Phenotypic and Functional Analyses of KIR3DL1$^+$ and KIR3DS1$^+$ NK Cell Subsets Demonstrate Differential Regulation by Bw4 Molecules and Induced KIR3DS1 Expression on Stimulated NK Cells[1]

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Recently, the Z27 mAb was shown to recognize the NK cell-activating receptor KIR3DS1, and several genetic studies suggest that the most probable ligands of KIR3DS1 are HLA class I molecules with the Bw4 motif. Despite these findings, the attempts to establish a functional interaction between KIR3DS1 and its potential ligand have been unsuccessful. Here, we study the proliferation and cytotoxicity of KIR3DS1$^+$ NK cells, compared with KIR3DL1$^+$ NK cells, according to the Bw4$^+$ or Bw4$^-$ allogeneic environment. Our results show for the first time that KIR3DS1 expression on NK cells can be induced after exposure to stimulator cells (221, K562, EBV-B cell lines, and B cells), polyinosinic-polycytidylic acid, IL-15, or IL-2. Furthermore, whereas KIR3DL1$^+$ NK cell proliferation and cytotoxicity were inhibited in a Bw4$^+$ but not a Bw4$^-$ context, KIR3DS1$^+$ NK cell functions were not influenced by the presence of Bw4 on target cells. Nevertheless, despite the absence of demonstrated regulation of KIR3DS1$^+$ NK cell functions by HLA-Bw4 molecules, we found a higher KIR3DS1$^+$ NK cell frequency and higher levels of KIR3DS1 expression in Bw4$^+$ compared with Bw4$^-$ individuals. Altogether, these results suggest that KIR3DS1 does not recognize HLA-Bw4 molecules in a physiological context, and they highlight the induced expression of KIR3DS1 observed on stimulated NK cells and the higher frequency of KIR3DS1$^+$ NK cells in Bw4$^+$ individuals. Because a protective *KIR3DS1-Bw4* association has been reported in viral infections, our results further the understanding of the role of KIR3DS1$^+$ NK cells in controlling viral infections. *The Journal of Immunology,* **2009, 182: 6727–6735.**

Essential components of the innate immune system, NK cells recognize and kill target cells with absent or altered expression of HLA class I molecules. This missing self recognition makes NK cells able to eliminate tumoral and virus-infected cells that lack self HLA class I molecules (1). Furthermore, in the allogeneic context of hematopoietic stem cell transplantation, NK cells from the donor can be alloreactive against recipient cells (2–4). This NK cell alloreactivity is mediated by a balance between activating and inhibitory signals (5) and leads to cytotoxicity and production of proinflammatory cytokines, such as IFN-γ (6). Receptors of HLA class I molecules involved in recognition of target cells partly belong to the killer cell Ig-like receptor (KIR)[3] family (7), which consists of 14 genes and 2 pseudogenes located on chromosome 19q13.4 (8). Within this cluster, some *KIR* genes display an allelic variability with phenotypic and functional consequences.

The *KIR3DL1/S1* locus presents a high degree of polymorphism (66 alleles) and is the most studied of the *KIR* loci (http://www.ebi.ac.uk/ipd/kir/). The receptors encoded by this gene have three extracellular Ig-like domains. KIR3DL1-inhibitory receptor has a long intracytoplasmic tail, containing an ITIM (9). On the contrary, KIR3DS1-activating receptor possesses a short intracytoplasmic tail and present a charged residue within its transmembrane domain that is responsible for its interaction with the activating adaptor protein DAP12 (10).

The expression of KIR3DL1, can be revealed by the binding of two mAbs, DX9 and Z27, with three levels of fluorescence intensity reflecting the receptor density at the cell surface: high (*KIR3DL1*001*, **002*, **008*, and **015* alleles); low (*KIR3DL1*005*, **006*, and **007* alleles); and null (*KIR3DL1*004* allele) (11–13). KIR3DL1 binds HLA class I molecules, from the HLA-A and HLA-B groups, having the serological motif Bw4 between positions 77 and 83: IALR (Bw4*80I), or TALR and TLLR (Bw4*80T; see Refs. 14 and 15). *KIR3DL1* allelic diversity, HLA-Bw4 motif variability, and the loaded peptide sequence may be responsible for differential binding in this KIR-HLA interaction (15, 16). For example, highly expressed *KIR3DL1* allotypes are stronger inhibitory receptors (17), and the Bw4*80I motif has a better affinity for KIR3DL1 than other Bw4 motifs (18). In addition, KIR3DL1$^+$ NK cells are only functional in Bw4$^+$ individuals (19–21).

Contrary to KIR3DL1, which recognizes HLA-Bw4 molecules, the ligand of KIR3DS1 is not determined, whereas a strong homology in their extracellular domain (>97%). Indeed, HLA-Bw4 tetramers loaded with various peptides did not succeed in binding

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[3] Abbreviations used in this paper: KIR, killer-Ig like receptor; MFI, mean channel fluorescence intensity; poly(IC), polyinosinic-polycytidylic acid.

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to KIR3DS1 (22). This absence of interaction in vitro does not exclude protection by the *KIR3DS1-Bw4* association as suggested by genetic studies for the acute hepatitis C virus (23, 24), the human papillomavirus (25, 26), and HIV-1 infections (27–29). The recent findings showing that KIR3DS1 is actually expressed on the cell surface and is detectable by flow cytometry with the KIR3DL1/S1-specific mAb, Z27, at a very low intensity of fluorescence (30–33), led to study KIR3DS1 expression and to investigate a potential interaction between KIR3DS1 and HLA-Bw4. Then, higher levels of KIR3DS1 expression were observed in HIV-1-exposed uninfected individuals (34), and a protective effect of the *KIR3DS1* and *HLA-Bw4*80I* association was shown on the inhibition of HIV replication in T cells mediated by autologous NK cells (35), without demonstrating a functional interaction between KIR3DS1 and HLA-Bw4 molecules.

The absence of an established and functional KIR3DS1-HLA-Bw4 interaction may be explained in part by the experimental approaches used, which were based on a KIR3DS1-transfected NKL cell line (35), Bw4$^+$ tetramers (22), or KIR3DS1 fusion proteins (30, 33). Thus, in this study, we investigated directly KIR3DS1$^+$ NK cells by flow cytometry from *KIR*- and *HLA*-genotyped PBMCs. Taking into account *KIR3DL1*, *KIR3DS1*, and *Bw4*, we studied frequencies, proliferation, and cytotoxic potential of this KIR3DS1$^+$ NK cell population in parallel to the well-characterized KIR3DL1$^+$ NK cell population in different Bw4$^+$ and Bw4$^-$ allogeneic environments.

Materials and Methods

Cells (PBMCs and cell lines)

PBMCs were isolated from citrate-phosphate-dextrose blood from healthy adult volunteers by gradient centrifugation on Ficoll-Hypaque (Lymphoprep, Axis-Shield; PoC). All blood donors were recruited at the Blood Transfusion Center (Établissement Français du Sang, Nantes, France) and informed consent was obtained from all individuals. EBV-B cell lines were obtained by EBV transformation of peripheral B cells using EBV supernatant harvested from the cell line B95–8 (American Type Culture Collection). FEB and DAX, both principal EBV-B cell lines used in the study, were obtained from two different HLA-typed blood donors: FEB (HLA-A*01-*01; -B*08 -*08; -Cw*07 -*07) and DAX (HLA-A*02 -*03; -B*44 -*44; -Cw*05 -*05). B cells were isolated from PBMCs obtained from healthy individuals as described previously (36). HLA class-I deficient 721.221 lymphoblastoid EBV-B cells, referred to as 221 cells, and HLA class I-deficient immortalized myelogenous leukemia line K562 were used as controls in functional assays. The 221 cell line and B*1513- and B*3901-transfected 221 cells (respectively named 221-Bw4 and 221-Bw6) were used to assess NK cell cytotoxicity in functional assays. Cells were cultured in RPMI 1640 (Life Technologies) containing glutamine (Life Technologies) and penicillin-streptomycin (Life Technologies) and supplemented with 10% FBS (Life Technologies).

HLA and KIR genotyping

Genomic DNA was extracted from PBMC (37) and *HLA-A* and *-B* genes and *KIR3DL1/S1* gene were typed via molecular techniques using PCR-sequence-specific primers methods under the conditions recommended by the manufacturers, as described previously (38, 39).

In vitro model of NK cell expansion

Amplification of NK cells was evaluated using an in vitro model based on a protocol described previously (36). Briefly, PBMC were cultured with irradiated allogeneic EBV-B cell lines at a ratio (PBMC:EBV-B cells) of 10:1, in CellGro SCGM medium (CellGenix) containing 10% human AB serum, 50 U/ml penicillin (Life Technologies), 50 μg/ml streptomycin (Life Technologies), and 150 IU/ml IL-2 (Chiron). Cell phenotyping was performed for all conditions of stimulation at days 0, 6, 9, and 16. To assess different stimuli, PBMCs were equally stimulated by irradiated B cells or K562 cells at the same PBMC-stimulator cell ratio (10:1), IL-15 at 5 ng/ml or polyinosinic-polycytidylic acid (poly(IC)) at 50 μg/ml.

Phenotypic analysis by flow cytometry

Cell surface phenotype was determined by four-color flow cytometry using the following mouse anti-human mAbs: anti-KIR3DL1/S1-PE (Z27), anti-KIR3DL1-FITC (DX9) (Beckman Coulter, Immunotech), anti-CD3-PerCP (SK7), and anti-CD56-allophycocyanin (B159), from BD Biosciences. Anti-NKG2A (Z199; Beckman Coulter, Immunotech) mAb was labeled with the FITC fluorochrome before use. Cells were also stained with the corresponding isotype-matched control mAb. Results were expressed as percentages of NK cells expressing KIR3DL1 (CD3$^-$CD56$^+$Z27$^{high/low}$) or KIR3DS1 (CD3$^-$CD56$^+$Z27^{dim}). The purity of sorted B cells was checked using the anti-CD19 (HIB19; BD Biosciences) mAb. Data were collected using a FACSCalibur (BD Biosciences) and analyzed using FlowJo 6.2 software (TreeStar).

CD107a mobilization detected by flow cytometry

KIR3DS1$^+$ and KIR3DL1$^+$ NK cell subsets were tested for their cytolytic potential with the CD107a mobilization assay after stimulation with 221, K562, 221-Bw4 (B*1513), and 221-Bw6 (B*3901) cell lines. Preincubated NK cells with CD107-PECy5 or CD107a-FITC (H4A3; BD Biosciences) were incubated with the different target cells for 5 h at an E:T ratio of 1:1, with brefeldin A (Sigma-Aldrich) at 10 μg/ml for the last 4 h. The cells were surface stained with Z27-PE, NKG2A-FITC, and NKp46-allophycocyanin (9E2; BD Biosciences) or Z27-PE, CD3-PerCP, and NKG2A-allophycocyanin (131411; R&D Systems). Degranulation (CD107a expression) of Z27$^+$NKG2A$^-$ cells was studied by four-color flow cytometry, using NKp46-allophycocyanin-specific mAb to target NK cells with the fourth fluorophore. Data were collected using a FACSCalibur and analyzed using FlowJo 6.2 software.

Statistical analyses

Repeated-measures ANOVA using linear mixed models described previously (36) were used to examine changes in NK cell frequency over time and across EBV-B cell lines (FEB and DAX). p values <0.05 were considered to be statistically significant. Comparisons of KIR3DS1$^+$ and KIR3DL1$^+$ NK cell frequencies from two different series of individuals were performed using Student's t tests. Values of $p < 0.05$ were considered to be statistically significant.

Results

Induction of KIR3DS1 expression on stimulated NK cells

Three different patterns of KIR3DS1 expression could be observed on resting NK cells from *KIR3DS1*$^+$-genotyped individuals by flow cytometry as illustrated in Fig. 1*A*; a Z27^{dim} population clearly distinguished itself from the Z27$^-$ population, evaluated by an isotype control for individual D1, a lower Z27^{dim} population associated with the Z27$^-$ population for individual D2, and no Z27^{dim} population for individual D3. In this study, we compared the frequencies of NK cells (CD3$^-$CD56$^+$) expressing KIR3DS1 (Z27^{dim}) by flow cytometry on resting cells (day 0) and cells stimulated (day 9) by allogeneic EBV-B cell lines. The study was conducted using PBMCs from healthy individua$^+$ls for whom the *KIR3DL1/S1* gene is presented in Table I. In *KIR3DL1*$^-$*/S1*$^+$ individuals illustrated by D4 (Fig. 1*B*), KIR3DS1 expression was weak and difficult to determine on resting NK cells (3.2%). However, after stimulation of D4 PBMC, the KIR3DS1 receptor was expressed on a high proportion of NK cells (38.2%) at a low fluorescent intensity. As a control, in the *KIR3DL1*$^+$*/S1*$^-$ individual D5 (Fig. 1*B*), no Z27^{dim} population was detectable in resting and stimulated NK cells (CD3$^-$CD56$^+$). Because KIR3DS1 is expressed on a large proportion of stimulated NK cells from individuals whose resting NK cells do not express KIR3DS1 (Fig. 1*C*), KIR3DS1 expression is induced on the stimulated NK cell surface. KIR3DS1 expression after stimulation by allogeneic EBV-B cells displayed a peak at ~9 days (data not shown) in all *KIR3DS1*$^+$*KIR3DL1*$^-$ or *KIR3DS1*$^+$*KIR3DL1*^{null} individuals studied ($n = 5$; Fig. 1*D*). This increased KIR3DS1$^+$ NK cell frequency after allogeneic EBV-B cell stimulation was also observed for all *KIR3DL1*$^+$*/S1*$^+$ individuals ($n = 5$) who expressed KIR3DL1 receptor with a low or high mean fluorescence intensity (Fig. 1*E*).

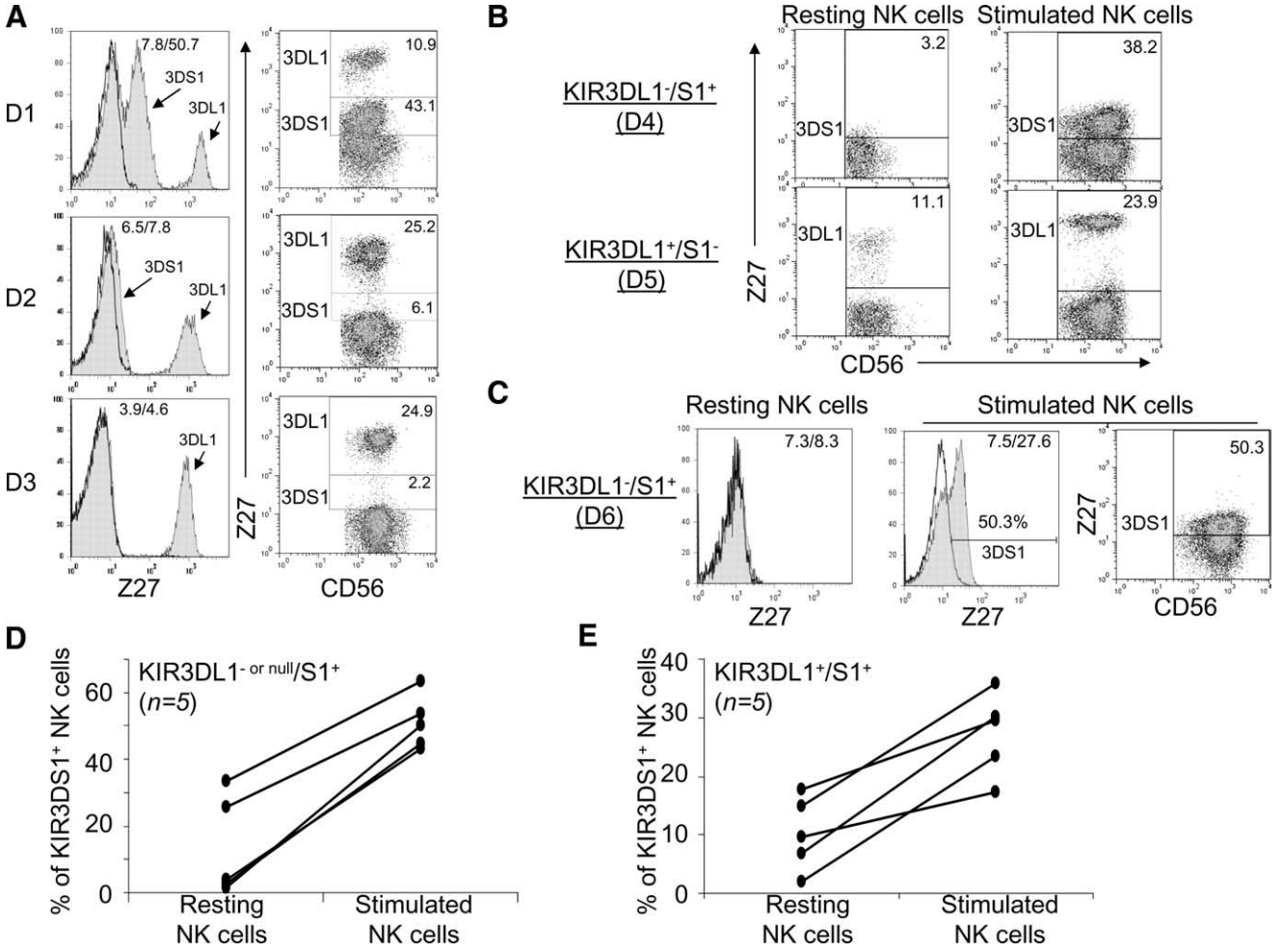

FIGURE 1. Induction of KIR3DS1 expression on NK cells. *A*, Three different patterns of KIR3DS1 expression were observed on resting NK cells from *KIR3DS1*$^+$-genotyped individuals by flow cytometry using Z27 mAb. Histograms and density plots are gated on CD3$^-$CD56$^+$ NK cells and presented for three patterns of KIR3DS1 expression on NK cells, from three representative healthy *KIR3DS1*$^+$ individuals. Cells were stained with the Z27 mAb (shaded histograms) and isotype control (open histograms). MFI values obtained with the isotype control and the Z27 mAb are indicated in histograms. The percentages of KIR3DL1$^+$ and KIR3DS1$^+$ NK cell subsets are indicated in the density plots. *B–E*, PBMCs were stimulated with an irradiated allogeneic EBV-B cell line and the KIR3DL1/S1 phenotype of resting (day 0) and stimulated NK cells (day 9) was determined by flow cytometry using Z27 mAb. *B*, Density plots are gated on resting or stimulated CD3$^-$CD56$^+$ NK cells. Data are from a *KIR3DL1*$^-$*/S1*$^+$ genotyped individual D4 and a *KIR3DL1*$^+$*/S1*$^-$-genotyped individual D5. The percentages of KIR3DL1$^+$ and KIR3DS1$^+$ NK cell subsets are indicated in the density plots. *C*, Histograms are gated on resting or stimulated CD3$^-$ CD56$^+$ NK cells. Cells were stained with the Z27 mAb (shaded histograms) and isotype control (open histograms). MFI values obtained with the isotype control and the Z27 mAb are indicated in histograms. A density plot is gated on stimulated CD3$^-$CD56$^+$ NK cells. The percentage of KIR3DS1$^+$ NK cell subset is indicated. Data are from a single individual representative of four (D4, D6, D8, and D14). *D*, KIR3DS1$^+$ NK cell (CD3$^-$CD56$^+$) frequency was determined for five *KIR3DL1*$^{-\ or\ null}$ (D4 and D6–D9) and *E*, five *KIR3DL1*$^+$ (D10–D14) individuals on resting NK cells and allogeneic EBV-B cell-stimulated NK cells.

Induction of KIR3DS1 expression on NK cells by various stimuli

Because cell phenotype can be modified by cryopreservation and fixation, we investigated both parameters on KIR3DS1 expression. Interestingly, we observed a lower KIR3DS1$^+$ NK cell frequency after one night and even 1 h of culture after thawing PBMCs in comparison with KIR3DS1$^+$ NK cells freshly isolated from peripheral blood, as illustrated for individual D15 (supplemental Fig. S1*A*).[4] In addition, cell fixation with 1% PFA before flow cytometric acquisition had a deleterious impact on KIR3DS1$^+$ NK cell frequency in freshly isolated and thawed cells (supplemental Fig. S1*A*). These results were confirmed in 10 *KIR3DS1*$^+$ genotyped individuals (D1, D2, and D15–D22, supplemental Fig. S1, *B* and *C*). Thus, to investigate the impact of different stimuli on KIR3DS1$^+$ NK cell frequency, we performed the study from overnight cultured PBMCs after thawing and determined the KIR3DS1$^+$ NK cell phenotype without cell fixation. Given that EBV-B cell stimulation of PBMCs induced a large increase in KIR3DS1$^+$ NK cell frequency, we assessed the impact of other stimuli on this latter cell population. To determine the impact of EBV on KIR3DS1 expression by NK cells, PBMC stimulations by an EBV-B cell line and their non-EBV-transformed counterpart cells were compared from four *KIR3DS1*$^+$ genotyped individuals (D4, D13, D14, and D23). A high frequency of KIR3DS1$^+$ NK cells was observed with EBV-B cells and B cells (Fig. 2, *A* and *B*) despite a less significant NK cell expansion with B cells (data not shown). Likewise, as illustrated for the *KIR3DL1*$^-$*/S1*$^+$ individual D17 in Fig. 2*C*, we studied the impact of the myelogenous K562 cell line in comparison with the lymphoblastoid EBV-B 721.221 cell line (221), poly(IC), and IL-15, on KIR3DS1 expression on NK cells from six

[4] The online version of this article contains supplemental material.

Table I. *HLA-Bw4 molecules and* KIR3DL1/S1 *genotypes of studied individuals*

Individual	HLA Typing[a]				No. of HLA-Bw4	*KIR* Typing	
	A*	A*	B*	B*		*3DL1*	*3DS1*
D1	0101	**2402**	0801	3906	1	+	+
D2	0301	2301	3508	5001	0	+	+
D3	0201	2601	1801	4002	0	+	+
D4	0101	0201	0801	**5101**	1	−	+
D5	0201	0301	1501	**4901**	1	+	−
D6	02	02	40	**51**	1	*null*[b]	+
D7	0201	3303	**4901**	**5801**	2	*null*[b]	+
D8	0201	1101	4002	**4402**	1	*null*[b]	+
D9	0201	0201	0801	**5101**	1	−	+
D10	02	02	07	35	0	+	+
D11	0301	0301	0801	1801	0	+	+
D12	0201	0201	3901	**4402**	1	+	+
D13	0101	0201	0801	4002	0	+	+
D14	3101	6801	1801	3901	0	+	+
D15	**2402**	**2402**	4901	5101	2	*null*[b]	+
D16	**2402**	3402	1402	3502	1	*null*[b]	+
D17	0201	0201	4002	**5101**	1	−	+
D18	02	03	**5101**	5501	1	+	+
D19	0205	**2402**	**3801**	**4402**	3	+	+
D20	0201	**2402**	1801	4002	1	+	+
D21	0201	0201	1801	3501	0	+	+
D22	03	11	15	40	0	+	+
D23	0201	3301	1402	1501	0	+	+
D24	0301	**2402**	0702	1401	1	+	−
D25	0201	3301	**5201**	**5801**	2	+	−
D26	1101	2301	3501	**4403**	1	+	−
D27	0201	**2402**	1501	1501	1	+	−
D28	0101	0201	0702	0801	0	+	−
D29	0301	6801	3502	1501	0	+	−
D30	0201	**2402**	0702	4002	1	+	−

[a] Genes and alleles coding for HLA-Bw4 molecules are indicated in bold.
[b] *KIR3DL1* gene is detected by PCR, but the cell surface expression of KIR3DL1 receptor is not detected by flow cytometry.

KIR3DS1$^+$ genotyped individuals (D2, D14–D17, and D19; Fig. 2*D*). As a control, PBMCs were cultured in parallel in culture medium containing IL-2. Under this condition, no NK cell amplification (6.5% of resting D17 NK cells and 4.2% of cultured D17 NK cells) was observed. In the opposite, NK cell (CD3$^-$CD56$^+$) frequency increased after stimulation by 221 (64.3%), K562 (76.7%), poly(IC) (18.6%), and IL-15 (28.0%). The frequency of KIR3DS1$^+$ NK cells (Z27$^+$DX9$^-$) increased not only with stimulator cells (221 or K562) regardless of their origin (lymphoid or myeloid) but also with poly(IC) and cytokines as IL-15 and IL-2 (medium). Of note, the magnitude of this increase is variable according to the studied individuals.

Impact of the Bw4 KIR ligand on KIR3DL1$^+$, but not KIR3DS1$^+$, NK cell expansion in vitro

The modulation of KIR2DL1$^+$ and KIR2DS1$^+$ NK cell frequencies by the allogeneic C2$^+$ or C2$^-$ environment that we have recently shown (36) raises the possibility that the HLA-Bw4 molecule could differentially modulate the in vitro KIR3DL1$^+$ and KIR3DS1$^+$ NK cell expansion. Using a model of NK cell expansion in vitro described previously (36), we investigated the impact of allogeneic HLA-Bw4 ligand on the changes in KIR3DL1$^+$ and KIR3DS1$^+$ NK cell frequency. PBMCs from *KIR3DL1/S1* genotyped individuals were stimulated with Bw4$^-$ or Bw4$^+$ irradiated allogeneic EBV-B cell lines. The frequency of NK cells (CD3$^-$CD56$^+$) expressing KIR3DL1$^+$ (Z27$^{low/high}$) and KIR3DS1$^+$ (Z27^{dim}) was determined by flow cytometry at days 0, 6, 9, and 16 after stimulation by either Bw4$^-$ or Bw4$^+$ EBV-B cell lines. A higher KIR3DL1$^+$ NK cell frequency was observed in the Bw4$^-$ condition (42% at day 16) than in the Bw4$^+$ condition (28.7% at day 16) of stimulation as illustrated for a representative *KIR3DL1$^+$/S1$^+$* genotyped individual D23 (Fig. 3*A*). However, no significant difference in KIR3DS1$^+$ NK cell frequency could be detected between these two Bw4$^-$ and Bw4$^+$ allogeneic conditions of stimulation (18.1 and 18.6%, respectively, at day 16). To confirm this observation, the analyses were conducted in 10 healthy *KIR3DL1$^+$*-genotyped individuals (D5, D11, D12, and D24–D30) for whom *KIR3DL1/S1* genes are indicated in Table I. KIR3DL1$^+$ NK cell frequency was significantly higher ($p = 0.003$) after stimulation of PBMCs with the Bw4$^-$ EBV-B cell line (FEB) compared with stimulation with the Bw4$^+$ EBV-B cell line (DAX) (Fig. 3*B*). Besides, 10 *KIR3DL1$^+$*-genotyped individuals were stimulated with EBV-B cell lines expressing other HLA-Bw4 allelic encoded molecules than those expressed by DAX and with other Bw4$^-$ EBV-B cell lines than FEB. Changes in KIR3DL1$^+$ NK cell frequency according to the presence or the absence of Bw4 confirmed that the KIR3DL1$^+$ NK cell frequency observed after stimulation is higher in a Bw4$^-$ than in a Bw4$^+$ environment (data not shown). As regards the KIR3DS1, stimulation of PBMCs from eight individuals (D4, D7, D8, D11 to D14 and D23) by Bw4$^-$ or Bw4$^+$ EBV-B cell lines similarly increased KIR3DS1$^+$ NK cell frequency after 9 days of culture (Fig. 3*C*). We can therefore conclude that the frequency of the KIR3DL1$^+$ NK cell pool is higher with Bw4$^-$ than with Bw4$^+$ EBV-B stimulator cells. However, the frequency of KIR3DS1$^+$ NK cells is similar with Bw4$^-$ and Bw4$^+$ allogeneic EBV-B cells, suggesting that KIR3DS1$^+$ NK cells do not preferentially respond to the presence of Bw4 ligand on stimulator cells.

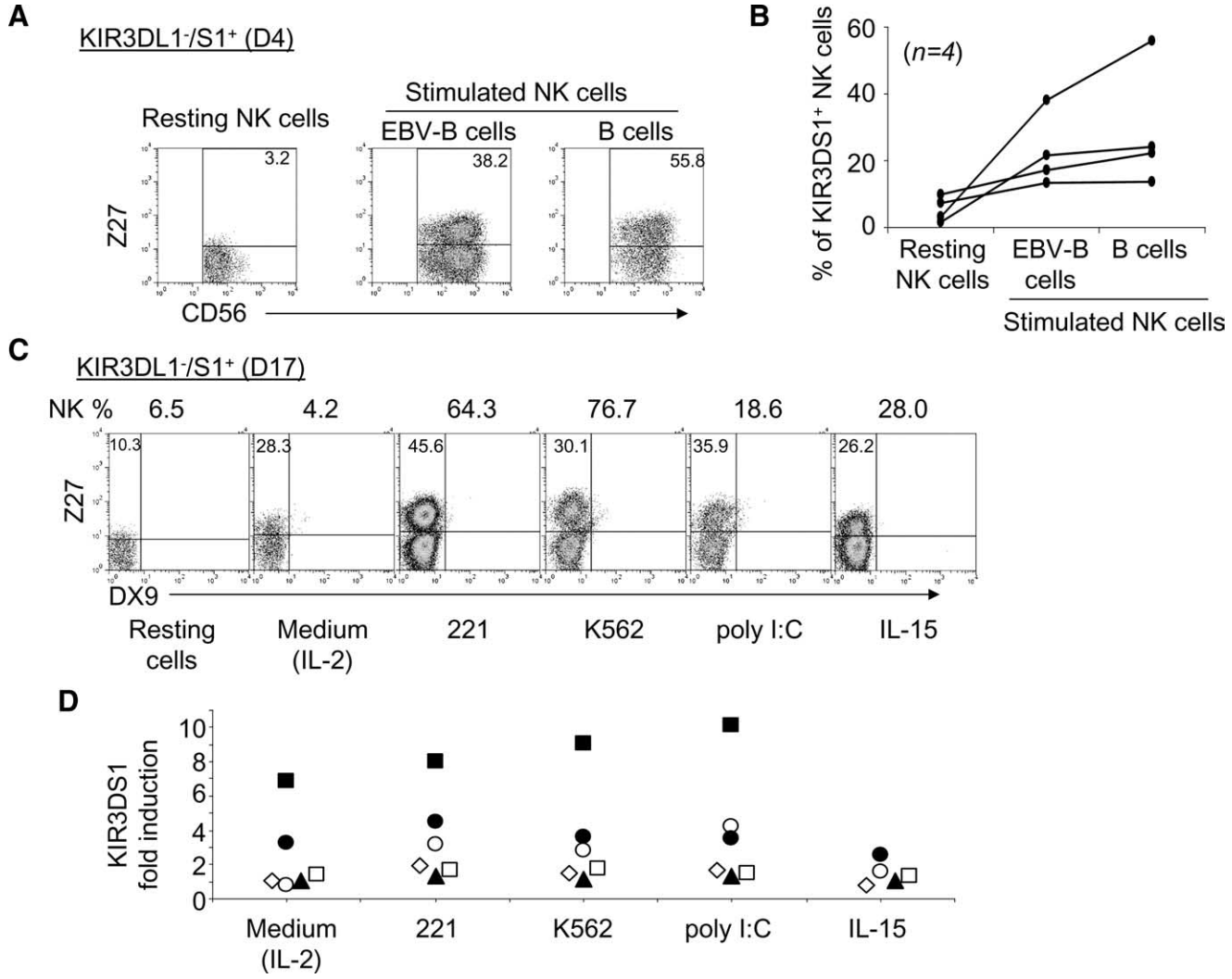

FIGURE 2. Induction of KIR3DS1 expression on NK cells by various stimuli. *A* and *B*, PBMCs from *KIR3DS1*$^+$-genotyped individuals were stimulated with an allogeneic EBV-B cell line and the corresponding B cells. The KIR3DS1 phenotype of resting and stimulated NK cells (CD3$^-$CD56$^+$) was determined by flow cytometry using Z27 mAb. *A*, Density plots presenting percentages of the Z27$^+$ cell subset are shown for resting (day 0) and stimulated (day 9) NK cells from a *KIR3DL1*$^-$*/S1*$^+$-genotyped individual D4. *B*, KIR3DS1$^+$ NK cell (CD3$^-$CD56$^+$) frequencies determined for four *KIR3DS1*$^+$ genotyped individuals (D4, D13, D14, and D23). *C* and *D*, PBMCs from *KIR3DS1*$^+$-genotyped individuals were stimulated with culture medium (IL-2), 221, K562, poly(IC), and IL-15. The phenotype of resting and stimulated cells was realized by flow cytometry. NK cells (CD3$^-$CD56$^+$) were stained with the Z27 and DX9 mAbs. *C*, Density plots presenting percentages of the KIR3DS1$^+$ (Z27$^+$DX9$^-$) NK cell subset are shown from a representative *KIR3DL1*$^-$*/S1*$^+$-genotyped individual D17. *D*, KIR3DS1$^+$ NK cell frequency fold induction (percentage of KIR3DS1$^+$-stimulated NK cells/percentage of KIR3DS1$^+$ resting NK cells) determined for six *KIR3DS1*$^+$ genotyped individuals. ▲, D2; ■, D14; □, D15; ◇, D16; ●, D17; ○, D19.

In contrast to KIR3DL1$^+$ *NK cells, activation of KIR3DS1*$^+$ *NK cell cytotoxicity is Bw4 independent*

Because the HLA-Bw4 molecule did not influence KIR3DS1$^+$ NK cell proliferation, we investigated whether this was also the case for the cytotoxic capacity of these cells. Degranulation experiments (CD107a expression), using 4-color flow cytometry, were performed from 10 *KIR3DL1/S1*-genotyped individuals (D1, D2, and D15 to D22) to determine the conditions of KIR3DL1$^+$ and KIR3DS1$^+$ NK cell cytotoxicity. First, because cell stimulation leads to induce KIR3DS1 expression on NK cells, PBMCs were amplified beforehand for 2 wk with the 221 cell line as stimulator cells to obtain a substantial KIR3DS1$^+$ NK cell population. Given that CD94/NKG2A expression on KIR3DS1$^+$ NK cells may inhibit the triggering signal of KIR3DS1 engagement with its ligand (40), only KIR3DS1$^+$NKG2A$^-$NKp46$^+$ NK cells were targeted (Fig. 4*A*). NKp46-specific mAb was used to target NK cells with only one marker (41). Degranulation of these selected cells was evaluated against HLA class I-deficient myeloid K562, lymphoid 221, 221-Bw6, and 221-Bw4 cell lines (Fig. 4*B*). As control, degranulation of KIR3DL1$^+$ NKG2A$^-$ NK cells (Fig. 4*A*) was determined against the same target cells used in this cytotoxic assay (Fig. 4*B*). Stimulation by the 221 and K562 cell lines activated degranulation of both KIR3DS1$^+$ NKG2A$^-$ and KIR3DL1$^+$NKG2A$^-$ NK cell subsets (Fig. 4*B*). However, while degranulation of KIR3DL1$^+$NKG2A$^-$ NK cells was inhibited by the 221-Bw4 cell line, no difference in degranulation of KIR3DS1$^+$NKG2A$^-$ NK cells was observed between stimulation with the 221-Bw4 and that with 221-Bw6 cell lines. Moreover, the same results were obtained with experiments of degranulation performed on resting KIR3DL1$^+$NKG2A$^-$ and KIR3DS1$^+$NKG2A$^-$ NK cells (data not shown), from resting PBMCs of five individuals. These degranulation assays did not allow us to ascertain a potential triggering engagement of KIR3DS1 with Bw4$^+$ target cells.

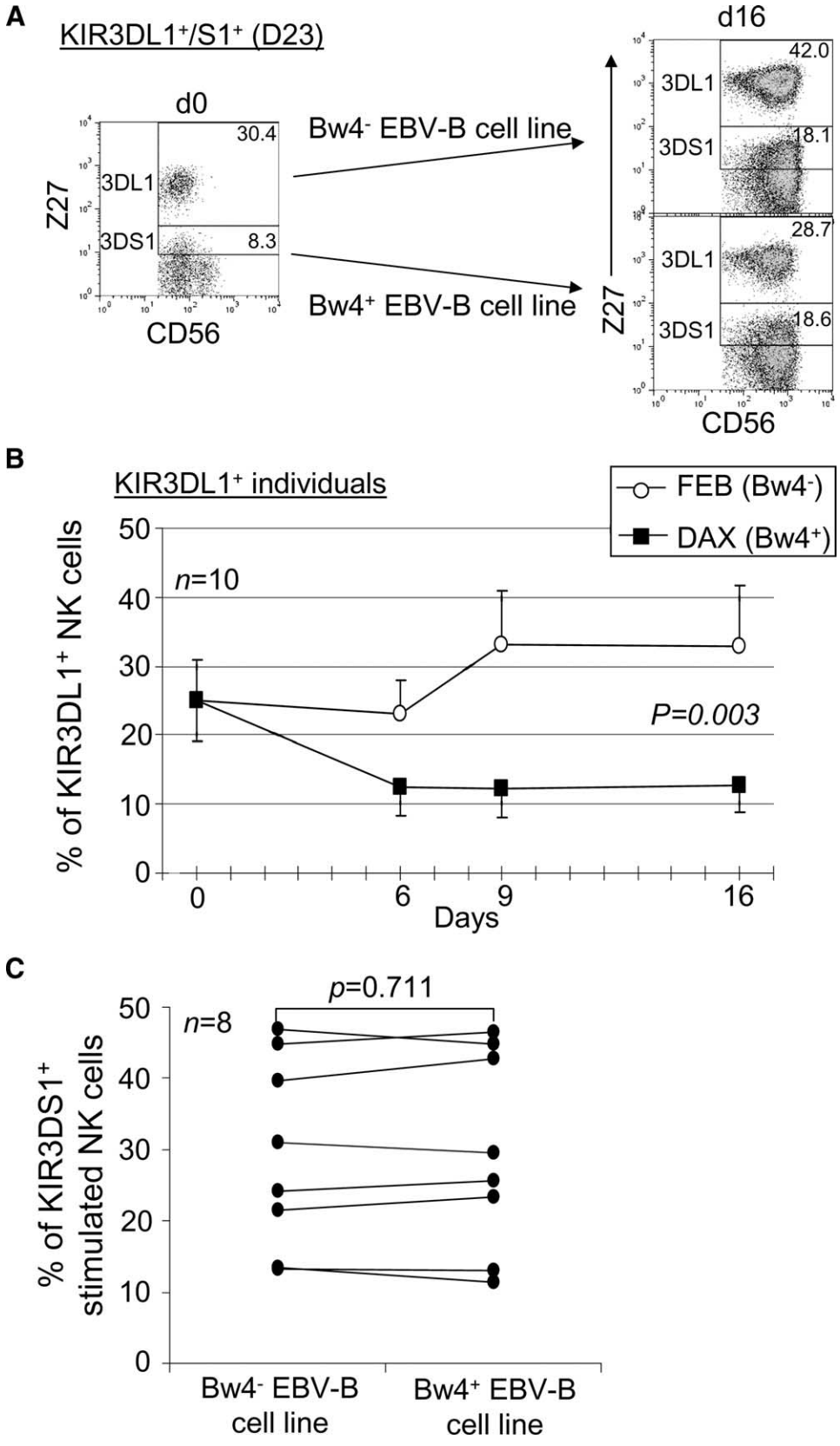

FIGURE 3. Impact of the Bw4 KIR ligand on KIR3DL1$^+$, but not KIR3DS1$^+$ NK cell expansion in vitro. PBMCs from genotyped individuals were cultured with irradiated Bw4$^-$ or Bw4$^+$ EBV-B cell lines. NK cells, defined as the CD3$^-$CD56$^+$ population, were stained with the Z27 mAb. KIR3DS1$^+$ cells were identified as the Z27^{dim} subset and KIR3DL1$^+$ cells as the Z27$^{low/high}$ subset. *A*, Density plots are gated on resting NK cells and on NK cells from *KIR3DL1*$^+$*/S1*$^+$-genotyped individual D23 after 16 days of stimulation by B*0801, B*0801 (Bw4$^-$Bw4$^-$), or B*3501, B*4403 (Bw4$^-$Bw4$^+$) EBV-B cell lines. Percentages of KIR3DL1$^+$ and KIR3DS1$^+$ NK cells are indicated in density plots. *B*, KIR3DL1$^+$ NK cell frequencies were observed by flow cytometry for Bw4$^-$ FEB and Bw4$^+$ DAX EBV-B cell conditions of stimulation at days 0, 6, 9, and 16. Means and significant differences are shown for 10 *KIR3DL1*$^+$*/S1*$^{+/-}$ genotyped individuals (D5, D11, D12, and D24–D30), $p = 0.003$. Repeated-measures ANOVA based on linear mixed models were used. *C*, D7, D8, D11, D12, and D13 PBMCs were stimulated with FEB (Bw4$^-$) and DAX (Bw4$^+$) EBV-B cell lines. D4 PBMCs were stimulated with B*1302, B*4402 (Bw4$^+$/Bw4$^+$) and B*0801, B*0801 (Bw4$^-$/Bw4$^-$) EBV-B cell lines. D14 PBMCs were stimulated with B*4002, B*4402 (Bw4$^-$/Bw4$^+$), and B*1402, B*1501 (Bw4$^-$/Bw4$^-$) EBV-B cell lines. D23 PBMCs were stimulated with B*3501, B*4403 (Bw4$^-$/Bw4$^+$) and B*0801, B*0801 (Bw4$^-$/Bw4$^-$) EBV-B cell lines. KIR3DS1$^+$ NK cell frequencies were observed by flow cytometry for Bw4$^-$ and Bw4$^+$ EBV-B cell conditions of stimulation at day 9. No significant difference was observed for eight *KIR3DL1*$^{+/-}$*/S1*$^+$ genotyped individuals (D4, D7, D8, D11–D14, and D23), $p = 0.711$. A paired Student *t* test was used.

Impact of autologous Bw4 ligand on KIR3DL1 *and* KIR3DS1 *expression by NK cells*

The expression of autologous KIR ligand has been shown to have an impact on the corresponding KIR$^+$ NK cell frequency in the peripheral blood (13, 19). HLA-Bw4 molecules are KIR3DL1 ligands, and genetic analyses have suggested that the HLA-Bw4 molecule could be the ligand of KIR3DS1. Thus, we hypothesized that the presence of *HLA-Bw4* could influence the frequency of KIR3DS1$^+$ NK cells, compared with KIR3DL1 as control. Phenotypic analyses were performed on *KIR3DL1*$^+$ ($n = 53$) and/or

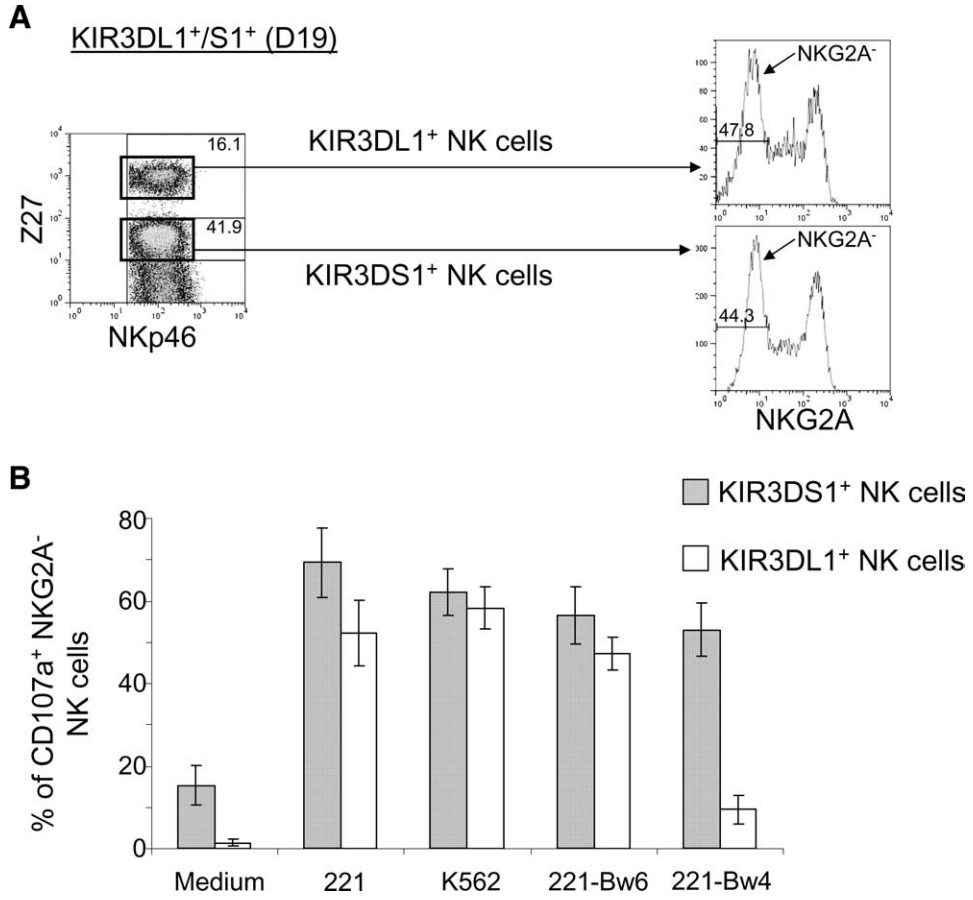

FIGURE 4. Contrary to KIR3DL1$^+$ NK cells, activation of KIR3DS1$^+$ NK cell cytotoxicity is Bw4 independent. PBMCs were amplified for 2 wk with the 221 cell line as stimulator cells to obtain a substantial KIR3DS1$^+$ NK cell population. *A*, KIR3DS1$^+$NKG2A$^-$NKp46$^+$ NK cells and KIR3DL1$^+$NKG2A$^-$ NK cells were targeted. A density plot is gated on NKp46$^+$ NK cells. Percentages of KIR3DS1$^+$ or KIR3DL1$^+$ NK cells are indicated. Histograms showing the NKG2A phenotype of KIR3DL1$^+$ NK cells and KIR3DS1$^+$ NK cells are gated on Z27^{high} NK cells and Z27^{dim} NK cells, respectively. Percentages of NKG2A$^-$ NK cells are indicated. Data are shown for a representative *KIR3DL1$^+$/S1$^+$* individual D19 of 10 individuals studied (D1, D2, and D15 to D22). *B*, Degranulation of both KIR3DS1$^+$NKG2A$^-$NKp46$^+$ ($n = 10$) and KIR3DL1$^+$NKG2A$^-$NKp46$^+$ ($n = 7$) gated cell subsets was evaluated against HLA class I deficient myeloid K562 ($n = 6$), lymphoid 221 ($n = 10$), 221-Bw6 ($n = 9$), and 221-Bw4 ($n = 10$) cell lines. The E:T ratio was 1:1. Degranulation assays were performed as described in *Materials and Methods*. CD107a expression was defined by flow cytometry after stimulation with 221, K562, 221-Bw6, and 221-Bw4 in parallel to the control (medium). Means and SDs are shown for 10 individuals studied (D1, D2, and D15 to D22).

KIR3DS1$^+$ ($n = 32$) genotyped PBMCs using KIR3DL1/S1-specific Z27 mAb. Surprisingly, no significant difference was observed between KIR3DL1$^+$ NK cell frequency in PBMCs from *Bw4*$^+$ ($n = 38$) and *Bw4*$^-$ ($n = 15$) individuals (Fig. 5*A*). Contrary to this, the frequency of KIR3DS1$^+$ NK cells was significantly higher ($p = 0.002$) in the presence of at least one HLA-Bw4 motif

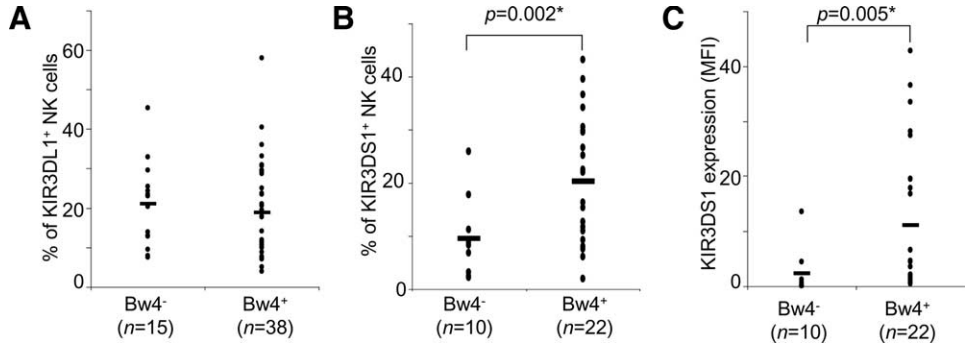

FIGURE 5. Impact of autologous Bw4 on KIR3DL1 and KIR3DS1 expression on NK cells. Flow cytometric analysis of NK cells from 53 *KIR3DL1*$^+$- and 32 *KIR3DS1*$^+$-genotyped individuals was performed with the anti-KIR3DL1/S1 mAb Z27. NK cells were described as CD3$^-$CD56$^+$KIR3DS1$^+$ NK cells as the Z27^{dim} cell subset and KIR3DL1$^+$ NK cells as the Z27$^{low/high}$ cell subset. *A*, The frequencies of KIR3DL1$^+$ NK cells were compared between Bw4$^-$ ($n = 15$) and Bw4$^+$ ($n = 38$) individuals, $p = 0.264$. *B*, The frequencies of KIR3DS1$^+$ NK cells were compared between Bw4$^-$ ($n = 10$) and Bw4$^+$ ($n = 22$) individuals, $p = 0.002$. *C*, KIR3DS1 expression (MFI) on NK cells is lower in Bw4$^-$ ($n = 10$) than in Bw4$^+$ ($n = 22$) individuals, $p = 0.005$. Student's *t* test with bilateral distribution and unequal variance was used for all statistical analysis.

(Fig. 5*B*). Because three patterns of KIR3DS1 expression were observed (Fig. 1*A*), we suggested that the level of KIR3DS1 expression, provided by the median fluorescence intensity (MFI), could be a more relevant indicator than the KIR3DS1$^+$ NK cell frequency, which itself is too difficult to assess. As a consequence, the MFI of the KIR3DS1$^+$ cells stained with Z27 mAb was evaluated after subtraction of the background fluorescence of the negative or isotype control. By this approach, the level of KIR3DS1 expression on NK cells was also significantly higher ($p = 0.005$) in the presence of at least one HLA-Bw4 molecule than in its absence (Fig. 5*C*).

Discussion

The recent interest in *KIR3DS1* can be explained, not only by the newly discovered possibility to detect its expression by flow cytometry (30–33) but also by the genetic associations favoring protection to AIDS (27–29, 42–44), clearance of hepatitis C virus (23) and resistance to cervical neoplasia (25, 26) or hepatocellular carninoma (24). In this study, we show for the first time that, even for individuals with a very low proportion of KIR3DS1$^+$ NK cells on resting PBMCs, the expression of KIR3DS1 is induced on NK cells after stimulation with stimulator cells (221, K562, EBV-B cell lines, and B cells), poly(IC), and cytokines as IL-15 and IL-2. The fact that these various stimuli can induce KIR3DS1 expression suggests that this activating receptor could possibly be considered as a marker of NK cell activation. The induction of KIR expression on NK cells is poorly described in literature. Certain allelic variants of *KIR2DL4* have been shown to be induced on cultured NK cells, either as a full-length and detectable protein, or as a secreted receptor (45). A higher KIR3DS1 expression on NK cells has been observed in vivo in the context of HIV infection (34). As a result, this induction of KIR3DS1 expression might play a protective role in the control of viral infections.

Some genetic studies (23–29, 46–49) have highlighted an association of the *KIR3DS1* with HLA-Bw4 molecules, suggesting that the HLA-Bw4 molecules are the best candidate as the KIR3DS1 ligand. However, we show in this present study that even KIR3DL1$^+$ NK cell frequency was significantly higher after 2 wk of coculture in a HLA-Bw4$^-$ allogeneic environment than in a HLA-Bw4$^+$ environment, the proliferation of KIR3DS1$^+$ NK cells is not regulated by the Bw4$^+$ environment. The expression of KIR3DS1 was equally induced regardless of the autologous or the allogeneic Bw4$^-$ or Bw4$^+$ environment. Moreover, degranulation assays, targeting amplified NKG2A$^-$KIR3DS1$^+$ NK cells, did not allow us to discern a triggering functional interaction between KIR3DS1 and the Bw4 molecule, even though these experimental conditions are closer to the in vivo context than those of previous studies (22, 30, 33, 35).

Despite the absence of demonstrated regulation of KIR3DS1$^+$ NK cell functions by HLA-Bw4 molecules, we found a statistically significant difference in KIR3DS1$^+$ NK cell frequency and the level of KIR3DS1 expression, between the absence and the presence of at least one autologous *HLA-Bw4*. On the other hand, no significant difference was observed in KIR3DL1$^+$ NK cell frequency according to the presence or absence of the autologous ligand Bw4. The discrepancy between these observations and previous reports (13, 32) can potentially be explained by different allelic distributions of *KIR3DL1/S1* and *HLA-Bw4* between our European cohort and American (32) and Japanese (13) cohorts (47–50). These data support an influence of HLA-Bw4 molecules in shaping the KIR3DS1$^+$ NK cell subset during development.

One hypothesis proposed in the literature still supporting HLA-Bw4 as a ligand for KIR3DS1 is that a viral or a tumoral peptide is loaded by HLA-Bw4 molecules and stabilizes a functional interaction with KIR3DS1. A previous study supported this loaded peptide model, with a reinforced lysis of Bw4$^+$ cell targets infected by HIV-1 (35), but without pinpointing a particular peptide. It has been recently reported that a T cell escape variant HIV peptide bound to HLA-Cw4 constitutes a better ligand for KIR2DL1 than the consensus peptide (51). Moreover, different publications underlined the impact of the loaded peptide on the KIR-HLA affinity as described for KIR3DL2 (52) and KIR3DL1 (15). Another hypothesis is raised by the Ly49H$^+$ (a murine activating KIR receptor counterpart) NK cell population which controls murine CMV infection by recognizing a murine MHC-like protein, m157 (53, 54). Thus, it is feasible to envisage that KIR3DS1 binds to a protein expressed by infected or tumor cells. This has already been shown for KIR2DS4, another activating KIR, which interacts functionally with a non-HLA ligand expressed only on tumoral cell lines (55).

Finally, although in the KIR2DL1/KIR2DS1 pair only one of these two homologous receptors can be functional, depending on the presence or absence of HLA-C2 ligand (36), the KIR3DL1 and KIR3DS1 receptors do not act in the same way. In fact, KIR3DL1$^+$ NK cell functions (degranulation and proliferation) are regulated by HLA-Bw4, but the regulation of KIR3DS1$^+$ NK cell functions is Bw4 independent in physiological conditions. However, we highlight a large induction of KIR3DS1 expression on NK cells after stimulation with various stimuli and a higher KIR3DS1$^+$ NK cell frequency in Bw4$^+$ individuals. Altogether, these data suggest that, in contrast to KIR3DL1, KIR3DS1 is not implicated in non-self recognition by NK cells and does not recognize HLA-Bw4 molecules in physiological context. These results further the understanding of the role of KIR3DS1$^+$ NK cells in controlling viral infections.

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Disclosures

The authors have no financial conflict of interest.

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