Characterization of the Anti-HLA Class I and II IgG Antibodies in Moroccan IVIg Using Regular Beads and Ibeads in Luminex Multiplex Single Antigen Immunoassay

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Abstract: Therapeutic Immunoglobulin Intravenous (IVIg), approved to treat a wide range of autoimmune and primary immunodeficiency diseases, contain mixture of polyreactive and polyclonal IgG purified from the pooled plasma of thousands of donors. The aim of this study is to characterize the profiles of anti-Human Leukocyte Antigen (HLA) class-I and class-II IgG antibodies in four lots of Moroccan IVIg preparations using Luminex Multiplex Single Antigen Bead Immunoassay and to compare it with the unique high frequency HLA types found in the Moroccan population. Anti-HLA class I IgG profiles were assessed using regular (Labscreen) Beads and iBeads. The regular beads are coated with all conformational and structural variants of HLA-I (HLA heavy chain (HC) with β2-microglobulin (β2m) with or without peptides, β2m-free HC with or without peptides or HC only), quite contrast to iBeads, which contained only native tissue-associated HLA HC with β2m and with or without peptides. The level of antibodies was measured as Mean Fluorescent Intensity (MFI). The reactivity of anti-HLA-I IgG antibodies to different alleles of HLA-I loci differed in their recognition of native HLA-I and other structural variants of the HLA-I. High MFI IgG antibodies in the IVIg corresponded with several high frequency HLA-I alleles (B*0801, B*5001, Cw*0602 and Cw*0702) and HLA-II haplotypes (DQA1*0201-DQB1*0201/DRB1*0301), which accounted for 50% of the total gene frequencies in the Moroccan population. HLA-I reactivity of the IVIg with iBeads confirms that the IgG reacting to normal tissue associated with peptide-associated or -free β2mHC. These findings caution the use of high dose IVIg for the carriers of the high frequency HLA types for it may cause tissue injury. The β2m-free-HC reactivity of IVIg indicates the potential of IVIg to bind to activated T and B cells that express these variants, to suppress antibody production. Such an immunomodulation by IVIg renders benefit for patients with autoimmune diseases and organ transplantation.

Keywords: Intravenous Immunoglobulin, HLA, Antibodies, Moroccan IVIg, Beads, Ibeads, MFI

1. Introduction

When Immunoglobulin (Ig) therapy was replaced by Intravenous Immunoglobulin (IVIg) with sera derived from multietnic population from Morocco, the IVIg was not affordable for most patients, until the Blood Transfusion and Hematology National Center and Fractionation and Biotechnologies French Laboratory (LFB-France) industry subcontracted IVIg production using plasma from Moroccan
blood donors [1], which included both allo-immunized and non-alloimmunized males and females. As a result, the cost of IVIg therapy is reduced by about 66% [1]. Consequently Moroccan IVIg therapy is administered to primary deficiency, autoimmune and neurological diseases, including Guillain-Barre syndrome, for which it was found to be safer and effective alternative to standard therapies [2]. However, very little effort has been made to characterize the composition of Moroccan IVIg, pooled and purified from the plasma of Moroccan population, which has remarkable ethnic and genomic diversities, which is reflected in their Human Leukocyte Antigen (HLA) typing profiles. As of March 2017, it has been reported that the Human major histocompatibility complex includes highly polymorphic proteins of classical HLA class I (HLA-A [n = 2747], -B [n = 3465], and -C [n = 2450]) proteins and HLA-II (HLA-DRA [n = 7], -DRB [n = 1711], -DQA1 [n = 34], -DQB1 [n = 761], -DPB1 [n = 23] and -DPB1 [n = 627] proteins [3]. Studies examining HLA-I types in Casablanca, the largest region of Morocco, revealed that 21% of the population were HLA-A2, 11% of the population were HLA-A1, 10% of the population were HLA-B44, 9.9% of the population were HLA-B5, 8.5% and 6.5% of the population were HLA-B502 and HLA-B503, respectively. Similarly, in the Amazigh ethnic group, the most frequent alleles were HLA-A*0201, A*0101; HLA-B*4403, B*4402, B*0801, B*5001, B*5002; HLA-Cw*0602, Cw*070102, Cw*0702, and Cw*0704, Cw*040101 [5]. Another study revealed high frequencies for DRB1*0701, DRB1*0301, DQA1*0501, DQA1*0201, and DQB1*0201. Three haplotypes (DRB1*0701-DQA1*0201-DQB1*0201, DRB1*0301-DQA1*0501-DQB1*0201, and DRB1*0301-DQA1*0501-DQB1*0201) are accounted for nearly 50% of the total gene frequencies. Many other studies on HLA typing of Moroccan population have confirmed the above mentioned high frequency types in the Moroccan population [7-10]. On the other hand, it is also well established that IgG antibodies against HLA-I and -II molecules do occur naturally as auto- or allo-antibodies in non-alloimmunized males [11-20]. The origin of these antibodies in “so-called” healthy individuals is still far from clear. It remains to be seen whether the HLA antibodies in the Moroccan healthy blood donors reflect the HLA types of the population and whether they are auto-antibodies, which means, directed against self-antigens, as has been reported elsewhere [21, 22]. High level of anti-HLA antibodies are reported in several autoimmune diseases [22, 23]. Therefore, it is felt that the Moroccan IVIg may reflect the HLA profiles of the Moroccan population. Examination of the anti-HLA IgG antibody profiles of Moroccan IVIg preparations may validate the above assumption. There is also yet another need to characterize HLA-I and -II antibodies in Moroccan IVIg, because IVIg may be administered to patients with Transfusion-related Acute Lung Injury (TRALI), while it is well established that the causal factor of TRALI is the presence of HLA antibodies in the patients. There are many reports on the occurrence of TRALI after IVIg administration [24-29]. Anti-HLA-II IgG observed in patients after plasma transfusion is implicated in TRALI [26]. The anti-HLA-II IgG binding to monocytes in patients with TRALI may induce activation of neutrophils that may penetrate the endothelium of lungs, causing destruction of the endothelial cells [24, 30]. Presence of HLA-II antibodies in allo-immunized females led to prevention of using blood from females for transfusion, Therefore, the avoidance of female blood has become routine as a preventive measure against TRALI in several countries [31, 32]. It was reported that this policy did indeed significantly reduce the incidence of TRALI both in large-scale surveillance studies and haemovigilance reports [32]. Furthermore, it is perplexing to note that IgG antibodies to allo–HLA proteins are common in sera after transfusions, organ transplantation, and autoimmune diseases [21-23, 33 - 35]. After all, Moroccan IVIg is prepared from plasma from over 40000 male and female healthy donors, but certainly with a history of infection, inflammation, injuries and unknown immune related diseases. Therefore, the primary specific objective of this investigation is to characterize IgG reactivity against HLA-I and -HLA-II in Moroccan IVIg. Such an investigation is necessary to determine whether the therapeutic application of IVIg should be preceded by HLA antibody screening. Commercially available IVIg preparations, depending on their polyreactive and their polyclonal antibody strength may contain variable amounts of IgG dimers in the range of 5-15% [36], although IgG dimer, unlike polymers, does not cause anaphylactic shock. Nevertheless IVIg preparations with high dimer content are less tolerated and can give rise to undesirable side effects such as fever, nausea and sometimes lowered blood pressure [37, 38]. Therefore, our second objective is to evaluate Moroccan IVIg formulation for dimer composition.

2. Materials and Methods

2.1. Source and Preparation of IVIg

In Morocco, IVIg is prepared from the whole blood of 40 000 male and female donors. The Moroccan IVIg is manufactured according to the Kistler-Nitschmann method, involving cryoprecipitation, multiple (11% 16% and 22%) ethanolic precipitations, viral inactivation, viral and prion filtration, acidic and enzymatic treatments, sterilization, and lyophilization. All four lots were received as lyophilized powder and were reconstituted with water for injection at a concentration of 50 mg/mL (or 5% proteins). All the IVIg preparations contained IgA (17mg/g of protein), traces of pepsin, sucrose and sodium. Most of the IVIg preparations from US and Europe are devoid of sucrose, due to adverse reports after IVIg administration to patients [38]. The source of IVIg is: (Immunoglobulilune Normale IV-LFB-CNTs (50 mg/ml) 2015, LFB Biomedicaments, Courtaboeuf Cedex, France). The details of the lots are as follows: Lot#1: 14L 00532; Lot#2: 14L 00534; Lot#3: 14L 01611; Lot#4: 14L 01617.
2.2. Determination of Monomer/Dimer Ratio with Fast Protein Liquid Chromatography (FPLC)

The four lots of IVIg preparations (50mg/ml) were initially diluted with normal saline (0.9% NaCl) to 10 mg/ml, and examined within 24 hours, by FPLC using a superdex G-200 column (1.5x50 cm) pre-equilibrated with 50 mM sodium phosphate/150mM sodium chloride at pH 7.2, using Amersham Biosciences AKTA Purifier FPLC System (includes: Box 900; CU-950 System Interface; pH/C-900 Conductivity Detector; Amersham UV-900 UV/Monitor; Amersham P-900 Pump). The flow rate was maintained at 3ml/tube after 0.5ml of IVIg was loaded onto the column. The ratio of monomer/dimer is determined as follows: [monomer/dimer] x 2. The percentage of monomers and dimers was determined as follows: monomer 2 x 100/[dimer+(monomer x 2)].

(1) Dimer % = (1/2)*dimer integral/ (1/2)*dimer integral+monomer integral; and
(2) Monomer % = (monomer integral)/ (monomer integral + (1/2)*dimer integral).

2.3. Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)

Molecular sizes of the protein fractions in the four lots of IVIg were assessed to ascertain the presence of monomeric IgG fractions, their degradation, if any, following a standard protocol [39] using MALDI-TOF MS. The IVIg preparations (10 mg/mL) were analyzed using a microflex LT Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) instrument (Bruker Daltonik GmbH, Bremen, Germany). The spectra were recorded in the linear positive mode at a laser frequency of 5.0 Hz within a mass ranging from 10 to 100 kDa and 100 kDa to 200 kDa. Parameter settings for microflex instruments were ion source 1 at 20 kV, ion source 2 at 18.0 kV, lens at 9.5 kV, pulsed ion extraction of 50 nS and no gating. Mass spectrometry samples were prepared following the protocol [40]. Briefly, 0.5 µL of sample was loaded onto a spot on the MALDI-TOF steel target plate, and 0.5 µL of the calibration standard (ProteoMass Apomyoglobin MALDI-MS Standard; Sigma-Aldrich) was loaded onto a separate spot.

2.4. Immunoassay with Single Antigen Beads SABs

HLA-I and -II IgG reactivity were analyzed using Luminex Multiplex Single Antigen Bead immunoassay. The data obtained with a dual-laser flow-cytometry Luminex xMAP® (http://www.luminexcorp.com/) (LABScanTM100; One Lambda, Canoga Park, CA) [41]. The single recombinant HLA-Ia and HLA-II (rHLA-Ia & rHLA-II) antigens in LS1A04-Lot 008 (for HLA-Ia) containing 97 HLA-I antigens (31 HLA-A, 50 HLA-B and 16 HLA-Cw) and in LS2 A01009 (Lot 9) (for HLA-II) containing 91 HLA-II antigens (29 HLA-DRB1, 7 HLA-DRB3, 4, 5, 29 HLA-DQ, 26 HLA-DP). The SAB assay includes built-in control beads, coated with human IgG (positive control) or albumin (human or bovine) (negative control).

For HLA class I, two kinds of beads were used. They are regular Labscreen beads and iBeads [41]. The beads supplied by the manufacturer may have 2 categories of HLA proteins attached to the beads: HLA heavy-chain polypeptide only and heavy-chain polypeptides in association with Beta-2 microglobulin (β2m). Realizing the heterogeneity of proteins, the manufacturer recently developed iBeads (provided as Felix beads for in-house experimental use), in which regular HLA-Ia antigen-coated microbeads are subjected to proprietary enzymatic treatment to remove or reduce the amount of heavy chains (also referred to as “denatured antigens” by the manufacturers) [42, 43].

Briefly, the four lots of IVIg were titrated from 50 to 0.8mg/mL (diluted in 1X PBS, pH 7.2), and 20 µL of sample were incubated with 2µL of beads for 30 minutes at room temperature and on a shaker. The beads were then washed three times with LabScreen® wash buffer. The HLA-I and -II reactivity were monitored by incubating 50 µL (at 5µg/mL) of PE-conjugated Goat anti-human IgG (Fab’) for 30 minutes. The beads were washed three times, and then resuspended in 1X PBS before acquisition. For each sample analysis, at least 100 beads were counted. The IgG reactivity against each HLA-I and -II antigens were recorded as Trimmed Mean Fluorescence Intensity (MFI), and the MFI values are normalized against the negative control (bead #1) and the negative control (1X PBS). The MFI cutoff used was 1,000 for a positive reaction.

2.5. Statistical Analysis

All data were analyzed using statistical software package for PC (version 13; Dell, Inc. Round Rock, Texas). Analyzed groups were tested for normal distribution using Shapiro-Wilk W testing. Data sets with normal distributions were analyzed by multifactorial ANOVA to identify overall condition effects. Significant differences were determined by post hoc comparisons of means using Tukey’s honest significant difference test. Significance was set at a confidence level of 95%. Data are presented as mean ± SEM.

3. Results

3.1. MALDI-TOF MS Profile of the Four IVIg Lots

The MALDI-TOF MS spectra analysis of the four different lots of IVIg revealed the presence of 6 main protein peaks (Figure 1). The major peak of ~149 kDa is corresponding to the molecular weight of IgG (~150 kDa). Prominent prevalence of the mass of 149 kDa suggests that the IgGs in Moroccan IVIg are intact with no obvious degradation.

Four lots (Lots 1-4) of Moroccan IVIg were mixed and crystallized with α-Cyano-4-hydroxycinnamic acid (4-HCCA) in formic acid: water: isopropanol (3:1:2) in a 1:9 ratio. The expected mass of Moroccan IgG antibody is ~150 kDa, the observed species is ~149 kDa. Each lot of Moroccan IVIg was collected in two spectrums ranging from 10 kDa to 200 kDa.
100 kDa and 100 kDa to 200 kDa (shown as one spectrum).

**Figure 1.** MALDI-TOF MS spectra of the four Moroccan IVIg lots.

### 3.2. FPLC Analysis of the Four IVIg Lots

The FPLC analysis of the four different lots of IVIg showed that all IVIg preparations contain monomeric, dimeric and polymeric IgG (Figure 2, Table 1). The highest peak corresponds to monomeric IgG, the second highest corresponds to dimeric IgG, and the lowest is indicative of polymeric IgG. Table 1 shows the percentage of monomer and dimer of IgG in all IVIg lots tested. IVIg lot #4 being the highest dimer percentage (11 %), followed by IVIg lot #3 (9.9 %), IVIg lot #1 (6.4 %), and IVIg lot #2 (5.8 %). The amount of polymeric IgG is negligible in all IVIg lots tested.

**Figure 2.** FPLC chromatogram of the four Moroccan IVIg lots #1; 2; 3; 4.

| IVIg Lots | Dimer Peak Area | Monomer Peak Area | Monomer/Dimer ratio | Monomer (%) | Dimer (%) |
|-----------|----------------|------------------|---------------------|-------------|-----------|
| #1        | 704.45         | 5111.28          | 14.51               | 93.6        | 6.4       |
| #2        | 113.06         | 925              | 16.36               | 94.2        | 5.8       |
| #3        | 2130.7         | 9737.48          | 9.14                | 90.1        | 9.9       |
| #4        | 2427.08        | 9831.51          | 8.1                 | 89          | 11        |

Percentage of monomer and dimer of the four Moroccan IVIg.

### 3.3. Anti-albumin Reactivity of the Four IVIg Lots

All the IVIg lots reacted with the negative control beads coated with albumin (Figure 3), indicating that the Moroccan IVIg preparations contain naturally occurring anti-albumin antibodies, almost a similar inference can be derived from a previous report monitoring anti-HLA antibodies of Moroccan sera [44]. Among the four IVIg lots, lot #1 showed the highest anti-albumin reactivity, followed by lot #2, then lot #3, and finally lot #4 showed the least reactivity.
All the four IVIg lots reacted with the negative control beads coated with albumin, lot #1 showed the highest reactivity.

### 3.4. Anti-HLA-I and Anti-HLA-II IgG Reactivity of the Four IVIg Lots Using Regular Labscreen Beads

All IVIg lots recognize most of the HLA class-Ia alleles and HLA-II molecules. All preparations reacted with all beads coated with HLA-I and HLA-II alleles. However, for HLA-A and HLA-B alleles, there were no alleles reactive to any of the IVIg lots at 1:32 dilution. In contrast, HLA-DRB345 and HLA-DP alleles showed reactivity even at highest dilution 1/64 (Table 2).

**Table 2. Number of HLA-Ia and HLA-II alleles reactive to the four Moroccan IVIg.**

| IVIg Lots | Dilutions | Number of HLA-Ia and HLA-II alleles reactive to IVIg |
|-----------|-----------|-----------------------------------------------------|
|           | A B Cw DRB1 DRB345 DQ DP |
| [1/64]    | 0 0 0 0 1 0 1 |
| [1/32]    | 0 0 0 7 2 0 10 |
| [1/16]    | 0 0 7 11 5 1 15 |
| # 1       | 2 3 15 22 5 10 21 |
| [1/8]     | 24 38 16 28 7 29 26 |
| [1/4]     | 29 48 16 29 7 29 26 |
| Neat      | 31 50 16 29 7 29 26 |
| [1/64]    | 0 0 0 0 1 0 0 |
| [1/32]    | 0 0 0 1 1 0 4 |
| [1/16]    | 0 0 3 4 2 0 6 |
| # 2       | 1 1 13 14 5 2 16 |
| [1/8]     | 6 20 16 26 7 20 22 |
| [1/4]     | 25 44 16 29 7 29 26 |
| Neat      | 31 50 16 29 7 29 26 |
| [1/64]    | 0 0 0 0 1 0 1 |
| [1/32]    | 0 0 0 1 5 0 8 |
| [1/16]    | 0 0 8 8 3 0 10 |
| # 3       | 1 4 14 20 5 6 19 |
| [1/8]     | 16 32 16 26 7 20 23 |
| [1/4]     | 28 47 16 29 7 29 26 |
| Neat      | 31 50 16 29 7 29 26 |
| [1/64]    | 0 0 0 0 1 0 1 |
| [1/32]    | 0 0 1 1 1 0 3 |
| [1/16]    | 0 0 8 9 5 0 14 |

All IVIg lots reacted to the panel of the 97 HLA-I (31 HLA-A, 50 HLA-B and 16 HLA-Cw) alleles coated on the regular Labscreen beads using Luminex single-antigen bead (SAB) assays. Figure 4 shows the mean MFI of the undiluted (neat) preparations of each IVIg lot (at 50mg/mL concentration) against the HLA-A, -B and -Cw loci. The combined allelic mean MFI of pooled HLA-A reactivity of lot #1, 2, 3, and 4 was 1865 ± 710, 1946 ± 893, 1560 ± 655, and 1566 ± 585, respectively. The mean combined allelic mean MFI of pooled HLA-B reactivity of lot #1, 2, 3, and 4 was 2090 ± 619, 2228 ± 693, 1786 ± 579, and 1706 ± 524, respectively. The combined allelic mean MFI of pooled HLA-Cw reactivity of lot #1, 2, 3, and 4 was 3656 ± 1116, 4189 ± 1467, 3099 ± 920, and 2982 ± 957, respectively. All IVIg preparations showed stronger reactivity to HLA-Cw alleles, followed by HLA-B alleles and then HLA-A alleles.

Data represent mean ± SEM. A two-way ANOVA showed a statistically significant interaction between antigens and lots (F (18, 724) = 5.98, p<.00001). There was a significant effect of antigens (F (6, 724) = 234.49, p<.0001) and lots (F (3,724) = 11.54, p<.00001). Tukey post Hoc tests reactivity of HLA-A and B was significantly lower than all other antigens tested (*P<.001).

Table 3 provides a detailed profile of HLA-I reactivity of different lots of IVIg as MFI of individual alleles of each HLA-I loci. It may be noted that the most prevalent anti-HLA-A IgG antibodies in all the Moroccan IVIg lots, as assessed by the MFI strength at neat and 1/2 dilution, are...
Table 3. HLA-A, HLA-B, and HLA-Cw allelic reactivity of the four lots of Moroccan IVIg determined using regular LALassay Beads.

| HLA-A | IVIg Lot 1 | IVIg Lot 2 | IVIg Lot 3 | IVIg Lot 4 | HLA-B | IVIg Lot 1 | IVIg Lot 2 | IVIg Lot 3 | IVIg Lot 4 | Dilution | Neat | Neat | Neat | Neat |
|-------|-------------|-------------|-------------|-------------|-------|-------------|-------------|-------------|-------------|-----------|--------|--------|--------|--------|
|       | (1/2)       | Neat        | (1/2)       | Neat        | (1/2) | Neat        | (1/2)       | Neat        | (1/2)       | Neat      | Neat    | Neat    | Neat    | Neat    |
| **A*0101** | 952 | 1865 | 547 | 1772 | 683 | 1389 | 984 | 1543 | **A*0102** | 862 | 1651 | 515 | 1662 | 778 | 1472 | 878 | 1398 | **A*0207** | 797 | 1683 | 553 | 1527 | 671 | 1324 | 871 | 1325 | **A*0206** | 917 | 1654 | 691 | 1946 | 874 | 1492 | 967 | 1451 | **A*1101** | 2064 | 1203 | 808 | 2346 | 972 | 1585 | 1337 | 1685 | **A*1102** | 735 | 1590 | 1456 | 576 | 1183 | 753 | 1395 | **A*2301** | 679 | 1368 | 1323 | 543 | 1112 | 764 | 1157 | **A*2402** | 1361 | 2127 | 884 | 2381 | 1004 | 1678 | 1390 | 1943 | **A*2403** | 1441 | 2271 | 847 | 2002 | 1131 | 1750 | 1578 | 1846 | **A*2501** | 1016 | 1739 | 790 | 1699 | 904 | 1615 | 975 | 1449 | **A*2601** | 1272 | 2363 | 810 | 2556 | 1017 | 1885 | 1178 | 1835 | **A*2901** | 1196 | 2384 | 890 | 2469 | 1072 | 1860 | 1322 | 1858 | **A*2902** | 1522 | 2859 | 1141 | 3365 | 1366 | 2431 | 1650 | 2320 | **A*3003** | 892 | 1708 | 542 | 1555 | 634 | 1219 | 910 | 1392 | **A*3002** | 1003 | 1832 | 783 | 1878 | 969 | 1560 | 1049 | 1566 | **A*3301** | 986 | 1746 | 424 | 1387 | 1013 | 2344 | 1685 | 2257 | **A*3302** | 2311 | 666 | 2887 | 1007 | 2600 | 1208 | 1951 | 1376 | 1909 | **A*3303** | 999 | 1899 | 964 | 2399 | 1205 | 1809 | 1171 | 1604 | **A*3401** | 1915 | 2818 | 1217 | 2696 | 1525 | 2140 | 1938 | 2292 | **A*3402** | 633 | 1354 | 526 | 1331 | 603 | 1183 | 738 | 1098 | **A*3601** | 1814 | 1618 | 581 | 1424 | 657 | 1124 | 922 | 1251 | **A*4301** | 1235 | 2339 | 828 | 2335 | 1032 | 1912 | 1714 | 1903 | **A*6601** | 1447 | 2543 | 890 | 2677 | 1112 | 2000 | 1438 | 1954 | **A*6602** | 1142 | 2409 | 817 | 2493 | 1031 | 1867 | 1682 | 1997 | **A*6603** | 961 | 1292 | 814 | 1527 | 508 | 1061 | 630 | 1158 | **A*7301** | 1196 | 2384 | 890 | 2469 | 1072 | 1860 | 1322 | 1858 | **A*7302** | 1522 | 2859 | 1141 | 3365 | 1366 | 2431 | 1650 | 2320 | **A*8001** | 3381 | 4716 | 2902 | 5608 | 3532 | 4362 | 3457 | 3883 | **A*8001** | 3381 | 4716 | 2902 | 5608 | 3532 | 4362 | 3457 | 3883 |

IVIg was diluted from neat to 1/64 and MFI was measured at all dilutions. Data is restricted to Neat and (1/2) dilution. The two most prevailing antibodies against each of the HLA-A, HLA-B, and HLA-Cw loci are shown in Bold and Italics, suggesting the most prevalent anti-HLA IgG in IVIg pooled from the Moroccan male and female donors.

In contrast to MFI of anti-HLA-I IgG antibodies, the MFI of anti-HLA-II IgG antibodies, MF1 of Cw*0702 and Cw*1702 are the most predominant in all lots of the IVIg preparations.
The HLA-DRB1 reactivity of lot #1, 2, 3, and 4 was 6108 ± 1215, 4050 ± 1089, 5345 ± 1166, and 5254 ± 1238, respectively. The HLA-DRB345 reactivity of lot #1, 2, 3, and 4 was 5330 ± 1525, 3997 ± 1851, 5090 ± 1681, and 4859 ± 1528, respectively. The HLA-DQ reactivity of lot #1, 2, 3, and 4 was 4047±976, 2634±915, 3951 ± 1066, and 3473 ± 922, respectively. The HLA-DR reactivity of lot #1, 2, 3, and 4 was 4655 ± 1439, 3681 ± 1369, 4609 ± 1508, and 4277 ± 1335, respectively. Essentially, all IVIg preparations showed stronger reactivity to HLA-DRB1 alleles, followed by HLA-DRB345 alleles, HLA-DR, and then HLA-DQ alleles.

Table 4 provides a detailed profile of HLA-II reactivity of different lots of IVIg as MFI of individual allele of each HLA-II loci. The most prevalent anti-HLA-DR IgG antibodies in all Moroccan IVIg lots, as assessed by the MFI strength at neat and at all dilutions (data shown only for 1/2 and 1/4 dilutions), are DRB3*03:03 and DRB1*01:01 (indicated by Bold italics). The lot # 2 is the least expressed MFI for anti-HLA-DRB IgGs. Similarly, the most prevalent anti-HLA-DQ IgG antibodies in all the IVIg lots are DQB1*03:01*DQA1*03:01 and DQB1*06:02*DQA1*01:02, while DQB1*03:01*DQA1*01:02 remains the least expressed in all lots of IVIg. Among anti-HLA-DRB IgG antibodies, MFI of DPB1*19:01*DPA1*01:03, DPB1*23:01*DPA1*02:01 and DPB1*28:01*DPA1*02:01 are the most predominant in all lots of the IVIg preparations.
Data is restricted to Neat, 1/2 and 1/4 dilutions. Two or three most prevailing antibodies against each of the HLA-DR, HLA-DQ, and HLA-DP loci are shown in Bold and Italics, suggesting the most prevalent anti-HLA IgG in IVlg pooled from the Moroccan male and female donors.

### 3.5. Unique Profile of Anti-HLA-I IgG in IVlg on iBeads

HLA-I alleles on regular Labscreen single antigen beads may occur as intact or native trimeric HLA (HLA heavy chain and β2m with peptide), as well as monomeric form such as heavy chain only, and dimeric form such as peptide-free heavy chain with β2m or β2m-free heavy chain with peptide [43]. Whereas on iBeads, they may occur mostly as trimeric form and β2m with peptide, as well as monomeric form such as peptide-free heavy chain with β2m or β2m-free heavy chain with peptide [43]. Whereas on iBeads, they may occur mostly as trimeric form and β2m with peptide, as well as monomeric form such as peptide-free heavy chain with β2m or β2m-free heavy chain with peptide [43]. Whereas on iBeads, they may occur mostly as trimeric form and β2m with peptide, as well as monomeric form such as peptide-free heavy chain with β2m or β2m-free heavy chain with peptide [43].

| DR Alleles | IVlg Lot 1 | IVlg Lot 2 | IVlg Lot 3 | IVlg Lot 4 |
|------------|-----------|-----------|-----------|-----------|
| Dilution   | (1/4)     | (1/2)     | Neat      | (1/4)     | (1/2)     | Neat      | (1/4)     | (1/2)     | Neat      |
| DQB1*03:01/DQA1*0*01:01 | 1020 | 1200 | 1300 | 1400 | 1500 | 1600 | 1700 | 1800 | 1900 |
| DQB1*03:02/DQA1*0*01:01 | 1030 | 1230 | 1330 | 1430 | 1530 | 1630 | 1730 | 1830 | 1930 |
| DQB1*03:03/DQA1*0*01:01 | 1040 | 1240 | 1340 | 1440 | 1540 | 1640 | 1740 | 1840 | 1940 |
| DQB1*03:04/DQA1*0*01:01 | 1050 | 1250 | 1350 | 1450 | 1550 | 1650 | 1750 | 1850 | 1950 |
| DQB1*03:05/DQA1*0*01:01 | 1060 | 1260 | 1360 | 1460 | 1560 | 1660 | 1760 | 1860 | 1960 |
| DQB1*03:06/DQA1*0*01:01 | 1070 | 1270 | 1370 | 1470 | 1570 | 1670 | 1770 | 1870 | 1970 |
| DQB1*03:07/DQA1*0*01:01 | 1080 | 1280 | 1380 | 1480 | 1580 | 1680 | 1780 | 1880 | 1980 |
| DQB1*03:08/DQA1*0*01:01 | 1090 | 1290 | 1390 | 1490 | 1590 | 1690 | 1790 | 1890 | 1990 |

An MFI of an allele with iBeads that is higher than that of the regular bead would indicate that the HLA reactivity in question is toward intact or native trimeric HLA. Percentage of increase refers to the same [43]. In other words, an MFI of an allele with iBeads that is lower than that of the regular bead would indicate that the affinity of the antibody is towards other HLA-I variants such as the β2m-free HC of HLA with or without peptides. Percentage of decrease refers to the same.

In this investigation, the reactivity lot 3 of Moroccan IVlg (Table 5) to intact HLA-I (regular beads) and to heavy-chain HLA (iBeads) are compared. The percentage difference between regular beads and iBeads was calculated for every allele. The observations were restricted to the undiluted (neat) IVlg. The two most prevailing antibodies against the HLA-A, -B, and -Cw on iBeads are shown in Bold
Italics) in the Table 5. The density of the peptide associated or peptide-free HLA heavy chains with β 2m coated on HLA-A; -B; -Cw antigen-coated iBeads in HLA-I Labscreen iBeads is 50%; 54%; 0% respectively.

Alternately, the density of β 2m-free HLA heavy chains coated on HLA-A; -B; -Cw antigen-coated beads in HLA-I Labscreen beads is 19%; 46%; 100% respectively. Therefore, not all antibodies bound to the Labscreen beads can recognize the trimeric HLA-I molecule, occurring naturally on tissues. The IgG reactivity against alleles (e.g., A*0301; A* 2901; A* 3402; A* 7401; B*1501; B*1503; B*1512; B*4501; B*4901; B*5001; B*5501) is mainly due to anti-HLA-I IgG binding to “intact” or “native” trimeric HLA (HLA heavy chain and β2m with peptide).

### Table 5. HLA-A, HLA-B, and HLA-Cw allelic reactivity of the lot 3 of Moroccan IVIg.

|rozen alleles           | MFI of the undiluted IVIg (neat) Lot 3 | MFI of the undiluted IVIg (neat) Lot 3 |
|------------------------|----------------------------------------|----------------------------------------|
| Alleles                | MFI of the undiluted IVIg (neat) Lot 3 | MFI of the undiluted IVIg (neat) Lot 3 |
| HLA-A                  | Regular                                | iBeads                                 | % HC+ β2M |
| A*0101                 | 1389                                   | 687                                     | 49        |
| A*0201                 | 1472                                   | 730                                     | 50        |
| A*0203                 | 1324                                   | 901                                     | 68        |
| A*0206                 | 1492                                   | 1093                                    | 73        |
| A*0301                 | 576                                    | 666                                     | 115       |
| A*1101                 | 1585                                   | 769                                     | 48        |
| A*1102                 | 1183                                   | 994                                     | 84        |
| A*2301                 | 1112                                   | 946                                     | 85        |
| A*2402                 | 1768                                   | 1036                                    | 59        |
| A*2403                 | 1750                                   | 1031                                    | 59        |
| A*2501                 | 1615                                   | 1301                                    | 81        |
| A*2601                 | 1885                                   | 1263                                    | 67        |
| A*2901                 | 1860                                   | 2244                                    | 121       |
| A*2902                 | 2431                                   | 1942                                    | 80        |
| A*3001                 | 1219                                   | 882                                     | 72        |
| A*3002                 | 1560                                   | 767                                     | 49        |
| A*3101                 | 1519                                   | 878                                     | 58        |
| A*3201                 | 1061                                   | 1052                                    | 99        |
| A*3301                 | 1931                                   | 939                                     | 49        |
| A*3303                 | 1809                                   | 843                                     | 47        |
| A*3401                 | 2140                                   | 1264                                    | 59        |
| A*3402                 | 1183                                   | 1354                                    | 115       |
| A*3601                 | 1124                                   | 960                                     | 85        |
| A*4301                 | 1718                                   | 1364                                    | 79        |
| A*6601                 | 2000                                   | 1500                                    | 75        |
| A*6602                 | 1867                                   | 1261                                    | 68        |
| A*6801                 | 959                                    | 820                                     | 86        |
| A*6802                 | 1785                                   | 1133                                    | 63        |
| A*6901                 | 1547                                   | 771                                     | 50        |
| A*7401                 | 731                                    | 863                                     | 118       |
| A*8001                 | 4362                                   | 1374                                    | 31        |
| HLA-Cw                 |                                       |                                         |           |
| Cw*0102                | 3199                                   | 832                                     | 26        |
| Cw*0202                | 2989                                   | 608                                     | 20        |
| Cw*0302                | 2716                                   | 1326                                    | 49        |
| Cw*0303                | 2609                                   | 1011                                    | 39        |
| Cw*0304                | 2832                                   | 911                                     | 32        |
| Cw*0401                | 3124                                   | 740                                     | 24        |
| Cw*0501                | 3295                                   | 688                                     | 21        |
| Cw*0602                | 3305                                   | 729                                     | 22        |
| Cw*0702                | 5333                                   | 1117                                    | 21        |
| Cw*0801                | 2752                                   | 1353                                    | 49        |
| Cw*1203                | 3147                                   | 952                                     | 30        |
| Cw*1402                | 3073                                   | 1218                                    | 40        |
| Cw*1502                | 2692                                   | 904                                     | 34        |
| Cw*1601                | 2202                                   | 812                                     | 37        |
| Cw*1701                | 5245                                   | 974                                     | 19        |
| Cw*1802                | 4594                                   | 725                                     | 16        |

MFI obtained with regular Labscreen Beads and iBeads were compared for every allele of HLA-I locus. The data is restricted to undiluted (neat) IVIg. The percentage difference between regular beads and iBeads is shown for every allele in
each locus. The two most prevailing antibodies, against each of the HLA-A, HLA-B, and HLA-Cw coated on iBeads, are shown in Bold and Italics.

4. Discussion

Four lots Moroccan IVIg preparations purified from pooled plasma of males and females of different ethnic groups and origins from different parts of the country were tested for reactivity to HLA-I, HLA-II and albumin using Luminex single antigen beads and for the antibody dimerization. In contrast to measuring MFI of serum anti-HLA IgG, the MFI of anti-HLA IgG reactivity of IVIg is not affected by the presence of IgM or other classes of antibodies. The MFI values of IVIg indeed reflect HLA affected by the presence of IgM or other classes of antibodies. The MFI values of IVIg indeed reflect HLA IgG, the MFI of anti-HLA IgG reactivity of IVIg is not affected by the presence of IgM or other classes of antibodies. The MFI values of IVIg indeed reflect HLA reactivity of IgG antibodies that binds to HLA coated beads.

Although it is known that the classical HLA-I and HLA-II molecules are highly polymorphic and represented by 2747 HLA-A, 3465 HLA-B, 2450 HLA-C, 1711 HLA-DRB, 34 HLA-DQA1, 761 HLA-DQB1, 23 HLA-DPA1 and 627 HLA-DPB1 proteins [3], the number of HLA coated beads available for monitoring antibodies are far less (97 HLA-A, B and Cw and 91 HLA-DRB/DB, DQA/DQB and DPA/DPB), thus imposing a limitation on characterizing the HLA antibodies prevailing in any population. In spite of the limitation, antibodies against high frequency alleles found in Moroccan population were observed.

The Moroccan IVIg preparations contain IgG antibodies against several high frequency HLA-I alleles found in the Moroccan population (A*0101, A*0201, B*0801, B*4403, B*4402, B*5001, Cw*0401, Cw*0602 and Cw*0702) [5]. The density of the antibodies as assessed by the levels of MFI for B*0801, B*5001, Cw*0602 and Cw*0702 are high parallel with their high frequency distribution. Similarly, the Moroccan IVIg had IgG antibodies against several high frequency HLA-II alleles found in the Moroccan population [6], which include DRB1*0701, DRB1*0301, DQA1*0501, DQA1*0201, and DQB1*0201 and haplotypes DQA1*0201-DQB1*0201/DRB1*0301, which nearly account for 50% of the total gene frequencies found in Moroccan Souss cohort. These findings caution administering high dose IVIg for the carriers of the HLA types, because they may experience adverse effects such as TRALI. This investigation emphasizes the need to carry out HLA typing of any patient who receives Moroccan IVIg and if Moroccan IVIg is administered to these carriers, a critical patient care is required at least for a week after administering high dose IVIg.

The HLA molecules on the tissues appear as different conformational variants. The most common configuration on normal tissues is considered to be an HLA trimer, which consists of a heavy chain (HC) (40-45 kDa) non-covalently associated with β2-microglobulin (β2m) (12 kDa) and an 8-10 amino acid long peptide that are bound in the HC groove. Frequently, the native HLA may also exist, devoid of the peptide, as a HC with β2m. Indeed, using Flow cytometric Cross match analyses, with epitope specific monoclonal antibodies, confirmed the prevalence of peptide-associated β2m–associated HLA HC (pepA-β2aHC), on resting T-cells [43]. In addition to the above structural variants, a pool of β2m-free HLA as β2-free HC (β2fHC) was observed in proliferating human lymphoid cells [45], and in activated human T and B cells [46, 47]. Binding of IVIg to activated T and B cells may bring about immunomodulation such as suppression of antibody production, which may be beneficial for patients with autoimmune diseases and organ transplants [48].

Jucaud and co-investigators [43] have carefully compared the conformational variants on the Labscreen regular Beads and iBeads, to confirm the striking differences between the two beads. They have confirmed that the two beads differ as follows:

1. The presence and the heterogeneity of density of peptide-associated-β2m-associated HLA-heavy-chain (pepA-β2aHC), peptide-free-β2aHC (pepF-β2aHC), and β2-free-HC (β2fHC) on the regular Labscreen Single antigen beads.
2. In contrast, iBeads harbored a high density of pepA-β2aHC and low density of pepF-β2aHC, but devoid of β2fHC.

High prevalence of IgG antibodies for 81% of HLA-A and 54% HLA-B alleles on iBeads confirms the presence of IVIg antibodies reacting to normal tissue associated pepa-β2aHC and pepF-β2aHC variants. Such antibodies that bind to HLA trimers found on normal tissues are at potential risk for inflammatory diseases, such as TRALI.

However, IVIg reactivity to β2fHC, which frequently found on activated T and B cells, and are responsible of increased production of antibodies in autoimmune diseases, may significantly suppress activated T and B cells involved, and thus alleviates autoimmune diseases. The dual functionality of HLA antibodies in IVIg emphasizes the need to modify the profiles of HLA antibodies to be directed against the HLA variants (β2fHC) found on activated immune cells and may possibly have a beneficial influence on the patients with autoimmune diseases. Possibly the antibody profile can be modified by adsorbing out IgG antibodies directed against pepA or pepF-β2aHC using bead preparations like that of iBeads. Anticipating such benefits of IVIg, the US Federal Food and Drug Administration has recommended IVIg [51] for; (1) Primary Immune Deficiencies (PID), (2) Idiopathic Thrombocytopenic Purpura (ITP), (3) Chronic Lymphocytic Leukemia (CLL), (4) Kawasaki Disease, (5) Bone Marrow Transplantation (BMT). Again comparing the characteristics of IVIg from different manufacturers (Alpha, Baxter, Bayer, Centeon, Novartis), FDA has noted that while all the preparations are well suited for PID but not for all other - above mentioned- disease conditions. The recent findings about HLA and IVIg has raised the necessity for screening HLA antibodies in different lots of Moroccan IVIg preparations and especially against the two kinds of HLA coated beads; one coated only with HLA-Trimer and the other one only with β2fHC. Such a critical evaluation and the selection of lots reacting mostly to β2fHC would minimize adverse reactions (of course, without sucrose) and promote the
utility of IVIg for autoimmune diseases, and PID that are highly prevalent in Morocco. The dimerization emphasizes the need to examine the IgG subclasses in Moroccan IVIg preparations, since subclasses are different in their ability for dimerization [52-54].

5. Conclusion

HLA-I and HLA-II reactive high MFI IgG antibodies in the Moroccan IVIg corresponded with several high frequency HLA-I alleles (B*0801, B*5001, Cw*0602 and Cw*0702) and HLA-II haplotypes (DQA1*0201-DQB1*0201/DRB1*0301), which accounted for 50% of the total gene frequencies in the Moroccan population. Measuring anti-HLA-I IgG antibodies was performed using regular (Labscreen) Beads coated with all conformational and structural variants of HLA-I (pepA- and pepF- β2aHC, pepA- and pepF- β2fHC) and iBeads coated with native tissue-associated HLA-I trimers (pepA-β2aHC > pepF- β2aHC). It was realized that the IVIg contains IgG antibodies against all of the structural variants. While HLA-A and HLA-B reactive antibodies in the four lots of IVIg were predominantly binding to native HLA-trimers, HLA-Cw reactive antibodies were mostly reactive to pepA and pepF - β2fHC. These findings caution use of high dose IVIg for the carriers of the high frequency HLA types for it may cause tissue injuries such as TRALI. The β2fHC reactivity of IVIg for the need to examine the IgG subclasses in Moroccan IVIg preparations, since subclasses are different in their ability for dimerization [52-54].

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