Cutting edge: selective expression of inhibitory or activating killer cell Ig-like receptors in circulating CD4+ T lymphocytes.

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Selective expression of inhibitory or activating killer cell Ig-like receptors in circulating CD4$^+$ T lymphocytes [1]

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Running title: KIR expression in CD4$^+$ T cells

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Apart from NK cells, TCRγδ and $CD8^+$ T cells, killer cell Ig-like receptors (KIR) expression was described on a minor subset of $CD4^+$ T cells. However, their functions remain to be elucidated in this latter lymphocyte population. We demonstrated that KIR2DL2/L3 (CD158b) and KIR2DS2 (CD158j) transcripts were synthesized by sorted $CD4^+CD158b/j^+$ T cells obtained from healthy individuals. In contrast, we observed that only the inhibitory or activating receptor was expressed at the cell surface according to the donor tested. In CD158b-expressing cells, KIR-triggering leads to an inhibition of the CD3-induced cell proliferation and Erk activation, and the receptor exhibits an activation-dependent tyrosine-phosphorylation and association with SHP-1. In CD158j-positive cells, KIR-engagement results in an enhanced CD3-mediated cell growth and Erk phosphorylation. Our results suggested that in contrast to NK cells, the functions of KIR in $CD4^+$ T lymphocytes might derive from a selective expression of their activating or inhibiting forms.

Introduction

The killer cell Ig-like receptors (KIRs)[4] belong to a family of structurally related cell membrane receptors that recognize MHC class I molecules. While KIRs are mainly expressed by functional circulating NK cells, they were also found on $CD8^+$ and TCRγδ T lymphocytes, and on a marginal $CD4^+$ T cell population (1-3). KIR molecules were classified according to their structure and functional properties into inhibitory and activating forms. Inhibitory receptors (KIR-L) are characterized by a long cytoplasmic tail where are located consensus ITIMs. Once phosphorylated, these latter allow the recruitment of downstream phosphatases, leading to the general shutdown of NK cell effector functions. In contrast, activating KIRs (KIR-S) encompass a short cytoplasmic tail and mainly use the ITAM-containing adaptor molecule DAP12 to deliver activating signals. For a given receptor, both forms are usually expressed on NK cells and the balance between their respective signaling pathway then determines the ability of NK cells to lyse specific targets (4).

The expression of NK receptors on effector/memory $CD4^+$ T cells was essentially described under chronic inflammatory conditions. Indeed, it has been reported that clonally expanded $CD4^+CD28^{null}$ T cells from patients with rheumatoid arthritis (RA) expressed KIRs (5), with a preferential expression of the activating receptor KIR2DS2/CD158j (6). In these cells, which do not express DAP12, CD158j was found to selectively activate the DAP12-independent JNK signaling pathway, and was therefore thought to contribute to the T cell-mediated autoimmune disease (7). Similarly, KIR expression was reported on $CD4^+CD28^{null}$ T cells from patients with acute coronary syndromes (ACS), with again a preference for the stimulatory variant CD158j (8). However, DAP12 was frequently transcribed by the ACS patients-derived clones, and CD158j-mediated cytotoxicity was exclusively observed in T-cell clones that expressed both CD158j and DAP12. More recently, we identified KIR3DL2/CD158k as a phenotypic marker of Sézary cells (9). We further reported the expression of CD158a and CD158b on the circulating $CD4^+$ malignant cells isolated from one Sézary syndrome (SS) patient. In this case, both KIR were found to display a co-activatory function through the recruitment of the JNK-dependent signaling pathway, although the receptors were efficiently expressed under their activating and inhibitory forms at the cell surface (10).

The expression of KIRs on $CD4^+$ T lymphocytes in healthy individuals was more controversial. It has been initially reported that among the $CD3^+KIR^+$ cells from healthy donors, 7±3% expressed CD4 (11). However, a recent report by van Bergen et al. established that, in healthy individuals, an average of 0.2% of $CD4^+$ T lymphocytes was stained with anti-KIR mAbs (3). In addition, KIR expression was first thought to be restricted to the $CD4^+CD28^-$ lymphocytes subset (12), until it was showed that the $CD4^+CD28^+$ cells displayed a broader repertoire of KIR than the $CD4^+CD28^-$ subpopulation (3). Finally, while the presence of KIR on $CD4^+$ T lymphocyte subsets was better documented, the precise function of these receptors remained to be established. Here we provide data regarding CD158b1/b2 (KIR2DL2/KIR2DL3) and CD158j (KIR2DS2) cell surface expression and function in normal $CD4^+$ T lymphocytes.

Materials and Methods

Antibodies

FITC-conjugated anti-CD4 (13B8.2) and PE-coupled anti-CD158b/j (GL183) and -CD28 (28.2) mAb, purified anti-CD16 (3G8) mAb, and their corresponding isotype-matched control mAbs, were from Beckman Coulter (Marseille, France). The specific anti-KIR2DL3/CD158j mAb (ECM41; IgM) was kindly provided by Pr A. Moretta (University of Genova, Italy), while anti-CD3 mAb (ANA 3, IgG1) was produced locally.

Cell sorting and amplification

PBMC were isolated from heparinized venous blood from healthy volunteers by density gradient centrifugation. To isolate the $CD4^+CD158b/j^+$ subpopulation, cells were simultaneously stained with FITC-conjugated anti-CD4 mAb and PE-coupled anti-CD158b/j (GL183) mAb for 30 minutes at 4°C. $CD4^+GL183^+$ cells were further sorted using a MoFlow cell sorter (Dako Cytomation, Trappes, France). Cells were cultured in RPMI 1640 medium, supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), 2 mM L-glutamine (all from Invitrogen, Cergy Pontoise, France), 10% heat-inactivated human serum (Institute of Biotechnologies, Reims, France), and 200 IU/ml rIL2 (a gift of Sanofi-Aventis, Labège, France). Mixed irradiated allogenic PBMC plus 2 µg/ml PHA (Sigma-Aldrich, St Quentin Fallavier, France) were added at the start of the culture and subsequently at 7- to 10-day intervals.

Flow cytometry

Cells were stained with PE-labeled anti-CD158b/j, or FITC-conjugated anti-CD4 or -CD28 mAb using a standard protocol. Alternatively, anti-KIR2DL3 mAb was used followed by goat anti-mouse IgM PE-labeled Ig. Appropriate isotype-matched mAbs were used as controls. Cell analyses were performed on an Epics flow cytometer (Beckman Coulter).

Proliferation assays

96-well plates were coated with suboptimal to optimal concentrations of anti-CD3 together with anti-CD16 (as isotype-matched control) or anti-CD158b/j mAb. Cells ($7.5x10^4$ in 200 µl of culture medium) were cultured for 4 days, and pulsed with 1 µCi of ^{3}H[TdR] for additional 16 h. Incorporation of ^{3}H[TdR] was measured in a liquid scintillation counter (Topcount, Packard Instruments). Results were expressed as the mean of triplicates ± SD.

KIR transcripts analysis

Total mRNA were extracted from $5x10^6$ cells using the TRIzol reagent according to the manufacturer's instructions (Invitrogen). Reverse transcription was performed using an oligo-$dT_{(12-18)}$ primer (Invitrogen) and the Powerscript reverse transcriptase (BD ClonTech, Le Pont de Claix, France). Specific primers for the amplification of KIR2DL2, KIR2DL3 and KIR2DS2 cDNA, as well as amplification conditions, were as described previously (13). β-actin cDNA amplification was performed in parallel as internal control.

Cell surface biotinylation, immunoprecipitation and immunoblotting

To detect KIR surface expression, cells were biotinylated with sulfo-NHS-LC-biotin prior to lysis (Perbio Sciences, Brebières, France) as previously reported (14). After washes, cells ($2x10^7$ per sample) were either left untreated or incubated with 1 mM sodium vanadate and 0.03% H_2O_2 for 2 min at room temperature. Cells were washed and resuspended in 1% NP40 lysis buffer (20mM Tris-HCl pH7.5, 150mM NaCl, 1% NP40, 1 mM Na vanadate, 10 mM NaF, 1 mM PMSF, 1 µg/ml aprotinin, and 1 µg/ml leupeptin) for 1h at 4°C. After a pre-clearing step on protein A sepharose beads, lysates were subjected to immunoprecipitation with 5 µg of control mIgG1 or anti-CD158b/j GL183 mAb. Immunoprecipitates were collected on protein G sepharose beads, separated by SDS-PAGE, and transferred onto a nitrocellulose membrane. To detect biotinylated proteins, membranes were directly probed with streptavidin-peroxidase and revealed with an ECL system (Perbio Sciences). Alternatively, blots were probed in series with an anti-phosphotyrosine mAb (4G10, Upstate Biotechnology Inc., Lake Placid, NY) and anti-SHP1 antibodies (Cell Signaling Tech., Ozyme, St Quentin en Yvelines, France), or anti-CD3ζ, -

DAP10 and -DAP12 antibodies (all from Santa Cruz Biotechnology, Santa Cruz, CA) followed by the appropriated HRP-conjugated secondary antibodies (Jackson Immunoresearch/Beckman Coulter).

For the detection of Erk phosphorylation, $CD4^+GL183^+$ T cells were incubated with control mouse IgG1, anti-CD3 or GL183 mAb alone or in combination, and then cross-linked with rabbit anti-mouse IgG antibodies (Jackson Immunoresearch). Activation was carried for 20 min at 37°C. Cells were harvested immediately after stimulation and lysed. Following SDS-PAGE and transfer to nitrocellulose membrane, the blot was probed with an anti-phospho-Erk1/2 mAb (Cell Signaling Tech.), stripped, and re-probed with an anti-Erk1/2 antibody (Cell Signaling Tech.). Quantification of phospho-Erk immunoblot was performed by densitometry analysis and a relative densitometric intensity (RDI) value was assigned to each band, the RDI of 1 being assigned to the maximal density value obtained under optimal CD3-mediated activation.

Results and Discussion

GL183 mAb triggers activating or inhibitory signals on CD4$^+$GL183$^+$ T lymphocytes from healthy individuals

By performing initial KIR expression analysis on sorted CD4$^+$ T lymphocytes from healthy individuals, we observed a preferential cell staining with the anti-CD158b/j mAb GL183, as reported elsewhere ((3) and data not shown). To further establish the function of CD158b/j on normal CD4$^+$ T cells, the CD4$^+$GL183$^+$ cell subset, that represented 0.05 to 0.2% of the circulating CD4$^+$ T lymphocytes (not shown), was isolated from the blood of 6 healthy donors, and the whole double-positive population from each individual was further expanded *in vitro* for no longer than 4 weeks. Immunostaining analyses confirmed that the sorted and amplified cells stably expressed CD4 and CD158b/j, as exemplified by the data obtained on cells arising from two representative donors (Fig. 1). Furthermore, among the CD4$^+$GL183$^+$ population, CD28 expression seemed to vary from one donor to another. Thus, the amplified CD4$^+$GL183$^+$ cell subset may consist in a mix of CD28$^+$ and CD28$^-$ cells, or exclusively in CD28^{dim} cells (Fig. 1). Finally, we ensured that the *in vitro*-derived cells were representative of the entire circulating CD4$^+$CD158b/j$^+$ T lymphocyte population, and therefore did not correspond to a clonal cell expansion, by performing cell staining analyses using specific anti-TCRVβ mAbs. This led to the detection of several sub-clones exhibiting distinct TCRVβ rearrangement for all donor tested (data not shown).

The influence of CD158b/j on the proliferation of CD4$^+$GL183$^+$ T lymphocytes was then evaluated for each individual. As demonstrated by the results obtained on two representative donors, the *in vitro*-amplified CD4$^+$CD158b/j$^+$ cells showed a dose-dependent proliferation upon CD3 triggering (Fig. 2A). However we observed a significant, but opposite, effect of CD158b/j engagement using GL183 mAb on the CD3-mediated proliferation of the cells tested. Thus, a dramatic inhibition of the CD3-induced cell proliferation was detected upon CD158b/j co-ligation on sorted cells corresponding to one set of donors (n=2/6 ; Fig. 2A, group 1). In contrast, CD158b/j triggering resulted in a significant increase in the CD3-mediated cell growth of the CD4$^+$GL183$^+$ subpopulation isolated from a second set of individuals (n=4/6;

Fig. 2A, group 2). Thus, depending on the donor, GL183-mediated KIR targeting up- or down-regulated the CD3-mediated proliferation of $CD4^+GL183^+$ T lymphocytes.

Selective expression of CD158b and/or CD158j on $CD4^+$ T lymphocytes

As a result of the high sequence identity existing within the extracellular domain of the inhibitory (KIR-L) and the activating (KIR-S) forms of a given KIR, the commercially available anti-KIR mAb usually recognize both structures. Thus, the generic anti-CD158b/j mAb GL183 efficiently targets KIR2DL2 (CD158b1), KIR2DL3 (CD158b2) and KIR2DS2 (CD158j) receptors. To establish if the detection of an activating or inhibitory function of CD158b/j on the CD3-induced cell proliferation was related to a donor-dependent expression of the KIR-L or KIR-S molecules, the analysis of their mRNA synthesis was first performed. A similar, but distinct, pattern of CD158b/j transcripts synthesis was observed according to the donors group tested. Indeed, KIR2DL2, KIR2DS2 and KIR2DL3 cDNA were detected in the $CD4^+GL183^+$ T cells isolated from a representative donor belonging to group 1 (n=2; Fig. 2B). Moreover, only KIR2DL2 and KIR2DS2 transcripts were amplified from the cells corresponding to a donor representative of group 2 (n=4; Fig. 2B). These results established that, regardless of the donor, the *in vitro*-derived cells possessed the transcriptional machinery for the concomitant expression of an inhibitory (CD158b1 and/or b2) and an activating (CD158j) KIR.

To assess which of the three KIR was efficiently expressed at the surface of $CD4^+GL183^+$ T lymphocytes, cells were surface-biotinylated and further subjected to an immunoprecipitation procedure using GL183 mAb. The immunoprecipitates obtained on cells corresponding to individuals from group 1 revealed an exclusive detection of inhibitory molecule(s) presenting an apparent molecular mass of 58 kDa (Fig. 2C, upper panel, group 1). Further flow cytometry analyses performed with a specific anti-KIR2DL3/CD158b2 mAb (15) showed that this inhibitory receptor was expressed at the cell surface (Fig. 2C, bottom panel). However, the lack of a specific anti-KIR2DL2 mAb did not allow to establish if the detection of KIR2DL2/CD158b1 transcript also corresponded to protein expression. In contrast, in $CD4^+GL183^+$ T cells corresponding to donors from group 2, only the activating KIR2DS2 receptor was

efficiently immunoprecipitated by GL183 mAb, as assessed by the detection of a unique 50 kDa protein (Fig. 2C, group 2). These data demonstrated that, according to the donor, and despite the simultaneous synthesis of both KIR-L and KIR-S mRNA, either an inhibitory CD158b or an activating CD158j receptor is expressed at the cell surface of $CD4^+GL183^+$ T lymphocytes. This therefore distinguishes $CD4^+KIR^+$ T cells from NK cells, since in the latter cell population a parallel synthesis of the KIR-L and KIR-S forms was usually reported for a given KIR. One can further hypothesize that in $CD4^+$ T cells the function of a KIR might be driven by the selective and regulated expression of its KIR-L or KIR-S form rather than by a differential affinity of each receptor form for a common ligand, as previously suggested for KIR2DS1/L1 and KIR2DS2/L2 (16).

Analysis of CD158b- and CD158j-mediated signaling pathways when expressed in $CD4^+$ T lymphocytes

To definitely demonstrate that the exclusive expression of either KIR2DL2/L3 or KIR2DS2 dictates the KIR function in $CD4^+$ T lymphocytes, CD158b- or CD158j-dependent signaling pathways were analyzed. Inhibitory KIR were shown to exert their activity through the phosphorylation of their intracellular ITIM(s) that subsequently allows their interaction with the phosphatase SHP-1 (17). We therefore investigated the tyrosine phosphorylation status of CD158b in $CD4^+GL183^+$ T cells isolated from a healthy donor classified in group 1. To this end, cells were left untreated or incubated with vanadate, and subjected to immunoprecipitation using GL183 mAb. Immunoblot analysis of the anti-CD158b precipitates with an anti-phosphotyrosine mAb allowed the detection of an activation-dependent phosphorylation of the KIR-L isoforms (Fig. 3A, left panel, group 1). Consequently, an interaction of the phosphorylated receptor with the phosphatase SHP-1 was evidenced in vanadate-treated cells (Fig. 3A, left panel, group 1). A similar experiment was performed on $CD4^+$ T cells expressing CD158j (Fig. 3A, right panel, group 2). Since stimulatory receptors were shown to initiate intracellular signals through their association with ITAM-containing adaptor molecules, among which DAP10, DAP12, or CD3ζ (17), a possible interaction of CD158j with one of these molecules was investigated. However, neither DAP10 nor DAP12 was found expressed in $CD4^+$ T cells, or detected in CD158j immunoprecipitates (Fig. 3A, right panel). Furthermore, no co-precipitation of CD3ζ with CD158j was observed following cell

activation. Thus, it seems that in KIR2DS2-expressing cells, the activating function of the receptor relays on its interaction with an unusual adaptor molecule which identity remains to be defined. Interestingly, a similar observation was done in $CD4^+$ T cell clones isolated from patients with RA or SS. Thus, in these diseases, a KIR2DS2-mediated DAP12-independent cell activation was also described (8, 10).

In order to ascertain that the exclusive expression of a KIR-L or KIR-S molecule functionally influences $CD4^+$ T cell activation process, the effect of CD158b or CD158j engagement on ERK signaling pathway was investigated. To this aim, $CD4^+$ T cells were stimulated with control mouse IgG1, anti-CD3 or -CD158b/j mAb, or a combination of anti-CD3 and anti-CD158b/j mAb. The corresponding cell lysates were analyzed by Western blot for the detection of phosphorylated ERK (Fig. 3B). Note that the concentration of anti-CD3 mAb used for activation (1 and 0.2 μg/ml for group 1 and group 2 donors, respectively) was the one previously showed to allow the detection of a maximal effect of KIR-engagement on CD3-mediated cell proliferation (see Fig. 2A). In KIR2DL2/L3-expressing cells, the crosslinking of CD3 with an optimal concentration of mAb (1 μg/ml) together with the KIR allowed the detection of phosphorylated ERK, the phosphorylation level being lower than the one reached upon optimal CD3-mediated activation (Fig. 3B, group 1). In addition, the use of a suboptimal concentration of anti-CD3 mAb (0.2 μg/ml) on KIR2DS2-positive cells resulted in a discrete ERK activation that is increased in the presence GL183 mAb (Fig. 3B, group 2). However, in these cells, the triggering of KIR2DS2/CD158j alone was not sufficient by itself to promote ERK activation, as previously reported (18). Thus, in normal $CD4^+$ T lymphocytes, CD158b or CD158j fulfills a co-receptor function and efficiently modulates the CD3-mediated ERK activation when engaged.

In conclusion, we showed that KIR are expressed as functional co-receptors in $CD4^+$ T lymphocytes from healthy individuals. We further demonstrated that, in this lymphocyte population, the detection of an inhibitory or activating KIR function might correspond to an exclusive and regulated expression of a KIR-L or KIR-S form. In this context, the nature of the delivered KIR-mediated signal would not relay on a commonly proposed model involving the receptors binding affinities to a common

ligand (16), but rather reflects the structural features of the expressed molecule. In our study, the overall $CD4^+KIR^+$ T cell subset isolated from each donor, while not representing a clonal cell population, was found to homogeneously express a single form of the receptor and consequently to uniformly answer to KIR triggering in terms of cell proliferation and ERK activation. One can therefore postulate that the preferential expression of an activating KIR on $CD4^+$ T lymphocytes might render a single individual more susceptible to inappropriate stimuli leading to the proliferation and growth escape of these cells, as observed in patients suffering from RA, ACS, or SS.

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Footnotes

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[4] Abbreviations used: ACS: acute coronary syndrome, KIR: killer-cell immunoglobulin like receptor, KIR-L: KIR inhibitory form, KIR-S: KIR activating form, RA: rheumatoid arthritis, SHP-1: src homology 2-containing phosphatase 1, SS: Sézary syndrome.

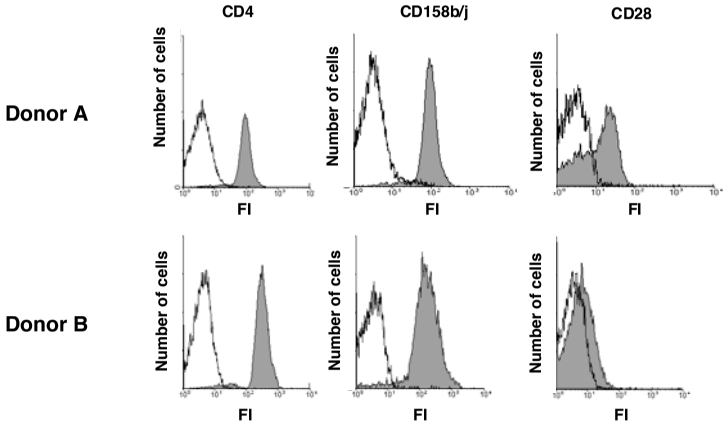
Figure Legends

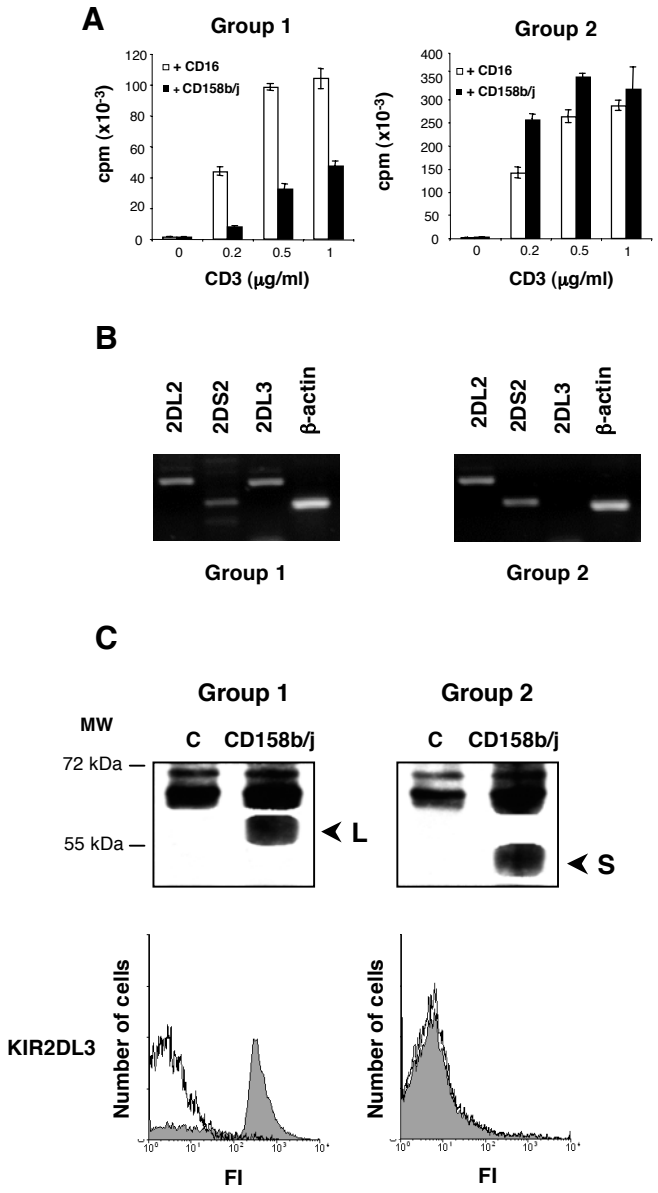
Figure 1. Phenotyping of sorted and *in vitro* expanded $CD4^+GL183^+$ T lymphocytes. *In vitro* expanded $CD4^+GL183^+$ cells were labeled using PE- or FITC conjugated anti-CD4, -CD158b/j (GL183) or CD28 mAb. The appropriate isotype-matched antibody was used as negative control. Shown are representative data corresponding to 2 of the 6 donors tested. FI: fluorescence intensity.

Figure 2. The expression and function of CD158b/j receptor in $CD4^+$ T lymphocytes is donor-dependent. (A) KIR triggering with GL183 mAb resulted in an enhanced or diminished CD3-induced cell proliferation in $CD4^+$ T lymphocytes. Expanded $CD4^+GL183^+$ T lymphocytes from healthy individuals were activated with increasing concentrations of anti-CD3 mAb plus isotype-matched anti-CD16 (white histogram) or GL183 anti-CD158b/j mAb (black). Concentrations of the anti-CD3 mAb used are indicated. Shown are representative data corresponding to individuals where the KIR was found to act as an inhibitory (group 1; n=2) or as an activating (group 2; n=4) co-receptor. Results are expressed as the mean of triplicates ± SD. (B) Analysis of KIR2DL2, KIR2DS2 and KIR2DL3 mRNA synthesis in $CD4^+GL183^+$ T lymphocytes. Total mRNA was extracted from cells obtained from donors belonging to either group 1 or group 2 (as defined in (A)) and processed for reverse transcription. cDNA amplification was performed using primers specific for KIR2DL2, KIR2DS2 or KIR2DL3 transcripts. Amplification of β-actin cDNA was done in parallel as internal control. Shown are data representative for the donors of each group (C) Exclusive membrane-expression of KIR-L or KIR-S molecules by $CD4^+$ T cells. *Top panel*: cells were surface-biotinylated prior to lysis. Immunoprecipitation was performed on cellular lysates using an anti-CD16 (C) or GL183 anti-CD158b/j mAb. Following protein separation by SDS-PAGE and transfer onto a nitrocellulose membrane, the immunoprecipitated receptors were revealed with streptavidin-peroxidase. The position of the short (S) and long (L) isoforms is indicated. Shown are data representative for donors belonging to group 1 (n=2) or group 2 (n=4). *Bottom panel*: cells were tested by flow cytometry analysis for membrane expression of KIR2DL3. FI: fluorescence intensity.

Figure 3. Analysis of CD158b/j signaling pathways in $CD4^+$ T cells. (A) $CD4^+GL183^+$ T cells from a representative healthy donor of group 1 (left panel) or group 2 (right panel) were either left untreated (-) or incubated in the presence of vanadate (+). Total cell lysates or/and anti-CD158b/j immunoprecipitates were prepared and transferred to nitrocellulose. The blot corresponding to the donor of group 1 was probed with an anti-phosphotyrosine mAb, stripped, and incubated with purified anti-SHP-1 polyclonal antibodies. The membrane from group 2 donor was revealed in series with anti-DAP10, DAP12, or -CD3ζ antibodies. Lysates of COS cells transfected with a control empty vector (V^-), or DAP10 or DAP12 coding construct (V^+), were used as controls. (B) Influence of CD158b/j triggering on Erk signaling pathway. Cells were incubated in the presence of control murine IgG1 (mIgG1), anti-CD3 and/or anti-CD158b/j mAb. Following activation, cell lysates were prepared and subjected to gel electrophoresis. Immunoblotting was performed sequentially using an anti-phospho-Erk1/2 mAb or anti-Erk1/2 antibodies. The concentrations of the antibodies used for cell activation are given in μg/ml. RDI: relative densitometric intensity.

Figure 1

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+ CD16
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cpm (x10-3)
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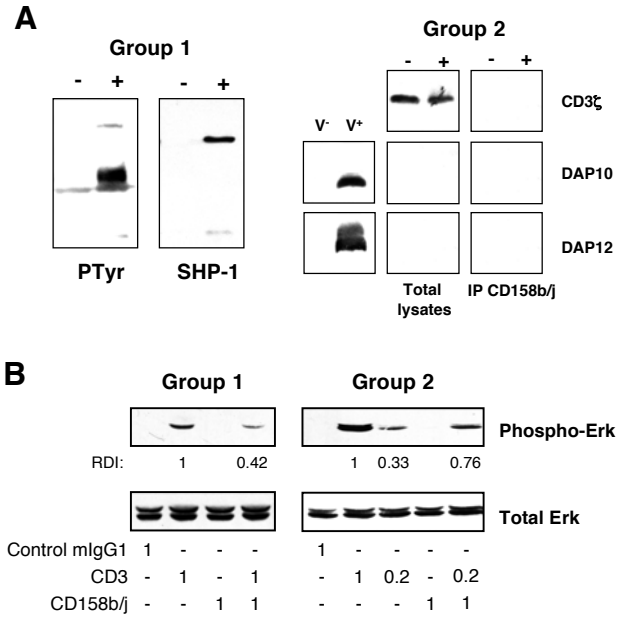
Figure 2

Figure 3


A
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Group 1
Group 2
Phospho-Erk
RDI: 1 0.42 1 0.33 0.76
Total Erk
Control mIgG1 1 - - - 1 - - - -
CD3 - 1 - 1 - 1 0.2 - 0.2
CD158b/j - - 1 1 - - - 1 1