## Evidence for idiotype-directed immunosurveillance is restricted to follicular lymphoma and attributable to somatic hypermutation

Antigen receptors of B cells (BCR) exhibit a virtually unlimited repertoire, created by V(D)J recombination through combinatorial and junctional diversity of genetic elements in B-precursor cells. B cells further increase the antigen affinity of their BCR through somatic hypermutation (SHM). VDJ recombination and SHM create unique and novel peptide sequences that are not encoded in germline (GL) and may, therefore, act as neoantigens for the adaptive immune system. Naturally occurring BCR-directed immunity could, therefore, influence expansion of individual B-cell clones. We have previously found evidence for HLA class I-dependent, BCR-directed immunosurveillance in follicular lymphoma (FL).[1] We here report lack of evidence for this mechanism in other indolent B-cell lymphomas and an apparent dependence of possible immunosurveillance on the high SHM rate in FL.

Lymphoma samples were obtained with written informed consent from the Freiburg University idiotype vaccination program[2-4] and the hematology biobank of Leiden University Medical Center with approval of the ethics committee of Freiburg University. HLA alleles were identified by serotyping and high-resolution genotyping (Olerup SSP kit, Qiagen, Hilden, Germany). Unbiased sequencing of full-length immunoglobulin heavy chain VDJ transcripts was performed as described.[1,2] IGHV genes and mutational status were identified for VDJ consensus sequences by the IMGT/V-QUEST algorithm (imgt.cines.fr). Peptide nonamers predicted to bind to HLA alleles represented in the patient cohorts were identified by the BIMAS algorithm (*http://www-bimas.cit.nih.gov/molbio/hla_bind/*).

The sum of the predicted scores of the 20 highest ranking peptides were compared between the VDJ sequence of any given patient's lymphoma ("self" VDJ) and the mean of the BCR of lymphomas arising in other patients ("non-self" VDJ) in 32 mantle cell lymphomas (MCL) and 12 marginal zone lymphomas (MZL) (Table 1) with Wilcoxon signed rank test (Prism 5.02; GraphPad Software, San Diego, CA, USA). In contrast to FL,[1] no evidence for selection of VDJ sequences for aggregate low predicted HLA binding scores on the respective patient's HLA complex was found (Figure 1). Subanalyses of complementarity-determining regions (CDR) 1-3 likewise failed to indicate any immunological selection of BCR in these lymphomas. Equivalent results were obtained when the analysis was restricted to MCL cases with less than 99% (n=14) (Figure 1) or less than 98% (n=8) (*data not shown*) sequence identity to GL IGHV.

Follicular lymphoma distinguishes itself from other B-cell malignancies by arrest in the germinal center maturation stage and expression of activation-induced deaminase.[5,6] Consequently, FL cells continuously undergo SHM and accumulate high IGHV mutation loads.[7,8] We investigated SHM-induced changes in HLA class I binding scores of mutated IGHV peptides in comparison to the corresponding GL peptides in 9 FL cases that shared expression of IGHV3-23 (Table 1). Forty-one of all possible 89 IGHV3-23 GL nonamers were predicted to bind with a BIMAS score of 1 or higher to at least one of the eleven BIMAS-analyzable HLA alleles of the 9 patients, resulting in a total of 96 analyzable HLA binding scores. Per FL case, a median of 33 (range 23-38) of these 41 GL peptides had been mutated by SHM. SHM led to 115 increases and 250 decreases of binding scores. The median change in the score of GL peptides was -1.6 (range -143 to +298). Since HLA binding of a nonamer is dependent on defined anchor residues,[9] reduction of HLA binding of a GL peptide through random SHM was expected to be more likely than strengthening of binding.

SHM-induced binding score alterations were compared for the 41 GL binders between self-HLA alleles (n=78 scores) and non-self alleles (n=287 scores). Peptide binding on self-HLA could thus actually occur in an FL-bearing patient, whereas predictions of presentations on an HLA allele not expressed by the respective patient are purely hypothetical. The median change of binding scores was -2.4 for self-HLA alleles and -1.5 for non-self alleles ($P$=0.035; two-tailed Mann Whitney test). An FL patient's HLA type, therefore, appears to exert selection pressure on BCR peptides that bind to HLA towards lesser HLA binding strength during SHM. However, it is unclear whether comparisons of scores between several HLA alleles are meaningful. Therefore, the following analyses were restricted to comparisons of different IGHV sequences on a common set of HLA alleles.

To study the aggregate effects of score-altering mutations of all HLA binding peptides in an FL idiotype, we calculated the sum of the SHM-induced score differences of every IGHV3-23 GL peptide on all analyzable self-HLA alleles. For every individual patient, this sum of score differences was compared to the hypothetical sum of score differences as calculated for each of the other eight non-self BCR FL on the same self-HLA alleles. Scores on HLA alleles that were shared between 2 patients were disregarded, since such HLA binding alterations would underlie the same immune selection pressure *in vivo*. Therefore, the sum of the peptide binding differences of the self FL BCR was compared with every non-self BCR in a matched-pair analysis with 69 pairs. No non-shared HLA alleles existed for three comparisons (*Online Supplementary Table S1*). The aggregate peptide HLA binding score of a patient's self-FL BCR was on average significantly more reduced than the hypothetical peptide binding of non-self BCR (Figure 2A). This bioinformatic evidence supports our hypothesis that HLA class I-mediated selection pressure shapes the evolution of SHM-induced changes of FL BCR peptides towards lesser potential immunogenicity.

Since tolerance towards GL-encoded peptides is assumed in the self-HLA context,[10] we hypothesized that reduction of HLA binding strength of a particular peptide would not contribute to immune selection pressure on SHM-induced BCR sequence alterations. Indeed, no difference was seen between self and non-self BCR on self-HLA when the comparative analysis was restricted to decreases of predicted HLA binding strength of GL IGHV3-23 peptides in self- or non-self HLA alleles (Figure 2B). Consistent with the assumption of tolerance against GL-encoded BCR peptides, this finding suggests that loss of HLA binding strength of such peptides is irrelevant for immune selection pressure.

Consequently, we hypothesized that only the relatively few mutations leading to increased HLA binding of GL peptides would create targets for immune counter selection. In addition, we also hypothesized that creation of neoepitopes through SHM at positions of GL peptides that do not bind HLA could also be relevant to immunosurveillance. In accordance with our hypothesis, SHM created significantly lower increases in HLA binding for self-BCR in comparison to non-self BCR undergoing SHM in unrelated HLA complexes (Figure 2C). However, the significance of this comparison might be over-estimated due to a large difference in

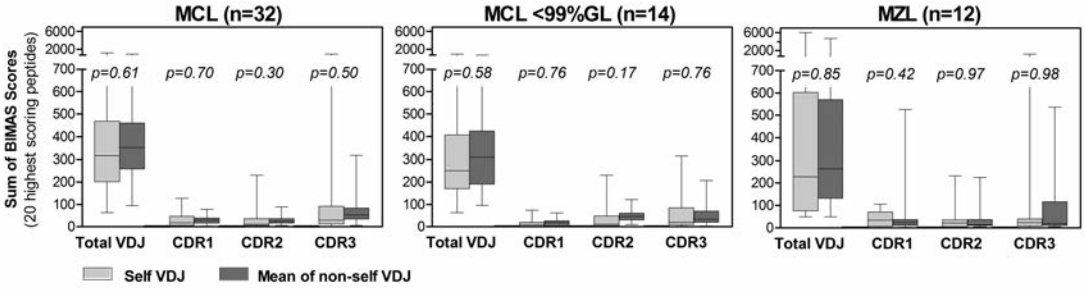


Figure 1. BIMAS sum scores of the 20 highest-scoring peptide nonamers located in the indicated parts of heavy chain VDJ sequences of mantle cell (MCL) and marginal zone (MZL) lymphomas. Symbols represent median, range, and 25% / 75% quartiles. Sum scores of self and the mean of non-self VDJ were compared by Wilcoxon matched-pairs signed rank test. A separate analysis was performed for MCL cases with less than 99% homology to the GL IGHV sequences.

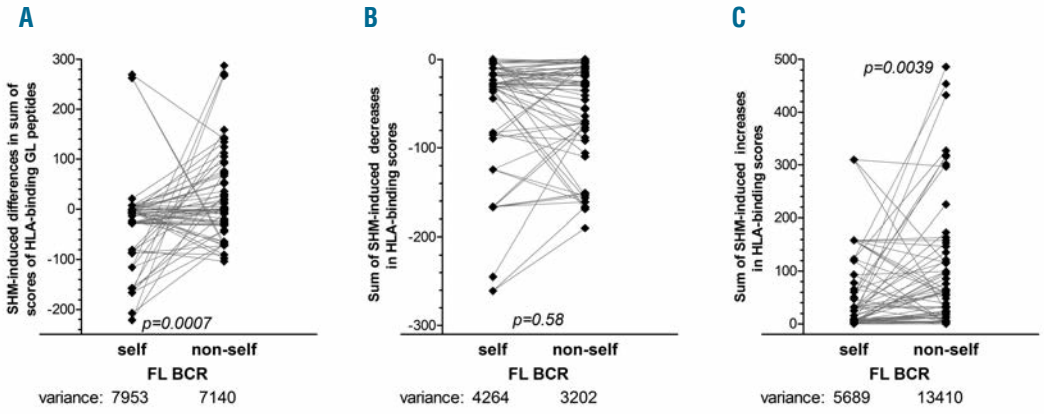


Figure 2. Comparisons between self and non-self BCR of aggregate BIMAS scores of BCR peptides with altered predicted HLA binding strength compared to their GL sequences through SHM in 9 follicular lymphoma cases expressing the *IGHV3-23* gene. For each pair-wise comparison, only HLA alleles of the patient in whom the self FL BCR had arisen and that were not shared with the patient in whom the respective non-self comparator FL BCR was observed were included. Comparisons were performed with Wilcoxon matched-pairs signed rank test. (A) Peptides with predicted HLA binding in GL. Data points in the self FL BCR column are calculated as: $(Sc^{MUTself1}\text{-}Sc^{GL1})+(Sc^{MUTself2}\text{-}Sc^{GL2})+...+(Sc^{MUTself\mathit{n}}\text{-}Sc^{GL\mathit{n}})$. $Sc^{GL\mathit{n}}$ is defined as the BIMAS score of the GL nonamer at IGHV3-23 position ***n*** for peptides with $Sc^{GL\mathit{n}} \geq 1$. $Sc^{MUTself\mathit{n}}$ is defined as the BIMAS score of the mutated nonamer at IGHV3-23 position ***n***. Data points in the non-self FL BCR column are calculated as $(Sc^{MUTnonself1}\text{-}Sc^{GL1})+(Sc^{MUTnonself2}\text{-}Sc^{GL2})+...+(Sc^{MUTnonself\mathit{n}}\text{-}Sc^{GL\mathit{n}})$. $Sc^{MUTnonself\mathit{n}}$ denotes the score of a non-self FL BCR mutated nonamer, i.e. a nonamer that was observed in the FL BCR of a different patient at IGHV3-23 position ***n***. (B) Peptides with decreased predicted HLA binding strength by SHM compared to their GL sequences. Data points are defined as in (A) with the additional requirement of $Sc^{MUTself\mathit{n}} < Sc^{GL\mathit{n}}$ and $Sc^{MUTnonself\mathit{n}} < Sc^{GL\mathit{n}}$. (C) Peptides with increased predicted HLA binding strength by SHM compared to their GL sequences. Data points are defined as in (A) with the additional requirement of $Sc^{MUTself\mathit{n}} > Sc^{GL\mathit{n}}$ and $Sc^{MUTnonself\mathit{n}} > Sc^{GL\mathit{n}}$ and without the requirement of $Sc^{GL\mathit{n}} \geq 1$.

variance stemming from the matched-pair design on the basis of non-shared HLA alleles. In contrast, variances were well balanced in the former comparisons (Figure 2A and B).

Overall, our results are compatible with a scenario of immunological tolerance to unmutated epitopes of IGHV genes. However, immune selection apparently acts against SHM-generated IGHV epitopes that acquire increased affinity for HLA binding; presumably through recognition by HLA class I-restricted T cells. Negative immune selection affects both GL peptides that bind HLA and acquire increased binding strength through SHM, and SHM-generated neoepitopes.

The CDR3 of an immunoglobulin chain contains random residues that are not encoded in GL and that are created during VDJ recombination. Since our analysis was based on comparisons of mutated BCR peptides and their corresponding GL sequences, only SHM occurring within the IGHV sequence could be analyzed. Since non-GL-encoded CDR3 motives contributed substantially to the global differences in HLA binding capacity of FL BCR,[1] the observed level of significance obtained from analysis of only IGHV sequences from 9 FL cases expressing the same IGHV is remarkable.

Evidence for HLA-mediated BCR-directed immunosurveillance was only detectable in FL. The lack of evidence for immunosurveillance in MCL and MZL, even when the analysis was restricted to hypermutated cases, could be explained by overall higher mutation loads of FL. On the other hand, active immunosurveillance could be facilitated by expansion in the germinal center microenvironment, a unique immunobiological characteristic of FL. The seminal observation that an activated T-cell signature within FL biopsies is associated with superior prognosis is compatible with this scenario.[11]

While T cells recognizing non-mutated frame work peptides can be isolated *in vitro*,[12] our results suggest that they fail to exert detectable effects *in vivo*. In contrast, we have shown that HLA class I-restricted T cells with specificity for

individual BCR circulate in FL patients.[13] In agreement with these and previous data on possible immunosurveillance *in vivo*,[1] active immunization of untreated FL patients with a highly immunogenic idiotype formulation induces T cells with specificity for mutated or individual BCR epitopes.[3,4] These T-cell responses were associated with objective clinical remissions and favorable long-term outcome.[4]

In conclusion, we provide further support for the exis-

**Table 1.** Immunoglobulin characteristics and HLA alleles as analyzed in BIMAS.

| Diagnosis | Patient code | Biopsy | IGHV | % homology | HLA A | | HLA B | |
|---|---|---|---|---|---|---|---|---|
| MCL | A150 | BM | 1-2*04 | 100.00 | A1 | A3 | B60 | B_4403 |
| | A225 | PE | 4-34*01 | 100.00 | A1 | A24 | B7 | |
| | LUMC2 | BM | 3-21*01 | 100.00 | A_0201 | A_0205 | | |
| | LUMC8 | PB | 3-9*01 | 100.00 | A_0201 | A24 | B7 | B40 |
| | A167 | LN | 4-34*01 | 99.66 | A1 | | B_3501 | B60 |
| | A122 | LN | 1-3*01 | 99.65 | A24 | A68.1 | B7 | B60 |
| | A124 | LN | 4-34*01 | 99.65 | A3 | A24 | B_5101 | |
| | A206 | LN | 1-2*02 | 99.65 | A1 | A3 | B7 | B62 |
| | A260 | PB | 1-3*01 | 99.65 | A1 | A24 | B7 | |
| | LUMC15 | PB | 4-59*01 | 99.60 | A_0201 | A68.1 | B_3501 | |
| | A164 | LN | 3-21*01 | 99.31 | A1 | A3 | B7 | B8 |
| | LUMC1 | PB | 3-21*01 | 99.31 | A_0201 | | B_5801 | |
| | LUMC5 | PB | 3-23*01 | 99.31 | A_0201 | A24 | | |
| | A026 | LN | 4-34*01 | 99.30 | A1 | | B7 | B60 |
| | B021 | BM | 3-30*18 | 99.30 | A1 | | B7 | |
| | LUMC3 | PB | 3-23*01 | 99.30 | A_0201 | A68.1 | | |
| | LUMC11 | PB | 3-21*01 | 99.30 | A_0201 | A3 | B7 | B8 |
| | LUMC12 | PB | 4-34*01 | 99.30 | A_0201 | A_0201 | B7 | |
| | LUMC7 | PB | 5-51*01 | 98.96 | A_1101 | A68.1 | | |
| | LUMC10 | PB | 3-21*01 | 98.96 | A3 | A_1101 | B7 | |
| | A021 | LN | 4-34*01 | 98.95 | A1 | | B8 | B_3901 |
| | A280 | PB | 4-59*01 | 98.59 | A1 | A_0205 | B8 | |
| | LUMC13 | PB | 3-23*01 | 98.56 | A_0201 | A24 | | |
| | A117 | PB | 4-34*01 | 98.25 | A1 | A24 | B_4403 | |
| | A226 | PE | 1-8*01 | 97.22 | A1 | A3 | B7 | B8 |
| | LUMC4 | PB | 4-39*07 | 96.88 | A1 | A_1101 | B_3701 | B_4403 |
| | LUMC14 | PB | 1-8*01 | 96.80 | A1 | A3 | | |
| | A095 | BM | 4-31*03 | 96.22 | A1 | A3 | B7 | B60 |
| | A020 | PB | 3-21*01 | 95.83 | A1 | A3 | B_5101 | |
| | A159 | PB | 6-1*01 | 94.61 | A3 | A24 | B7 | |
| | A013 | PB | 3-5*01 | 92.36 | A1 | A1 | B8 | |
| | A216 | PB | 3-23*01 | 90.53 | A24 | | B7 | |
| MZL | A244 | PB | 3-30-3*01 | 100.00 | A3 | | B_3501 | |
| | A215 | PB | 3-30*03 | 97.22 | A1 | | B14 | |
| | A223 | BM | 4-39*01 | 96.56 | A3 | | B_3801 | |
| | A241 | PB | 3-74*01 | 95.83 | A_0201 | A3 | B_3501 | |
| | A271 | LN | 3-48*03 | 95.83 | A1 | A24 | B7 | B7 |
| | A157 | PB | 1-69*01 | 95.49 | A3 | A1 | B7 | |
| | A201 | PB | 3-7*01 | 95.14 | A3 | A3 | B7 | B62 |
| | A183 | LN | 1-69*01 | 94.79 | A24 | | | |
| | A002 | BM | 3-30*03 | 93.75 | A1 | | B8 | |
| | A130 | LN | 4-34*01 | 91.93 | A1 | A1 | | |
| | A176 | PB | 1-69*06 | 91.67 | A1 | A1 | B7 | B_5101 |
| | A333 | KM | 3-7*01 | 90.28 | A1 | | B7 | |
| FL | A192 | BM | 3-23 | 90.62 | | | B14 | B_4403 |
| | A112 | LN | 3-23 | 89.24 | A3 | | B_3501 | |
| | A126 | LN | 3-23 | 88.54 | A1 | A_0201 | | |
| | A170 | LN | 3-23 | 87.85 | A_0201 | A_1101 | B_3501 | |
| | A019 | BM | 3-23 | 87.15 | A1 | A_0201 | B7 | |
| | A189 | LN | 3-23 | 84.38 | A1 | A_0201 | B14 | |
| | A299 | LN | 3-23 | 82.29 | A24 | | B62 | |
| | A125 | LN | 3-23 | 81.75 | A_0201 | | B7 | |
| | A173 | LN | 3-23 | 80.56 | A_0201 | A_3101 | B_3501 | |

Comparison of mutation load (Mann Whitney test):

| Comparison | *p* |
|---|---|
| FL *vs.* MCL | <0.0001 |
| FL *vs.* MCL(<99%) | <0.0001 |
| FL *vs.* MCL(<98%) | 0.0002 |
| FL *vs.* MZL | 0.0002 |

*Cases are ranked by IGHV homology. LN: lymph node; BM: bone marrow; PB: peripheral blood.*

tence of naturally occurring, T-cell-mediated immunosurveillance in FL, and for concepts seeking to exploit this phenomenon therapeutically. While the BCR of a clinically manifest lymphoma is apparently insufficiently immunogenic for efficacious immune control of the expanding tumor, active immunization may enhance the failing immunosurveillance to suppress the FL clone. We suggest that interventional trials of active immunization should focus on FL and the induction of T-cell responses against SHM-generated neoepitopes.

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