Published in final edited form as:

Hum Immunol. 2020 ; 81(2-3): 73–78. doi:10.1016/j.humimm.2019.12.006.

Identification of a recurrent pattern of false-positivity by Luminex HLA MHC class I single antigen bead testing

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Abstract

Previously, a distinct MHC class II Luminex-single antigen bead (SAB) pattern was described and attributed to antibodies targeting denatured antigens. In this study, we describe a distinct MHC class I reactivity pattern observed in 1.8% (105/5992) of samples resulted in 2017. The pattern displays reactivity to the following Luminex-SABs: HLA-A*33:03, A*36:01, A*80:01, B*54:01, B*53:01, C*06:02, C*07:02, C*18:02, C*14:02, C*03:03, C*03:04, and C*15:02. This pattern was identified in patients with no sensitization history, negative FlowPRA results, and antibody to self-antigen(s). Epitope analysis failed to reveal a common determinant(s) to explain this pattern of reactivity. Additionally, we found this pattern to be prevalent in female patients (62%) and also those with systemic lupus erythematosus (62%). Given these findings, we speculate this pattern likely represents false-positive reactivity, possibly due to antibody targeting denatured antigens or a specific peptide, molecular mimicry, autoimmunity, or a combination thereof.

Keywords

HLA antibody; MHC class I; Luminex assay; Single antigen bead

1. Introduction

Advances in clinical HLA testing over the last 25 years have translated into improvements in organ allocation. Indeed, solid phase assays to identify specific HLA antibodies led to increased allocation of deceased donor kidneys to more highly sensitized transplant candidates by way of virtual crossmatching [1]. However, solid phase assays have some well-known limitations which can make interpretation of results challenging [2]. In particular, single antigen bead (SAB) testing detects naturally occurring antibodies to cryptic epitopes likely unmasked during the manufacturing process of the beads (e.g. denatured antigens) [3,4]. Importantly, antibodies to cryptic/denatured HLA antigens do not correlate with the poor graft outcomes associated with antibodies targeting intact HLA antigen [5].

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Author contributions

All authors reviewed the literature, wrote and contributed to the manuscript

Conflict of interest disclosure

The authors of this manuscript have no conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.humimm.2019.12.006>.

Reactivity to denatured antigens should thus be viewed as false-positive reactions. Failure to identify such reactions could lead to improper assignment of unacceptable antigens, thereby potentially precluding an otherwise compatible transplant.

In 2013, Grenzi et al. described a distinct, recurring MHC class II Luminex-SAB pattern in serum samples from renal transplant candidates [6]. They observed a specific pattern of bead positivity (HLA-DRB1*09:01, DRB3*01:01, DRB3*02:02, DRB3*03:01, DPB1*02:01, DPB1*20:01 and DPB1*28:01) in patients without prior sensitization, and even in some who carried these antigens (i.e. reactivity against self-antigen). They also found this pattern to be associated with female gender and with a diagnosis of systemic lupus erythematosus (SLE). The authors concluded that this pattern was likely due to detection of epitopes exposed in/on denatured antigens, as flow cytometric studies performed with intact antigens were negative.

Similarly, we identified a distinct, recurring MHC class I Luminex-SAB pattern in our laboratory showing reactivity to the following SABs: HLA-A*33:03, A*36:01, A*80:01, B*54:01, B*53:01, C*06:02, C*07:02, C*18:02, C*14:02, C*03:03, C*03:04, and C*15:02. In this report the frequency of this particular reactivity and the patient characteristics in which it occurs are described.

2. Materials and methods

Routine review of serum samples from renal transplant patients revealed a recurring pattern of reactivity with the MHC class I Luminex-SAB assay (LABScreen® Single Antigen, One Lambda, Canoga Park, CA; lot 10). The pattern included the following beads: HLA-A*33:03, A*36:01, A*80:01, B*54:01, B*53:01, C*06:02, C*07:02, C*18:02, C*14:02, C*03:03, C*03:04, and C*15:02. After approval from our institutional review board, all MHC class I SAB results performed in 2017 on sera from renal transplant candidates/recipients were reviewed retrospectively for the presence of the aforementioned pattern and the associated mean fluorescence intensity (MFI) values of the corresponding beads. We also reviewed results from lot 11 SAB in patients demonstrating the aforementioned pattern to determine if the pattern persisted.

Additionally, corresponding FlowPRA (One Lambda, Canoga Park, CA), MHC class II SAB [LABScreen® Single Antigen, One Lambda, Canoga Park, CA; lot 11 and 12 (lot change occurred in April 2017)], and allogeneic flow cytometric crossmatches [FCXM (FACSCanto II, BD Biosciences San Jose, CA)] were reviewed in all patients demonstrating the aforementioned MHC class I Luminex-SAB pattern. Reactivity to self-antigen(s) was assessed by comparing SAB reactivity to each patient's HLA typing (LABType™ SSO Typing Test, One Lambda, Canoga Park, CA). The assay methods performed were previously described [7]. Further investigation was performed to determine the prevalence of this pattern and specific characteristics of patients in which it was observed. Finally, the transplant status and transplant outcomes of the cohort were evaluated, including clinical concern for AMR (as noted in the electronic medical record) and subsequent development of de novo donor-specific antibody (DSA).

3. Results

In total, MHC class I SAB data generated by 5992 serum samples from 3027 patients was retrospectively reviewed. We observed 105 (1.8%) samples from 58 patients displaying the recurring MHC class I SAB pattern (see Fig. 1a for examples). Along with the beads mentioned above, the pattern includes a long trailing tail of reactivity on the SAB panel where the broad reactivity decreases in strength from the left to right on the histogram. The average MFI values of the pattern antigens (i.e. antigens comprising the MHC class I pattern described) was as follows: A*33:03 (922), A*36:01 (832), A*80:01 (829); B*54:01 (2448), B*53:01 (1954); C*06:02 (2663), C*07:02 (2517), C*18:02 (2187), C*14:02 (1256), C*03:03 (1217), C*03:04 (1030), and C*15:02 (805).

Twenty-nine percent (n = 17) of these patients had no history of a potential sensitizing event (e.g. blood transfusion, pregnancy, transplant). Of the samples demonstrating this pattern, 83% had reactivity against self-antigen involving 1 or more of the alleles in question, with HLA-C*07:02 being the most common target (Table 1). We found that 62% of patients with this pattern had a diagnosis of SLE, of which 81% were female and 29% were male.

Reviewing the FlowPRA results to look for architecture corresponding to this pattern, we found that 50% of FlowPRA samples reviewed had a particular pattern of either a widened or right-shifted peak (see Fig. 1b for examples). In general, FlowPRA demonstrating true HLA antibody is reflected with distinct peaks that are clearly moved to the right of the negative control as demonstrated in the inset of Fig. 2. Widened peaks or scenarios where the whole peak is right-shifted frequently do not represent true HLA antibody(ies) and instead are due to high background reactivity. In our laboratory's experience, such results are typically seen in patients with autoimmune diseases. Of note, 31% of cases were a mixture of both true HLA antibody and antibodies to pattern antigens. When true HLA antibodies were present, they preceded the MHC class I pattern with higher MFI values (i.e., the left side of the histogram).

To examine potential clinical relevance of the reactivity in the MHC class I pattern, allogeneic FCXMs that had been previously performed were also retrospectively reviewed. Allogeneic FCXM data were assessed in the 58 patients displaying this distinct MHC class I pattern but lacking true HLA specificities. In patients without SLE (n = 22) and excluding patients with non-pattern DSA (i.e., antibody targeting antigens not identified in the MHC class I pattern), we observed that 11 samples from 6 patients were all negative by FCXM (Table 2). Ten of these samples included donor cells that expressed 1 or more pattern antigens at an average MFI of 1693. In patients with SLE, allogeneic FCXM was performed on 23 samples from 16 patients (Table 3). We observed that 10 samples were positive with B-cells only, with an average delta molecules of equivalent soluble fluorochrome (dMESF) of 27,632. All 10 of these samples were from donors expressing 1 or more of the pattern antigens at an average MFI of 2690. In this SLE cohort, another 3 samples were B-cell (average dMESF = 35,632) and T-cell positive (average dMESF = 2625) by FCXM. Only 1 of these samples came from a donor expressing a pattern antigen, at an average MFI of 1427. The remaining 10 samples from this cohort were negative by FCXM, with 9 of the

samples from donors expressing 1 or more of the pattern antigens at an average MFI of 2463 (Table 3).

Examining the transplant status and outcomes in this patient cohort revealed that 29% (17/58) had been transplanted since 2017. Nine of the patient's had negative B- and T-cell crossmatches, while 8 had B-cell positive, T-cell negative crossmatches, a pattern not associated with poor outcomes in the absence of DSA [7]. In terms of outcome, thirteen (76%) patients have not experienced clinical concern for antibody-mediated rejection (AMR) or developed DSA during the follow up period [average follow-up time 355.71 days (range: 21–920)]. Of the four patients that did develop DSA, two were not mismatched for pattern-antigens. Of the remaining two, one developed MHC class II DSA and the other developed multiple MHC class I and II DSAs, of which the pattern antigen (C7) was the DSA with the lowest MFI value.

Review of the 58 patients displaying the pattern revealed that 39 (67%) had serum samples tested on what is now our current lot (lot 11). Of these, 35 display a recurrent pattern with a similar long tail of trailing reactivity but different from the above reported pattern. The pattern with lot 11 is characterized by reactivity of the following beads: A*74:01, B*15:15 (B63), B*27:05, B*15:13 (B77), B*81:01, and all the C locus beads except the C*01:02, C*12:03, and C*15:02 beads (see Fig. 3). The C locus reactivity is interesting in that it is almost the complete opposite of the well characterized C*01:02, C*12:03, and C*15:02 denatured pattern [8].

Like Grenzi et al.'s cohort, [6] a substantial proportion of our cohort also had SLE; as such, we examined our patient cohort's corresponding MHC class II SAB results for the presence of the MHC class II pattern described in their report. Though we did not observe the same MHC class II pattern, we observed another distinct, recurring MHC class II Luminex-SAB pattern with the following beads: HLA-DRB1*04:03, *08:01, *09:01, *13:01, *14:01, DQA1*04:01/DQB1*02:01, DQA1*02:01/DQB1*04:01, DQA1*02:01/DQB1*04:02, DPA1*01:03/DPB1*28*01, DPA1*02:02/DPB1*13:01, DPA1*03:01/DPB1*13:01, DPA1*03:01/DPB1*20:01, DPA1*01:03/DPB1*23:01, and DPA1*04:01/DPB1*28:01 (see Fig. 4). This pattern was identified in 1.9% of 4073 samples from 57 patients tested with MHC class II Luminex-SAB lot 12. Similar to our findings in the samples positive for the MHC class I SAB pattern, about one-third of these patients had no history of a prior potential sensitizing event. Of the positive samples, 87% had reactivity against self-antigen involving 1 or more of the alleles in question and 32% of these had a diagnosis of SLE.

4. Discussion

Solid phase antibody testing revolutionized HLA testing and led to great improvements for transplant candidates/recipients, from organ allocation to post-transplant care. However, understanding the limitations of such testing is imperative for accurate interpretation of the results. A previous report described a specific recurring pattern with MHC class II Luminex-SAB assays, likely due to detection of denatured antigens on the bead surface [6]. Here, we observed another recurring pattern in results from the MHC class I Luminex-SAB assay. We found that among nearly 6000 samples, 1.8% displayed the described MHC class I pattern.

With MFI values reaching up to 17,000, the corresponding beads could easily be interpreted as true positives and be assigned as unacceptable antigens. Using the OPTN CPRA calculator (<https://optn.transplant.hrsa.gov/resources/allocation-calculators/cpra-calculator/>), a cPRA of ~80% is attained by assigning the antigens contained within the MHC class I pattern we identified. According to renal allograft offer rates based on cPRA, patients with cPRA of 80–89% receive only 2.7% of transplant post the new kidney allocation scheme (KAS) [9]. Thus, if this reactivity was interpreted as antibodies to intact HLA antigens, a patient's donor pool would be drastically reduced. As such, it is important to identify such patterns as false-positive reactions to avoid restricting donor access and inappropriately precluding donors who are compatible.

There are aspects to this MHC class I pattern we observed that strongly suggest it is false-positive reactivity. First, the pattern exhibits a long trailing tail of positivity on the SAB histogram, reflecting panreactivity. Such high background is seen in sera of patients receiving intravenous immunoglobulin (IVIG), in patients with an infection, and experiencing a systemic inflammatory event [10]; none of which were identified in this patient cohort. Second, examination of the HLA specificities within the pattern revealed that 83% of the patients typed for at least one of the alleles in the pattern, meaning reactivity was detected against self-antigens the majority of time. Whenever reactivity to self-antigen is observed, further investigation is warranted. Third, reviewing sensitization history, approximately one-third of patients had no history of a prior potential sensitizing event; as such, we would not expect to see such degree of bead reactivity in these patients. Fourth, we examined allogeneic FCXM data that had previously been performed on these patients. If the antibody was directed to intact antigens, the FCXM with cells expressing the corresponding antigens should be positive, which was not always the case. Fifth, despite the broad-spectrum MHC class I SAB reactivity, only one patient of those transplanted developed AMR with multiple MHC class I and II DSAs identified. Only one of these DSAs was against a pattern antigen (C7), which had the lowest MFI value of all the DSAs. Sixth, epitope analysis failed to identify a common determinant(s) that could explain the reactivity of the pattern.

In terms of patient characteristics, we found that 62% of patients with the MHC class I SAB pattern were women and another 62% had a diagnosis of SLE. This is similar to the findings reported by Grenzi et al in patients with their characterized MHC class II pattern [6]. Considering this high prevalence of SLE and knowing that patients with SLE can often have B-cell positive allogeneic FCXM not attributable to HLA antibody [11], we examined allogeneic FCXM data from these patients separately. Expectedly, we found, 10 were B-cell positive, but 10 were completely negative. Again, this is despite most of these donor cells expressing at least 1 pattern antigen, some with MFI values that we would generally predict to yield reactivity on a FCXM. That being said, most of the mismatches between recipient and donor were C-locus antigens contained within the pattern described. Thus, we may not expect positive flow crossmatches in these cases due to the lower expression of C-locus antigens as compared to HLA-A, B and DR antigens [12]. Notably, 3 samples were B-cell and T-cell positive with average dMESFs of 35,632 and 2625, respectively (Table 3). One of these samples came from a donor expressing a pattern antigen, at an average MFI of 1427. In our experience a positive FCXM with high dMESFs (~35,000) is inconsistent with MHC

class I antibody at an MFI of 1427; further suggesting that this reactivity is unlikely due to true HLA antibody. Hence, this antigen was not considered to be clinically relevant and was not reported as DSA.

When reviewing the corresponding MHC class II Luminex SAB results anticipating we would see the previously described pattern reported by Grenzi et al. [6], we instead found a different recurring MHC class II Luminex-SAB pattern. Similar to our findings in the samples positive for the MHC class I SAB pattern, the class II pattern presented clues of false-positivity including 1) a long tail of pan-reactivity; 2) reactivity to self-antigen in 87% of patients (similar rate to patients with MHC class I SAB pattern); 3) lack of a prior potential sensitizing event in approximately one-third of the patients (similar rate to patients with the MHC class I SAB pattern); and 4) absence of a shared epitope that could explain this pattern.

Furthermore, reactivity of some of the beads in the MHC class II SAB pattern would have to be due to allele-specific antibodies (eg: DRB1*04:03) and to antibodies directed towards unique epitopes encompassing individual alpha and beta chains [13]. An example of a possible combinatorial antibody within this MHC class II SAB pattern is the reactivity with the DQA1*02:01/DQB1*04:01 bead, but not the DQA1*03:03/DQB1*04:01. Given that there is no DQA1*02:01 antibody present and the differential reactivity in the presence of DQB1*04:01, only a combinatorial antibody could explain this reactivity. While the identification and prevalence of these antibodies is not well established, it is highly unlikely that all these patients would have been exposed to that particular combination of DQA/DQB alleles. Though up to 30% of highly sensitized patients have been reported to have allele-specific antibodies [14], this cohort is not highly sensitized as evidenced by a lack of prior potential sensitizing events, 50% of the patients with negative FCXM with donor cells, and negative FlowPRA results. Moreover, the probability of multiple patients sharing the same exact allele-specific pattern of reactivity is likely low.

Like the lot-to-lot variation observed with the MHC class I pattern, the MHC class II SAB pattern is slightly different in the current testing lot (One Lambda, Inc; lot 13): DRB1*14:54, DRB1*12:01, DQA1*04:01/DQB1*02:01, DQA1*02:01/DQB1*04:01, DQA1*03:01/DQB1*03:02, DPA1*01:03/DPB1*19:01, DPA1*03:01/DPB1*20:01, DPA1*01:03/DPB1*28:01 (Supplemental figure). These findings indicate that false-positive, recurring Luminex-SAB patterns can change from lot-to-lot, suggesting this may be related, at least in part, to bead manufacturing. It is quite possible that the MHC class II pattern we observed is a lot variant of the pattern described by Grenzi et al. Such patterns have implications for the quality control process when changing to new SAB lots.

The American Society For Histocompatibility and Immunogenetics (ASHI) Standard D.4.1.3.5 requires that “prior to reporting results new lots or shipments of reagents, satisfactory performance must be verified and documented.” [15]. Lot-to-lot variability of these false-positive patterns must be considered when comparing lot-to-lot results and may be more readily apparent in high-volume testing laboratories. If samples from SLE patients are included in new verification, reconciliation of the results with two different lots may not be possible. It may also be worthwhile to perform a separate verification using samples with

suspected reactivity to denatured antigen to identify when changes in these patterns occur. Though smaller laboratories may not have the capability to perform such extensive verification studies, all laboratories should be vigilant of these lot-to-lot variations as they examine pattern changes in their patient populations over time. It is equally important to consider that different laboratories may see different patterns depending on their patient populations.

When false-positive reactivity is suspected, testing sera using multiple platforms (SAB, phenotypic beads) and different vendors may be helpful. Differential reactivity and susceptibility to false-positivity observed between vendors may vary; and differences in reactivity between phenotypic beads (FlowPRA and flow specificity) and single antigen beads (SAB) is likely due, at least in part, to the different antigen content and source. Phenotypic beads are generated using EBV transformed cells and present haplotypes in more native confirmation; whereas, single antigen beads are manufactured using HLA transfected cell lines. These differences in manufacturing may result in variations in antigen and/or peptide confirmation and antibody interactions.

In summary, we believe this to be the first published report of a distinct MHC class I Luminex-SAB pattern, not reflective of true HLA antibody. This pattern appears to be prevalent in patients with SLE and we speculate it represents false-positive reactivity, possibly due to antibody targeting denatured antigens or a specific peptide, molecular mimicry, autoimmunity, or a combination thereof. Notably, all patients who had both the MHC class I and class II SAB patterns had a diagnosis of SLE. Considering the prevalence of SLE in patients with these patterns, autoimmunity may play a key determinant in these reactivity patterns, at least for a certain subset of patients. And though we cannot provide the mechanism to explain these patterns, our findings highlight the importance of recognizing such patterns, as assigning false-positive specificities can negatively impact organ allocation access. Since recurring patterns, such as the one we describe, are well known [6,16,17], test manufacturers may consider including a disclaimer on package inserts alerting users to the possibility of these false positive reactivities, especially among patients with autoimmune disease. In conclusion, it is paramount that HLA laboratories work together to identify and report these nonspecific reactions in an effort to improve product manufacturing and thereby improve patient care.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A) Examples of the recurring class I single antigen bead pattern in testing lot 10 (One Lambda, Inc) from 4 samples, which includes beads HLA-A*33:03, -A*36:01, -A*80:01, -B*54:01, -B*53:01, -C*06:02, -C*07:02, -C*18:02, -C*14:02, -C*03:03, -C*03:04, and -C*15:02 (boxes) and the long tail of reactivity (bracket). B) Examples of FlowPRA from corresponding sera demonstrating the Class I pattern on SAB testing.

True HLA Antibodies with Recurring Class I Single Antigen Bead Pattern (Lot 10)

Fig. 2.
Example of the recurring class I single antigen bead pattern in a patient with true HLA antibodies. Inset: FlowPRA results from the same sample demonstrating true HLA antibodies.

Recurring Class I Single Antigen Bead Pattern (Lot 11)

Fig. 3.

Example of class I pattern on testing lot 11 (One Lambda, Inc) characterized by reactivity of the following beads: A*74, B*15:15 (B63), B*27:05, B*15:13 (B77), B*81:01, and all the C locus beads except the C*01:02, C*12:03, and C*15:02 beads (boxes) and the long tail of reactivity (bracket).

Recurring Class II Single Antigen Bead Pattern (Lot 12)

Fig. 4.

Example of the newly identified recurring class II single antigen bead pattern in testing lot 12 (One Lambda, Inc), which includes beads HLA-DRB1*04:03, *08:01, *09:01, *13:01, *14:01, DQA1*04:01/DQB1*02:01, DQA1*02:01/DQB1*04:01, DQA1*02:01/DQB1*04:02, DPA1*01:03/DPB1*28*01, DPA1*02:02/DPB1*13:01, DPA1*03:01/DPB1*13:01, DPA1*03:01/DPB1*20:01, DPA1*01:03/DPB1*23:01, and DPA1*04:01/DPB1*28:01 (boxes) and the long tail of reactivity (bracket).

Table 1

Frequency of self-antigen in reactive samples.

Class I SAB	No. (%) of samples with reactivity
C*07:02	48 (45%)
B*53:01	24 (23%)
C*06:02	14 (13%)
C*03:04	14 (13%)
A*33:03	11 (10%)
C*18:02	4 (4%)
C*15:02	4 (4%)
A*36:01	3 (3%)
C*14:02	2 (2%)
A*80:01	1 (1%)

SAB: single antigen bead.

Table 2

Allogeneic FCXM: Non-SLE patients.

FCXM	n (11)	Avg B dMESF (range)	No. with pattern antigen	Avg MFI (range)
B-/T-	11	n/a	10	1693 (0–4535)
B+/T-	0	n/a	n/a	n/a
B+/T+	0	n/a	n/a	n/a

FCXM: flow cytometry crossmatch, SLE: systemic lupus erythematosus, Avg: average, dMESF: delta molecules of equivalent soluble fluorochrome, No: number, MFI: mean fluorescence intensity.

Table 3

Allogeneic FCXM: SLE patients.

FCXM	n (23)	Avg B dMESF (range)	Avg T dMESF (range)	No. with pattern antigen	Avg MFI (range)
B-/T-	10	n/a	n/a	9	2463 (0–9102)
B+/T-	10	27,632 (1972–79,836)	n/a	10	2690 (383–8224)
B+/T+	3	35,632 (21,298–45,988)	2625 (1354–3713)	1	1427 (1389–1465)

FCXM: flow cytometry crossmatch, SLE: systemic lupus erythematosus, Avg: average, dMESF: delta molecules of equivalent soluble fluorochrome, No: number, MFI: mean fluorescence intensity.