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Epitope characterization of a monoclonal antibody that selectively recognizes KIR2DL1 allotypes

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Killer immunoglobulin-like receptor (*KIR*) genes code for a family of inhibitory and activating receptors, finely tuning NK cell function. Numerous studies reported the relevance of *KIR* allelic polymorphism on KIR expression, ligand affinity, and strength in signal transduction. Although *KIR* variability, including gene copy number and allelic polymorphism, in combination with HLA class I polymorphism, impacts both KIR expression and NK cell education, only a precise phenotypic analysis can define the size of the different KIR^{pos} NK cell subsets. In this context, reagents recognizing a limited number of KIRs is essential. In this study, we have characterized the specificity of an anti-KIR mAb termed HP-DM1. Testing its binding to HEK-293T cells transfected with plasmids coding for different KIRs, we demonstrated that HP-DM1 mAb exclusively reacts with KIR2DL1. Using site-directed mutagenesis, we identified the four amino acids relevant for HP-DM1 recognition: M44, S67, R68, and T70. HP-DM1 mAb binds to a conformational epitope including M44, the residue crucial for HLA-C K80 recognition by KIR2DL1. Based on the HP-DM1 epitope characterization, we could extend its reactivity to all KIR2DL1 allotypes identified except for KIR2DL1*022 and, most likely, KIR2DL1*020, predicting that it does not recognize any other KIR with the only exception of KIR2DS1*013. Moreover, by identifying the residues relevant for HP-DM1 binding, continuously updating of its reactivity will be facilitated.

KEYWORDS
killer immunoglobulin-like receptors, monoclonal antibodies, natural killer cells, polymorphism, site-directed mutagenesis

1 | INTRODUCTION

Natural killer (NK) cells are potent effectors of the innate immune system relevant for controlling viral infections and tumor development.[1–3] Moreover, NK cells play a significant role in reproduction by contributing to maternal-fetal immune tolerance.[4–7] NK cell activities are finely regulated by an array of signals transduced by germ-line encoded surface molecules with either inhibitory or activating functions.[8–13] During the education/ licensing process, NK cells become fully functional by the expression of at least one inhibitory receptor recognizing self HLA class I molecule(s).[14–16] Through this process, NK cells spare healthy cells, expressing physiological levels of HLA class I molecules ("self-tolerance"), and eliminate pathological cells that down-regulate HLA class I ("missing self-recognition") and/or overexpressing or expressing de novo ligands for activating receptors ("induced self-recognition").[16–21] NK cells sense HLA class I expression via killer Ig-like receptors (KIR) and the CD94/NKG2 heterodimer, recognizing the highly polymorphic HLA class I classical allotypes and the non-classical HLA-E molecules, respectively.[9,11,22] The best characterized inhibitory KIRs (iKIRs) include KIR2DL1 and KIR2DL2/L3, which bind HLA-C molecules carrying Lysine or Asparagine at position 80 (K80 or N80) respectively; KIR3DL1 recognizing both HLA-B and HLA-A allotypes sharing the Bw4 epitope, and KIR3DL2 sensing HLA-A*03, -A*11, and HLA-F in an open conformation.[8,9,11,23–25] Thus, iKIRs specific for HLA-C sense virtually all allotypes that, except for five, are characterized by K80 or N80 [Immuno Polymorphism Database (IPD)/HLA release 3.46, 2021]. On the contrary, most HLA-B and HLA-A allotypes are not ligands of iKIR molecules. The characterization of activating KIR (aKIR) ligands needs to be further clarified. Among them, KIR2DS1, KIR2DS2, and KIR3DS1 have extracellular domains similar to their inhibitory counterparts (i.e., KIR2DL1, KIR2DL2/L3, and KIR3DL1), with which they share the HLA ligands.[25–27]

KIRs are coded by a gene family located on chromosome 19q13.4 consisting of 13 genes and 2 pseudogenes closely packed in a head-to-head orientation and inherited as a haplotype.[28–30] More than 80 different KIR haplotypes, differing in gene content variability and allelic polymorphism, have been identified.[28,30–33] Despite this, a conserved characteristic of almost all KIR haplotypes is the presence of two regions, referred to as centromeric and telomeric based on their orientation, separated by a hot spot of recombination.[30–36] HLA-C specific iKIR genes are located in the centromeric region, whereas those recognizing HLA-B and HLA-A are in the telomeric one. KIR2DL2 and KIR2DL3 are alleles of the same locus (i.e., KIR2DL2/ L3), and virtually all individuals are characterized by at least one KIR2DL2/L3 allele coding for a surface receptor. Differently, KIR2DL1 is present only in a fraction of the centromeric regions, and the percentage of KIR2DL1 negative individuals varies among the different ethnic groups ranging from 3% to 5% in Caucasian populations.[31,37,38]

KIR expression on the NK cell surface is determined by genetic (i.e., KIR gene variability and HLA polymorphism), environmental (e.g., exposure to viral infections), and stochastic factors.[39–50] Moreover, phenotypical analyses revealed that the clonally distributed expression of individual KIRs leads to a great diversity of NK cell subsets displaying different KIR combinations. Most studies on KIR phenotype had been primarily performed using anti-KIR mAbs and cytofluorimetric approaches.[40,43,48,51–53] However, because of the high similarity between KIRs encoded by different genes, the high level of polymorphism (especially among KIR genes coding for iKIRs), and the elevated amino acid identity between iKIR and aKIR recognizing the same ligands, the number of reagents specific for a single KIR is somewhat limited.

In this study, we characterized the reactivity of HP-DM1, an anti-KIR mAb selectively recognizing KIR2DL1 allotypes. Using the site-directed mutagenesis approach, we have identified the four residues relevant for HP-DM1 binding. The characterization of this reagent paves the way for its use, alone or in combination with other anti-KIR antibodies, leading to more accurate identification of the different NK subpopulations. These studies may be relevant in basic research and clinical applications such as hematopoietic stem cell transplantation, autoimmune diseases, and pregnancy complications.

2 | METHODS AND MATERIALS

2.1 | Immunization and fusion

BALB/c mice were immunized with a human NK clone expressing CD94, ILT2, and KIR2DL1 to obtain mAbs specific for receptors involved in the regulation of NK cell cytotoxicity. Hybridomas were selected by their ability to modulate NK cell functions in reverse antibody-dependent cellular cytotoxicity (R-ADCC) assays against the P815 mastocytoma cell line, as previously described.[54,55] Their specificity was assessed by flow cytometry of polyclonal NK cell populations.

2.2 | Polyclonal NK cell populations

Peripheral blood mononuclear cells were isolated by Lympholyte-H density gradient centrifugation (Cedarlane, Burlington, Canada) from buffy coats of

healthy donors, provided by the blood transfusion center of IRCCS Ospedale Policlinico San Martino (Genoa, Italy), following approved internal operational procedures (IOH78). The donor *KIR* genotypes were analyzed as previously described.[52] NK cells were purified using the RosetteSep method (StemCell Technologies, Vancouver, BC) and cultured on irradiated feeder cells in the presence of 2 μg/ml phytohemagglutinin (Sigma-Aldrich, Irvine, UK) and 600 IU/ml rIL-2 (Proleukin, Chiron Corp., Emeryville, CA) to obtain activated polyclonal NK cells. NK cells were stained first with HP-DM1 mAb followed by anti-IgG1-PE (Southern Biotechnology, Birmingham, AL), washed twice, and incubated with EB6B-APC (anti-KIR2DL1/S1 and anti-KIR2DL3 allotypes carrying E35 and R50, Beckman Coulter, Brea, CA). 143211-PE mAb (anti-KIR2DL1/S5, R&D Systems, Minneapolis, MN) in combination with EB6B-APC was also used. Flow cytometric analysis was performed on Gallios flow-cytometer (Beckman Coulter), and data were analyzed using FlowJo Version 10.7 (BD Biosciences, San José, CA).

2.3 | Transfection

HEK-293T cells were transiently transfected using the linear polyethylenimine derivative jetPEI transfection reagent (Polyplus, New York, NY) following the manufacturer's instruction. The pcDNA 3.1 plasmids used in the study code for: *2DL1*002*, **003*, **004*, **012*; *2DL2*001*; *2DL3*002*, **005*; *2DS1*001*, **002*; *2DS2*001*, *2DS4*001*, and *2DS5*002*. Forty-eight hours after the transfection, the cells were tested with a panel of anti-KIR2D mAb followed by anti-IgG-PE or anti-IgM-PE (Southern Biotechnology) by immunofluorescence and flow cytometric analysis (FACSCalibur and Cell Quest software, BD Biosciences). The mAbs used in these experiments were: 143211 (R&D Systems, Minneapolis, MN), 11PB6 (anti-KIR2DL1/S1 and anti-KIR2DL3 allotypes carrying E35 and R50, Miltenyi Biotec, Bergisch Gladbach, Germany), HP-MA4 (anti-KIR2DL1/S1/S3/S5, Biolegend, San Diego, CA), GL-183 (anti-KIR2DL2/L3/S2, Beckman Coulter), and FES172 (anti-KIR2DS4, Beckman Coulter).

2.4 | Mutagenesis

Mutagenesis was performed using the Gene Tailor Site-Directed Mutagenesis System (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. The primers include sequence coding for the amino acid we wanted to replace (Table S1). The plasmids encoding the mutated

*KIR*s were sequenced using a d-Rhodamine Terminator Cycle Sequencing kit and a 3100 ABI automatic sequencer (PerkinElmer, Wellesley, MA) to confirm the required substitutions. The produced *2DL1*002* mutants included the following substitutions: M44K, M44T, R50H, S67G, R68P, T70M, T70R, T70K, V90L, and M44T+S67G. Moreover, we generated *2DS1*001* R70T and *2DS1*002* K70T.

3 | RESULTS

3.1 | HP-DM1 mAb selectively recognizes KIR2DL1

To obtain mAbs specific for receptors involved in the regulation of NK cell cytotoxicity, we generated hybridomas from BALB/c mice immunized with a phenotypically characterized human NK clone. A mAb, hereafter termed HP-DM1 (IgG1), was selected for inhibiting the NK cell clone cytotoxicity in R-ADCC and its reactivity on NK cells appeared comparable to that of the anti-KIR2DL1/S1 EB6B mAb. Further studies with polyclonal NK cells derived from donors with known *KIR* repertoires showed that HP-DM1 mAb displayed a reactivity similar to the anti-KIR2DL1/S5 143211 mAb. Double stainings, combining HP-DM1 with the anti-KIR2DL1/S1 EB6B mAb or 143211 with EB6B mAb, were performed (Figure 1A; hereafter, KIR will be omitted by the acronyms). In particular, in *2DL1*^{pos}/*2DS1*^{neg}/*2DS5*^{neg} donors, 2DL1^{pos} NK cells were co-stained by these mAbs; in *2DL1*^{neg}/*2DS1*^{pos}/*2DS5*^{neg} individuals, no staining of HP-DM1 and 143211 was observed, while EB6B identified the 2DS1^{pos} NK cells; in *2DL1*^{pos}/*2DS1*^{pos}/*2DS5*^{neg} donors, both 2DL1^{neg} 2DS1^{pos} (HP-DM1^{neg} or 143211^{neg} and EB6B^{pos}) and 2DL1^{pos} ± 2DS1^{pos} (co-stained by all the three mAbs) populations were detected (Figure 1A).

To better define the HP-DM1 recognition pattern, we tested its reactivity on HEK-293T cells transiently transfected with plasmids coding for different lineage III KIR2D (namely, the KIR2D receptors composed of D1 and D2 domains; Figure 1B and Figure S1). For comparison, we selected three different mAbs recognizing 2DL1 molecules (i.e., 143211, 11PB6, and HP-MA4) whose reactivity has already been characterized.[51–53,56] Remarkably, HP-DM1 mAb stained exclusively *2DL1*002* transfected cells, showing a pattern different from the other three mAbs (Figure 1B). Indeed, HP-DM1 mAb did not stain 2DS5*002, which conversely is recognized by 143211 and HP-MA4 mAbs. 11PB6 mAb brightly stained transfected cells expressing 2DL1*002, 2DS1*001, 2DL3*005, as previously reported.[53,56] Using 11PB6, we also obtained unexpected dull staining of 2DS5*002^{pos} transfectants,
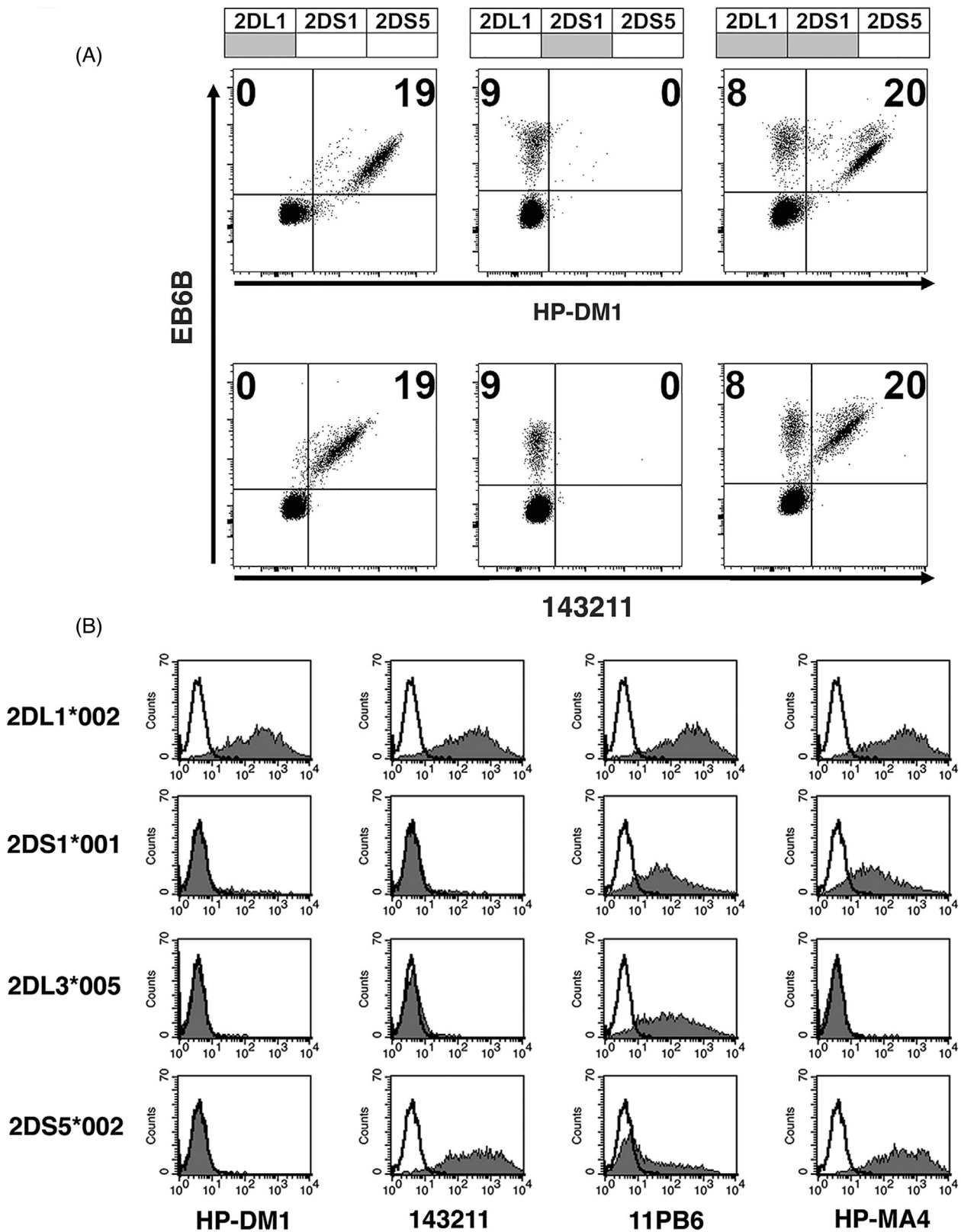
(A)

(B)

FIGURE 1 HP-DM1 mAb selectively recognizes KIR2DL1 transfected cells. (A) Double fluorescence analyses were performed using activated NK cell populations derived from representative donors with defined *KIR* gene profiles (positive genes are identified as gray, negative as white boxes). Cells were stained with HP-DM1 and EB6B-APC (upper panels) or with EB6B-APC and 143211-PE mAbs (lower panels). (B) HEK-293T cells, transiently transfected with plasmids coding for the indicated *KIR2D* alleles, were stained with HP-DM1, 143211, 11PB6, and HP-MA4 mAb, followed by the PE-conjugated goat anti-mouse IgG (filled profiles). Empty profiles represent staining with the second reagent alone. A representative of five independent experiments is shown

probably because of the high level of 2DS5 expression. Our data confirmed that HP-MA4 mAb recognizes 2DL1*002, 2DS1*001, and 2DS5*002,[53] and extended its characterization showing that it does not recognize 2DL3*005. None of the selected mAbs reacted with 2DL2*001, 2DL3*002, 2DS2*001, and 2DS4*001 (Figure S1).

These data indicated that HP-DM1 mAb is a unique reagent selectively recognizing 2DL1.

3.2 | Amino acid residue T70 is crucial for HP-DM1 reactivity

To better define the HP-DM1 mAb reactivity, we investigated the residues relevant for its binding. Since a high level of amino acid identity characterizes the extracellular regions of 2DL1 and 2DS1, we firstly focused our analysis on the amino acid positions that differ between these two KIRs. The alignment of the sequences of four tested HP-DM1 positive 2DL1 allotypes (namely 2DL1*002, *003, *004, and *012) and two HP-DM1 negative 2DS1 (i.e., 2DS1*001 and *002) revealed that the HP-DM1 epitope should include residue(s) located in the D1 domain (Figure 2). Indeed, 2DL1*004, strongly recognized by HP-DM1 mAb, has a D2 domain identical to 2DS1*001 and 2DS1*002 (Figure 2). The comparison led us to identify Threonine 70 (T70) and Valine 90 (V90) as possible relevant residues for HP-DM1 binding. Thus, we introduced in the 2DL1*002 backbone the amino acid present at these positions in 2DS1 allotypes and determined the effect of the mutations on HP-DM1 mAb reactivity. The replacement of T70 with either Lysine (K) (present in most of the 2DS1 allotypes) or
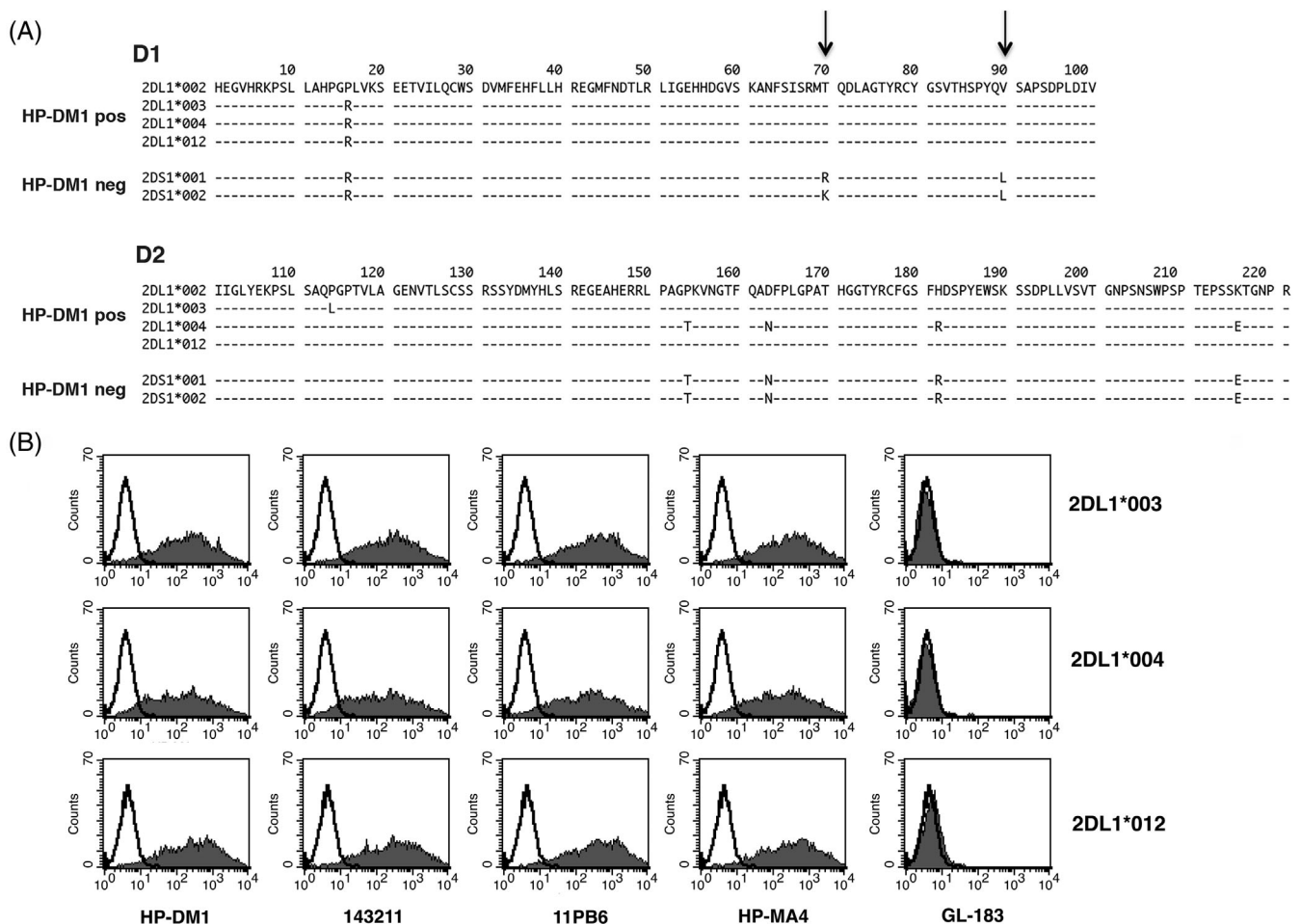

FIGURE 2 HP-DM1 epitope includes residue(s) located in the first KIR2DL1 domain. (A) Alignment of KIR2D extracellular regions of three 2DL1 and two 2DS1 allotypes of known HP-DM1 mAb reactivity. The numbers indicate the amino acid positions present in the mature proteins. Arrows indicate the amino acid positions considered for HP-DM1 mAb epitope characterization. (B) HEK-293T cells transiently transfected with the plasmid coding for the 2DL1*003, *004, and *012 allotypes were surface stained with the indicated mAbs followed by the PE-conjugated goat anti-mouse IgG second reagent (filled profiles). Empty profiles represent staining with the second reagent alone. A representative of five independent experiments is reported

Arginine (R) (found in 2DS1*001) completely abrogated HP-DM1 mAb reactivity, while the replacement of V90 with Leucine did not (Figure 3A). In these experiments, we used 11PB6 as control mAb to confirm the surface expression of the 2DL1 mutants. Conversely, the replacement in 2DS1 backbones of either R70 or K70 with T determined a strong HP-DM1 mAb recognition (Figure 3B). The data showed that T70, a residue present in all the 2DL1 allotypes identified so far, is crucial for HP-DM1 binding.

3.3 | HP-DM1 epitope also includes M44, S67, and R68

Taking into account the relevance of T70 for HP-DM1 mAb binding, we focused on the amino acids present in the other KIRs belonging to lineage III that are not recognized by HP-DM1. As all 2DL2/L3, 2DS2, and 2DS4 allotypes are characterized by a Methionine at position 70 (M70), we examined if it negatively affects HP-DM1 mAb binding by the generation of the *2DL1*002* T70M mutant. As shown in Figure 3C, replacing T70 with M in 2DL1 partially reduced HP-DM1 mAb reactivity, suggesting that other residues may contribute to the HP-DM1 epitope. This possibility was further supported by the observation that, although all the 2DS5 allotypes are characterized by T70, *2DS5*002* transfected cells were not stained by HP-DM1 mAb (Figure 1B). First, we investigated why 2DL2/L3, 2DS2, and 2DS4 molecules did not react with HP-DM1 mAb. Comparing the D1 amino acid sequences of the KIRs mentioned above with 2DL1, we identified four additional residues (M44, R50, S67, and R68) present only in 2DL1 (Figure S2). To analyze the possible involvement of these positions, we generated four novel *2DL1*002* mutants (i.e., M44K, R50H, S67G, and R68P). As shown in Figure 4A, M44K and R68P abrogated HP-DM1 recognition, while S67G sharply decreased HP-DM1 staining. On the contrary, the replacement of R50 with H did not interfere with HP-DM1 mAb binding. These data demonstrated that M44, S67, and R68 are relevant for HP-DM1 binding, explaining why this mAb does not react with 2DL2, 2DL3, 2DS2, and 2DS4 allotypes. In these experiments, we used 11PB6 mAb to control the surface expression of the transfected mutants. As expected, since 11PB6
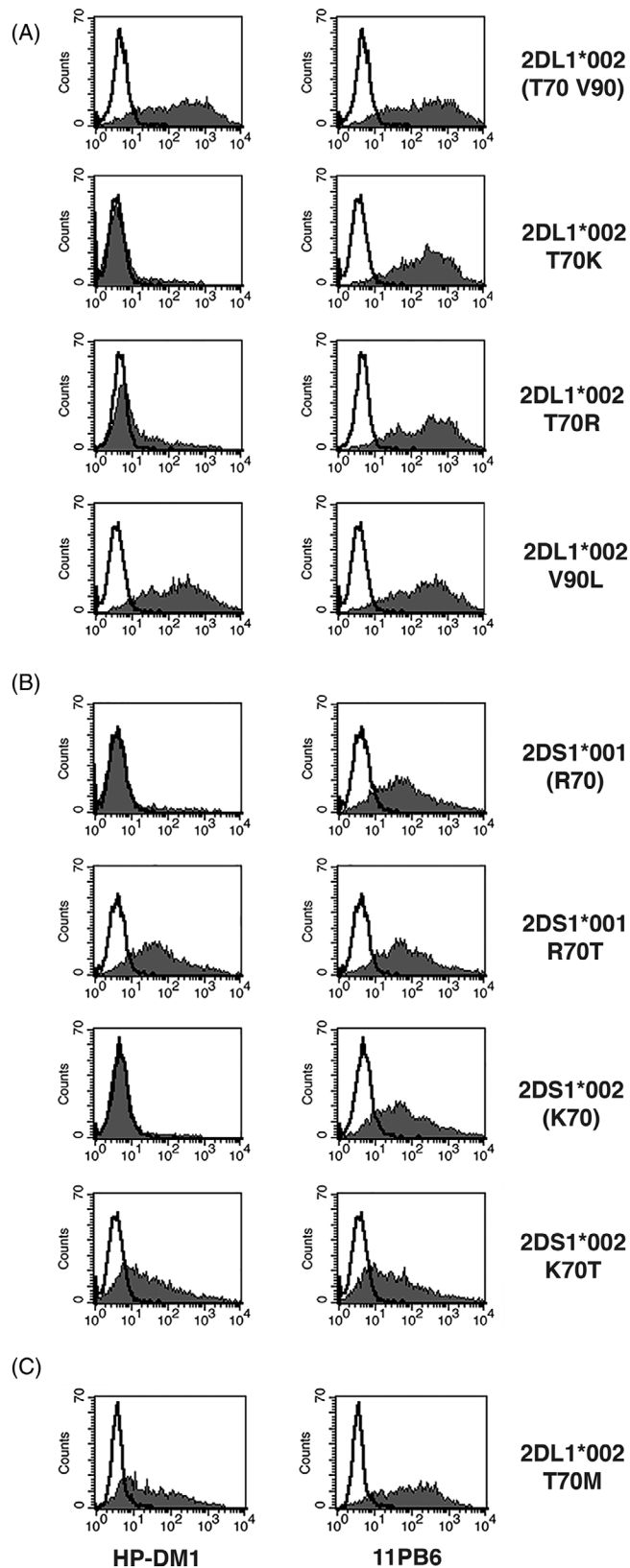

FIGURE 3 Threonine 70 is part of HP-DM1 epitope. (A, B, and C) HEK-293T cells transiently transfected with the indicated plasmids were surface stained with the HP-DM1 and 11PB6 mAb followed by the PE-conjugated goat anti-mouse IgG second reagent (filled profiles). Empty profiles represent staining with the second reagent alone. The residues present in wild-type KIR are indicated in brackets. A representative of five independent experiments is shown
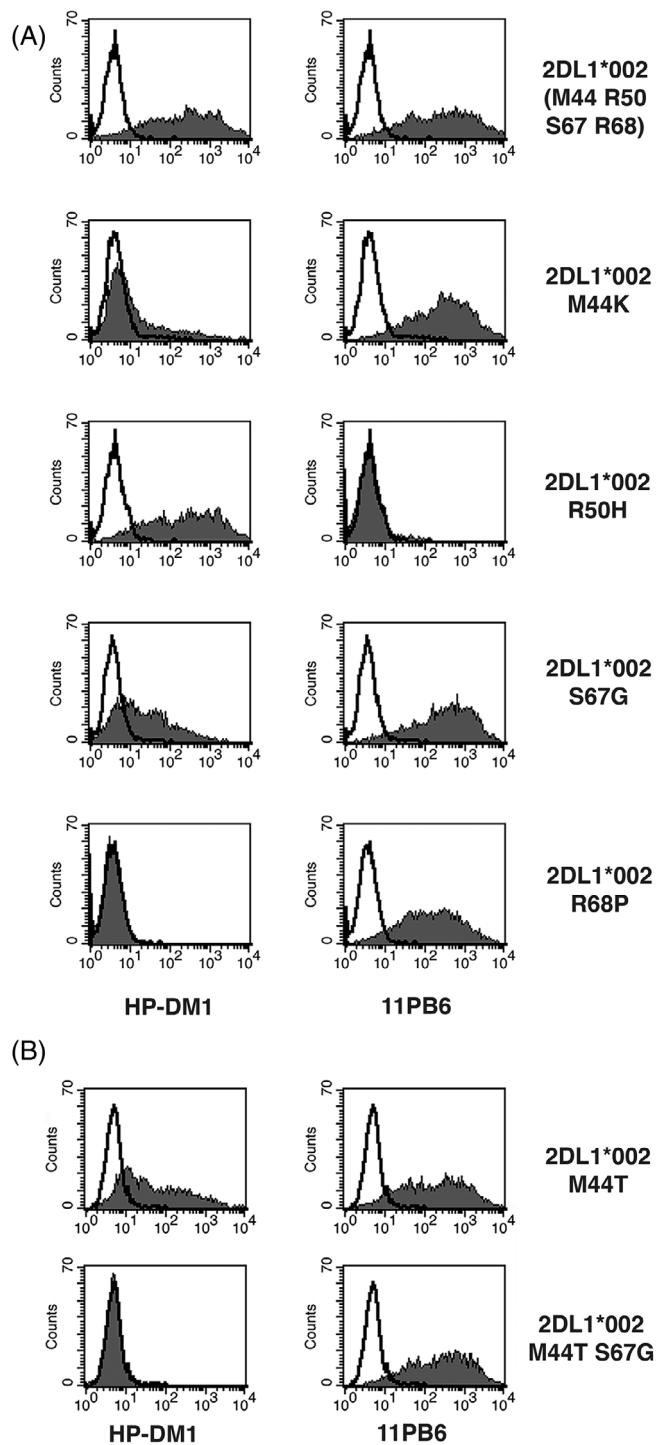
FIGURE 4 HP-DM1 recognition is also influenced by M44, S67, and R68. (A, B) HEK-293T cells transiently transfected with the indicated plasmids were surface stained with the HP-DM1 and 11PB6 mAbs followed by the PE-conjugated goat anti-mouse IgG second reagent (filled profiles). Empty profiles represent staining with the second reagent alone. A representative of four independent experiments is shown

epitope includes Glutamic Acid and Arginine at positions 35 and 50, respectively, this mAb labeled all the _2DL1*002_ mutants except for _2DL1*002_ R50H.[52]

Then we examined the four residues critical for HP-DM1 binding (i.e., M44, S67, R68, and T70) in 2DS5 allotypes (Figure S2). Since all 2DS5 allotypes, except for _2DS5*014_, are characterized by R68 and T70 (that are relevant for HP-DM1 binding), G67 (that marginally affects the HP-DM1 staining), and Threonine 44 (T44), we generated _2DL1*002_ M44T mutant to test if T44 could abrogate HP-DM1 mAb recognition. As shown in Figure 4B, M44T replacement decreased, but not abolished, HP-DM1 mAb staining. Finally, we tested if the combined presence of T44 and G67 (the two amino acid residues that reduce HP-DM1 mAb binding) had an additive effect. To this end, we generated the _2DL1*002_ M44T S67G double mutant, which was no longer recognized by HP-DM1 mAb (Figure 4B). Taken together, our data indicated that M44, S67, R68, and T70 are the four amino acids relevant for HP-DM1 recognition (Table 1).

The identification of the residues relevant for HP-DM1 mAb binding allows predicting that this mAb exclusively recognizes all 2DL1 allotypes present in the IPD-KIR database (release 2.10.0, 2020) except for 2DL1*022 and, most likely, 2DL1*020. Indeed, 2DL1*022 codes for a mature protein identical to the HP-DM1 negative 2DL1*002 M44K mutant (Figure S3 and Figure 4), and 2DL1*020 encodes for a receptor that carries G67 that we know to decrease the HP-DM1 binding (Figure S3 and Figure 4).

3.4 | The HP-DM1 and 143211 mAb epitopes partially overlap

Finally, we investigated the residues relevant for 143211 mAb specific binding to 2DL1 and 2DS5. To this end, we used an approach similar to that previously described for HP-DM1. We focused on T70 (Figure S4), a residue present in the extracellular portions of 2DL1 and 2DS5 but absent in the other lineage III KIRs. We tested the 143211 mAb reactivity on _2DL1*002_ T70M, T70R, and T70K mutants. As shown in Figure 5, the replacement of T70 with either R or K virtually abrogated the 143211 mAb recognition, as also occurred for HP-DM1 mAb (see Figure 3A), while the substitution with M had no effect. Since all the tested KIRs characterized by M70 have P68 (Figure S4), we further investigated if position 68 influenced the 143211 binding. The results obtained analyzing the reactivity of _2DL1*002_ R68P mutant revealed that the presence of P68 almost completely abrogated 143211 staining (Figure 5). In this set of experiments, 11PB6 mAb was chosen as transfection control mAb and the obtained profiles (not shown) were similar to those reported in Figures 3A,C and 4A. Being both T70 and R68 residues relevant for HP-DM1 binding, we conclude

| Clone name	KIR specificity	Residues relevant for mAb binding	References
HP-DM1	2DL1 (except for 2DL1*020, *022), 2DS1*013	M44, S67, R68, T70	This study
EB6B	2DL1, 2DS1, 2DL3 E35 and R50	E35, R50	8,51,52,56,57
11PB6	2DL1, 2DS1, 2DL3 E35 and R50	E35, R50	51,52
143211	2DL1, 2DS5	R68, T70	This study[51–53,56]
HP-MA4	2DL1, 2DS1, 2DS3, 2DS5	N.D.	53
GL-183	2DL2, 2DL3, 2DS2	N.D.	51,56,57
FES172	2DS4	N.D.	58

Note: ND, not determined.
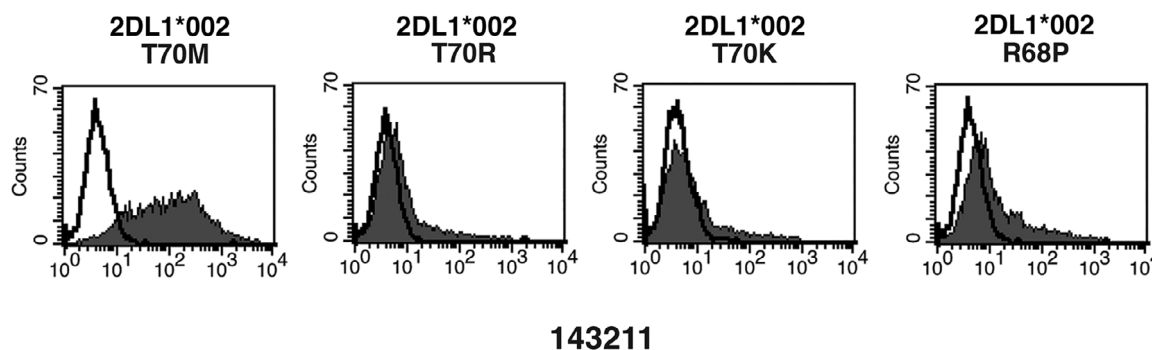
143211

FIGURE 5 Identification of the residues relevant for 143211 mAb binding. HEK-293T cells transiently transfected with the indicated *2DL1*002* mutants were surface stained with the 143211 mAb followed by the PE-conjugated goat anti-mouse IgG second reagent (filled profiles). Empty profiles represent staining with the second reagent alone. A representative of five independent experiments is shown

that the amino acid positions required for HP-DM1 and 143211 mAb recognition partially overlap (Table 1).

4 | DISCUSSION

Recently, progress has been achieved in understanding the impact of allelic *KIR* variability, *KIR* gene copy number, *KIR* ligand polymorphism (i.e., HLA class I), and viral infections on KIR expression in different individuals.[14,40,42–46,48,50] The cytofluorimetric analysis represents the leading approach to define the presence and proportions of the different NK cell subsets. However, the panel of available anti-KIR monoclonal antibodies is limited, and they often recognize allotypes encoded by more than one *KIR* gene. As far as we know, only anti-KIR mAbs selectively recognizing 2DS4, 3DL1, 2DL3, 2DL4, and 2DL5 molecules are available. The major obstacle in KIR-specific antibody production is the high degree of amino acid identity among different KIRs, resulting in a low number of specific residues, further complicated by the allelic polymorphism that characterizes each locus of this multigene family. In this study, we characterized the specificity of the HP-DM1 mAb, which differs from that

of the other available anti-KIR mAbs, identifying the residues relevant for its binding. Our approach was based on assessing HP-DM1 reactivity performed by cytofluorimetric analysis of HEK-293T cells transfected with plasmids coding for different KIRs or 2DL1 mutants. Introducing into the *2DL1*002* backbone the amino acids present in HP-DM1 negative allotypes, we identified four residues, namely M44, S67, R68, and T70, relevant for HP-DM1 reactivity. The characterization of HP-DM1 epitope allowed us to predict the mAb reactivity with all KIR allotypes present in the IPD-KIR database. In particular, we inferred that HP-DM1 mAb should recognize all the currently identified 2DL1 allotypes except for 2DL1*022 and, most likely, 2DL1*020. Lack of HP-DM1 binding to 2DL1*022 was demonstrated by the negative staining obtained testing the *2DL1*002* M44K mutant, which encodes for a mature protein identical to 2DL1*022 (Figure 4 and Figure S3). Since the dimorphism M/K at position 44 determines the HLA-C K80 and N80 specificity, respectively,[59] the 2DL1*022 allotype recognizes HLA-C N80 molecules.[60–62] 2DL1*022 arose by non-synonymous point mutation most likely from *2DL1*001* and was detected in sub-Saharan African populations (ranging ~17.2% in KhoeSan), but not in

other ethnic groups.[60,61] Considering the 2DL1*022 distribution, the inability of HP-DM1 to bind 2DL1*022 would be relevant whenever sub-Saharan African individuals are analyzed. Moreover, even in the presence of 2DL1*022, HP-DM1 mAb can be a useful reagent since it allows the restricted identification of the NK cell subsets expressing iKIRs recognizing an HLA-C K80 ligand. 2DL1*020 was also detected in sub-Saharan African populations and codes for a receptor characterized by G67 that we proved to diminish (although not abolish) the recognition of HP-DM1 on transfectants. Unfortunately, we did not have the opportunity to confirm this prediction by testing NK cells derived from a 2DL1*020^{pos} donor. Being the level of KIR expression on NK cells lower than that obtained after plasmid transfection, we tentatively included 2DL1*020 allotype among the molecules not recognized by HP-DM1. A similar analysis on 2DS1 alleles, revealed that the HP-DM1 mAb failed to bind all 2DS1 except for 2DS1*013, which is the only one carrying T70. The HP-DM1 binding to 2DS1*013 was verified by positive staining of the mutant 2DS1*002 K70T, which encodes for a mature protein identical to 2DS1*013 (Figure 3 and Figure S3). No information on the 2DS1*013 frequency or the ethnic origin of the donor in which this allele was found is available. Furthermore, none of the 2DL2, 2DL3, 2DS2, 2DS4, and 2DS5 described allotypes include all the amino acids relevant for HP-DM1 binding. Although it has been documented that 2DS3 does not reach the cell surface,[53,63] we also verified whether HP-DM1 could stain the allotypes encoded by this locus. All 2DS3 alleles in the database are characterized by R70, which prevented HP-DM1 binding (Figure 3). The data obtained in this study indicated that HP-DM1 mAb is a unique reagent. Indeed, the other available 2DL1 binding mAbs also react with other KIRs. In particular, EB6B and 11PB6 also recognize 2DS1 and 2DL3 allotypes carrying E35 and R50, while 143211 also binds 2DS5.[8,52,53,56] Moreover, since the number of KIR alleles is expected to increase, based on the introduction of next-generation sequencing approaches, knowledge on the residues required for HP-DM1 binding will allow continuing to predict/revise its reactivity.

The positions of the residues relevant for HP-DM1 binding (i.e., 44, 67, 68, and 70) indicated that the epitope recognized by this mAb is conformational. This hypothesis is supported by the analysis of the 2DL1 crystal structure, showing that M44, R68, and T70 (three out of four residues involved in the HP-DM1 binding) are in the solvent-accessible area at close contact with each other.[64] Moreover, mutational studies have demonstrated the relevance of 2DL1 M44 and T70 in HLA-C K80 recognition.[59,65] Consequently, HP-DM1 should mask receptor/ligand contacts and become a suitable reagent also for

functional studies, allowing the analysis of the 2DL1 contribution in cell target recognition. This prediction was supported by experimental evidence.[66] In this study, characterizing the 143211 epitope, we revealed that the two residues involved in the 143211 recognition (i.e., R68 and T70) were also relevant for the HP-DM1 binding. This information should be considered when performing stainings with the simultaneous use of 143211 and HP-DM1 mAbs.

The more precise detection of 2DL1^{pos} NK cells can be relevant for both basic research and clinical studies. In the haploidentical hematopoietic stem cell transplantation setting, patients receiving the graft from an NK alloreactive donor (i.e., with a population of NK cells expressing only an inhibitory receptor for HLA molecules present in the donor but absent in the recipient) have a better outcome.[67,68] Among the alloreactive NK populations identified using this model (namely, KIR/KIR-L mismatch in graft versus host direction), one is characterized by the expression of 2DL1 receptors only.[69,70] In this context, HP-DM1 should allow a better definition of the alloreactive NK population. Moreover, the use of the HP-DM1 mAb in combination with other available anti-KIR mAbs allows more precise detection of the different KIR^{pos} NK cell subsets.[66] Pregnancy disorder studies are another field that could take advantage of the availability of HP-DM1. In the first trimester of gestation, uterine NK cells are present in the decidua playing a relevant role in the placentation. Several studies demonstrated that women with a high risk of pregnancy disorders have two KIR A haplotypes (namely two copies of 2DL1 alleles coding for "strong receptors") and bear an HLA-C K80 fetus.[71,72]

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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