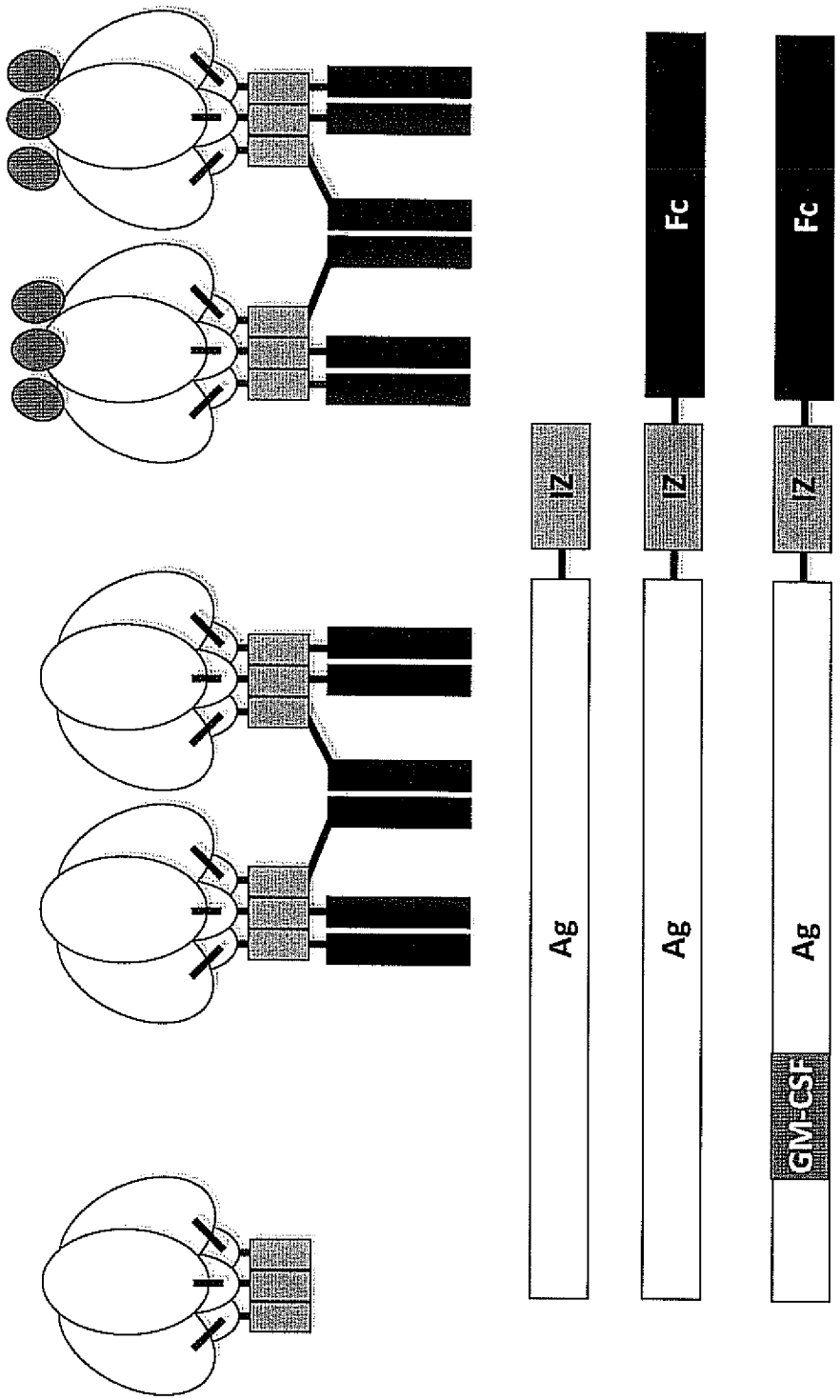



Figure 35

5ng/ml gp120
Env-IL4-hFC
Env-IL4
Env-IL2-hFC
Env-IL2
Env-hFC
Env-GMCSF-hFC
Env-GMCSF
Env
Env

Figure 36

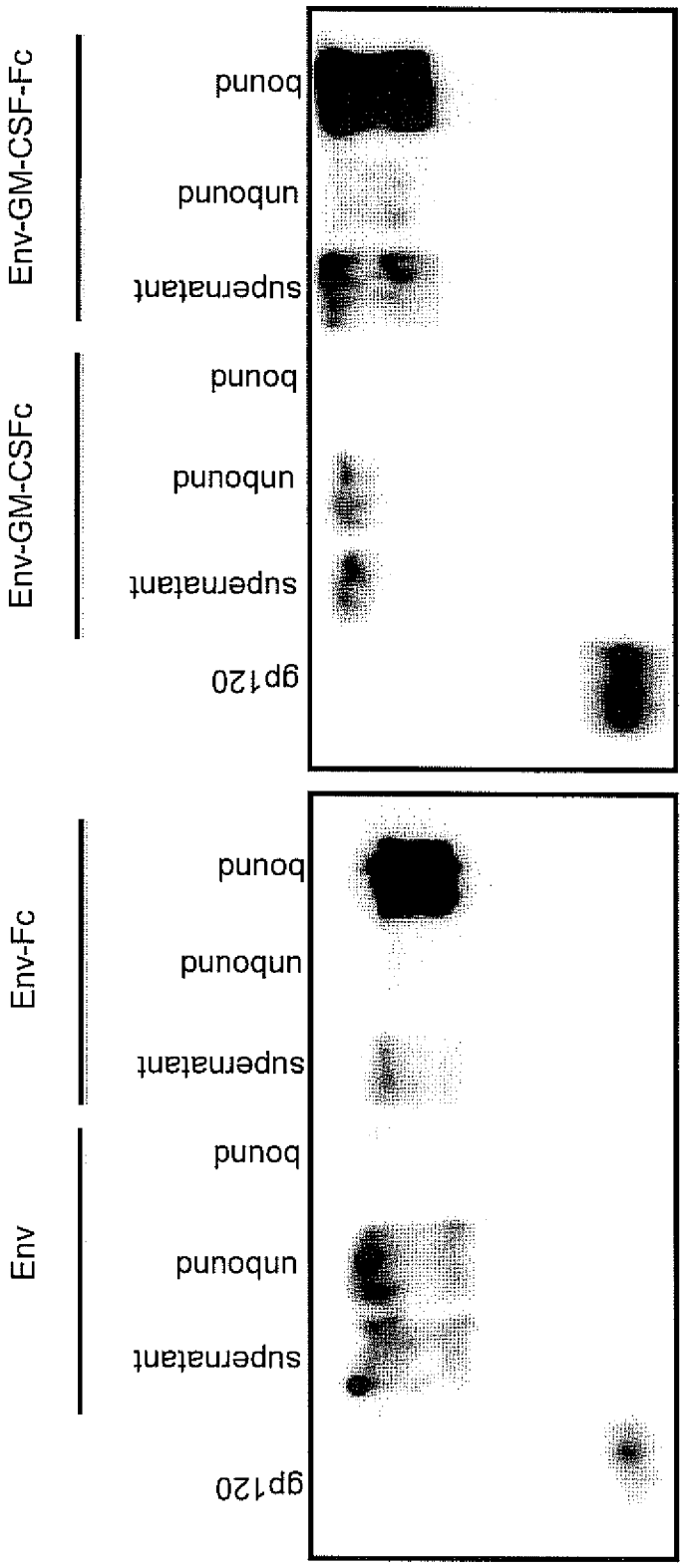

Env-GM-CSF-Fc
punoq
punoqun
supernatant
Env-GM-CSFc
punoq
punoqun
supernatant
gp120
Env-Fc
punoq
punoqun
supernatant
Env
punoq
punoqun
supernatant
gp120

Figure 37

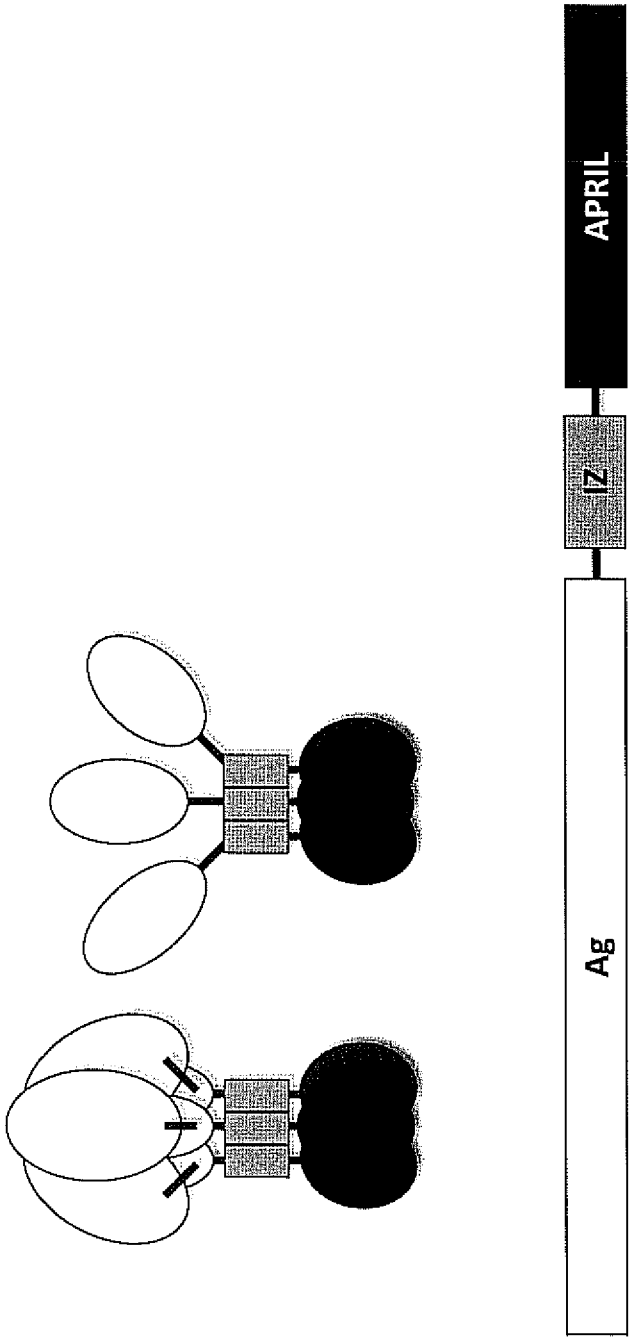



Figure 38

Fig. 39A

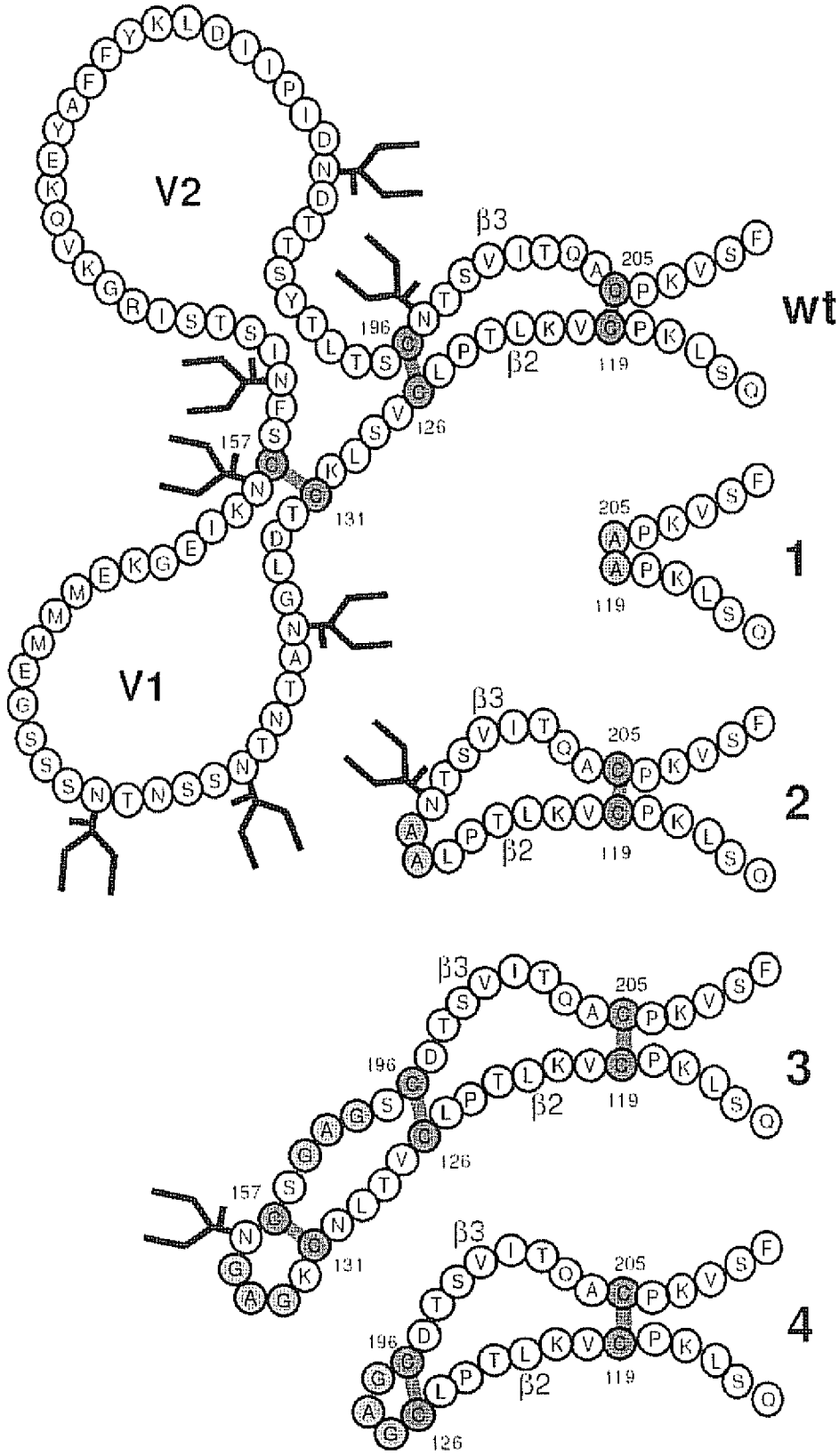


Fig. 39A, contd.

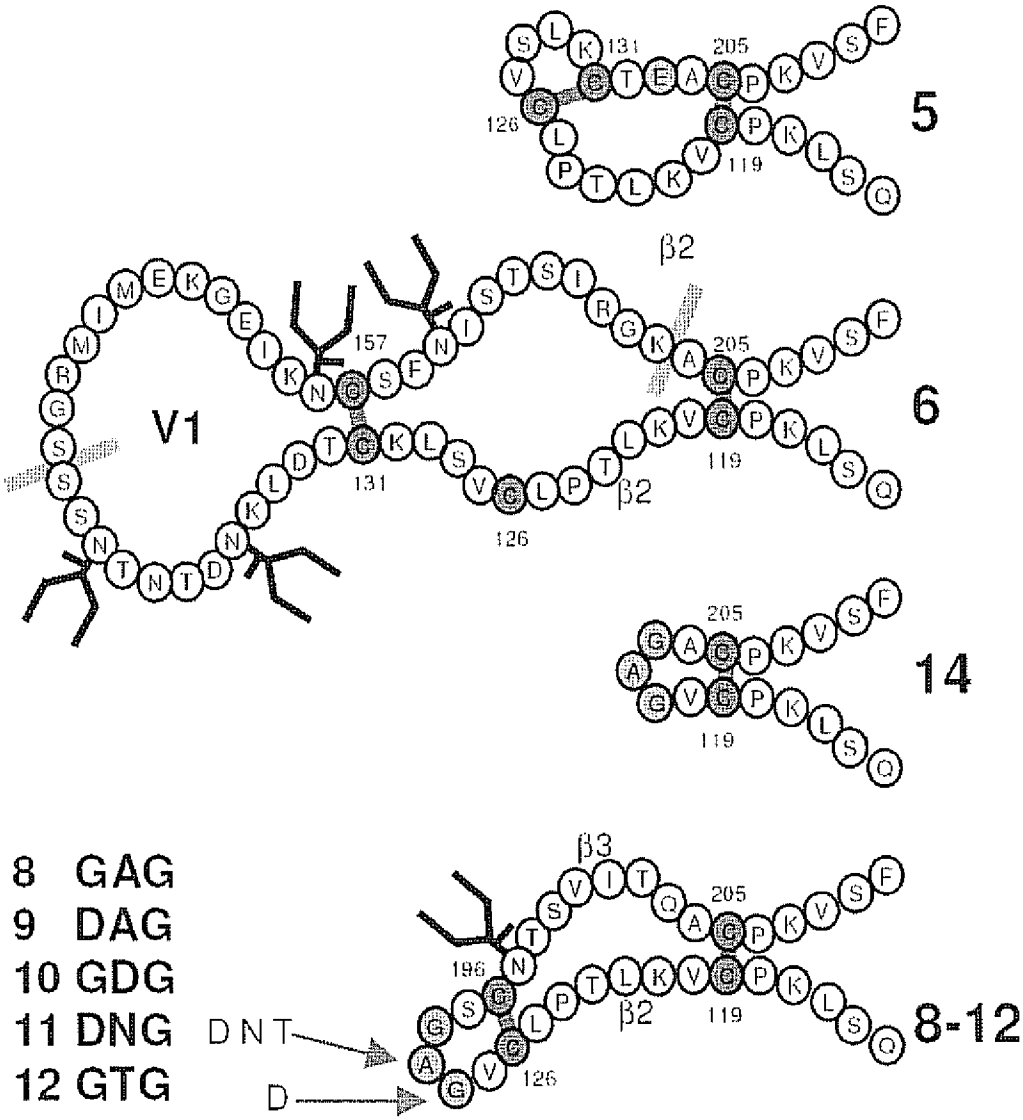


Fig. 39B

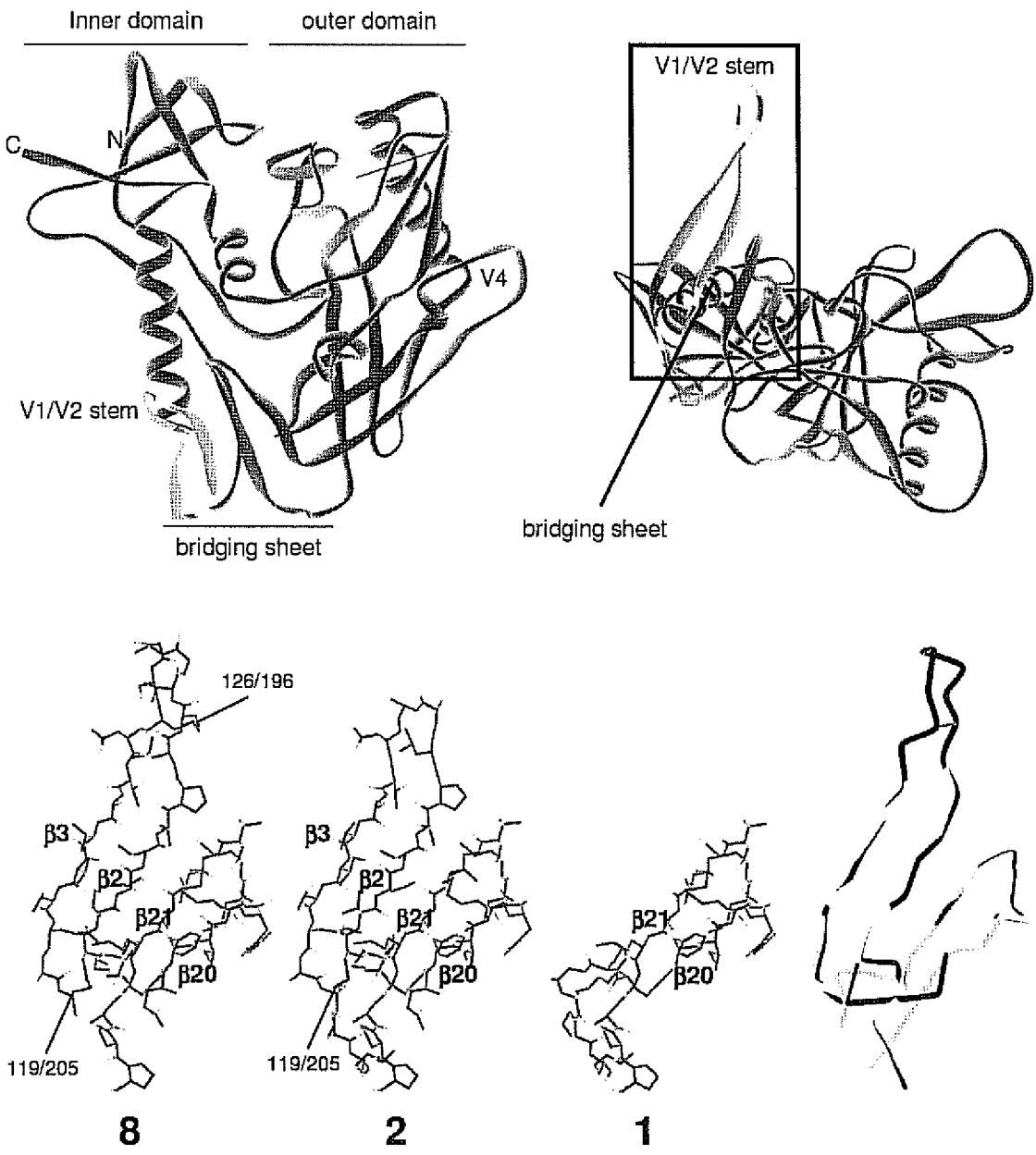

Inner domain
outer domain
C
N
V4
V1/V2 stem
bridging sheet
V1/V2 stem
bridging sheet
126/196
β3
β2
β21
β20
119/205
8
β3
β2
β21
β20
119/205
2
β21
β20
1

Fig. 39C

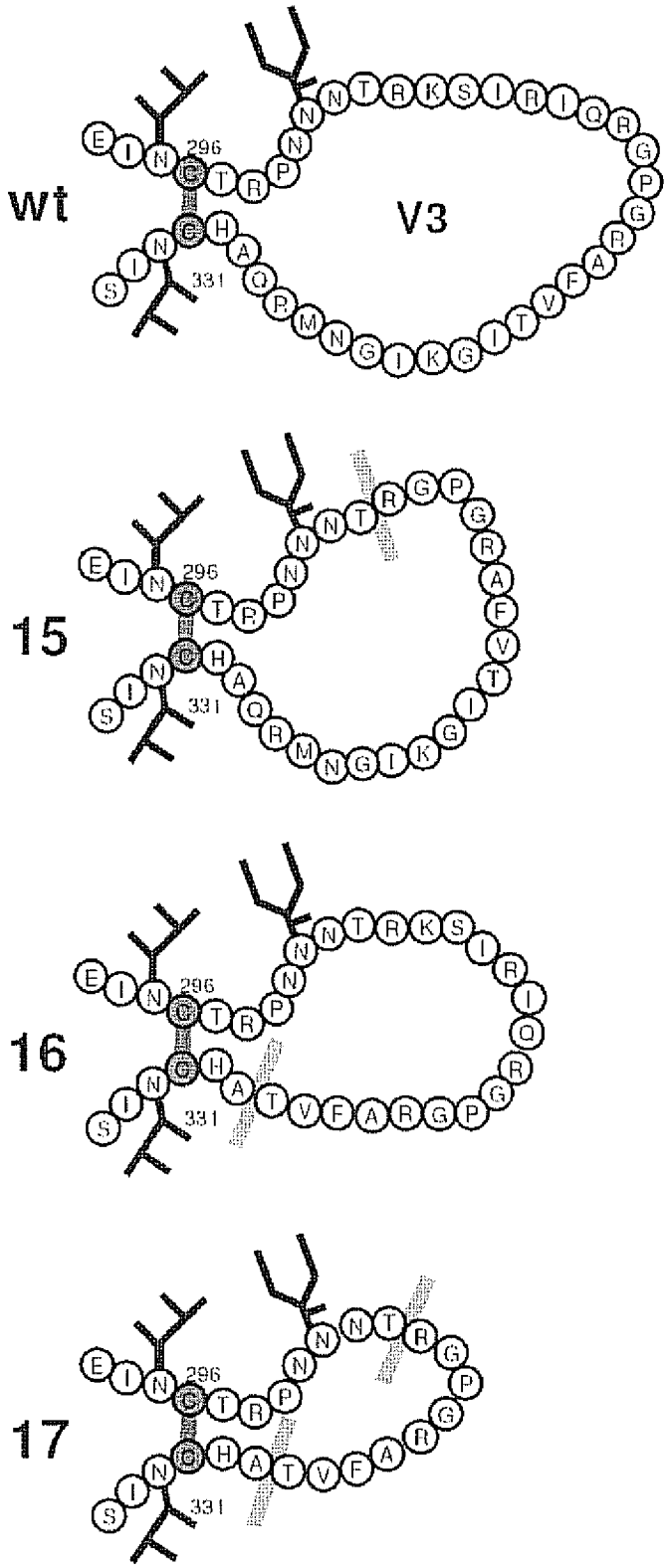
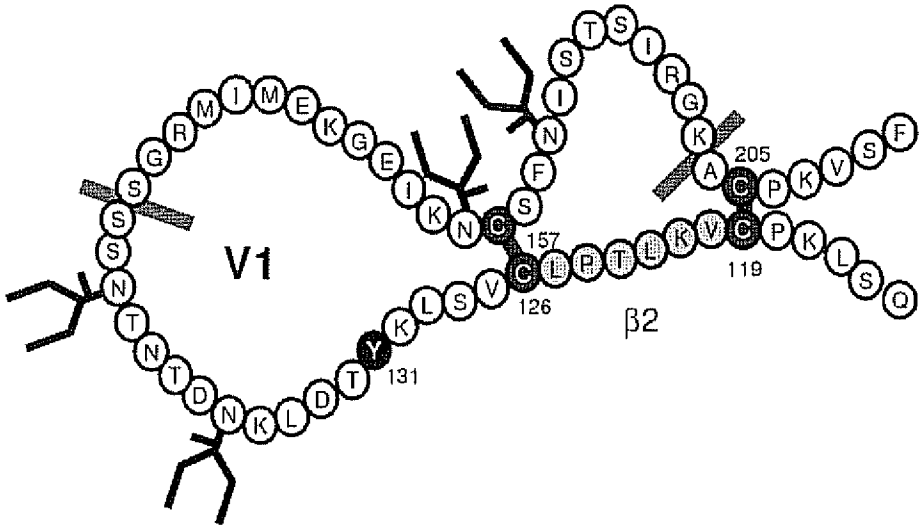
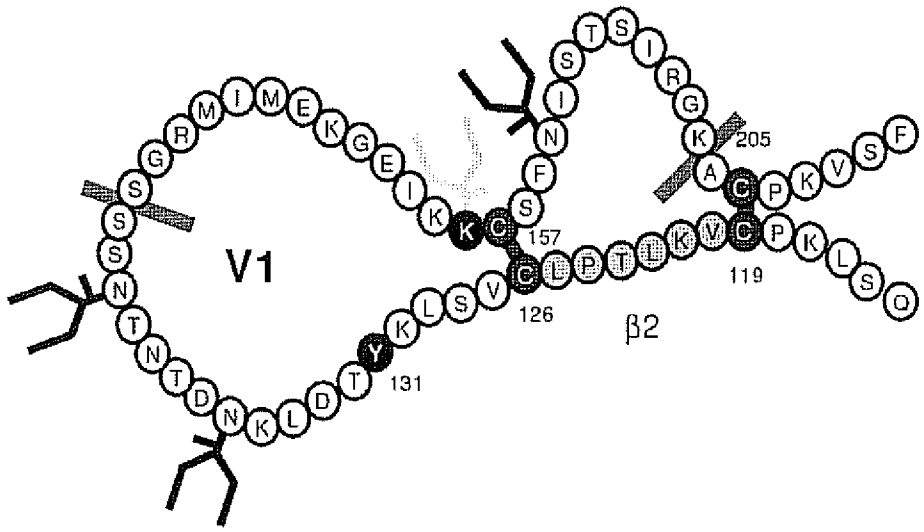


Fig. 39D




**6C** (d38)




**6A** (d99)

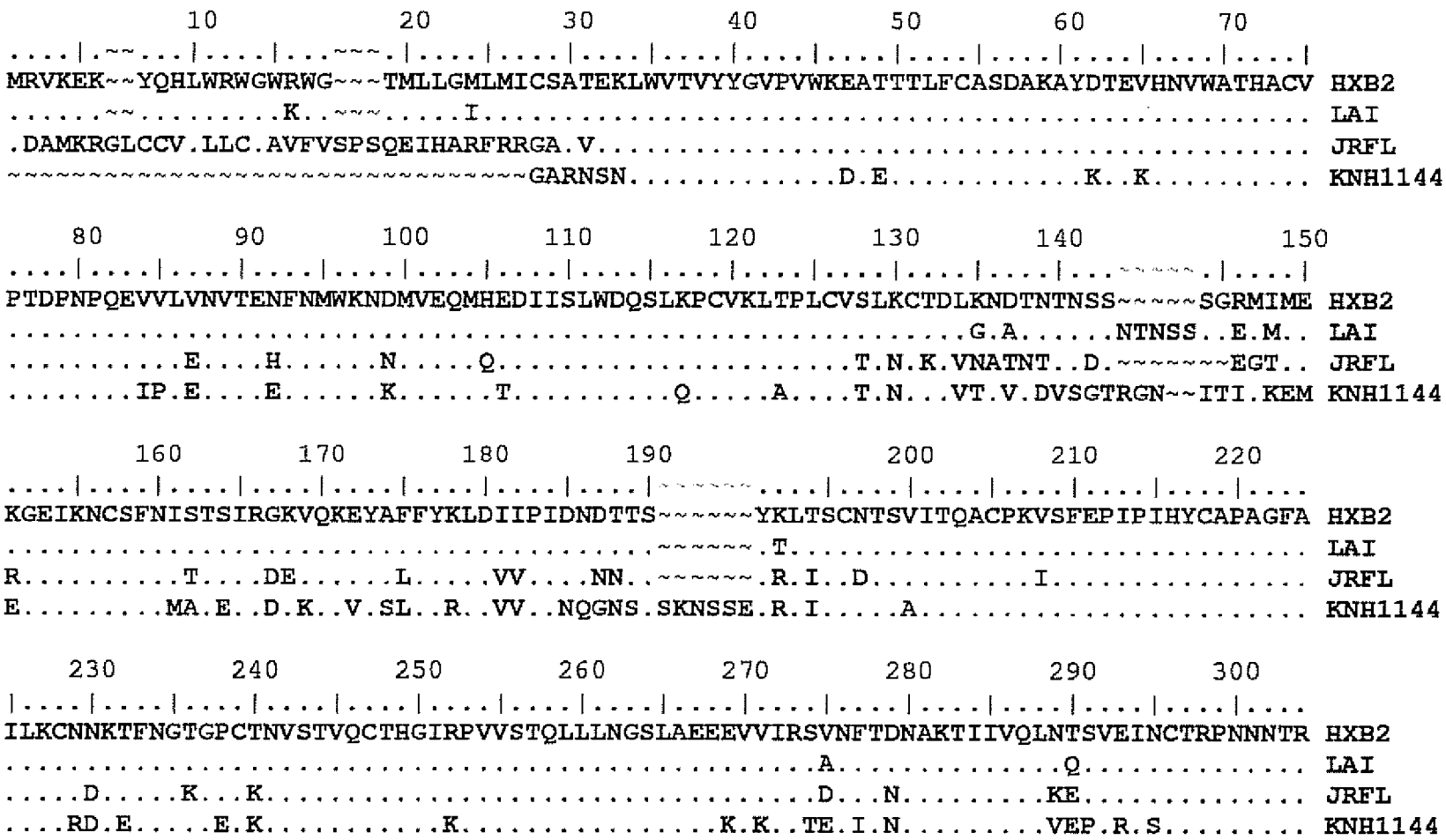

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LAI
JRFL
KNH1144
80 90 100 110 120 130 140 150
PTDPNPQEVVLVNVTENFNMWKNDMVEQMHEDIISLWDQSLKPCVKLTPLCVSLKCTDLKNDTNTNSS~~~~~SGRMIME HXB2
LAI
JRFL
KNH1144
160 170 180 190 200 210 220
KGEIKNCSFNISTSIRGKVQKEYAFFYKLDIIPIDNDTTS~~~~~~YKLTSCNTSVITQACPKVSFEPIPIHYCAPAGFA HXB2
LAI
JRFL
KNH1144
230 240 250 260 270 280 290 300
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LAI
JRFL
KNH1144

Fig. 40

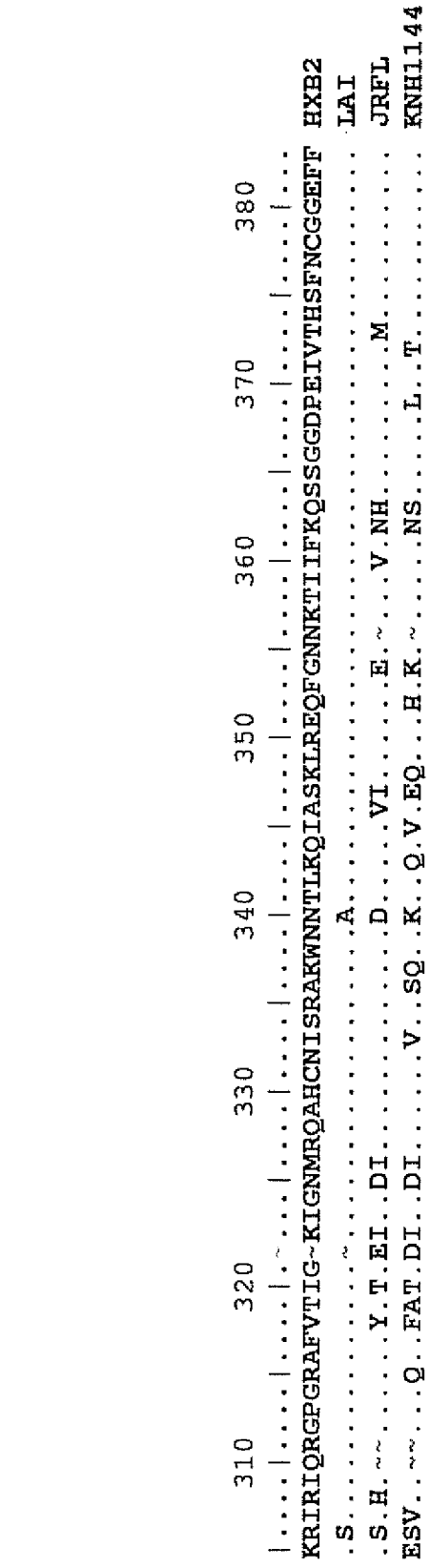

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.S.....A..... LAI
.S.H.~~.....Y.T.EI..DI.....D.....VI.....E.~....V.NH.....M..... JRFL
ESV..~~....Q..FAT.DI..DI.....V..SQ..K..Q.V.EQ...H.K.~.....NS.....L..T..... KNH1144
390 400 410 420 430 440 450 460
YCNSTQLFNSTWFNSTWSTEGSNNTEGSDTITLPCRIKQIINMWQKVGKAMYAPPISGQIRCSSNITGLLLTRDGGNSNN HXB2
.....F.....E.....N.. LAI
.....N.~~~N.....~N.....E.....R.....INE. JRFL
...TSG.....NT~~~.MS..S...TNG....Q.....RT.Q.I.....Q.V...E.....EEK. KNH1144
470 480 490 500 510
ES~EIFRPGGGDMRDNWRSELYKYKVVKIEPLGVAPTKAKRRVVQREKR HXB2
G.~..... LAI
GT~..... JRFL
STN..... KNH1144

Fig. 40, contd.

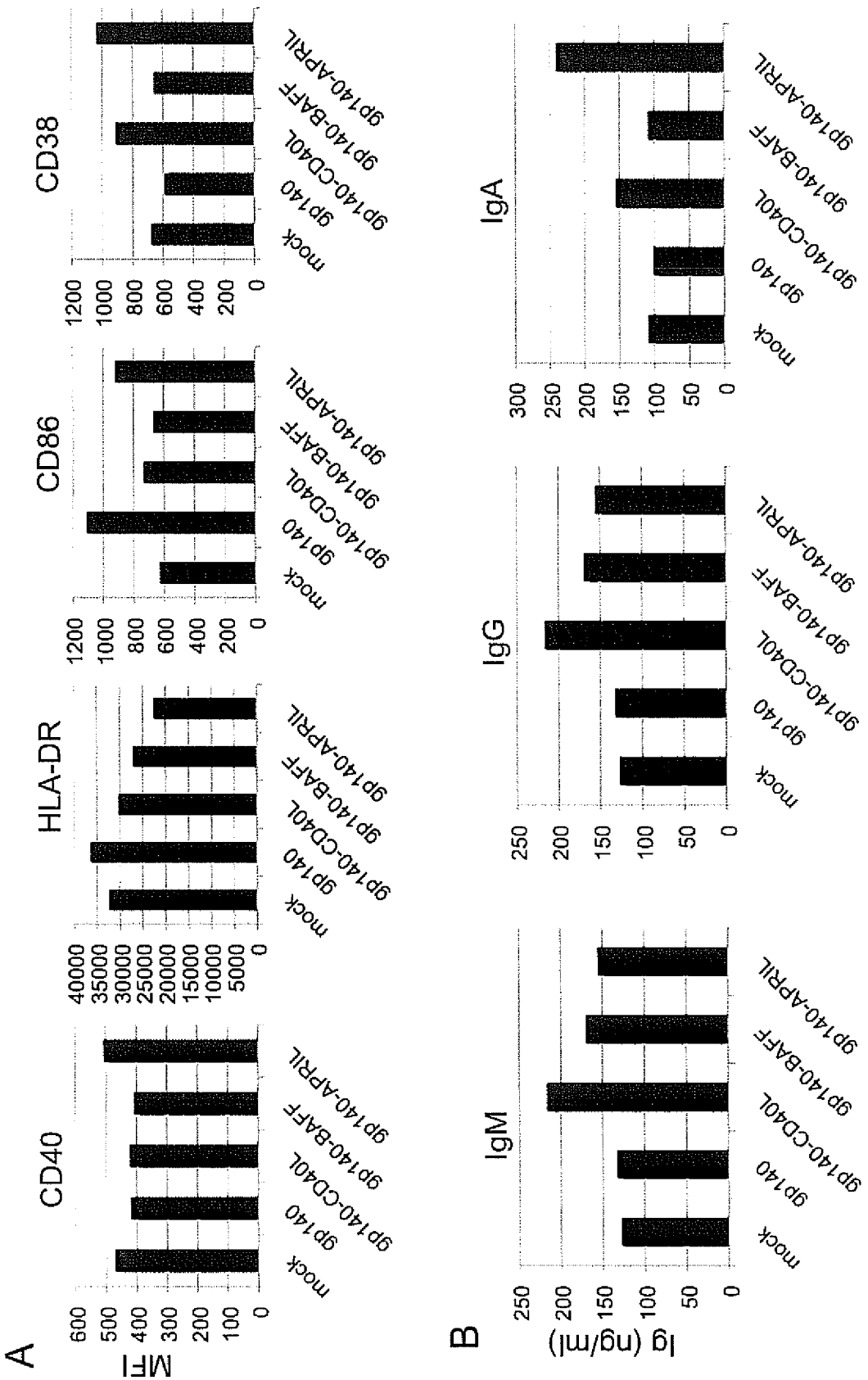

A
MFI
CD40
HLA-DR
CD86
CD38
mock
gp140
gp140-CD40L
gp140-BAFF
gp140-APRIL
B
Ig (ng/ml)
IgM
IgG
IgA

Figure 41

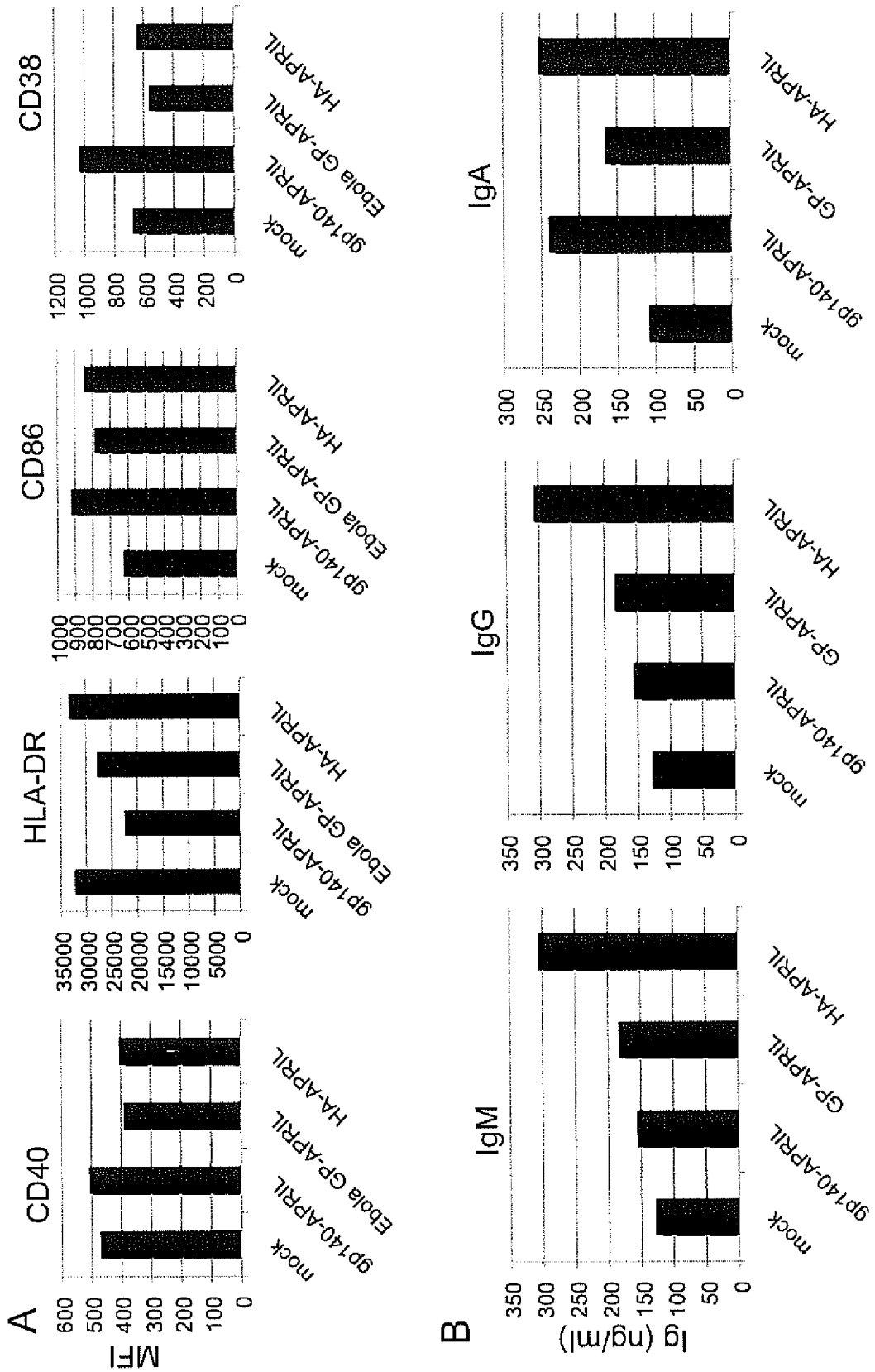

A
MFI
CD40
HLA-DR
CD86
CD38
mock
gp140-APRIL
Ebola GP-APRIL
HA-APRIL
B
Ig (ng/ml)
IgM
IgG
IgA

Figure 42

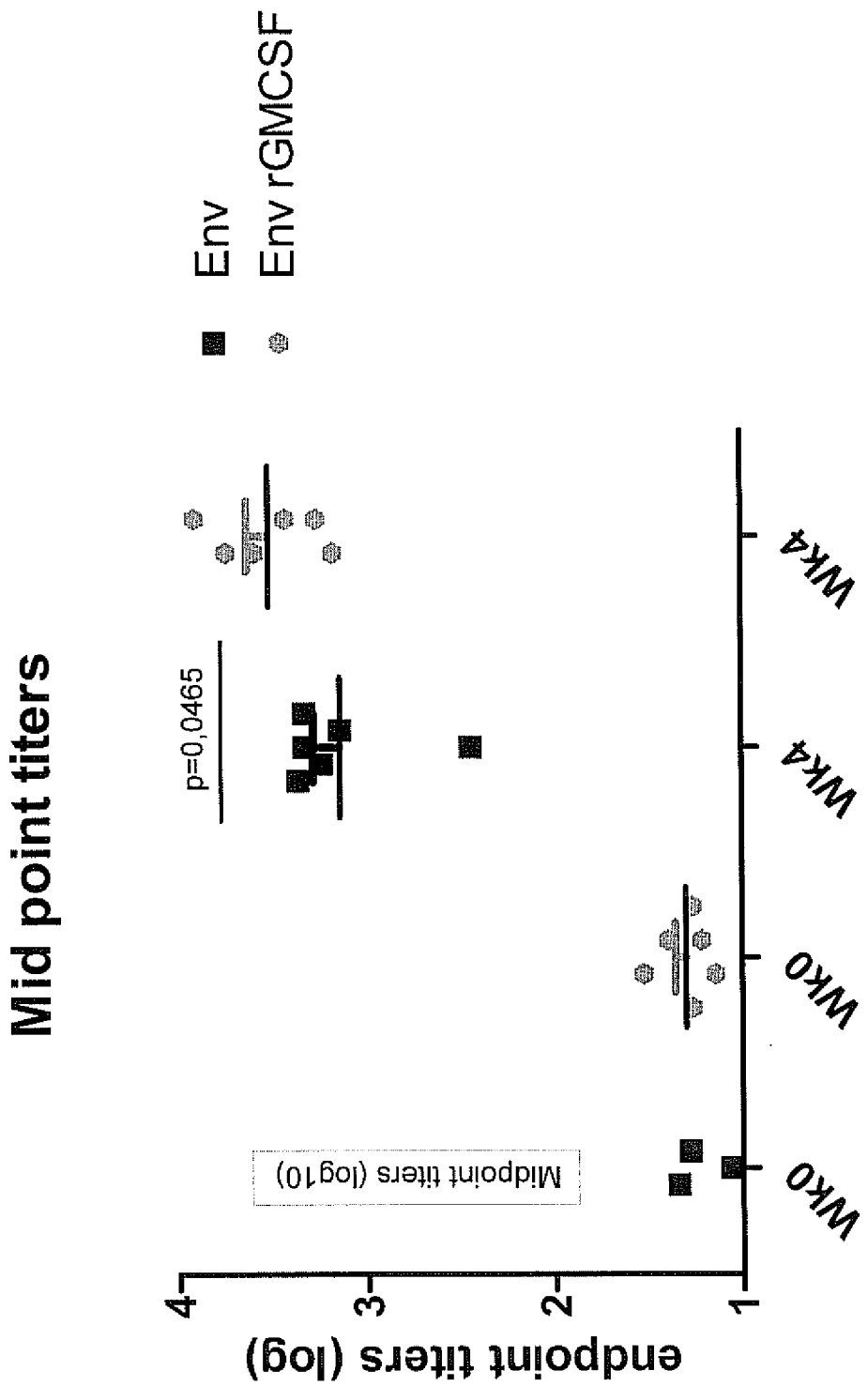

Mid point titers
Env
Env rGMCSF
p=0,0465
endpoint titers (log)
Midpoint titers (log10)
WK0
WK4
WK4
WK4

Figure 43

Figure 44

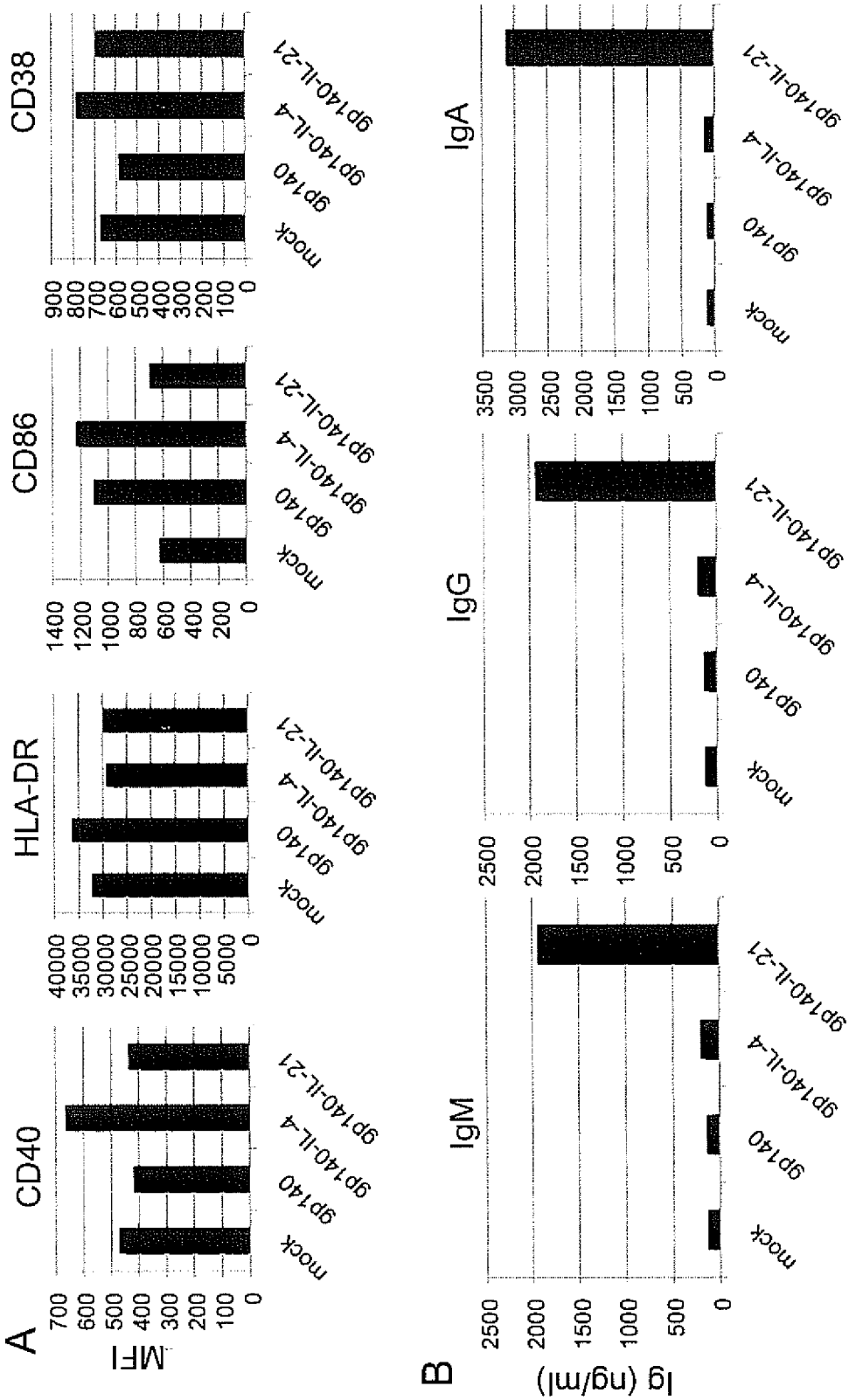

A
MFI
CD40
HLA-DR
CD86
CD38
mock
gp140
gp140-IL-4
gp140-IL-21
B
Ig (ng/ml)
IgM
IgG
IgA

Figure 45

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Figure 46

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CDS 368..1925                            /label="H5"
ORIGIN
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```

Figure 47

```
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/vntifkey="4 "                                  rep_origin complement(978..1645)
/label="Col\E1\origin "                         /vntifkey="33"
CDS 380..798                                    /label="mouse_interleukin_4"
ORIGIN
CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGA
GATAGGGTTGAGTGGCCGCTACAGGGCGCTCCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGTTTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGT
GCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCGCGACGTAATACGACTCACTATAGGGCGAATTGGCGGAAGGCCGTCA
AGGCCACGTGTCTTGTCCAGGTACCAAGCTTACACCTCTCTGTGTGGGATCTGGGCACGGATGTGACAAAAACCATCTCCGGGAAATCATCGGCATTCTGAACGAAGTGACTGGCGAGGG
AACACCCTGTACTGAAATGGACGTCCCCAATGTGCTCACTGCCACCAAAAACACAACCGAGAGCGAACTGGTGTGTAGAGCCTCAAAAGTGCTGAGAATCTTCTACCTGAAACACGGCAA
AACCCCATGTCTGAAAAAAAACTCCTCTGTCCTCATGGAACTCCAGCGACTGTTTAGGGCCTTTCGGTGTCTGGATAGTAGCATCTCTTGTACAATGAATGAGTCCAAATCCACCTCACT
CAAGGACTTTCTGGAATCTCTCAAATCAATCATGCAGATGGACTACTCCGGATCTGGCTCTTGTAACACGTCGAGCTCTGGAGCACAAGACTGGCCTCATGGGCCTTCCGCTCACTGCCC
GCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAACATGGTCATAGCTGTTTCCTTGCGTATTGGGCGCTCTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGGTA
AAGCCTGGGGTGCCTAATGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCT
CAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTC
TCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCG
CCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCT
TGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCG
CTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTT
AAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAAT
GCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTG
CAATGATACCGCGAGAACCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTA
ATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCG
GTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTA
TGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCC
CGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGA
TGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAAT
GTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCA
CATTTCCCCGAAAAGTGCCAC//
```

Figure 48

```
LOCUS 0900560_IL-4_Human_pMA 2810 bp DNA circular CDS complement(1822..2682)
/label="AmpR "                  /vntifkey="4"
rep_origin complement(1007..1674)   /label="Col\E1\origin"
/vntifkey="33 "                 CDS complement(378..829)
/label="IL-4_Human "            ORIGIN
CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGA
GATAGGGTTGAGTGGCCGCTACAGGGCGCTCCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGTTTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGT
GCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCGCGACGTAATACGACTCACTATAGGGCGAATTGGCGGAAGGCCGTCA
AGGCCTAGGCGCGCCATGAGCTCGACGTGTTACAAGAGCCAGATCCGCTAGAGCATTTTGAGTATTTTTCCCTCATAATGGTTTTCAGCCGTTCGAGAAAATTCTCCAGTGTAGACTGAT
TGGCCTCATTTCACAGGGCAAGAGTTCAGGCCAGCGAGTCCCCACAGGTTTCTATCCAGTCGTTTGAGGAACCGGATGAGCTGTTTGTGTCTATGAAACTGCTGGGCGGTGGCTCCGAGGC
ATCGGGTGTCCTTCTCATGATGGGAGTAGAACTGTCTCAGCACAGTAGCGGCTCGACAGAATGTTTCCTTTTCGGTGGTGTTTTTGGAAGCGGCGCAAGATGTCGGTCACGGTGAGTTCGG
TACAGAGTGTTTTCTGCTCAGTGAGTGAGTTCAGGGTTTTGATAATCTCCTGGAGTGTGATGTCACATTTGTGCCCAGATCCCACACAGAGAGGTGTAAGCTTGGTACCTCTTAATTAAC
TGGCCTCATGGGCCTTCCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAACATGGTCATAGCTGTTTCCTTGCGTATTGGGCGCTCTCCGCTTCCTCGCTCA
CTGACTCGCTGCGCTCGGTCGTTCGGGTAAAGCCTGGGGTGCCTAATGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCC
CCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCT
GCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCA
CGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGC
GAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGG
TAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTC
TGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATA
TGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACG
GGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAACCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGC
AACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTC
GTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAA
GTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATA
GTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAG
GATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGC
AAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTA
GAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC//
```

Figure 49

```
LOCUS 1029126_mouse_interleukin_21_pMA-T 2805 bp DNA circular
CDS complement(1817..2677)                /label="AmpR"
/vntifkey="4 "                            rep_origin complement(1002..1669)
/label="Col\E1\origin "                   /vntifkey="33"
CDS 380..822                              /label="mouse_interleukin_21"
ORIGIN
CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGA
GATAGGGTTGAGTGGCCCCTACACGGGCGCTCCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGTTTCGGTGCGGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGT
GCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCGCGACGTAATACGACTCACTATAGGGCGAATTGGCGGCGAAGGCCGTCA
AGGCCACGTGTCTTGTCCAGGTACCAAGCTTACACCTCTCTGTGTGGGATCTGGCATGGGACCCGATAGACTGCTCATTAGACTCCGGCACCTCATCGATATTGTGGAACAGCTGAAAAT
CTACGAGAACCACCTCGACCCTGAACTCCTGTCTGCCCCACAGGATGTGAAGGGCCATTGTGAACACGCCGCCTTTCGCTTGTTTCCAGAAGGCCAAACTGAAACCCTCCAACCCTGGCAA
TAACAAAACCTTTATCATCGACCTGGTGGCACAGCCTCCGACGGAGACTGCCTGCTAGACGGGGCGGAAAAAACAGAAACACATTGCCAAATGCCCCTCTTGTCACTCCTACGAAAAAGG
GACCCCAAAAGAATTTCTCGAACGCCTGAAATGGCTGCTCCCAGAAAATGATTCACCAGCACCTGAGTTCTAGGGGGAGCGGATCATGTTAACACGTTCGAGCACAAGACTGGGCC
TCATGGGCCTTCCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAACATGGTCATAGCTGTTTCCTTGCGTATTGGGCGCTCTCCGCTTCCTCGCTCACTGAC
TCGCTGCGCTCGGTCGTTCGGGGTAAGCCTGGGGTGCCTAATGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTG
ACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGC
TTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAAC
CCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGT
ATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCT
CTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACG
CTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGT
AAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGG
GCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAACCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTT
TATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGT
TTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGG
CCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTA
TGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCT
TACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAA
AGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAA
ATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC//
```

Figure 50

```
LOCUS 1029124_h_Interleukin_21_pMA-T 2808 bp DNA circular
CDS complement(1820..2680)                /label="AmpR"
/vntifkey="4 "                            rep_origin complement(1005..1672)
/label="Col\E1\origin "                   /vntifkey="33"
CDS 380..825                              /label="h_Interleukin_21"
ORIGIN
CTAAATTGTAAGCGTTAATATTTTGTTAAAATTCGCGTTAAATTTTTGTTAAATCAGCTCATTTTTTAACCAATAGGCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGACCGA
GATAGGGTTGAGTGGCCGCGCTCACAGGGCGCACCCATTCGCCAATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGTTTCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAGGGGGATGTG
GCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGAGCGCGCGTAATACGACTCACTATAGGGCGAATTGGCGGAAGGCCGTCA
AGGCCACGTGTCTTGTCCAGGTACCAAGCTTACACCTCTCTGTGTGGGATCTGGCATCGCAGGGACAGGATAGGCATATCATTCGGATAGCAGCTCATCGTGGATCAGCTCAA
AAACTACGTGAACGACCTGGTGCCCGAGTTCCTCCCCGCTCCTGAGGATGTGGAAACCAACTGTGAGTGGAGTGCTTTTTCCTGCTTTCAGAAGGCACAGCTCAAATCCGCCAATACCGG
CAACAATGAACGGATCATCAACGTGTCCATCAAAAACTCAAACGGAAACCCCCCTCTACAAATGCTGGCCGACGGCAGAAACACCGCCTCACTTGTCCTTCATGTGACTCCTACGAAAA
AAAACCTCCCAAGGAGTTTCTCGAACGCTTCAAATCACTGCTCCAGCACCTGAGTAGTATTCATCAGCACCTGAGGGCGAGCGGATCATGTAACACGTTCGAGGCACAAGACTG
GCCTCATGGGCCTTCCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAACATGGTCATAGCTGTTTCCTTGCGTATTGGGCGCTCTCCGCTTCCTCGCTCACT
GACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCA
CTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGC
CGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACG
AACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGA
GGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTA
GCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTG
ACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATG
AGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGG
AGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAACCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAA
CTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGT
CGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGT
TGGCCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGT
GTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGA
TCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAA
AAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGA
AAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCAC//
```

Figure 51

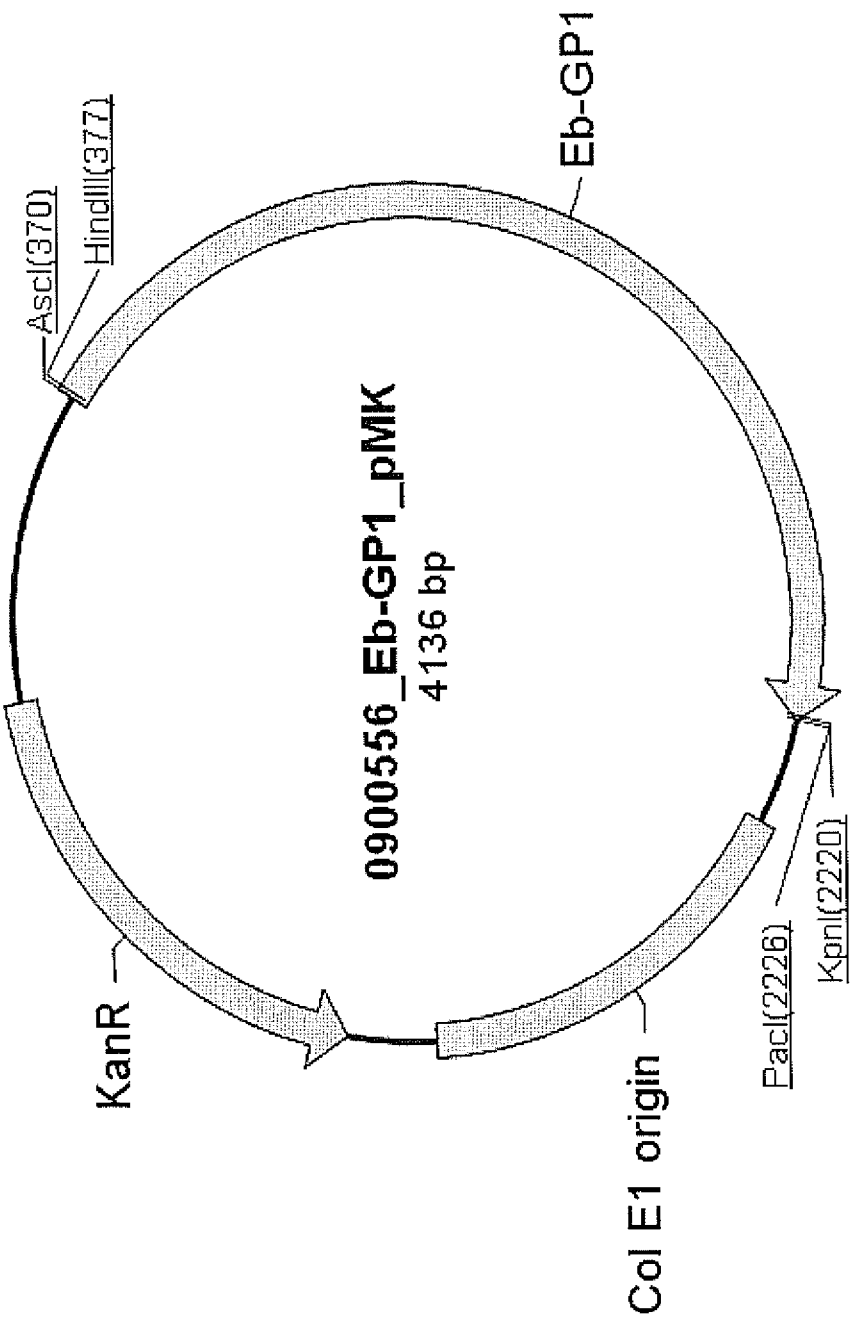

Eb-GP1
AscI(370)
HindIII(377)
0900556_Eb-GP1_pMK
4136 bp
KanR
Col E1 origin
PacI(2226)
KpnI(2220)

Figure 52

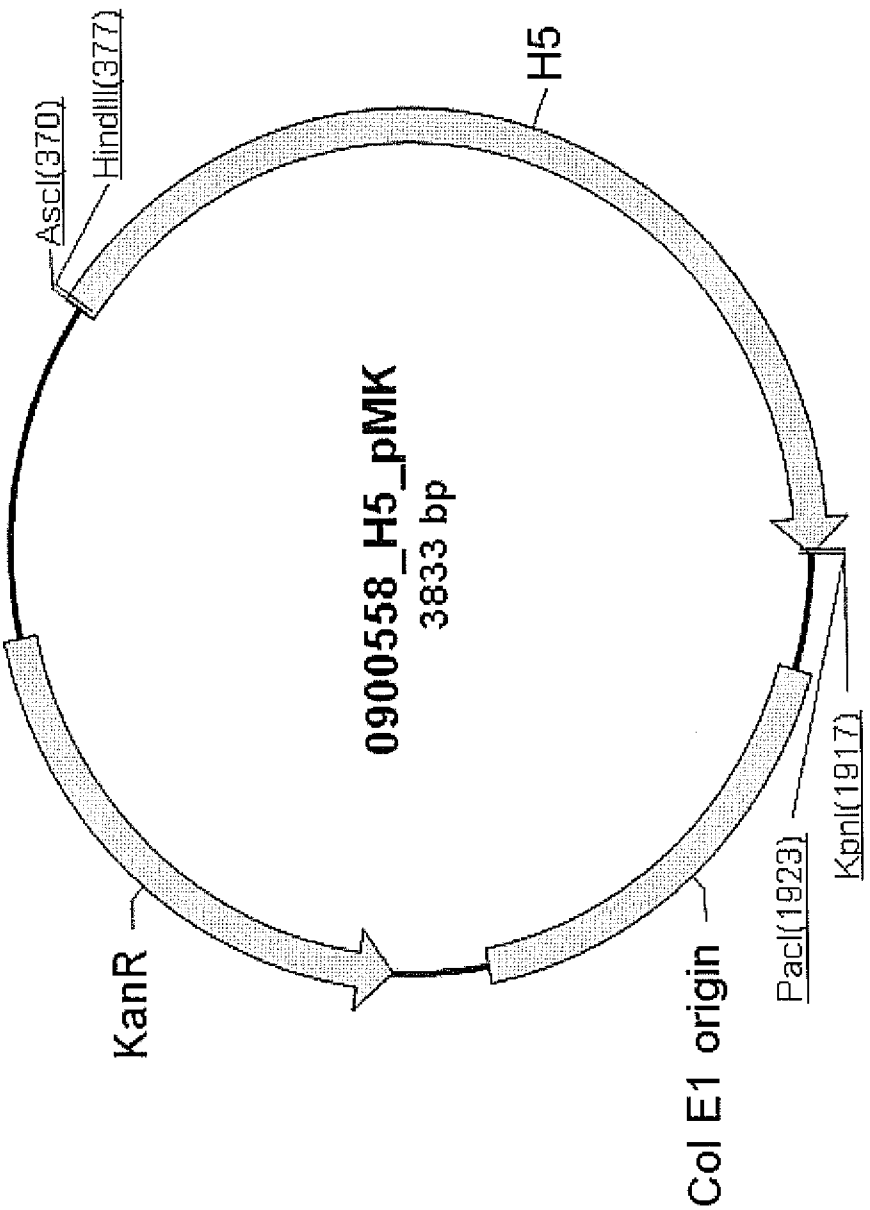

AscI(370)
HindIII(377)
H5
0900558_H5_pMK
3833 bp
KanR
Col E1 origin
PacI(1923)
KpnI(1917)

Figure 53

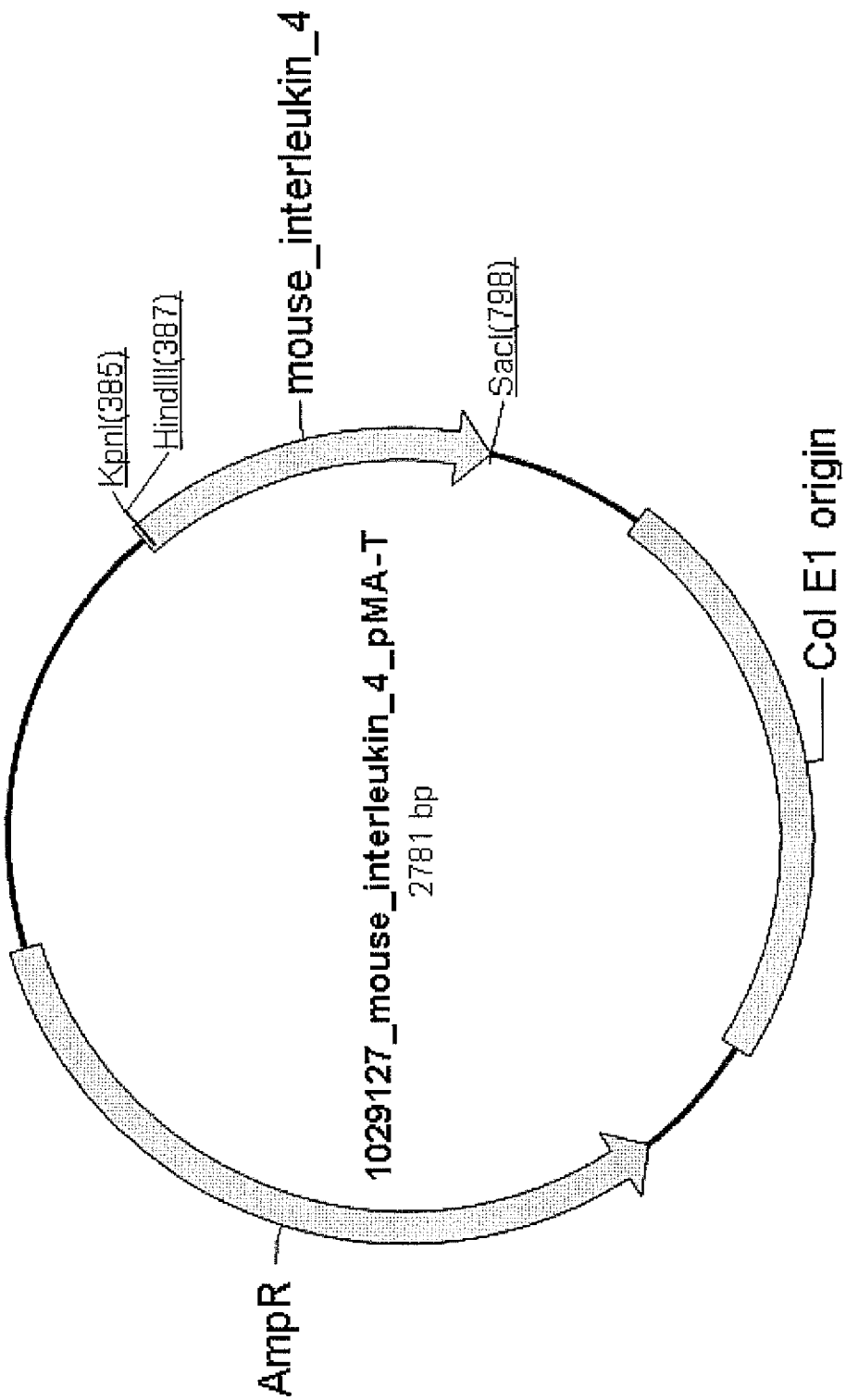

mouse_interleukin_4
KpnI(385)
HindIII(387)
SacI(798)
Col E1 origin
AmpR
1029127_mouse_interleukin_4_pMA-T
2781 bp

Figure 54

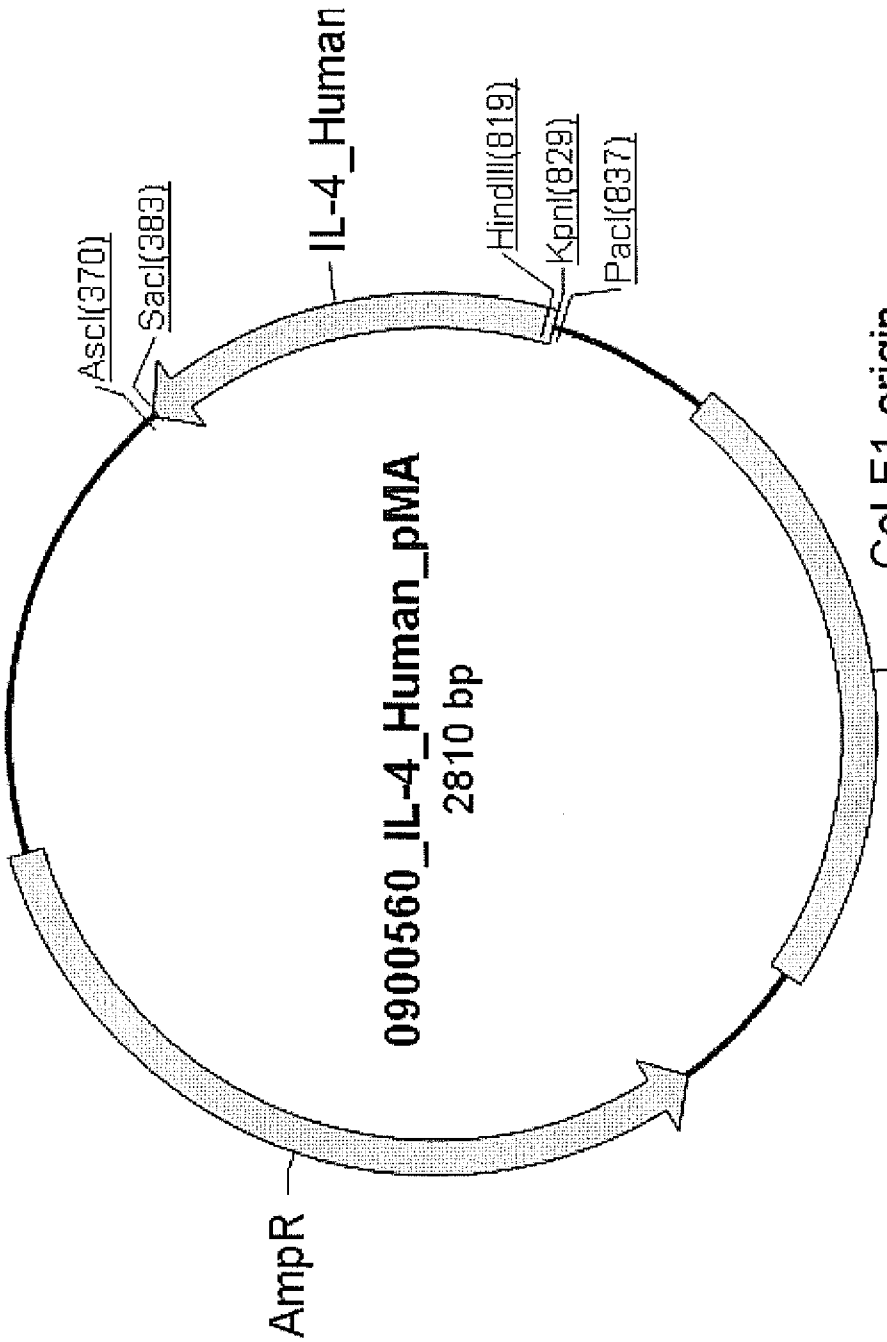

IL-4_Human
AscI(370)
SacI(383)
HindIII(819)
KpnI(829)
PacI(837)
0900560_IL-4_Human_pMA
2810 bp
Col E1 origin
AmpR

Figure 55

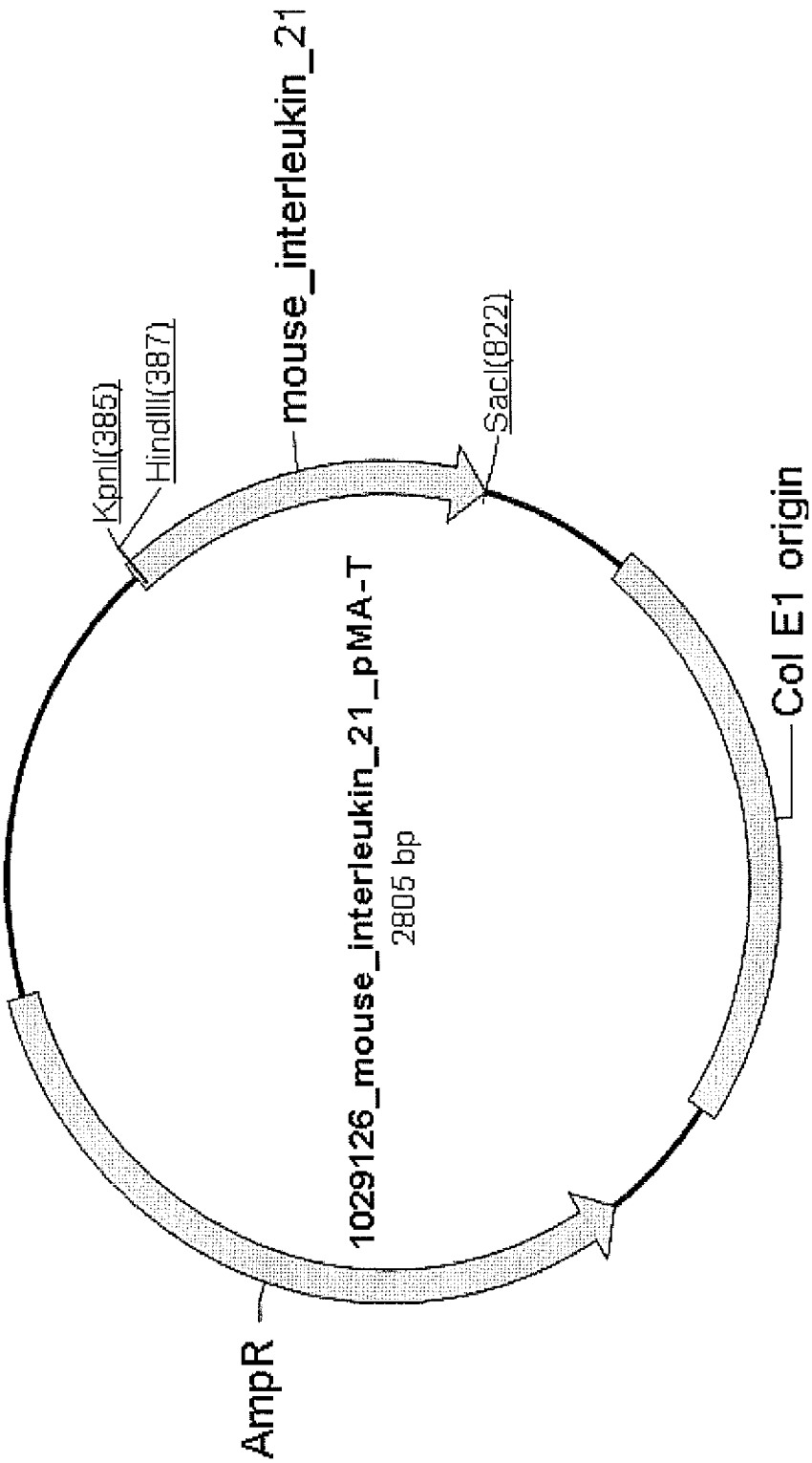

mouse_interleukin_21
KpnI(385)
HindIII(387)
SacI(822)
1029126_mouse_interleukin_21_pMA-T
2805 bp
Col E1 origin
AmpR

Figure 56

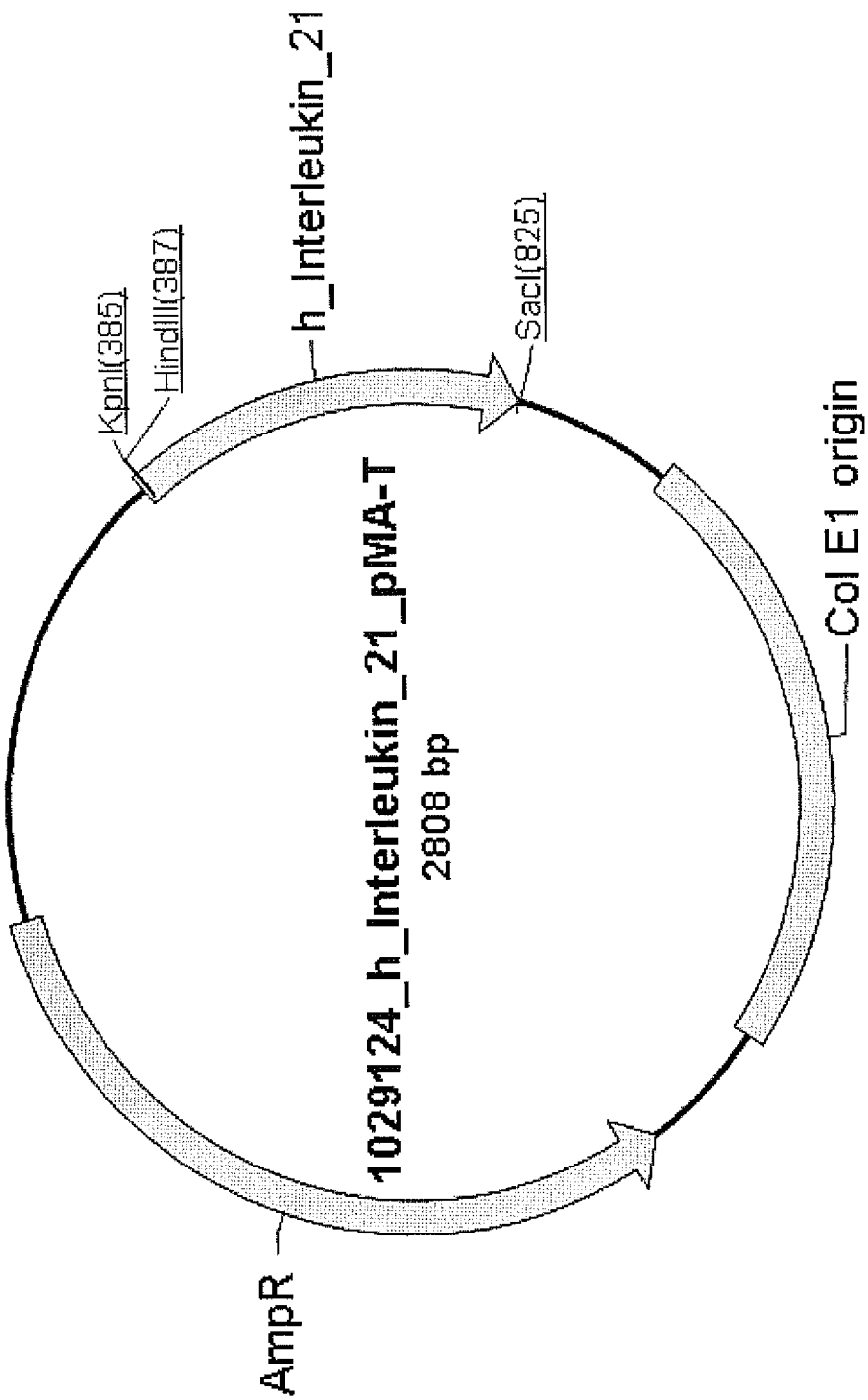

h_Interleukin_21
KpnI(385)
HindIII(387)
SacI(825)
1029124_h_Interleukin_21_pMA-T
2808 bp
Col E1 origin
AmpR

Figure 57

# PATENT COOPERATION TREATY

# PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

| Applicant's or agent's file reference<br>P90832PC00 | **FOR FURTHER ACTION** | see Form PCT/ISA/220<br>as well as, where applicable, item 5 below. |
|---|---|---|
| International application No.<br>PCT/NL2011/050159 | International filing date *(day/month/year)*<br>07/03/2011 | (Earliest) Priority Date *(day/month/year)*<br>05/03/2010 |
| Applicant<br>ACADEMISCH MEDISCH CENTRUM BIJ DE UNIVERSITEIT... | | |

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 10 sheets.

☐ It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**
   a. With regard to the **language,** the international search was carried out on the basis of:
      ☒ the international application in the language in which it was filed
      ☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))
   b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6*bis*(a)).
   c. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (See Box No. II)

3. ☒ **Unity of invention is lacking** (see Box No III)

4. With regard to the **title,**
   ☐ the text is approved as submitted by the applicant
   ☒ the text has been established by this Authority to read as follows:

   B-CELL STIMULATING FUSION PROTEINS OF A AN ANTIGEN WITH BAFF OR APRIL

5. With regard to the **abstract,**
   ☐ the text is approved as submitted by the applicant
   ☒ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings,**
   a. the figure of the **drawings** to be published with the abstract is Figure No. _____
      ☐ as suggested by the applicant
      ☐ as selected by this Authority, because the applicant failed to suggest a figure
      ☐ as selected by this Authority, because this figure better characterizes the invention
   b. ☒ none of the figures is to be published with the abstract

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL2011/050159

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [X] on paper

   [X] in electronic form

   b. (time)

   [ ] in the international application as filed

   [ ] together with the international application in electronic form

   [X] subsequently to this Authority for the purpose of search

2. [X] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/NL2011/050159

**Box No. IV Text of the abstract (Continuation of item 5 of the first sheet)**

The invention relates to the fields of molecular biology, medicine, virology and vaccine development. Because the different forms of the presently available vaccines all have their specific drawbacks, there is a need for alternative vaccine strategies. The current invention provides means and methods for such alternative vaccine strategies. It provides a fusion protein of an antigen (e.g. HIV gp120 or gp160) with the extracellular domain of APRIL or BAFF, or with GMCSF or IL-21, as well as therapeutic applications of said fusion protein in vaccination.

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL2011/050159

**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

see additional sheet(s)

**Remark on Protest**

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2011/050159

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/62 C07K14/705 C07K14/16 A61K38/19 A61K39/00 A61K39/12
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | KOLFSCHOTEN GEERTRUIDA M ET AL: "TWE-PRIL; a fusion protein of TWEAK and APRIL.", BIOCHEMICAL PHARMACOLOGY, vol. 66, no. 8, 15 October 2003 (2003-10-15), pages 1427-1432, XP002638816, ISSN: 0006-2952 abstract; figure 1 ----- | 1,2 |
| X | WO 2007/067681 A2 (UNIV LOUISVILLE RES FOUND [US]; SHIRWAN HAVAL [US]; ELPEK KUTLU G [US]) 14 June 2007 (2007-06-14) claims 1,3 ----- -/-- | 1,2, 13-15,17 |

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 26 May 2011 | 25/08/2011 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | van Heusden, Miranda |

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Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2007/030803 A2 (ZYMOGENETICS INC [US]; MOORE MARGARET D [US]; FOX BRIAN A [US]) 15 March 2007 (2007-03-15) claims 1-3,11,18,21,22 | 1,2,6-9, 14,17 |
| X | LYU MI-AE ET AL: "The rGel/BLyS fusion toxin specifically targets malignant B cells expressing the BLyS receptors BAFF-R, TACI, and BCMA", MOLECULAR CANCER THERAPEUTICS, vol. 6, no. 2, February 2007 (2007-02), pages 460-470, XP002591576, ISSN: 1535-7163 page 463, right-hand column, paragraph 3 - paragraph 4 | 2,5, 7-10,13, 14 |
| X | QIONG SHEN ET AL: "Construction and expression of a new fusion protein, thymosin [alpha]1-cBLyS, E. coli", BIOTECHNOLOGY LETTERS, SPRINGER NETHERLANDS LNKD- DOI:10.1007/S10529-004-7333-3, vol. 27, no. 3, 1 February 2005 (2005-02-01), pages 143-148, XP019231075, ISSN: 1573-6776 page 145, right-hand column, paragraph 2 page 147, left-hand column, paragraph 1 | 2,7-9, 13,14,17 |
| X | CAO P ET AL: "Construction and characterization of a bi-functional EGFP/sBAFF fusion protein", BIOCHIMIE, MASSON, PARIS, FR LNKD- DOI:10.1016/J.BIOCHI.2005.11.009, vol. 88, no. 6, 1 June 2006 (2006-06-01), pages 629-635, XP025066550, ISSN: 0300-9084 [retrieved on 2006-06-01] figure 1 | 2,7-9,13 |
| A | MELCHERS M ET AL: "P05-05. Enhanced immunogenicity of HIV-1 envelope glycoprotein trimers fused to CD40 ligand", RETROVIROLOGY, BIOMED CENTRAL LTD., LONDON, GB LNKD- DOI:10.1186/1742-4690-6-S3-P81, vol. 6, no. Suppl 3, 22 October 2009 (2009-10-22), page P81, XP021064199, ISSN: 1742-4690 the whole document | 1-3,5-18 |

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | MELCHERS M ET AL: "Enhancing the Immunogenicity of HIV-1 Env Trimers by Fusion to CD40 Ligand", AIDS RESEARCH AND HUMAN RETROVIRUSES, MARY ANN LIEBERT, US, vol. 24, no. suppl. 1, 16 October 2008 (2008-10-16), page 23, XP009135994, ISSN: 0889-2229 abstract | 1-3,5-18 |
| A | BARDI J. S.: "A selection of editing", NEWS & VIEWS, vol. 4, no. 15, 3 May 2004 (2004-05-03), pages 1-8, XP002591577, page 4, paragraph 5 - page 5, paragraph 2 | 1-3,5-18 |
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| A | CHEN L ET AL: "BAFF enhances B-cell-mediated immune response and vaccine-protection against a very virulent IBDV in chickens", VACCINE, ELSEVIER LTD, GB LNKD-DOI:10.1016/J.VACCINE.2008.12.040, vol. 27, no. 9, 25 February 2009 (2009-02-25), pages 1393-1399, XP026194433, ISSN: 0264-410X [retrieved on 2009-01-13] the whole document | 1-3,5-18 |
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | NIMAL S ET AL: "Enhancement of immune responses to an HIV gp120 DNA vaccine by fusion to TNF alpha cDNA", VACCINE, ELSEVIER LTD, GB, vol. 24, no. 16, 12 April 2006 (2006-04-12), pages 3298-3308, XP025151249, ISSN: 0264-410X, DOI: DOI:10.1016/J.VACCINE.2006.01.020 [retrieved on 2006-04-12] the whole document | 1-3,5-18 |
| A | CRAXTON ANDREW ET AL: "Macrophage- and dendritic cell-dependent regulation of human B-cell proliferation requires the TNF family ligand BAFF.", BLOOD, vol. 101, no. 11, 1 June 2003 (2003-06-01) , pages 4464-4471, XP002638817, ISSN: 0006-4971 the whole document | 1-3,5-18 |
| A | DARIDON ET AL: "BAFF, APRIL, TWE-PRIL: Who's who?", AUTOIMMUNITY REVIEWS, ELSEVIER, AMSTERDAM, NL, vol. 7, no. 4, 1 February 2008 (2008-02-01), pages 267-271, XP022493634, ISSN: 1568-9972, DOI: DOI:10.1016/J.AUTREV.2007.05.002 the whole document | 1-3,5-18 |

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Information on patent family members

International application No
PCT/NL2011/050159

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
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| | | AU 2006321889 A1 | 14-06-2007 |
| | | CA 2632663 A1 | 14-06-2007 |
| | | CA 2632682 A1 | 14-06-2007 |
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| | | EP 1960525 A2 | 27-08-2008 |
| | | US 2008220478 A1 | 11-09-2008 |
| | | US 2010297701 A1 | 25-11-2010 |

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/** 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1(completely); 2, 3, 5-18(partially)

   A fusion protein comprising an antigen and a ligand which is a compound comprising at least the extracellular domain of APRIL or a compound having at least 80% sequence identity thereto. A nucleic acid molecule encoding said fusion protein, a method of producing said fusion protein recombinantly, an oligomeric complex or a virus comprising said fusion protein, an immunogenic composition comprising said fusion protein, therapeutic applications of said fusion protein, a non-human animal provided with said fusion protein and a method of obtaining an antibody specific for said fusion protein.

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2. claims: 2-18(partially)

   A fusion protein comprising an antigen and a ligand which is the cytokine GMCSF or a compound having at least 80% sequence identity thereto. A nucleic acid molecule encoding said fusion protein, a method of producing said fusion protein recombinantly, an oligomeric complex or a virus comprising said fusion protein, an immunogenic composition comprising said fusion protein, therapeutic applications of said fusion protein, a non-human animal provided with said fusion protein and a method of obtaining an antibody specific for said fusion protein.

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3. claims: 2-18(partially)

   A fusion protein comprising an antigen and a ligand which is the cytokine IL-21 or a compound having at least 80% sequence identity thereto. A nucleic acid molecule encoding said fusion protein, a method of producing said fusion protein recombinantly, an oligomeric complex or a virus comprising said fusion protein, an immunogenic composition comprising said fusion protein, therapeutic applications of said fusion protein, a non-human animal provided with said fusion protein and a method of obtaining an antibody specific for said fusion protein.

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