Review Article

Endocytic receptor for pro-coagulant factor VIII: Relevance to inhibitor formation

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Summary

The immunogenicity of therapeutic factor VIII (FVIII) in patients with haemophilia A remains a critical issue in patient management. This review describes the immunological processes involved in the activation of the immune system against FVIII, with a particular focus on the role of endocytic receptors for the recognition of FVIII by antigen-presenting cells.

Keywords

Haemophilia A, factor VIII, antigen-presenting cells, endocytic receptors, allo-immunisation

Immune response to FVIII in patients with haemophilia A

The response of the immune system to therapeutic exogenous factor (F)VIII is believed to develop as a classical immune response to an external antigen. In the course of a specific immune response, antigens that circulate in the organism are recognised and internalised by antigen-presenting cells (AgPCs). When present in tissues or in the lymph, the antigens are endocytosed by immature dendritic cells (DCs), which mature while migrating to the draining lymph nodes. If circulating in the blood, the antigens are believed either to be captured by DCs in the blood or to accumulate preferentially in the spleen where they encounter different types of AgPCs such as DCs, macrophages and B lymphocytes in the marginal zone and macrophages in the red pulp. The AgPCs process the antigen in their endosomes and antigen-derived peptides as-sociate with MHC class II molecules to be exposed at the surface of the cells and presented to neighbouring CD4+ T lymphocytes. For naïve CD4+ T cell to be activated, AgPCs need to mature at the time of presentation of the antigen. Maturation of AgPCs is provided by "danger signals", which originate from the antigen itself or from the environment, that trigger receptors such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) on AgPCs. Activated antigen-specific T lymphocytes then provide help to antigen-specific B lymphocytes that in turn differentiate into immunoglobulin-secreting plasmocytes or memory B cells (1).

Information regarding the chain of events that lead to the production of anti-FVIII antibodies following the administration of exogenous FVIII has been provided both by clinical observations in patients with haemophilia A, and experimentally using a mouse model of severe haemophilia A. Thus, the nature of anti-FVIII antibodies and the requirement for a help from FVIII-specific CD4+ T cells in their generation are now well characterised. The nature of the AgPCs involved in the recognition of therapeutic FVIII is, however, more speculative.

FVIII-specific IgG in haemophilia A

Anti-FVIII antibodies found in patients are of the IgG subclass, with a preference for the IgG1 and IgG4 isotypes, and use both lambda and kappa light chains (2, 3). Antibodies to FVIII are polyclonal in each individual and contain antibodies against functional and non-functional domains of the protein (4). The epitopes recognised by the anti-FVIII IgG of the patients are found on the light and/or heavy chains of the molecule and the immuno-dominant domains of FVIII are C2 and A2 (5). Anti-FVIII IgG towards epitopes in the a3A3 domain have also been described (5–8). Most anti-FVIII IgG neutralise the pro-coagulant activity of FVIII by steric hindrance, i.e. by binding to the epitope for which they are specific on the FVIII molecule, they prevent its interaction with other molecules of the coagulation cascade. Antibodies that pre-vent the association of FVIII with von Willebrand factor (VWF), activated FIX, FX and phospholipids have thus been reported (6, 9–17). Anti-FVIII IgG that do not prevent the interaction of FVIII with.
with actors of the coagulation cascade have also been found: these do not inhibit FVIII activity per se but promote the formation of immune complexes that might alter the catabolism of therapeutic FVIII in treated patients (18). Our research group has shown the presence, in inhibitor-positive patients with severe haemophilia, of anti-FVIII antibodies endowed with proteolytic activity that hydrolyse and inactivate FVIII (19–21).

Importance of CD4+ T lymphocytes

The presence of FVIII inhibitors of the IgG isotype in the plasma of patients and mice treated with FVIII, together with the presence of somatic mutations in the V genes encoding human anti-FVIII IgG, suggest a help from helper CD4+ T cells in the development of the anti-FVIII immune response (3, 22). Indeed, CD4+ T lymphocytes have been isolated from inhibitor-positive patients that proliferate when stimulated in vitro with FVIII or FVIII-derived peptides (23–29). T-cell receptors of T lymphocytes that were found to proliferate in the presence of FVIII, preferentially use Vβ genes of the BV2, BV3 and BV9 families (30). Interestingly, patients with a history of high inhibitor titers were found to lose their FVIII inhibitor following infection by HIV (31–33). This was associated with a drop in CD4+ T cell counts, and the lack of an anamnestic immune response upon subsequent intravenous administration of therapeutic FVIII.

Additional evidence in favor of a need for a direct cross-talk between AgPCs and CD4+ T cells originates from experiments in FVIII-deficient mice wherein the abrogation of T cell help using antibodies to CD40-ligand (CD154), CD80 or CD86, or by using CTLA4-Ig constructs, that impairs the cross-talk between AgPCs and T cells, prevented the anti-FVIII immune response (34–38).

Nature of the antigen presenting cells that endocytose therapeutic FVIII

Different types of AgPCs may be implicated in the uptake of therapeutic FVIII in the patients, among which dendritic cells, macrophages and B lymphocytes are the most potent. In patients that have never been exposed to FVIII (i.e. previously untreated patients or PUPS), DCs are probably the major cell type that endocytoses the therapeutically administered FVIII, processes it and activates FVIII-specific CD4+ T lymphocytes. Indeed, DCs have the capacity to stimulate naïve T cells (39). A role for macrophages in endocytosing therapeutic FVIII and presenting it to CD4+ T cells and triggering primary immune responses should not be excluded however. In patients who have already developed anti-FVIII IgG, memory FVIII-specific B cells that circulate in the organism may be the main cells to endocytose FVIII and present it to T lymphocytes, thus refueling the primed anti-FVIII immune response. Furthermore, follicular DCs present in the germinal centers of the spleen, might also capture FVIII in the form of immune complexes, and sustain the differentiation of FVIII-specific B cells (40, 41).

Using naïve FVIII-deficient mice, we have recently documented the importance of the spleen in the induction of the anti-FVIII immune response (42). Intravenously delivered exogenous FVIII was found to accumulate at high concentration in the spleen of the mice for at least 45 minutes, and removal of the spleen drastically reduced the amplitude of the anti-FVIII immune response. In the spleen, FVIII was found to co-localise with macrophages; accumulation of FVIII in the spleen was independent from the presence of VWF, its chaperone molecule in the circulation (43). Interestingly, FVIII specifically targeted the marginal zone of the spleen where it co-localised with metallophilic macrophages (42). Accordingly, the selective in vivo elimination of macrophages and CD11c-positive CD8 alpha-negative DCs abrogated the onset of the anti-FVIII immune response. Our unpublished data indicate that, in primed animals, FVIII co-localised with follicular B cells in the spleen. Thus, the view the immune system of a patient has of therapeutic FVIII is intimately linked to its experience of the molecule (44). Depending on the naïve or primed status versus FVIII of the immune system of a patient, the immune-competent cells that capture the infused FVIII are either cells of the innate immune system, in particular DCs and macrophages, that lack adaptive receptors for the antigen, or cells of the adaptive immune system (B lymphocytes), that express at their surface high-affinity B-cell receptors (BCR). Consequently, the nature of the receptors that bind to circulating FVIII and mediate its internalisation by AgPCs, and, accordingly, the moieties of FVIII which are “recognised” and responsible for the immunogenicity of FVIII, evolves along the treatment of the patients.

Endocytic receptors for FVIII

Endocytosis of FVIII may have two consequences: mere accumulation in lysosomes and degradation, or delivery to early then late endosomes, processing and presentation in association with MHC class II molecules to effectors of the immune system. The catabolic or immunological fate of FVIII depends on the site of FVIII internalisation, on the nature of the cells that internalise it and on the receptors that mediate the endocytosis process. Most of the knowledge accumulated to date on endocytic receptors for FVIII is related to FVIII catabolism, and several catabolic receptors that are specific for different moieties of the FVIII molecule have been characterised (Table 1). We have recently identified a receptor that promotes FVIII internalisation by immune competent cells. The different endocytic receptors and their relevance in FVIII catabolism or immunogenicity are discussed below.

Low-density lipoprotein receptor-related protein and receptors of the low-density lipoprotein receptor family

The low-density lipoprotein receptor-related protein (LRP), also known as the α2-macroglobulin receptor or CD91, is a member of
The LDL receptor family of endocytic receptors that mediates the binding of multiple ligands and their transport from the cell surface to the endosomal or lysosomal compartment (45). CD91/LRP and other members of the LDL receptor (LDLR) family like LDLR, VLDL and megalin, have demonstrated affinity for FVIII with an $K_D$ at equilibrium in the range of 20 nM (46, 47). CD91/LRP expressed on transfected fibroblasts was demonstrated to mediate at least 50% of FVIII uptake and degradation (48). In parallel, the co-administration of FVIII with the receptor-associated protein (RAP), a specific antagonist of LRP and members of the LDLR family, was shown to significantly increase the half-life of human FVIII in mice (49). Accordingly, disruption of the expression of LRP in conditional knock-out mice has confirmed the relevance of LRP for clearance of circulating FVIII (50). LRP/CD91 and other members of the LDLR family recognise protein structures of FVIII in its heavy and light chains. The binding sites for LRP on the light chain of FVIII are protected when FVIII interacts with VWF. Interestingly, heparan sulfate proteoglycans (HSPGs) have been shown to assist LRP for FVIII catabolism (51). Thus, the simultaneous blocking of LRP and HSPGs in model cells completely prevented FVIII internalisation and degradation, and synergised in prolonging FVIII half-life in vivo up to 5.5-fold.

The expression of CD91 has been reported on various cell types including monocytes, macrophages and B lymphocytes (52). We recently asked the question whether LRP/CD91 on AgPCs may mediate the internalisation of FVIII leading to presentation to $CD4^+$ T lymphocytes and/or catabolism. In brackets, species wherein the implication of the receptors was described (mice: in vivo experiments).

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Catabolism†</th>
<th>Endocytosis leading to presentation of $CD4^+$ T cells†</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRP/CD91 and LDLR</td>
<td>Yes (mice, murine cell lines)</td>
<td>No (human dendritic cells)</td>
<td>(48, 49, 53, 59, 72)</td>
</tr>
<tr>
<td>HSPGs</td>
<td>Yes (mice, murine and human cell lines)</td>
<td>No (human dendritic cells)</td>
<td></td>
</tr>
<tr>
<td>ASGPR</td>
<td>Yes (mice)</td>
<td>No (human dendritic cells)</td>
<td></td>
</tr>
<tr>
<td>Mannose receptors/CD206</td>
<td>?</td>
<td>Yes (human dendritic cells)</td>
<td>(60)</td>
</tr>
</tbody>
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†Indicates whether receptors have been implicated in endocytosis followed by presentation to $CD4^+$ T lymphocytes and/or catabolism. In brackets, species wherein the implication of the receptors was described (mice: in vivo experiments).

**FVIII glycans**

FVIII is a heavily glycosylated molecule (54). It harbors 25 potential consensus sites for asparagine (Asn)-linked glycosylations, 19 of which are located within the B domain (55). Initial studies on recombinant full-length FVIII demonstrated that complex glycans are found on the B domain, and that exposed high-mannose chains are present outside the B domain (56). Similar glycosylation profiles were demonstrated for recombinant FVIII produced in Chinese ovaly hamster (CHO) cells and in baby hamster kidney (BHK) cells, and plasma-derived FVIII (57). Thus, all FVIII products present with high mannos structures, that contain bi-, tri- or tetra-antennary complex type chains, and are located on Asn239 and on Asn2118 (57, 58). In the case of glycans on the B domain of FVIII, more than 95% of the complex sugars chains terminate with galactoses that are capped with sialic acids. The implication of some endocytic lectin receptors in the catabolism and immunogenicity of FVIII has been investigated in the recent years, and is summarised below.

**Asialoglycoprotein receptors**

The asialoglycoprotein receptor (ASGPR) is a member of the C-type family of lectins. The ASGPR is abundantly expressed in the liver and plays a role in the binding and removal of glycoproteins from the circulation. To this end, the ASGPR selectively binds glycoproteins via their non-reducing terminal B-D-galactose (Gal) or N-acetyl-D-galactosamine (GalNAc) sugar residues in a calcium-dependent manner. Gal and GalNAc sugar moieties on plasma glycoproteins are generally protected by sialic acid residues. Hence, a
relatively limited number of naturally occurring endogenous ligands for ASGPR have been identified to date (59). FVIII was demonstrated to interact with ASGPR in vitro, through glycans on its B domain (59). Accordingly, B domain-deleted FVIII failed to interact with ASGPR. The injection of asialo-orosomucoid, a high-affinity antagonist of ASGPR, reduced the clearance of infused FVIII from the blood of mice, thus demonstrating that native FVIII presents with glycans which are not protected by sialic acids and that ASGPR contributes in modulating its catabolism.

As in the case of LRP, we examined the involvement of ASGPR on the endocytosis of FVIII by human DCs. An excess of galactose, that saturates ASGPR on the cells, did not prevent the internalisation of FVIII and the presentation of FVIII-depicted peptides to CD4+ T lymphocytes (60). Indeed, the absence of a role of desialylated glycans on FVIII in its recognition by ASGPR on immunocompetent AgPCs was already suggested by the fact that both B domain-deleted (BDD) and full-length therapeutic FVIII demonstrate similar degrees of immunogenicity in vivo (61, 62).

Mannose-specific receptors

We have established an assay wherein the internalisation of FVIII by human monocyte-derived DCs may be followed as well as the subsequent presentation of endocytosed FVIII to FVIII-specific T lymphocytes (63). Using this assay, we investigated a role for mannosylated sugars in FVIII internalisation by DCs. Indeed, mannosylated glycans have been reported on FVIII which are located on Asn239 and on Asn2118 (57, 58). The polycarbohydrate mannan, a model competitive ligand for mannose sensitive uptake (64), reduced the uptake of FVIII-FITC by 60 ± 19% and inhibited in a dose-dependent manner and up to 80% (60). A human FVIII-specific T cell clone (22). Comparison of the endocytosis of full length FVIII and BDD-FVIII by DCs revealed that the uptake of both forms of FVIII is saturated at the same concentration of mannan, thus confirming the involvement of mannose-terminating glycans located outside the B domain in the uptake of FVIII. Removal of mannosylated sugars from BDD-FVIII by mild enzymatic deglycosylation using EndoF1 also reduced the internalisation of FVIII by DCs and the activation of T cells (60).

Together, these data suggest that mannosylated sugars on FVIII mediate its internalisation by DCs leading to its presentation to FVIII-specific T lymphocytes. Interestingly, the immunogenicity of full-length and B domain-deleted FVIII are merely identical in patients with haemophilia A, irrespective of the cell type used for the production of the molecules. It is thus tempting to speculate that removal of mannosylated glycans from both sites of mannosylation on FVIII by site-directed mutagenesis may be an approach towards the reduction of the immunogenicity of therapeutic FVIII. While Asn239 is not systematically mannosylated, Asn2118 on the light chain of FVIII consistently harbours mannose-ending glycans (58). Available data suggest that removal of mannosylated glycans on FVIII by site directed mutagenesis might be a strategy to obtain less immunogenic FVIII products (65). Of note, the high-mannose glycan at position Asn2118 has been proposed to create an aromatic/hydrophobic interface that facilitate the A3-C1 domain interaction (66). Hence, site-directed replacement of Asn2118 to disrupt the Asn-glycosylation site may be associated with loss in FVIII structure thus impairing its pro-coagulant potency.

The macrophage mannose receptor binds to FVIII

The macrophage mannose receptor (MMR/CD206) was identified as one of the receptors involved in the mannose-dependent endocytosis of FVIII by DCs. MMR is a divergent ion-dependent type I C-type lectin expressed on many macrophage subtypes, monocyte-derived dendritic cells, and hepatic endothelium (67). MMR is the founding member of a family of molecules sharing the same basic domain structure: an NH2-terminal cystein-rich domain, a fibronectin type II domain and a variable number of C-type lectin carbohydrate recognition domains (CRDs), eight in the case of MMR, followed by a transmembrane domain and a COOH-terminal intracellular domain (68, 69). MMR is implicated in innate immunity as it is able to recognise a wide range of Gram-negative and Gram-positive bacteria, yeasts, parasites and mycobacteria (70). It has also a role in adaptive immunity and participates to antigen processing. In addition, MMR regulates the catabolism of a large number of mammalian glycoproteins of the reproductive and inflammatory cascades (71). FVIII endocytosis by human DCs was partially inhibited by co-incubation of DCs in the presence of a monoclonal anti-MMR antibody (60).

In collaboration with Dr. Luisa Martinez-Pomares, we demonstrated that FVIII binds in a mannose-dependent manner to MMR in ELISA and by surface plasmon resonance. Interestingly, VWF, which prevents FVIII endocytosis by DCs in a dose-dependent manner in vitro (63), inhibited the binding of FVIII to MMR, although up to 80% (60). Because VWF is known to interact with the light chain of FVIII, this observation suggests that VWF protects the binding of the mannosylated glycan located on Asn2118 of FVIII to MMR, while allowing the mannosylated glycan on Asn239 to interact with the receptor. MMR is expressed on hepatocytes in mice; our preliminary data suggest that, in mice, MMR participates in the catabolism of FVIII under physiological conditions. Whether the same is true in patients with haemophilia A remains to be investigated.

Conclusion

The immune response to FVIII will develop only provided several conditions are met. Firstly, the FVIII-specific effector T and B cells should not have been eliminated during ontogeny, and natural tolerance to the antigen should not have been established. This is thought to be the case in severe patients with haemophilia A. Indeed, the immune system of these patients has not been educated towards FVIII during its ontogeny and FVIII-reactive T and B cells persist in the circulation. Secondly, presentation of the antigen by
AgPCs to naïve T cells has to occur in the context of “danger” for the functional activation of T cells. The nature of the “danger” signals which are provided to AgPCs when the immune response to FVIII is initiated remains unknown and is the subject of intense research (44). Of interest, we failed to show an interaction between FVIII and TLR2 (M. Teyssandier and S. André, unpublished data) and, to our knowledge, a direct interaction between NLRs and FVIII has not been reported. Lastly, upstream from the activation of immune effectors, FVIII has to be recognised by AgPCs, internalised, processed and presented to antigen-specific T cells. As summarised in the present review, receptors have been identified that mediate FVIII uptake leading to FVIII removal and degradation. This is the case of ASGPR, LRP and receptors of the LDLR family. Other endocytic receptors, such as the MMR lectin receptor, allow the processing of FVIII by human AgPCs and presentation of FVIII-derived peptides to T cells in an MHC II-dependent manner. The delineation of the importance of mannose-6-phosphate glycoforms on both the light and heavy chains of FVIII to its recognition by cells of the immune system paves the way towards the development of engineered FVIII molecules with reduced immunogenicity.

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