Transplant Immunology 73 (2022) 101642

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Alloreactive memory B cell detection by flow cytometric cross match using polyclonally activated memory B cell culture supernatants

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ARTICLE INFO

Keywords:
Flow cytometric cross match
Alloreactive memory B cell
Culture supernatants
Polyclonal activation

ABSTRACT

In addition to alloantibodies, alloreactive memory B cell (mBC) evaluation has a potential for immunological risk assessment during transplantation processes. For the alloreactive mBCs evaluation currently, direct Flow Cytometric (FC) analysis using the HLA tetramer staining is an option. Evaluation of alloantibodies produced by the polyclonally stimulated alloreactive mBCs in *in vitro* culture system seems to be another useful approach, but this needs further downstream applications. In this study, we investigated the usefulness of the Flow Cytometric Cross Match (FCXM-supernatant) in which *in vitro* polyclonally activated mBCs culture supernatants and potential donor's lymphocytes being used for the mBC detection. FCXM-supernatant assays were performed between culture supernatants of polyclonally activated mBCs obtained from 4 allosensitized multiparous women and 14 renal transplant patients, and their non-alloimmunized spouses' or donors' lymphocytes, and *vice versa*. HLA typing was performed by SSP method. Anti-HLA antibodies produced by *in vitro* activated alloreactive mBCs were also evaluated by the Luminex assays. The success of *in vitro* polyclonal activation of mBCs was evaluated by a total IgG ELISA test and antibody secreting cell analyses by FC. Donor specific alloreactive mBCs were detected by FCXM-supernatant in 45% of the 18 allosensitized cases. Detection rate was 85% (6 out of 7) in the strongly allosensitized cases. No alloreactive mBCs was detected in control cases without allosensitization. FCXM-supernatant negative results of the allosensitized cases were related to low level of allosensitization and insufficient polyclonal stimulation evaluated by total IgG antibody tests of the supernatants. We herein report a practical methodology for alloreactive mBC detection as a donor specific manner using the FCXM-supernatant assay so that this would easily be transformed into a routine test performed in tissue typing laboratories.

1. Introduction

Alloantibodies, mainly anti-HLA antibodies, are the major targets for the pre- and post-transplant immunological assessments especially in the renal transplant patients [1,2]. In addition to alloantibodies in serum, alloreactive memory B cell (mBC) evaluation may have a potential to improve the immunological assessments [3–7]. Alloreactive mBCs by differentiating into alloantibody producing cells on antigen re-encounter or bystander activity may increase the alloantibody levels associated with higher risk of antibody-mediated rejection and poor allograft outcome [8–10]. Both variety and quantity of alloreactive mBCs have been of interest during the last two decades. Literature searches revealed that there are two different strategies for peripheral alloreactive mBC assessment. One is based on direct analysis of alloreactive mBCs by FC using the HLA tetramer staining. The main disadvantages of this approach are usage of synthetic HLA molecules, low incidence of alloreactive mBCs in circulation and requirement of additional steps to show the alloantibody secretion capability of the detected cells [11,12]. The other approach needs *in vitro* polyclonal activation of mBCs turning them into antibody (Ab) secreting cells. Thus, alloreactive

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https://doi.org/10.1016/j.trim.2022.101642
Received 10 February 2022; Received in revised form 30 May 2022; Accepted 31 May 2022
Available online 3 June 2022

mBCs can be evaluated indirectly by Ab detection [4,8,13]. Alloreactive mBCs can be detected and quantified by the HLA-specific ELISPOT analysis using synthetic HLA molecules or by the donor-specific mBC ELISPOT analysis using the cell lysates. Both are very complex assays having difficulties to be a routine test [13,14]. Moreover, Luminex that uses synthetic HLA molecules is a reliable for identification and quantification but an expensive test requiring concentration of Abs from the culture supernatants of the *in vitro* polyclonally activated mBCs [6,15]. Because of the technical difficulties, alloreactive mBCs evaluation cannot be routinely performed yet.

On the other hand, Flow Cytometric Cross Match (FCXM) is a widely used sensitive method for donor specific alloantibody detection or screenings [16–19]. There is limited number of cases evaluated by FCXM using the supernatants of activated mBC in the literature [5,15]. Therefore, we planned this work to extend the knowledge on usefulness of the FCXM assay for peripheral mBCs detection. In this assay, following to *in vitro* polyclonal activation of peripheral alloreactive mBCs, alloantibodies accumulated in culture supernatants were detected by the conventional FCXM assay using the donor or potential donor's lymphocytes.

2. Materials and methods

2.1. Study design

Allosensitized 4 multiparous women and 14 renal transplant patients selected by the FCXM-serum and Luminex analyses and their spouses or donors as controls, 36 cases in total, were included in this study. HLA typing was performed by the SSP method. FCXM-supernatant assays were performed twice for each case using the culture supernatants of polyclonally stimulated and unstimulated peripheral mBC collected at day 6 and at day 12, and using their paired corresponding lymphocytes. In addition, culture supernatants collected at day 12 were also screened for anti-HLA Abs by Luminex analysis and strongly positive two samples were later on evaluated by Luminex-SAB. Cell cultures were finalized at day 12 and collected cells were analyzed for ASC-like cells by FC. Total IgG Ab levels in the culture supernatants collected at day 12 were detected by ELISA. The study was approved by the Tekirdağ Namık Kemal University Ethical Committee (Document Number: 2017/21/02/04 and Document Number: 2019/10/01/10) and informed consents were obtained from all the cases included in this study.

2.2. Cases

For the detection of couples consisted of the allosensitized multiparous women and non-allosensitized spouses, women with the three or more pregnancies history from Department of Gynecology and Obstetrics, Faculty of Medicine, Tekirdağ Namık Kemal University were invited to study. Renal transplant patients from the Department of Nephrology and Transplantation Unit, Faculty of Medicine, University of Medical Sciences were included in the study. Using the FCXM and Luminex tests, four multiparous women with their spouses and 14 renal transplant patients with their kidney donors were finally selected for the further experimental procedures of this study (Table 1). It was confirmed by FCXM that the multiparous women's spouses had no alloantibodies against their wifes/partners and renal transplant patients' donors had no alloantibodies against their renal recipients.

2.3. Determination of the allosensitizations by FCXM-serum and Luminex

FCXM-serum tests were performed as total lymphocyte cross-match analysis according to standard procedures and analyzed with FACS Calibur (Becton Dickinson, USA). PBMNC were isolated from heparinized peripheral blood samples using the standard Ficoll-Hypaque density gradient centrifugation technique. The lymphocyte-Alloantibody binding was determined with anti-IgG-FITC (Jackson Immunoresearch

Table 1
Luminex and FCXM results performed with serum and supernatant samples of the cases.

Case No	Multiparous Woman (MW) / Renal Tx	Luminex-Serum Class 1 / Class 2	Luminex-Supernatant Day 12 Class 1 / Class 2	FCXM-Serum Positivity (MCS)	FCXM-Supernatant Day 6 / Day 12 Positivity (MCS)
1a	Renal Tx	+/+	+/+	+++ (61,68)	++/++ (5,33 / 4,83)
1b	Donor of Case 1	−/−	−/−	− (<1)	−/− (<1 / <1)
2a	Renal Tx	+/+	−/−	+ (5,19)	−/− (1,10 / <1)
2b	Donor of Case 2	−/−	−/−	− (1,06)	−/− (1,06 / 1,07)
3a	Renal Tx	+/−	−/−	++ (13,67)	+/+ (1,96 / 1,85)
3b	Donor of Case 3	−/−	−/−	− (<1)	−/− (1,04 / 1,02)
4a	Renal Tx	−/−	−/−	+ (3,86)	−/− (1,29 / 1,41)
4b	Donor of Case 4	−/−	−/−	− (<1)	−/− (<1 / <1)
5a	Renal Tx	NA	+/+	+++ (60,94)	+++/+++ (480,86 / 517,65)
5b	Donor of Case 5	−/−	−/−	− (<1)	−/− (1,11 / 1,11)
6a	Renal Tx	+/+	−/−	+ (3,52)	−/− (1,09 / 1,23)
6b	Donor of Case 6	−/−	−/−	− (<1)	−/− (1,20 / 1,20)
7a	Renal Tx	+/+	+/+	+++ (137,04)	+++/+++ (57,1 / 68,0)
7b	Donor of Case 7	−/−	−/−	− (<1)	−/− (1,19 / 1,21)
8a	Renal Tx	−/+	−/−	+ (2,42)	−/− (1,07 / 1,15)
8b	Donor of Case 8	−/−	−/−	− (1,70)	−/− (1,17 / 1,07)
9a	Renal Tx	−/+	−/+	++ (12,42)	+/+ (1,77 / 1,70)
9b	Donor of Case 9	−/−	−/−	− (<1)	−/− (1,05 / 1,04)
10a	Renal Tx	−/+	−/−	+ (2,11)	−/− (1,22 / 1,22)
10b	Donor of Case 10	−/−	−/−	− (<1)	−/− (1,03 / 1,03)
11a	Renal Tx	+/+	- /+	++ (13,95)	−/− (1,08 / 1,08)
11b	Donor of Case 11	−/−	−/−	− (1,05)	−/− (1,04 / 1,04)
12a	Renal Tx	−/+	−/−	+ (1,95)	+/+ (1,73 / 1,79)
12b	Donor of Case 12	−/−	−/−	− (1,17)	

(*continued on next page*)

Table 1 (*continued*)

Case No	Multiparous Woman (MW) / Renal Tx	Luminex-Serum Class 1 / Class 2	Luminex-Supernatant Day 12 Class 1 / Class 2	FCXM-Serum Positivity (MCS)	FCXM-Supernatant Day 6 / Day 12 Positivity (MCS)
					−/− (1,04 / 1,04)
13a	Renal Tx	+/+	−/−	+ (5,01)	−/− (1,15 / <1)
13b	Donor of Case 13	−/−	−/−	− (<1)	−/− (1,16 / 1,13)
14a	Renal Tx	+/−	−/−	+ (2,58)	−/− (1,25 / 1,21)
14b	Donor of Case 14	−/−	−/−	− (<1)	−/− (1,22 / 1,11)
15a	MW	+/+	−/−	+ (4,03)	+/+ (1,87 / 2,13)
15b	Spouse of Case 15	−/−	−/−	− (1,25)	−/− (<1 / 1,16)
16a	MW	+/+	−/+	+ (5,43)	−/− (<1 / 1,15)
16b	Spouse of Case 16	−/−	−/−	− (1,01)	−/− (<1 / <1)
17a	MW	+/+	−/+	+ (3,25)	−/− (1,50 / 1,57)
17b	Spouse of Case 17	−/−	−/−	− (<1)	−/− (<1 / 1,30)
18a	MW	+/+	+/−	+++ (64,76)	++/++ (33,87 / 37,22)
18b	Spouse of Case 18	−/−	−/−	− (<1)	−/− (1,09 / 1,44)

NA: Not-available. MCS: Median Channel Shift.

Laboratories, USA) in the FCXM-serum tests. Confirmed negative and positive control serum samples from the previous studies were also included. The cells were resuspended with 400 μL of FACS flow solution and transferred into flow tubes for analysis at a flow cytometry (FACS Calibur, BD Biosciences, CA, USA). The Ab binding score was determined as the median fluorescence intensity (MFI) value on the histogram. FCXM result was considered positive when sample to negative control MFI value ratio was greater than 1,6. Cases with (+) level of positivity in the FCXM assay were considered as having low level of sensitization, while cases with (++ and +++) level of positivity in the FCXM assay were considered as having high level of sensitization.

Luminex screening test were performed according to standard procedures and acquired from a luminex flow analyzer (LABScan 100) and analyzed using the Match IT! Antibody software version 1.3.0 (Lifecodes, Immucor). Sample positivity were detected both for Class I and Class II anti-HLA Abs according to the lot-specific cut-off values.

2.4. HLA typing

HLA types for the HLA-A, HLA-B, HLA-DR of the cases included in the study were detected by the sequence-specific primer (SSP) method using a commercial kit (Olerup SSP HLA Typing Kits, Sweden). According to the protocol, DNA isolations were performed by a commercial kit (Roche, Germany). Following to PCR, PCR products were run in 2% agarose gel and band profiles were evaluated by the start score software.

2.5. In vitro polyclonal stimulation of the peripheral mBC and experimental setup

After the confirmation of the allosensitizations and the HLA typing assays, selected cases were invited for collection of the fresh blood samples for later steps of the study. Twenty mL of peripheral blood samples were collected into the heparinized tubes. PBMNC were again isolated using the standard Ficoll-Hypaque density gradient centrifugation technique. Following to cell counting step, 5×10^4 cells were transferred into the wells containing 1 mL of DMEM medium for the unstimulated control, and 5×10^4 cells were transferred into the wells containing 1 mL of DMEM medium including the polyclonal stimulation cocktail consisting of 500 ng/mL α-CD40 monoclonal antibody (BioLegend, CA, USA), 2.5 μg/mL Toll-like receptor-9 (TLR-9) ligand oligodeoxynucleotides (ODN)-2006 cytosine–phosphate–guanine (CpG) (Hycult Biotechnology, the Netherlands), 600 IU/mL interleukin (IL)-2 (BioLegend, CA, USA), 25 ng/mL IL-10 (BioLegend, CA, USA) 100 ng/mL IL-21 (BioLegend, CA, USA) for the polyclonal stimulation as described previously [20], and cultured at 37 °C in a 5% CO_2 humidified incubator.

0.5 mL of the culture supernatants were replaced with freshly prepared 0.5 mL of DMEM including the polyclonal stimulation cocktail at day 6 and collected 0.5 mL of supernatants were stored at −20 °C for later analyses. Cells were harvested and supernatants were collected at day 12. Supernatants were used for the allogeneic Ab detection by FCXM-supernatant and Luminex screening tests. Two samples having relatively higher amounts of allogeneic Abs in FCXM-supernatant and Luminex screening tests were analyzed by Luminex-SAB.

2.6. Evaluation of the in vitro polyclonal stimulation of peripheral mBCs

Because an increase in the ASC-like cell ratio and elevation in total IgG Ab production upon polyclonal stimulation may indicate the success of the *in vitro* polyclonal stimulation procedure, FC analyses for the ASC-like cells and ELISA for total IgG Ab detection were performed in order to evaluate the success of the assay. Additionally, it was observed that the cell clusters possibly developed from clonal expansions of the mBC can be observed with the *in vitro* polyclonally stimulated cells culture under the inverted microscope (Supplementary 1).

2.7. ASC-like cell ratios by FC after the in vitro polyclonal stimulation of peripheral mBCs

Anti-CD19-PE, Anti-CD27-APC, Anti-CD38-PerCP monoclonal Abs (Biolegend, CA, USA) were used in the FC analyses. Shortly, Fluorescent labeled Abs were added to the tube containing the cells collected at the day 12, followed by shaking, mixing, and then incubating for 15 min at 20–25 °C; then the mixture was incubated for 15 min at 20–25 °C after shaking and mixing. Following this, 2 mL PBS, was added, followed by centrifugation at 8000 rpm for 1 min. The supernatant was discarded, and the procedure was repeated twice. Subsequently, 200 μL PBS was added to resuspend the pellet, and the samples were tested *via* a flow cytometry (FACS Calibur, BD Biosciences, CA, USA). CD19+ B lymphocytes were grouped according to their expression status for CD27 and CD38. ASC-like cells were determined as CD19 + CD27 + CD38++. The numbers of the ASC-like cells were compared between polyclonally stimulated and unstimulated samples of the cases and the controls.

2.8. Total IgG Ab analyses by ELISA after the in vitro polyclonal stimulation of peripheral mBCs

Total IgG Ab levels were measured by an in-house-indirect ELISA test. Optical Density (OD) values were obtained at 450 nm wavelength in a microplate reader (MultiSkan FC, Thermo Fisher Scientific). Total IgG Ab levels were compared between polyclonally stimulated and unstimulated samples for the cases and the controls.

2.9. Alloantibody detection in the polyclonal stimulated mBCs cultures by FCXM-supernatant

FCXM-supernatant tests were performed using the culture supernatants and corresponding lymphocytes in order to detect alloantibodies and to reveal accordingly the alloreactive mBC presence. FCXM-supernatant tests were performed as total lymphocyte cross-match test according to standard procedure similar to that of FCXM using serum samples and analyzed with a FC (FACS Calibur, Becton Dickinson, USA). FCXM-supernatant tests were considered positive when the ratio of MFI value of the stimulated supernatant sample to the MFI value of the unstimulated supernatant sample was greater than 1,6. FCXM-supernatant positivity levels (+, ++, and +++) were determined according to the MFI values as similar to that of conventional FCXM test. Fresh medium was also used as negative control sample in the FCXM-supernatant tests.

Additionally, the effects of the concentration procedures such as lyophilization and ultra-filtration using the ultra-centrifugal filters (Milipore, Ireland) on MFI values of the FCXM-supernatant test were evaluated for supernatant samples of the randomly selected two allosensitized cases.

2.10. Anti-HLA Ab detection in culture supernatants by Luminex

In addition to the FCXM-supernatant analyses performed using the supernatants and the corresponding lymphocytes, culture supernatants were screened for the presence of anti-HLA Abs by Luminex using Lifecodes Lifescreen Deluxe Kit (Immucor Transplant Diagnostics, Stamford, CT). Because the MFI values of the anti-HLA Abs in the polyclonally stimulated mBC culture supernatants in the Luminex assay were much less than those of serum samples, Luminex-SAB assay was applied to selected samples. In the polyclonally stimulated mBC culture supernatants, two of the cases (case 5a and case 7a in the Table 1) with relatively higher MFI values for alloantibodies at Luminex and FCXM-supernatant assays were chosen for the Luminex-SAB assay. These two samples were tested using LabScreen HLA class I and class II single antigen beads (One Lambda, Canoga, CA) to identify anti-HLA Ab specificities.

2.11. Statistical analyses

Normality of the data were tested by Kolmogorov-Smirnov test. Independent samples *t*-test and The Mann–Whitney *U* test were used for comparisons between different groups. Correlations were assessed by Pearson correlation test in SPSS statistical analysis program. *P* value <0.05 was considered statistically significant.

3. Results

3.1. Alloantibody detection in culture supernatants by FCXM and Luminex

Within the 18 FCXM-serum positive cases included in the study, we detected alloantibodies in 8 (45%) cases by the FCXM-supernatant in the polyclonally stimulated mBC culture supernatants collected at the day 6 and day 12 (Table 1). This detection rate of FCXM-supernatant test for alloantibodies in the polyclonally stimulated mBC culture supernatants was as high as 85% (6 out of 7) when the strongly alloimmunized (FCXM-serum ++ and +++ positive) cases considered (Table 1). Similar to the FCXM-supernatant test, Luminex was able to detect anti-HLA Abs in the polyclonally stimulated mBC culture supernatants collected at the day 12 in 8 (45%) out of 18 allosensitized patients included in the study (Table 1). On the other hand, the number of the common cases in which both the FCXM-supernatant and Luminex test were able to detect the alloantibodies was 5 (Table 1). None of the control cases had positivity for the alloantibodies in the polyclonally stimulated mBC culture supernatants by either FCXM-supernatant or Luminex tests. When all the cases included in the study were collectively assessed, a strong correlation was found for the paired FCXM results obtained by the serum and supernatant samples (Pearson correlation test, $r = 0.882$, p ⟨0,01). Representative FCXM-supernatant test results are shown in Fig. 1. The FCXM-supernatant results similar to the Fig. 1A were considered as (+) positive with Median Channel Shift (MCS) level of 1,96, similar to the Fig. 1B and C considered as (++) positive with MCS levels of 5,33 and 57,1, respectively, and similar to the Fig. 1D considered as (+++) positive with MCS of 480,86.

When the concentration procedures effects on MFI values of the FCXM-supernatant test were evaluated, we found that MFI values were 2.44-times higher in the lyophilized supernatant samples and 5.2-times higher in the filtered supernatant samples using the ultra-centrifugal filters.

3.2. Changes in total IgG Ab level and ASC-like cell number after polyclonal stimulation

The FC analysis revealed elevated ASC-like cell numbers in the polyclonally stimulated mBCs compared to the unstimulated mBCs at the end of day 12. The mean and SD values of ASC-like cell ratios of the stimulated to the unstimulated mBCs were 2.73 ± 1.77 and 1.51 ± 0.75 for the allosensitized cases and non-allosensitized controls, respectively. ASC-like cell ratio of the allosensitized cases was significantly higher than non-allosensitized group (Mann Whitney *U* test $p = 0.008$).

Comparison of the total IgG Ab levels in the supernatants collected at the day 12 revealed same level of stimulation of the mBC of allosensitized and non-allosensitized cases. Total IgG Ab levels were approximately 10-fold higher (10.52 for the allosensitized cases and 10.66 for the non-allosensitized cases) in the polyclonally stimulated mBC culture supernatants compared to unstimulated mBC culture supernatants. Total IgG Ab levels were significantly higher (3.7-fold, Independent Samples *t*-Test, $p = 0.007$) in the FCXM-supernatant positive cases (Mean ± SD; 15.80 ± 6.71) than the FCXM-supernatant negative cases (Mean ± SD; $4,27 \pm 3,94$) in the polyclonally stimulated mBC culture supernatants (Fig. 2). When the cases with the low level of allosensitization considered, total IgG Ab levels were significantly higher (4.03-fold Independent Samples t-Test, $p = 0.002$) in the FCXM-supernatant positive cases (Mean ± SD; 17.26 ± 1.69) than the FCXM-supernatant negative cases (Mean ± SD; 4.27 ± 3.94) (Fig. 3). Total IgG Ab ELISA test results of the three different cases and the standard graphic are given in supplementary 2.

3.3. Anti-HLA Ab species in culture supernatants by Luminex-SAB

Two cases (case 5a and case 7a in the Table 1) with relatively higher MFI values for the anti-HLA Abs in the polyclonally stimulated mBC culture supernatants in Luminex and FCXM were chosen for the Luminex-SAB assay. Luminex-SAB assay with the polyclonally stimulated mBC culture supernatants revealed the specific anti-HLA Ab species (Table 2).

4. Discussion

The assessment of alloreactive mBC as a part of humoral alloimmunity is expected to improve the success of transplantation. Because of the technical limitations, there is no routinely applicable assay for the alloreactive mBC assessment currently. HLA tetramer staining by FC, ELISPOT and Luminex based approaches are amongst the favorites, but less known about the usefulness of the FCXM for this purpose. Therefore, we aimed to investigate the value of the FCXM technique in the alloreactive mBC assessment in this study.

Because of the difficulties obtaining the biological samples such as bone marrow or lymph nodes in which alloreactive mBCs might be more abundant and there is a consensus on the peripheral blood is the best for

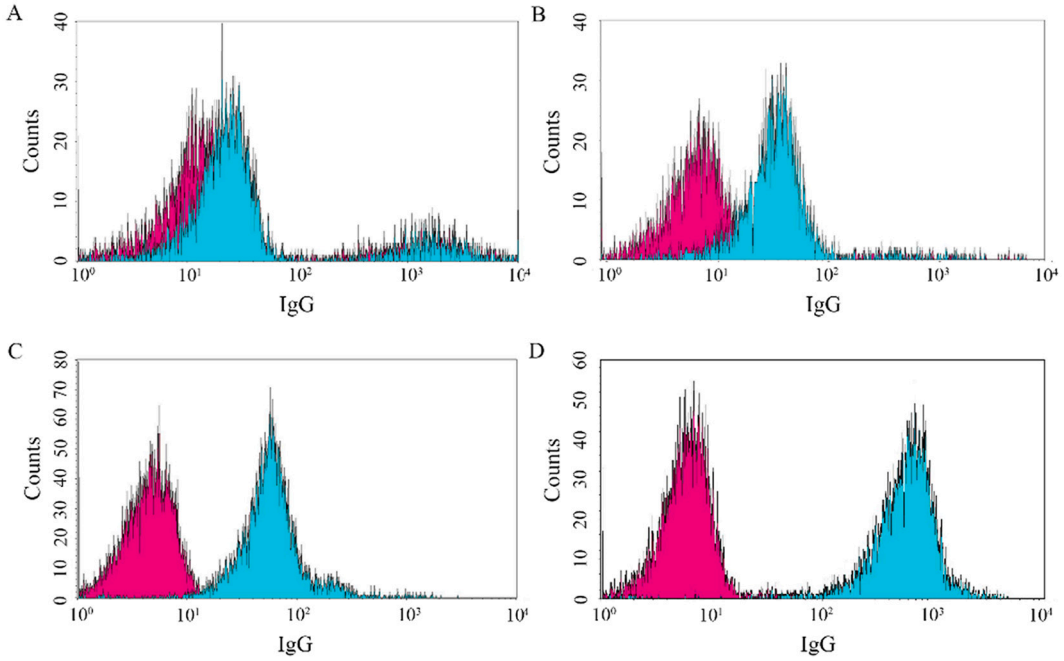

Fig. 1. FCXM-supernatant Histograms. FCXM-supernatant test result accepted as (+) positive (MCS: 1,96) (A); FCXM-supernatant test result accepted as (++) positive (MCS: 5,33 and 57,1, respectively) (B and C); and FCXM-supernatant test result accepted as (+++) positive (MCS: 480,86) (D).

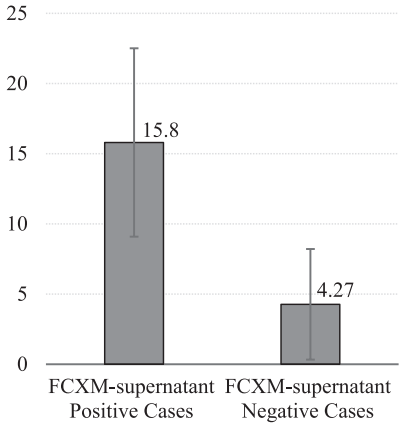

Fig. 2. Total IgG Ab Levels in the 12-day Stimulated Supernatant Samples of the FCXM-supernatant Positive and Negative Allosensitized Cases.

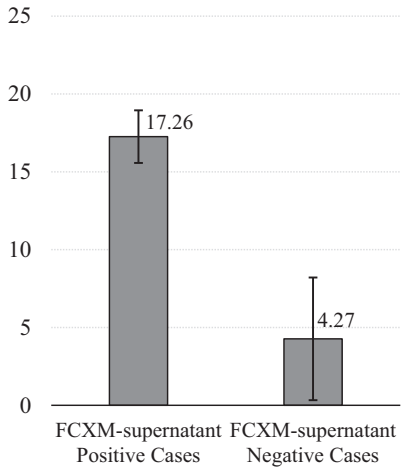

Fig. 3. Total IgG Ab Levels in the 12-day Stimulated Supernatant Samples of the FCXM-supernatant Positive and Negative Cases with the Low Level of Allosensitization.

alloreactive mBC assessment todays. On the other hand, how much the mBC repertoire in the peripheral blood can represent or complement the humoral alloimmune potential is not still entirely clear. Nevertheless, we used the peripheral blood samples to assess alloreactive mBC due to its accessibility in this study.

Beyond the biological material sampling, if the alloreactive mBC assessment will become a clinically relevant test, we had better use the equipment and techniques that already exist in tissue typing laboratories. Cost, simplicity and reliability are the other issues to take into account. When we consider all of these, FCXM-supernatant based approach offers some advantages. Unlike to other assays that use synthetic HLA molecules to detect the anti-HLA Abs, FCXM-supernatant uses natural forms of HLA and also other alloantigen molecules on the surface of lymphocytes which is a unique property for the FCXM-supernatant and the donor-specific HLA-ELISPOT assays. This can be an explanation for the FCXM-supernatant positive but Luminex-supernatant negative 3 cases (case 3a, case 12a and case 15a in Table 1) detected in our study. On the other hand, there are Luminex-supernatant positive but FCXM-supernatant negative 3 cases (case 11a, case 16a and case 17a in Table 1) detected in our study. This may be attributed to the higher sensitivity and/or specificity in detection of anti-HLA Abs of Luminex. It was reported that the donor-specific HLA-ELISPOT assay can also quantify the alloreactive mBCs [13]. Even though the MFI values obtained from the FCXM-supernatant assay may indicate the quantity of alloreactive mBC, it is difficult to have a comment on its capability for mBC quantification without a parallelly performed assay that is able to reliably quantify alloreactive mBCs.

There are two reports in which the FCXM-supernatant assay performed in a small study groups in the literature. Lucia et al. reported that only one out of 10 samples was negative in FCXM assay performed with the mBC supernatants. Karahan et al. used the HLA typed PBMNCs of healthy individuals together with the 10-day stimulated mBC culture supernatants in the FCXM assay in their study. They reported that FCXM

Table 2
Luminex-SAB results of the case 5a and case 7a.

Case No.	Class I anti-HLA Abs	Class II anti-HLA Abs	Miss-matches
Case 5a	**A*01:01** A*03:01 A*11:01 A*11:02 A*23:01 **A*24:02 A*24:03** A*25:01 A*26:01 A*32:01 A*33:01 A*33:03 A*34:02 A*36:01 A*66:01 A*66:02 A*68:01 A*68:02 A*69:01 A*80:01 B*07:02 B*07:03 B*08:01 B*13:02 B*14:01 B*14:02 B*15:12 B*15:16 **B*18:01** B*27:03 B*27:05 B*27:08 B*37:01 B*38:01 B*39:01 B*40:01 B*40:02 B*41:01 B*42:01 B*44:02 B*44:03 B*45:01 B*47:01 B*48:01 B*54:01 B*55:01 B*56:01 B*57:01 B*58:01 B*59:01 B*67:01 B*73:01 B*81:01 B*82:02 C*01:02C*02:02C*04:01C*04:03C*05:01C*06:02C*15:02C*17:01C*18:01	DRB1*09:01 DRB3*01:01 DRB3*03:01 DQA1*01:03 DQB1*03:02 DQB1*03:03 DQB1*06:01 DQB1*06:02 DQB1*06:03 DQB1*06:04	**A*01, A*24 B*18**, B*24 DRB1*11
Case 7a	A*02:02 A*02:03 A*02:05 A*03:01 A*11:01 **A*30:01** A*31:01 A*33:03 A*66:01 A*66:02 A*68:01 A*68:02 A*74:01		**A*30,** B*07

tests were positive either for the T-FCXM or for the B-FCXM in all the IgG isolated supernatant samples of the cases having alloantigen exposure. FCXM tests were negative for the cases without alloantigen exposure. They also claimed that FCXM test results were important to show binding capability of the alloantibodies, produced in a cell culture upon *in vitro* polyclonal stimulation, to the natural HLA molecules on surface of lymphocytes. Karahan et al. reported that the HLA-specific mBCs were detectable following to the ultra-centrifugal filtration in 64% of the immunized individuals. They also detected the HLA-specific mBCs following to concentration of the supernatant using the protein G affinity purification method in 82% of the immunized individuals [15]. We detected alloreactive mBCs by the FCXM-supernatant assay without any concentration step in 45% all of the alloimmunized individuals included in our study. We were not able to use IgG Ab purification in our study. Instead, we evaluated the technical benefits of the lyophilization and ultra-filtration procedures on MFI values of the FCXM-supernatant positive cases. 2.44-times higher MFI values in the lyophilized samples and 5.2-times higher MFI values in the ultra-filtered samples show that such concentration procedures may increase the detection rate of the FCXM-supernatant test especially in the samples with low levels of alloantibodies.

Our results showing approximately 10-fold higher levels of total IgG Ab in the polyclonally stimulated mBC culture supernatants in both allosensitized and non-allosensitized cases suggest that allosensitization does not have impact on level of polyclonal stimulation. In detailed analyses of the total IgG Ab levels, we noticed that the FCXM-serum weakly (+) positive but FCXM-supernatant negative samples were found to have lower total IgG Ab levels (Mean ± SD; 4.27 ± 3.94) indicating insufficient stimulation of the alloreactive mBCs in these cases. On the other hand, FCXM-supernatant test results of all the FCXM-serum strongly (++ and +++) positive cases with lower total IgG Ab levels were positive. Furthermore, when only the cases with the low level of allosensitization detected by FCXM-serum test considered, total IgG Ab levels were approximately 4.03-fold higher in the FCXM-supernatant positive cases (Mean ± SD; 17.26 ± 1.69) than those of the FCXM-supernatant negative cases (Mean ± SD; 4.27 ± 3.94). Consequently, the success of the polyclonal stimulation is closely related with the FCXM-supernatant test positivity and the total IgG Ab ELISA test may predict the success of the *in vitro* polyclonal stimulation.

Unlike to the similarity detected in total IgG levels of allosensitized and non-allosensitized cases in our study, ASC-like cell ratio of the allosensitized cases was unexpectedly higher than non-allosensitized cases. The question of how or can polyclonal stimulation increase the ASC-like cell number in allosensitized cases by leaving the total IgG levels equal between allosensitized and non-allosensitized cases remains to be answered.

Since previous studies indicated the insufficient quantity of the alloantibodies in polyclonally activated mBCs culture supernatants, we selected only two supernatant samples with the highest MFI value in the FCXM-supernatant test for the Luminex-SAB assay to search for the presence of the anti-HLA Abs against to the mismatched HLA groups. Luminex-SAB assay showed three different anti-HLA Abs and only one anti-HLA Ab that may have been produced against the mismatched HLA groups of the donors following to polyclonal activation of mBCs of the case 5a and the case 7a, respectively (Table 2). Because the aim of our study was not to compare the anti-HLA Ab species between serum and supernatant samples, we do not have the Luminex-SAB results for the serums of the case 5a and case 7a. It is possible to say that Luminex-SAB assay can successfully reveal the anti-HLA Ab species in the supernatants of the cases with (+++) level of positivity in the FCXM-supernatant test.

FCXM-supernatant test as a FC based alloreactive mBC detection assay is not suitable in its current form for profiling of mBC. Nonetheless, there is still a possibility for mBC profiling by FCXM-supernatant assay using the well HLA-characterized lymphocytes. Profiling the circulating alloreactive mBC pool, which can be achieved by HLA tetramer staining and Luminex-based assays with the limitation of the included HLA molecules, can potentially increase the accuracy of pre- and post-transplant risk assessment and can also shape the waiting list. On the other hand, revealing the alloreactive mBCs as a donor specific manner may merely improve the successful monitoring of donor specific antibodies during pre- and post-transplant period [6]. It seems that the donor-specific HLA-ELISPOT and FCXM-supernatant assays are two of techniques that can reveal the alloreactive mBCs as a donor specific manner [13]. In spite of lack of capability for a reliable quantification, it can be speculated that the FCXM-supernatant assay is one step ahead than the donor-specific HLA-ELISPOT due to its simplicity.

Complete concordance between day 6 and day 12 FCXM-supernatant assay results obtained in our study shows that 6-day culture period of PBMNC is good enough to get reliable results in the FCXM-supernatant assay.

Finally, we believe that when it comes to detection of the donor specific alloreactive mBCs, FCXM-supernatant assay with some modifications would be a candidate for becoming a routine test.

Declaration of Competing Interest

None.

Acknowledgements

This study was supported by the TUBITAK; The Scientific and Technological Research Council of Turkey (Grant number: 217S630) and by the Research Fund of the Tekirdağ Namık Kemal University (Project Number: NKUBAP.01.GA.21.301). We would like to thank Drs. Frans H.J. Claas and Gonca E. Karahan from Leiden University Medical Center, and Dr. Fahri Uçar from Akdeniz University Tissue Typing Laboratory for their supports.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.trim.2022.101642.

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