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Chapter 9

Towards a rationale for inhibitor development in nonsevere hemophilia A

C.L. Eckhardt, A.S. van Velzen, D.P. Hart, J. Voorberg, K. Fijnvandraat
ABSTRACT

Recent evidence shows that inhibitor development in patients with nonsevere hemophilia A is more prevalent than previously anticipated. Specific missense mutations in the factor VIII gene (F8) are associated with an increased risk of inhibitor formation. The etiological mechanisms underlying this association are presently unexplained. In this review we discuss our current immunological perspective, integrating recent evidence from a large epidemiological study and novel findings on the repertoire of factor VIII derived peptides that is presented on MHC class II. We propose a model for the immune reactivity to mismatched factor VIII in a subset of patients with nonsevere hemophilia A.
INTRODUCTION

The development of allo-antibodies against therapeutic FVIII concentrates (inhibitors) is a major complication of treatment in patients with hemophilia A.\(^1\) In approximately 25% of patients with severe hemophilia A, inhibitory antibodies arise that are directed towards exposed sites within the A2, A3 and C2 domain of factor VIII.\(^2\)\(^3\)

Although circulating endogenous factor VIII is present in patients with nonsevere hemophilia A (factor VIII plasma level [FVIII:C], 1-40 IU dL\(^{-1}\)), they may also form inhibitors after treatment with factor VIII concentrates.\(^4\) Inhibitors impair the management of bleeding by interfering with factor VIII activity or by accelerating its clearance. This causes substantially increased morbidity and cost-of-care.\(^4\)\(^5\) In nonsevere patients these antibodies may also neutralize endogenous factor VIII, resulting in conversion from a mild to a severe phenotype (FVIII:C, <1 IU dL\(^{-1}\)) with spontaneous bleeding.\(^4\)

Both genetic and treatment-related factors contribute to inhibitor development in hemophilia A.\(^6\) The F8 mutation is a strong genetic determinant of inhibitor risk, increasing the risk from <10% in individuals with missense mutations to 88% in individuals with large gene deletions.\(^2\)\(^-\)\(^10\) Genetic factors beyond the F8 gene that are associated with inhibitor risk include polymorphisms in the immunoregulatory genes encoding IL-10, TNF\(\alpha\) and CTLA4.\(^11\)\(^-\)\(^13\) More recently, results from the Hemophilia Inhibitor Genetic Study revealed over 50 additional single nucleotide polymorphism that are associated with inhibitor development in severe hemophilia A.\(^14\) However, these studies are in severe hemophilia A and currently there are no equivalent studies on nonsevere hemophilia A.

Recent findings from the INSIGHT study demonstrated that inhibitor development is more prevalent in nonsevere hemophilia A than previously appreciated, revealing a lifetime cumulative incidence of inhibitors of 13%.\(^9\) Inhibitor risk was strongly associated with F8 mutation; 19 specific F8 missense mutations were associated with clinically relevant antibody development among the total of 214 F8 missense mutations that were present in this cohort. The inhibitor risk in patients carrying these 19 mutations was comparable to the inhibitor risk of patients with severe hemophilia A.\(^9\) Why do these missense mutations predispose for inhibitor development?

In this paper we propose an immunological perspective on the interplay between F8 genotype, presentation of factor VIII peptides on MHC class II type in the etiology of inhibitor development in nonsevere hemophilia A. Factor VIII derived peptides are presented on MHC class II. We hypothesize that patients with missense mutations in F8 that correspond to critical anchor residues of these MHC class II presented peptides have an increased risk of inhibitor development. This hypothesis may provide an explanation for the high prevalence of inhibitors in nonsevere hemophilia A patients with specific F8 missense mutations.
IMMUNE TOLERANCE TOWARDS ENDOGENOUS FACTOR VIII

CD4+ T cells play a central role in the regulation of the immune response against factor VIII through their ability to support the generation of B cells producing high affinity antibodies.15 The circulating T cell repertoire is shaped by positive and negative selection in the thymus.16

Positive selection ensures immune responsiveness to foreign antigens, whereas negative selection ensures tolerance to self-antigens (to avoid autoimmunity). Positive selection controls the propensity of double positive CD4+ and CD8+ T cells to bind to peptide MHC complexes on antigen presenting cells. These double positive CD4+ CD8+ T cells mature into single positive CD4+ or CD8+ T cells that then migrate into the thymic medulla.17 Negative selection ensures that T cells binding with high affinity to peptide MHC class II are eliminated in the thymus.16

THE ROLE OF F8 MUTATION IN THE RECOGNITION OF ‘WILD-TYPE’ FACTOR VIII

Nonsevere hemophilia A is generally caused by a missense mutation in F8, of which over 500 causative mutations are reported in the Haemophilia A Mutation Structure, Test and Resource Site (HAMSTeRs) database or on the CDC Hemophilia A Mutation Project (CHAMP) database.18,19 Due to the expression of endogenous factor VIII and its presentation on thymic medullary dendritic cells, CD4+ T cells binding with high affinity to factor VIII peptide MHC are efficiently eliminated by negative selection in the thymus in patients with nonsevere hemophilia A. By this mechanism central tolerance towards endogenous factor VIII is achieved.

However, central tolerance is only established for peptides derived from the mutated endogenous factor VIII protein and not for therapeutic (sequence-mismatched) wild type factor VIII. Despite the fact that the endogenous factor VIII protein differs by only a single amino acid substitution from exogenous administered factor VIII concentrate for the majority of nonsevere hemophilia A patients, some specific F8 missense mutations are highly immunogenic. When patients with these mutations are exposed to wild type factor VIII this provokes a CD4+ T cell response directed towards wild type factor VIII derived peptides that span the F8 mutation position (Figure 1).

Evidence for a CD4+ T cell response against wild type factor VIII has been obtained from three independent studies. First, Jacquemin and colleagues isolated three factor VIII specific T cell clones from peripheral blood of an inhibitor patient with nonsevere hemophilia A caused by the p.Arg2169His substitution.20 Subsequent analysis revealed that these CD4+ T cell clones recognized peptides p.Ile2163-p.Thr2180 and p.Ile2158-p.Thr2173 both containing p.Arg2169. Peptide binding studies demonstrated that peptide p.Ile2163-p.Thr2180 was...
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Figure 1. CD4+ T cell response in a nonsevere hemophilia A patient with the p.Arg2169His mutation and HLA-DRB1*0101/*1301 haplotype upon exposure to ‘wild-type’ factor VIII (p.Arg2169)

The circulating T cell repertoire is shaped by positive and negative selection in the thymus. T cells that bind with high affinity to the endogenous factor VIII derived peptide (p.His2169) that is presented on MHC-class II on thymic epithelial cells (TEC) are eliminated in the thymus from the T cell repertoire (negative selection), whereas T cells that bind transiently with low affinity to peptide MHC complexes escape and migrate to the periphery (positive selection). Upon administration therapeutic factor VIII, ‘wild-type’ factor VIII containing p.Arg2169 is recognized and taken up by antigen-presenting cells (APC) in which it is processed into peptide fragments which are presented on the surface of APC in association with MHC-class II to CD4+ T cells. In the context of appropriate co-stimulatory signals non-censored CD4+ T cells reactive with the presented ‘wild-type’ factor VIII peptide (p.Arg2169) may become activated eventually resulting in the production of factor VIII specific antibody by B cells.

capable of binding to multiple HLA-DR molecules including the commonly expressed DRB1*0101, DRB1*0401 and HLA-DRB1*1501. Interestingly, it was noted that although the variant peptide p.Arg2169His bound with high affinity to different HLA-DR molecules, it did not support proliferation of all isolated CD4+ T cell clones. These findings indicate that the p.Arg2169 to His substitution fundamentally influences recognition of the peptide-MHC class II complex by the T cell receptor.

In a more recent study James and co-workers analyzed CD4+ T cell responses in two unrelated patients with the p.Arg612 to Cys mutation. Using MHC class II tetramers they...
identified CD4+ T cells reactive to peptide p.Glu608-p.Phe627, spanning the F8 missense mutation position in these patients. Peptide binding studies revealed that p.Glu608-p.Phe627 was capable of binding to DRB1*0101, DRB1*1101 and DRB1*1501. The variant peptide containing the p.Arg612 to Cys substitution bound with lower affinity to HLA-DRB1*0101, DRB1*1101 and DRB1*1501. Interestingly, these alleles were present in nine out of ten inhibitor patients in a cohort of patients with a p.Arg612Cys mutation.

In a third study CD4+ T cells reactive with p.Ser2213-p.Asn2232 peptide were identified in a mild hemophilia A patient with the p.Ala2220Pro substitution. DRB1*0101 restricted T cell clones did not proliferate in response to the p.Ser2213-p.Asn2232 peptide containing the Pro substitution at position 2220. Peptide binding studies revealed that the p.Pro2220 containing peptide bound with a lower affinity to HLA-DRB1*0101.

Taken together, the findings reported in these three studies emphasize that inhibitor development in patients with nonsevere hemophilia A is supported by a CD4+ T cell response directed towards wild-type factor VIII amino acid sequences spanning the F8 mutation position. Critically, this links the immunological response to factor VIII derived peptides determined by the underlying F8 missense mutation presented to the T cell repertoire in the context of MHC class II allele profile.

RATIONALE FOR INHIBITOR DEVELOPMENT IN NONSEVERE HEMOPHILIA A – COMBINATION OF F8 MUTATION AND HLA TYPE

Recent findings on F8 mutations associated with inhibitor development from the INSIGHT study and new results on the repertoire of factor VIII derived peptides presented on MHC class II provide fundamental information to further support the hypothesis that inhibitor development in nonsevere hemophilia A is due to F8 missense mutation-induced incomplete central tolerance to administered wild-type factor VIII. Activation of CD4+ T cells depends on the appropriate presentation of factor VIII-derived peptides on MHC class II molecules. The identification of putative factor VIII derived CD4+ T cell epitopes is important to understand why some allogenic wildtype peptides lead to inhibitor development while others do not. Two recent studies by Van Haren and colleagues identified 47 MHC class II presented peptides of factor VIII on monocyte-derived dendritic cells from eight unrelated healthy donors. The factor VIII peptide sequences were distributed throughout the domains of the factor VIII molecule, and were presented differentially by different donors (Figure 2). These data emphasize that factor VIII contains a restricted number of peptide-ligands that can be presented on MHC class II. In a parallel approach Steinitz and co-workers employed HLA-DRB1*1501 transgenic mice to
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**Figure 2.** F8 missense mutations associated with inhibitor development in nonsevere hemophilia (INSIGHT study) and their relation to the distribution of factor VIII core peptides in eight different donors.9,25

The factor VIII protein two-dimensional structure is schematically represented (B-domain deleted). F8 missense mutations that are located within HLA-DRB1-presented peptides are indicated in red. Factor VIII derived HLA-DRB1-presented peptides are presented by one or more different donors and are represented as rectangles for each individual donor. The total number of donors by which the HLA-DRB1-presented peptide is presented is indicated in color: grey: one donor; green: two donors; orange: three donors; red: four donors; blue: five donors; purple: seven donors.

Identify T cell epitopes on factor VIII.27 Actual presentation of peptides on human dendritic cells derived from a homozygous HLA-DRB1*1501 individual was verified.27

Interestingly, seven of the 19 F8 missense mutations (p.Phe1794Val, p.Arg1800Gly, p.Arg2016Trp, p.Asp2093Gly, p.Phe2120Cys, p.Tyr2124Cys and p.Arg2169His) that were associated with inhibitor development in nonsevere hemophilia in the INSIGHT cohort were clustered at five of the 47 core peptides as described by van Haren and colleagues (Figure 2)9,24,25 Two core peptide ligands were located at the A3 domain (p.1794-1802,

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<th>F8 mutations associated with inhibitors (INSIGHT)</th>
<th>HLA-DR bound peptides from human mDC</th>
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<tr>
<td>A1</td>
<td>DRB1<em>0101 DRB1</em>0701 DRB1<em>1501 DRB1</em>0401 DRB1<em>0102 DRB1</em>1502 DRB1<em>1502 DRB1</em>0701 DRB1<em>0301 DRB1</em>1501 DRB1*0101</td>
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<tr>
<td>p.Leu2125Pro</td>
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<td>p.Arg2126Glu</td>
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<td>p.Asn2127Ser</td>
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<tr>
<td>A2</td>
<td>DRB1<em>0101 DRB1</em>0701 DRB1<em>1501 DRB1</em>0401 DRB1<em>0102 DRB1</em>1502 DRB1<em>1502 DRB1</em>0701 DRB1<em>0301 DRB1</em>1501 DRB1*0101</td>
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<tr>
<td>p.Pro1778Gln</td>
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<td>p.Pro1793Gly</td>
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<td>p.Arg1800Gly</td>
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<tr>
<td>A3</td>
<td>DRB1<em>0101 DRB1</em>0701 DRB1<em>1501 DRB1</em>0401 DRB1<em>0102 DRB1</em>1502 DRB1<em>1502 DRB1</em>0701 DRB1<em>0301 DRB1</em>1501 DRB1*0101</td>
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<td>p.Pro2009Glu</td>
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<td>p.Arg2016Trp</td>
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<tr>
<td>C1</td>
<td>DRB1<em>0101 DRB1</em>0701 DRB1<em>1501 DRB1</em>0401 DRB1<em>0102 DRB1</em>1502 DRB1<em>1502 DRB1</em>0701 DRB1<em>0301 DRB1</em>1501 DRB1*0101</td>
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<tr>
<td>p.Glu2171Asp</td>
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<td>p.Thr2178Glu</td>
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<td>p.Lys2180Glu</td>
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p.2015–2023) and three core peptide ligands were located at the C1 domain (p.2092–
have been previously identified immunodominant T cell epitopes.20,28

In the limited number of donors with their specific MHC class II profile that was analysed,
the majority of peptides was only identified by one of the donors. Due to the small number
of donors tested, the donor-specificity of these peptides cannot yet be established with
certainty.24 However, two of the core ligands were presented by seven donors (p.1794–1802)
and three out of eight donors (p.2120–2128). This suggests that these specific core peptides
ligands, overlapping four inhibitor associated F8 mutations (p.Phe1794Val, p.Arg1800Gly, 
p.Phe2120Cys) are highly promiscuous and can be present by multiple HLA-DR molecules.

It is important to note that not only the position of the missense mutation seemingly
determines the inhibitor risk, but that this risk may also be influenced by the type of amino
acid substitution. In a recent study by Schwaab and colleagues the association between
amino acid change and inhibitor development in patients with missense mutations was
investigated.10 Amino acids were divided into four classes according to their chemico-
physical properties: small/hydrophobic, neutral, acidic and basic. In 720 hemophilia A
patients with missense mutations (46% mild and 22% moderate hemophilia A) including
36 patients with inhibitors, the risk of inhibitor development was significantly higher if the
substituted amino acid belonged to another physical-chemical class than the original
residue. Binding of peptides to MHC class II is critically dependent on anchor residues at
the P1, P4, P6 and P9 position of the core peptide sequence. We hypothesize that non-
conservative amino acid changes at these specific positions may have the most impact
on the MHC class II binding. Non-conservative changes at these positions may reduce the
MHC class II binding capacity of endogenously presented peptides resulting in a failure
of elimination of peptide-reactive T cells from the repertoire. Upon treatment with wild-type
factor VIII, the corresponding wild-type peptide may be presented on MHC class II and
trigger proliferation of CD4+ T cells thereby initiating an immune response to factor VIII. Yet,
there is insufficient data to confirm this as a potential mechanism.

In addition, the specific amino acid replacement by a cysteine may result in aberrant
disulphide-bridge formation, resulting in a conformational change of factor VIII.29,30 The effect
of a cysteine replacement on the antigenicity of factor VIII was demonstrated by Suzuki and
co-workers. They demonstrated that the binding of monoclonal antibody NMC-5 to the C2
domain of factor VIII was profoundly increased after replacement of p.Arg2178 by a Cys.31
Similar to T cells self-reactive being eliminated, self-reactive B cells are eliminated as well
resulting in a pool of naïve B cells with limited self-reactivity.32 In patients with a p.Trp2248 to
Cys substitution, the antigenicity of the endogenous protein is likely to differ strongly from the
wild type protein. The consequence may be that B cells recognizing wild type C2 domain
are not efficiently eliminated. Factor VIII reactive B cells are capable of internalizing factor
VIII and present factor VIII derived peptides on MHC class II abundantly expressed on B cells. This may elicit a CD4+ T cell response, inducing B cell affinity maturation by somatic hypermutation and Ig class switching. Eventually this results in the generation of high affinity antibodies directed towards factor VIII. Evidence for a B cell response specifically directed against wild type, exogenous but not endogenous "mutant" factor VIII has been presented for nonsevere hemophilia A patients carrying p.Arg612 to Cys and p.Arg2169 to His substitutions.33-35

LIMITATIONS

Although the observations from the INSIGHT study and the results on the repertoire of factor VIII derived peptides presented on MHC class II by van Haren and colleagues partially support the hypothesis that inhibitor development in nonsevere hemophilia A is due to F8 missense mutation-induced incomplete central tolerance to administered wild-type factor VIII, they also provide observations that contradict the hypothesis. These observations can be clustered into the following two categories: a.) not all inhibitor related F8 mutations from the INSIGHT cohort overlap with the core peptides, and b.) not all F8 mutations that overlap with the HLA-DR bound peptides were associated with inhibitor development. In this section we will discuss potential explanations for these observations.

Although seven out of 19 F8 missense mutations that were associated with inhibitor development in nonsevere hemophilia in the INSIGHT cohort were clustered at core peptides, the other 12 F8 missense mutations did not overlap with peptides that were identified to be presented by MHC class II.9,24,25 Only a limited number of donors with a restricted HLA-DR profile was analysed by van Haren and co-workers and the HLA-types of the patients included in the INSIGHT cohort were not available.9,24,25 Extension of the repertoire of HLA-DR presented peptides is likely to reveal additional overlap between HLA-DR presented peptides and missense mutations linked to inhibitor development in nonsevere hemophilia A. The majority of CD4+ T cell responses are restricted by HLA-DR; however systematic analysis revealed that 19% and 17% of allergen specific CD4+ T cells are restricted by HLA-DQ and HLA-DP, respectively.36,37 It is therefore reasonable to assume that 20-40% of factor VIII specific CD4+ T cell responses are also restricted by HLA-DP and HLA-DQ. The repertoire of HLA-DP and HLA-DQ binding peptides derived of factor VIII has yet to be determined. However, a hemophilia A patient with a certain missense F8 mutation, heterozygous at HLA-DR alleles will have 10s of potential wild type derived peptides straddling the mutation position. We are a long way of understanding how many of these potential peptides get to the MHC surface, but also a long way from understanding which of
the 10s of potential peptides are preferred for presentation. The limited data in the literature to date is a small part of this extensive puzzle.

A second finding that contradicts our hypothesis is that not all F8 mutations that overlap with HLA-DR bound peptides are associated with inhibitor development in the INSIGHT cohort. There could be several reasons for this observation. First, as most of the patients included in the INSIGHT cohort had limited number of exposures to therapeutic factor VIII, they are still at risk to develop an inhibitor in the future. In addition, patients that developed transient inhibitors or non-inhibitory antibodies that do not interfere with the coagulant function of factor VIII and are undetected by the Bethesda assay might have been misclassified as non-inhibitor patients as well. Second, although promiscuity may be demonstrated to multiple MHC alleles, individual patients have a very limited set of MHC alleles that may not be capable of binding the specific peptides spanning a given F8 mutation. Moreover, not all peptides found to bind HLA alleles in vitro do necessarily induce T-cell responses in vivo. Finally, according to the immunological “danger model” appropriate co-stimulatory signals, arising from tissue injury and inflammation, are necessary to activate and proliferate antigen reactive CD4+ T cells. If therapeutic factor VIII is administered in the absence of adequate co-stimulatory danger signals, an immune response is then less likely to occur. Polymorphisms in genes involved in the danger signalling pathway may influence the risk of nonsevere patients upon factor VIII administration at times of immunological danger. Further supporting evidence for this has yet to be obtained.

In summary, currently available data suggest that inhibitor development in nonsevere hemophilia A is due to lack of central tolerance to administered exogenous factor VIII, arising from peptides spanning the F8 missense mutation being presented by MHC class II. Additional factors may influence the propensity of inhibitor development, such as polymorphisms in immunoregulatory genes, number of cumulative exposures to therapeutic factor VIII, factor VIII product type and circumstances at the time of factor VIII exposure that form immunological challenges at time of exposure. Large cohort studies are required to identify these factors and clarify the immunological mechanisms underlying inhibitor development in nonsevere hemophilia A.

CONCLUSION

In contrast to patients with severe hemophilia A, patients with nonsevere hemophilia A carry a lifelong risk of developing inhibitors, adding a substantial risk of morbidity and mortality. A better understanding of the immunological mechanisms underlying inhibitor development in these patients may help to personalize inhibitor risk prediction and may create the opportunity to develop and implement preventive measures in susceptible patients. Until
we start to analyse actual or predicted antigen presentation at a personalised level of F8 mutation and HLA repertoire, we will not unpick the mechanism whether restricted CD4+ T cell responses to mismatched factor VIII are a common pathogenic mechanism for inhibitor formation in nonsevere hemophilia A.
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