HIV-1 envelope glycoproteins with costimulatory domains for vaccine applications
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Summary

Currently, no HIV-1 envelope glycoprotein (Env) subunit vaccine can induce antibodies (Abs) of the quality and quantity required for providing protective immunity. Our research focused on designing chimeric proteins, containing Env fused to costimulatory molecules, with the aim of simultaneously activating specific immune cells with Env and a costimulatory molecule in order to achieve enhance Env immunogenicity. We focused on costimulatory molecules with beneficial immune modulatory functions and/or well-defined adjuvant effects.

One molecule that can be used as an adjuvant is granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF is produced by a variety of cells such as fibroblasts, activated T lymphocytes, macrophages, epithelial, mesothelial, endothelial cells as well as some tumor cells. GM-CSF stimulates proliferation of various immune cell types during inflammation and prevents their apoptosis.

In chapter 2 we report on chimeric molecules in which GM-CSF was embedded within the V1V2 domain of trimeric Env. Env expression was only mildly affected by the replacement of the V1V2 with GM-CSF. The overall antigenicity of Env was not affected by the modification, with exception of the CD4 binding site (CD4BS) and CD4-induced (CD4i) epitopes, which are located close to the inserted cytokine domain. Importantly, GM-CSF located in the V1V2 retained its functionality and stimulated proliferation of a GM-CSF dependent cell line, albeit with decreased activity compared to recombinant GM-CSF. The immunogenicity of Env-GM-CSF was evaluated in mice using the mouse GM-CSF homologue. Sera of mice immunized with Env-GM-CSF had higher titers of anti-Env Abs compared to the Env control sera and were more efficient in neutralizing the neutralization-sensitive SF162 Env pseudotyped virus. Furthermore, the T cell responses were also enhanced in the Env-GM-CSF immunized mice.

In chapter 3 we have followed several strategies to increase the activity of GM-CSF, such as i) inserting smaller GM-CSF domains; ii) modifying the disulfide-bonded architecture of the GM-CSF domain; iii) removing the N-linked glycosylation sites of GM-CSF; iv) optimizing the flexible linkers between the Env and GM-CSF domains; and v) embedding GM-CSF within cleaved Env trimers. We observed that extending the linker length between the Env and the GM-CSF domain increased the GM-CSF activity by 6-fold compared to unmodified Env-GM-CSF chimera. Moreover, we showed that incorporation of GM-CSF
within cleavage competent Env further improved the GM-CSF activity. We have not tested the immunogenicity of these improved Env-GM-CSF molecules yet.

Another molecule with appealing functions that caught our attention was interleukin-21 (IL-21). IL-21 plays important roles both in innate and adaptive immune responses. It is produced by various types of T cell populations including activated CD4$^+$ T cells, natural killer T (NKT) and in particular T-follicular helper cells that are autocrinely activated by IL-21 in the germinal centers where the proliferation, differentiation, somatic hypermutation (SHM) and class switching processes of mature B cells take place during a normal immune response against an infection. Furthermore, in response to IL-21, CD8$^+$ T lymphocytes are activated to become killer cells by increasing their granzyme B and perforin expression. IL-21’s beneficial effects such as augmenting Ab production, class switching in B cells and driving differentiation of B cells to Ab secreting plasma cells are all desirable properties in the context of an HIV-1 vaccine.

We have designed a chimeric Env-IL-21 molecule by insertion of IL-21 into the V1V2 domain of Env (chapter 4), similar to what we did with GM-CSF in chapter 2. The insertion of IL-21 into the V1V2 domain reduced the expression of Env. The overall Env antigenicity was similar to wild type Env but the binding of CD4BS and CD4i Abs was decreased, similar to what we observed with the GM-CSF insertion. The IL-21 domain of the Env-IL-21 chimera was able to activate human B cells, increase their immunoglobulin (Ig) production and drive their differentiation to a plasmablast-like cell phenotype in vitro. Moreover, incorporation of IL-21 into cleavage competent Env preserved IL-21 bioactivity. In an attempt to improve the functionality of Env-IL-21, we replaced a homologous and structurally more stable region of IL-4 with the one from IL-21. This hybrid Env-IL-21/4 molecule was expressed more efficiently than Env-IL-21, but the IL-21 activity was not measurably improved in B cell experiments. Strategies to improve the activity of IL-21 by modulating the IL-21 receptor interactions decreased the activity of IL-21.

To test the immunogenicity of Env-IL-21, mice and rabbits were immunized with chimeric Env’s with the mouse and the rabbit homologues of IL-21, respectively (chapter 5). Contrary to our expectations, inclusion of IL-21 in Env did not improve the anti-Env Ab responses as Env-IL-21 immunized animals had lower anti-Env responses. One reason for the decreased immunogenicity of Env-IL-21 could be that IL-21 induces auto-antibodies (auto-Abs) that suppressed the activity of Env-IL-21, although auto-Abs against GM-CSF and IL-21 did not interfere with the therapeutic effect of GM-CSF and IL-21 in cancer patients.
We evaluated whether the cytokines incorporated in Env induced auto-Abs in the immunized animals. Indeed, we detected high levels of anti-GM-CSF and anti-IL-21 Abs in the sera of animals immunized with Env-GM-CSF and Env-IL-21, respectively, whereas animals immunized with wild type Env lacked such auto-Abs. In order to test whether insertion of GM-CSF or IL-21 within the body of Env (in the V1V2 domain) exacerbated the generation of Abs targeting GM-CSF or IL-21, we also designed chimeric Env-GM-CSF and Env-IL-21 constructs in which GM-CSF and IL-21 were fused to the C-terminus of the Env. These constructs also induced anti-GM-CSF or anti-IL-21 Abs in both mice and rabbit indicating that these anti-GM-CSF and anti-IL-21 anticytokine responses were independent of the location of the costimulatory molecule in the chimeric protein. Furthermore, these auto-Ab responses were not dependent on the animal species, as they were observed in both mice and rabbits.

Another interesting molecule is a proliferation-inducing ligand (APRIL), which promotes B and T cell proliferation and long-term survival of plasma cells. Furthermore, APRIL promotes Ig class switch recombination giving rise to IgG- and IgA-secreting cells, and has been suggested to be the major promoter of IgA production under antigen exposure, thus contributing to mucosal Abs. Processes supported by APRIL such as (i) increasing Ab breadth and potency by supporting SHM, (ii) enhancing Ab longevity by supporting long-lived plasma cells, and (iii) enhancing mucosal immunity by supporting class switching to IgA are highly desirable for an HIV-1 vaccine aimed at inducing protective Abs.

We studied an Env-APRIL molecule that was generated by fusion of APRIL to the C-terminus of the Env (chapter 6). We found that Env-APRIL bound efficiently to the two APRIL receptors, BCMA and TACI and signalled through these receptors leading to downstream intracellular signalling events. Env-APRIL containing rabbit APRIL induced significantly higher binding anti-Env responses in rabbits compared to wild type Env. Being cautious of the possibility of generating auto-Abs because of the results described in chapter 5, we measured the induction of anti-APRIL Abs. We detected minimal anti-APRIL Abs in the sera of mice and rabbits immunized with Env-APRIL. Furthermore, an Env-CD40L molecule of similar design did not induce significant anti-CD40L responses.

We also studied whether the heterologous GCN4-based trimerization domain (IZ) that is often used to stabilize trimeric vaccine antigens (including our studies described in chapters 2-6) or therapeutic proteins was immunogenic (chapter 7). Abs targeting the IZ domain were detected in sera from mice, rats and rabbits that were immunized with Env fused to IZ. In
order to immunogenically silence the IZ domain, we introduced four strategically placed \(N\)-
linked glycans into IZ. The glycosylated IZ domain allowed efficient trimerization of Env as well as influenza hemagglutinin (HA), generated significantly lower anti-IZ Ab responses, but did not affect the anti-Env or anti-HA Ab responses.

We used SOSIP.R6-IZ gp140 from the subtype B JR-FL strain as the antigen in studies described in chapters 2-7 of this thesis. Despite the presence of the stabilizing SOSIP modifications and the enhanced R6 cleavage site, SOSIP.R6-IZ, although mostly trimeric, is predominantly uncleaved and a poor mimic of the native Env spike. Although this Env antigen was appropriate for studying the hypothesis that fusion of Env to costimulatory molecules might improve Env immunogenicity, ideally one would wish to corroborate these findings with better recombinant mimics of the native-spike. Fortunately, a better mimic of the native-spike, BG505 SOSIP.664 gp140, became available during the course of these studies. It also became clear that in contrast to JR-FL SOSIP.R6, BG505 SOSIP.664 was not structurally perturbed by additions to the C-terminus such as costimulatory molecules.

We have fused human APRIL to the C-terminus of BG505 SOSIP.664 to generate BG505 SOSIP.664-APRIL (chapter 8) and found that the fusion preserved the superior antigenicity of BG505 SOSIP.664. The APRIL domain also retained its functionality in binding and signalling through the APRIL receptors BCMA and TACI. We immunized rabbits with BG505 SOSIP.664 and BG505 SOSIP.664-APRIL using the rabbit APRIL homologue and observed that inclusion of APRIL increased the anti-Env Ab titers. After the first boost, the anti-Env Ab responses waned slower in the BG505 SOSIP.664-APRIL immunized rabbits and the half-life of Ab response was improved. In contrast to the results described in chapter 6 for JR-FL SOSIP.R6-IZ-APRIL, we found that APRIL fused to the C-terminus of BG505 SOSIP.664 induced some levels of anti-APRIL Abs. Whether BG505 SOSIP.664-APRIL improves the induction of neutralizing Abs remains to be studied.

In this thesis, we have evaluated the functionality and immunogenicity of several chimeric Env-costimulatory molecules, in particular Env fused to GM-CSF, IL-21 and APRIL. All molecules appeared to be functional in activating specific human immune cells \textit{in vitro}. However, the responses in animals using species-matched cytokines were variable. We did not observe a beneficial effect \textit{in vivo} of IL-21, CCL28 and CXCL13. Furthermore, we observed that Env-GM-CSF lead to enhanced Env immunogenicity in mice and Env-APRIL in rabbits, but not \textit{vice versa}. This shows that the adjuvant effect of such molecules can vary per species and only human experiments can provide a final answer. A potential problem with
using costimulatory (self) molecules could be induction of auto-Abs. We observed that in particular GM-CSF and IL-21 are prone to induce such responses, APRIL much less so. Whether such responses are harmful should be studied carefully in different animal species, as well as humans receiving these molecules in a therapeutic setting. We believe that once promising chimeric Env molecules are proven to be effective and safe, they should be tested in humans. In toto, we think that fusion of Env to costimulatory molecules provides an opportunity to improve Env immunogenicity and might contribute to inducing protective humoral immunity against HIV-1.