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Functional Killer Ig-Like Receptors on Human Memory CD4+ T Cells Specific for Cytomegalovirus

Jeroen van Bergen,²* Engelina M. C. Kooy-Winkelaar,*, Henrike van Dongen,† Floris A. van Gaalen,‡ Allan Thompson,*, Tom W. J. Huizinga,† Mariet C. W. Feltkamp,‡ René E. M. Toes,† and Frits Koning*

Although very few CD4+ T cells express killer Ig receptors (KIR), a large proportion of CD4+ T cells with a late memory phenotype, characterized by the absence of CD28, does express KIR. Here, we show that KIR expression on CD4+ T cells is also associated with memory T cell function, by showing that the frequency of CMV-specific cells is higher in CD4+KIR+ T cells. In addition, engagement of an inhibitory KIR inhibited the CMV-specific proliferation of these CD4+KIR+ memory T cells, but had no detectable effect on cytokine production. Our data reveal that, in marked contrast with CD8+ T cells, the activity of a subset of CMV-specific CD4+ T cells is modulated by HLA class I-specific KIR. Thus, the CMV-induced down-regulation of HLA class I may in fact enhance memory CMV-specific CD4+ T cell responses restricted by HLA class II.


Human memory T cell differentiation is a dynamic and stepwise process, in which several stages can be distinguished based on the expression of CD27, CD28, CD45RA, and CCR7 on the cell surface (1–5). During the final stages of this process, characterized mainly by the loss of the co-stimulatory receptor CD28, T cells acquire a surface phenotype commonly associated with NK cells, such as the acquisition of killer Ig-like receptors (KIR)³ (6, 7). However, it is less clear whether KIR are frequently found on T cells responding to previously encountered Ags. CD8+ T cells specific for CMV rarely express KIR (8, 9). In fact, among CD8+ T cells, CMV specificity and KIR expression are inversely correlated (8, 9). It has not been determined whether expression of KIR is a common feature of CMV-specific CD4+ T cells.

The KIR represent the largest family of human NK receptors, with multiple inhibitory and activating members (10). The inhibitory receptors bind classical HLA class I molecules, while the physiological ligands for the activating KIR are mostly unknown. By FACS, KIR expression is detected on the large majority of NK cells, ~5% of CD8+ T cells (6), and 0.2% of CD4+ T cells (7). The role of KIR in regulating NK cell responses is reasonably well understood, but their role in T cell responses has remained largely enigmatic (11). As virus-specific CD4+KIR+ T cell clones have not been described, it is not known whether and how KIR-HLA interactions would affect the function of virus-specific CD4+ T cells.

CMV is a continuously activating β-herpesvirus that usually causes a life-long asymptomatic infection, but has a great impact on its host’s immune system. The presence of CMV has a striking impact on the surface phenotype of circulating NK and T cells. Compared with CMV seronegative donors, NK cells in CMV-seropositive donors more frequently express the activating NK receptor NKG2C (12). Furthermore, CMV seropositivity is strongly associated with the presence of late memory T cells (13, 14). In fact, only CMV-seropositive donors possess significant numbers of CD4+CD28− T cells and many of these T cells respond to CMV (14). In CMV-seropositive donors, one tenth, on average, of the total memory T cell population responds to CMV Ags in vitro (15). The CMV-specific T cell pool becomes even larger in old age (16, 17) and may then become harmful, because CMV seropositivity is associated with a reduced response to other viruses (18, 19), as well as increased mortality (20). Thus, this immune-obsession with CMV may be at the expense of other useful immune responses.

CD4+CD28− T cells may also contribute to various chronic inflammatory diseases. This subset is significantly expanded compared with age-matched controls (up to 70%) in ankylosing spondylitis (21), unstable angina (22), Wegener’s granulomatous (23), active Crohn’s disease (24), multiple sclerosis (25), and rheumatoid arthritis (RA) (26). In RA patients, the size of the CD28− subset of CD4+ T cells correlates with disease severity. Patients with severe extra-articular disease display significantly higher frequencies of CD4+CD28− T cells compared with patients in whom the disease is limited to their joints (26). CD4+CD28− T cells from RA patients express functional KIR (27) and have been reported to proliferate in response to autologous PBMC (28). Taken together, these findings suggest that CD4+CD28− T cells contribute to RA disease progression and that this process can be modulated by KIR.

In this study, we investigated the frequency and function of KIR on CMV-specific CD4+ T cells. In addition, we compared the CMV reactivity of CD4+CD28− and CD4+CD28+ T cells in healthy donors and in RA patients.

*Department of Immunohematology and Blood Transfusion, ²Department of Rheumatology, and ³Department of Medical Microbiology, Leiden University Medical Center, Leiden, the Netherlands.

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2 Address correspondence and reprint requests to Jeroen van Bergen, Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Albinusdreef 2, 2333 ZA, Leiden, the Netherlands. E-mail address: J.van_Bergen@lumc.nl

3 Abbreviations used in this paper: KIR, killer Ig-related receptor; RA, rheumatoid arthritis.

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Materials and Methods

Blood samples

After informed consent, heparinized and/or serum clotted-activated venous blood was collected from healthy blood bank donors and RA patients. Several patients evaluated at the Leiden Early Arthritis Clinic (for description, see Ref. 29) were included in this study if RA was diagnosed based on the 1987 American College of Rheumatology criteria (30). The CMV serostatus of the blood bank donors was unavailable. To quantitatively determine CMV serostatus of the RA patients, anti-CMV IgG was measured in serum using the AXSYM microparticle enzyme immunoassay (Abbott Laboratories) according to the manufacturer’s instructions. Measurements were calibrated relative to a standard serum. These studies have been reviewed and approved by the Leiden University Medical Center medical ethical review committee.

Antibodies

For the analysis of T cell surface marker expression by FACS, the following mAbs were used: CD3-Pacific Blue, CD4-PerCP/PerCP-Cy5.5/allophycocyanin, TCRβ-FITC, and CD28-FITC (all from BD Biosciences). The PE-conjugated KIR mAb mix recognized KIR2DL1, KIR2DS1 (clone EB6; Coulter Immunotech), KIR2DL2, KIR2DL3, KIR2DS2 (clone GL183; Coulter Immunotech), KIR3DL1 (clone DX9; BD Biosciences), and KIR3DS2 (clone FES172; Coulter Immunotech). For intracellular staining, IFN-γ-allophycocyanin (BD Biosciences) was used. Isotype-matched control mAbs were from BD Biosciences.

For functional blocking of interactions between the TCR and HLA class II, protein A-purified mAbs specific for HLA-DR (B11.2; Ref. 31), HLA-DQ (SPV-L3; Ref. 32), and HLA-DP (B7/21; Ref. 33) were used. For blocking interactions between KIR and HLA class I, a KIR2DL2/KIR2DS2/KIR2DL3-specific GL183 mAb (Coulter Immunotech) was used. For stimulation of T cell clones with plate-bound mAbs, anti-CD3 (OKT3; Janssen-Cilag) was used to mimic TCR triggering and anti-KIR2D (NKVSF1, which recognizes KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1, KIR2DS2, and KIR2DS4; a gift from Dr. Poggi, National Institute for Cancer Research, Genoa, Italy) was used to cross-link the inhibitory KIR2DL3. Isotype-matched control mAbs were purchased from R&D Systems.

Antigens

Viral Ags were inactivated whole mAbs specific for HLA-DR (B11.2; Ref. 31), HLA-DQ (SPV-L3; Ref. 32), and HLA-DP (B7/21; Ref. 33). For blocking interactions between KIR and HLA class I, a KIR2DL2/KIR2DS2/KIR2DL3-specific GL183 mAb (Coulter Immunotech) was used. For stimulation of T cell clones with plate-bound mAbs, anti-CD3 (OKT3; Janssen-Cilag) was used to mimic TCR triggering and anti-KIR2D (NKVSF1, which recognizes KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1, KIR2DS2, and KIR2DS4; a gift from Dr. Poggi, National Institute for Cancer Research, Genoa, Italy) was used to cross-link the inhibitory KIR2DL3. Isotype-matched control mAbs were purchased from R&D Systems.

Analysis of Ag-specific CD4 T cells in peripheral blood

For the detection of Ag-specific IFN-γ responses, PBMC were isolated from Ficoll-isopaque gradient and stimulated with CMV lysate for 8 h at 37°C. From two additional RA patients, CD28 KIR*, CD28 KIR*, and CD28 KIR* T cells were also cloned randomly. To this end, PBMC from these patients were stained with CD4-Pacific Blue, CD4-allophycocyanin, CD28-FITC (all from BD Biosciences), and the PE-conjugated KIR mAb mix and plated at 1 cell/well using a FACSCanto II flow cytometer (BD Biosciences) with the addition of brefeldin A during the final 4 h. The cells were stained with CD4-PE-Cy5 and IFN-γ-allophycocyanin as described above and analyzed by flow cytometry. The percentage of IFN-γ+ cells among CD4+ and CD8−negative cells was calculated, thus excluding IFN-γ-producing CD8+ T cells in the CFSE-positive PBMC from the analysis.

CMV-specific CD4 T cell clones were generated by incubating the cell lines from this patient with CMV lysate (10 µg/ml) in the presence of irradiated (3000 rad) autologous PBMC for 3 days. The culture was stained with CD4-allophycocyanin and KIR mAb mix-PE, combined with CD28-FITC to detect Ag-activated T cells. CD4 KIR2D T cell clones were generated by incubating the cell lines from this patient with CMV lysate (10 µg/ml) in the presence of irradiated (3000 rad) autologous PBMC for 3 days. The culture was stained with CD4-allophycocyanin and KIR mAb mix-PE, combined with CD28-FITC to detect Ag-activated T cells. CD4 KIR2D T cell clones were generated by incubating the cell lines from this patient with CMV lysate (10 µg/ml) in the presence of irradiated (3000 rad) autologous PBMC for 3 days. The culture was stained with CD4-allophycocyanin and KIR mAb mix-PE, combined with CD28-FITC to detect Ag-activated T cells. CD4 KIR2D T cell clones were generated by incubating the cell lines from this patient with CMV lysate (10 µg/ml) in the presence of irradiated (3000 rad) autologous PBMC for 3 days. The culture was stained with CD4-allophycocyanin and KIR mAb mix-PE, combined with CD28-FITC to detect Ag-activated T cells.

The third RA patient (patient 3) was a 76-year-old female with the HLA genotype of the patient was KIR2DL1, KIR2DL2, KIR2DL3, KIR2DS1, KIR2DS2, KIR2DS3, KIR3DL1, KIR3DL2, KIR3DS1, KIR3DL3, KIR2DS4*001, and also included a truncated KIR2DS4 variant (KIR2DS4*003*007). Therefore, in this patient the possible inhibitory HLA-KIR interactions were HLA-Cw*07-KIR2DL2 and KIR2DS4 in the T cell responses in Ag-stimulated samples. Values subtracted from the IFN-γ responses in Ag-stimulated samples. Values greater than 0.02% (two SDs) after background subtraction were considered positive.

Although this assay is widely used to quantify CD4 T cell responses, additional tests were performed to validate this method in our laboratory, using PBMC from healthy donors. First, CD4 T cell responses to recombinant CMV pp65 protein were detected only in donors responding to whole CMV lysate, whereas responses to recall mix were similar between donors that did or did not respond to CMV lysate.

Isolation of CD4 T cell lines and clones from RA patients

PBMC from a 74-year-old male RA patient, who had been diagnosed with RA when he was 64 years of age, were stained with the KIR mAb mix combined with CD28-FITC and CD4-allophycocyanin. CD28 KIR2DL1 (3000 cells), CD28 KIR2DL1 (3000 cells), and CD28 KIR2DL1 (3000 cells) CD4+ T cells were isolated using a BD FACS Vantage flow cytometer using the CellQuest Pro software (BD Biosciences). The T cells were expanded twice, using mixed irradiated allogeneic PBMC supplemented with 1 µg/ml PHA and 20 unit/ml IL-2, resulting in 10–100 million cells of each type.

To test the CMV reactivity contained within these CD4 T cell subsets, the expanded bulk cell lines were incubated for 6 h with CFSE-labeled and HLA-matched PBMC, either preloaded with CMV lysate or not, in the presence of brefeldin A during the final 4 h. The cells were stained with CD4-PE-Cy5 and IFN-γ-allophycocyanin as described above and analyzed by flow cytometry. The percentage of IFN-γ+ cells among CD4+ and CD8−negative cells was calculated, thus excluding IFN-γ-producing CD8+ T cells in the CFSE-positive PBMC from the analysis.

CMV-specific CD4 T cell clones were generated by incubating the cell lines from this patient with CMV lysate (10 µg/ml) in the presence of irradiated (3000 rad) autologous PBMC for 3 days. The culture was stained with CD4-allophycocyanin and KIR mAb mix-PE, combined with CD28-FITC to detect Ag-activated T cells. CD4 KIR2D T cell clones were generated by incubating the cell lines from this patient with CMV lysate (10 µg/ml) in the presence of irradiated (3000 rad) autologous PBMC for 3 days. The culture was stained with CD4-allophycocyanin and KIR mAb mix-PE, combined with CD28-FITC to detect Ag-activated T cells. CD4 KIR2D T cell clones were generated by incubating the cell lines from this patient with CMV lysate (10 µg/ml) in the presence of irradiated (3000 rad) autologous PBMC for 3 days. The culture was stained with CD4-allophycocyanin and KIR mAb mix-PE, combined with CD28-FITC to detect Ag-activated T cells. CD4 KIR2D T cell clones were generated by incubating the cell lines from this patient with CMV lysate (10 µg/ml) in the presence of irradiated (3000 rad) autologous PBMC for 3 days. The culture was stained with CD4-allophycocyanin and KIR mAb mix-PE, combined with CD28-FITC to detect Ag-activated T cells.
Functional assays with T cell clones

HLA-matched PBMC (100,000 irradiated; 3000 rad) were incubated for 2 h in the presence of CMV lysate or control lysates from other herpes viruses (EBV, HSV1, HSV2, VZV) in IMDM/10% human serum in each well of a 96-well flat-bottom plate. After this preincubation step, 20,000 cells per well of the T cell clones were added. To interfere with KIR2DL3-HLA interactions, the T cells were preincubated for 30 min with 5 μg/ml GL183 Ab or an isotype-matched control Ab, while the Ab-binding sites on the PBMCs were blocked by preincubating these with 100 μg/ml purified mouse IgG during the 2-h preincubation step with CMV lysate.

For stimulation with plate-bound Abs, non-tissue culture treated plates were coated with 0–10 μg/ml anti-CD3 in the presence of 3 μg/ml anti-KIR2D or isotype-matched control Ab. After overnight coating and blocking at room temperature, 20,000 cells per well of the T cell clones were added.

T cell proliferation was determined by measuring the incorporation of [3H]thymidine during the final 16 h of a 96-h culture period. Cytokine concentrations in supernatants at 48 h were measured using the Luminex Human Cytokine Group I kit (Bio-Rad).

Statistics

The SPSS software (version 12.0.1) was used to calculate p values. For comparisons of FACS-based measurements between groups of individuals, the Mann-Whitney U test (exact, one-tailed) was used. For comparisons between paired FACS measurements, the Wilcoxon signed rank test (exact, one-tailed) was applied.

Results

CMV reactivity of CD4⁺CD28⁻ T cells from healthy donors

In CD4⁺ T cells from healthy donors, CMV reactivity is associated with the absence of CD28 expression (14). Because we...
previously showed that a large proportion of CD4⁺CD28⁻ T cells expresses KIR and that purified CD4⁺KIR⁺ T cells respond to CMV (7), we determined the relative frequencies of CMV-specific cells among CD4⁺KIR⁻ and CD4⁺KIR⁺ T cells (Fig. 1A).

Approximately half (13 of 27) of the healthy donors responded to CMV in these assays, consistent with the seroprevalence of CMV in northwestern Europe (Fig. 1B). In these CMV responders, on average 1.0% (range, 0.04–3.5%) of CD4⁺ T cells produced IFN-γ in response to CMV. CMV-specific T cells were significantly more frequent among CD4⁺KIR⁺ compared with CD4⁺KIR⁻ cells, on average 2.4-fold (Fig. 1B: range, 1.0- to 4.1-fold, \( p = 0.004 \)). In line with previous results (14), CMV responses were also considerably more frequent among CD4⁺CD28⁻ cells compared with CD4⁺CD28⁺ cells, on average 30-fold (Fig. 1B: range, 3- to 81-fold, \( p = 0.002 \)). This was not due to a greater propensity of CD4⁺KIR⁺ or CD4⁺CD28⁺ T cells to produce IFN-γ, as IFN-γ responses to other recall Ags (from M. tuberculosis, C. tetani, and C. albicans) did not display this bias (data not shown). The dominant factor associated with CMV reactivity appeared to be CD28. Among CD4⁺CD28⁻ T cells, the responses of KIR⁻ and KIR⁺ cells were comparable (data not shown). However, in most donors the CD4⁺CD28⁻ KIR⁺ subset was too small to reliably compare responses between CD4⁺CD28⁻ KIR⁻ and CD4⁺CD28⁺ KIR⁺ cells. In conclusion and in sharp contrast with CD8⁺KIR⁺ T cells (8, 9), these experiments revealed that a relatively large proportion of CD4⁺KIR⁻ T cells responded to CMV.

![CMV reactivity of KIR⁺ and CD28⁻ CD4⁺ T cell lines and clones from rheumatoid arthritis patients.](image)

**FIGURE 3.** CMV reactivity of KIR⁺ and CD28⁻ CD4⁺ T cell lines and clones from rheumatoid arthritis patients. A–C, KIR (mAbs EB6, GL183, DX9, FES172) and CD28 expression on CD4⁺ T cells from three RA patients. Cells from all four CD4⁺ T cell subsets (CD4⁺CD28⁻ KIR⁺, CD4⁺CD28⁺ KIR⁺, CD4⁺CD28⁻ KIR⁻, and CD4⁺CD28⁺ KIR⁻) were isolated by FACS. From the patient material depicted in A (patient 1), bulk lines were generated (3000 cells/subset, except for CD28⁻ KIR⁻ which had 100 cells), while eight T cell clones were randomly cloned from each subset of the patients depicted in B (patient 2) and C (patient 3). D, The CMV reactivity of the resulting bulk cultures from A was tested by incubating the cells with CFSE-labeled HLA matched PBMC, either preloaded with CMV lysate (black bars) or not (white bars). CMV-specific IFN-γ producing CD4⁺ T cells were detected by intracellular cytokine staining. CMV-specific cells within the stimulator PBMC were excluded by gating out CFSE-positive cells. E and F, CMV-specific proliferation of T cell clones with the indicated phenotypes, isolated from the CD4⁺ T cells shown in B and C, respectively, was determined by stimulating the cells with irradiated HLA-matched PBMC, either preloaded with CMV lysate or with EBV, HSV1, and HSV2 lysates, and by measuring [³H]thymidine incorporation (cpm) during the final 18 h of a three-day incubation period. Stimulation indices (SI) were calculated by dividing the median cpm value upon antigenic stimulation by the median cpm value obtained without Ag, and SI values >3 (dotted line) were considered positive. Each closed circle represents a single clone. All clones and lines had maintained the CD28 and KIR phenotype that was the basis of their selection after multiple rounds of in vitro restimulation (not shown).
FIGURE 4. Inhibitory KIR impair the recognition of CMV by CD4⁺ T cell clones from rheumatoid arthritis patients. A CD4⁺KIR2DL3⁺ T cell clone (A–D) and a CD4⁺KIR⁻ clone (E–H), both isolated from an RA patient (patient 1), were stained with mAbs to CD4 and KIR2DL3 (MoAb GL183). NK receptor-specific Abs recognizing other KIR (mAbs EB6, DX9, FES172), CD94, NKG2D, or LILRB1 did not stain these clones (data not shown). B and F, Both T cell clones were tested for reactivity against fully HLA-matched PBMC loaded with a variety of herpes virus lysates in the presence or absence of HLA class II-specific Abs. C and G, The clones were preincubated with an Ab that blocks the interaction between KIR2DL3 and HLA-C (MoAb GL183), or an isotype-matched control Ab, and subsequently tested for reactivity against fully HLA-matched PBMC expressing HLA-C ligands (HLA-Cw*07/HLA-Cw*12) for the inhibitory KIR2DL3 loaded with CMV lysate, or HLA-DR matched PBMC that did not express ligands for KIR2DL3 (D and H). A single experiment is shown, representative of three separate experiments. I and J, The same experiment was performed with a CD4⁺CD28⁺KIR2DL3⁺ clone from the second RA patient (patient 2) offered (I) HLA class I and II-matched PBMC that did express KIR2DL3-ligand HLA-Cw*07 or (J) HLA class I-mismatched, HLA class II-matched control PBMC that were homozygous for HLA-Cw*04, which does not bind KIR2DL3. K, This experiment was also done with a CD4⁺CD28⁺KIR2DL3⁺ clone from the third RA patient (patient 3) and HLA class I- and II-matched PBMC that expressed HLA-Cw*07.
CD4⁺CD28⁻ cells from CMV-seropositive patients with high (>10%) and low (<10%) CD4⁺CD28⁺ T cell frequencies were comparable (data not shown). In line with these findings, CMV-specific cells were on average 26-fold (range, 2- to 94-fold, \( p = 0.004 \)) more frequent among CD4⁺CD28⁻ cells compared with CD4⁺CD28⁺ cells (Fig. 2C). Similar to healthy donors (Fig. 1), CMV-specific T cells were on average 4.2-fold (range, 0.3- to 15-fold) more frequent among CD4⁺KIR⁺ cells compared with CD4⁺KIR⁻ cells, but this difference was not statistically significant (\( p = 0.07 \), Fig. 2C). In conclusion, in CD4⁺ T cells from RA patients, like in healthy donors, CMV reactivity was strongly associated with the absence of CD28 and possibly also with the presence of KIR.

**CD4⁺CD28⁻ and CD4⁺KIR⁺ T cell lines and clones isolated from RA patients respond to CMV**

To confirm that CD4⁺CD28⁻ and CD4⁺KIR⁺ T cells from RA patients respond to CMV, CD4⁺ T cell subsets were isolated from an RA patient and tested for CMV reactivity. This male patient was CMV-seropositive and about a third of his CD4⁺ T cells were CD28⁻ (Fig. 3A). CD4⁺CD28⁻ KIR⁺, CD4⁺CD28⁻ KIR⁻, CD4⁺CD28⁺ KIR⁺, and CD4⁺CD28⁺ KIR⁻ cells were isolated by FACS, expanded in vitro and subsequently tested for CMV reactivity. CMV-specific IFN-γ production was detected only in the CD4⁺CD28⁻ cell lines and was most prominent in the CD4⁺CD28⁻ KIR⁺ subset of this donor (Fig. 3D).

To more rigorously test the CMV reactivity of these subsets in RA patients, CD4⁺ T cells were randomly cloned from the four CD4⁺ T cell subsets from two additional CMV⁺ RA patients. In one patient (Fig. 3, B and E), all CD28⁻ KIR⁺, most CD28⁻ KIR⁻, and a few CD28⁺ KIR⁺, but none of the CD28⁺ KIR⁻, clones responded to CMV (Fig. 3E). In the other patient (Fig. 3, C and F), half of the CD28⁻ KIR⁺ but virtually none of the other clones responded to CMV (Fig. 3F). These results confirmed that in RA patients, CMV reactivity of CD4⁺ T cells is strongly associated with the absence of surface CD28, and that CD4⁺ KIR⁺ T cells can respond to CMV. Clearly, the frequency of CMV-specific T cells among CD4⁺CD28⁻ T cells in RA patients is so high that Ag-independent cloning results in the isolation of CMV-specific cells.

**Expression of functional inhibitory KIR on CMV-specific CD4⁺ T cell clones**

Two CMV-specific T cell clones were isolated from the CD28⁻ KIR⁺ and CD28⁻ KIR⁻ cell lines of the first RA patient (Fig. 3, A and C). These clones shared identical TCRα and TCRβ sequences, but one clone expressed KIR2DL3, but not other KIR, while the other clone did not express any KIR (data not shown). This confirmed that KIR expression can be acquired after TCR rearrangement (37). KIR2DL3, expressed at high levels on the CD4⁺KIR⁻ clone (Fig. 4A), binds HLA-C molecules characterized by an asparagine at position 80 of their H chain, including the HLA-Cw*07 and HLA-Cw*12 alleles present in the patient’s genotype. Both clones proliferated specifically in response to CMV lysate, but not to lysates derived from other herpes viruses. This response was restricted via HLA-DR (Fig. 4, B and F).

Next, the role of KIR2DL3 in target cell recognition was investigated. The CD4⁺KIR⁺ and the CD4⁺KIR⁻ T clone displayed similar proliferative responses to CMV-loaded and fully HLA-matched PBMC (Fig. 4, C and G). In the presence of a KIR-specific Ab that blocked the interaction between KIR2DL3 and HLA-Cw*07/H11001, but not of a control Ab, proliferation of the CD4⁺KIR⁺, but not the CD4⁺KIR⁻ clone, was significantly enhanced (Fig. 4, C and G). This enhancement was not observed when the T cells were stimulated with CMV-loaded PBMC that did express the appropriate HLA-DR molecule but lacked a ligand for KIR2DL3 (Fig. 4, D and H). To demonstrate that this effect was not restricted to this particular patient, we performed similar experiments with additional T cell clones. Two CD4⁺CD28⁻ KIR2DL3⁺ clones from the two other RA patients (Fig. 3, E and F) yielded virtually identical results (Fig. 4, I–K). Overall, the interaction between the inhibitory KIR and HLA class I reduced by ~10-fold the Ag sensitivity of the HLA class II-restricted and CMV-specific CD4⁺KIR⁺ T cell clones (Fig. 4, C, I, and K).

Next, we tested whether triggering of inhibitory KIR also impaired TCR-induced cytokine production. To this end, the CMV-specific CD4⁺KIR⁺ and CD4⁺KIR⁻ T cell clones were stimulated with plate-bound Abs specific for CD3 and KIR. Although T cell proliferation was markedly impaired by KIR2DL3 engagement, the production of the cytokines TNF-α and IL-4 was not detectably affected (Fig. 5). Thus, engagement of inhibitory KIR selectively influenced a subset of T cell effector functions.

**FIGURE 5.** KIR2DL3 engagement changes the functional profile of a CD4⁺ T cell clone from a RA patient. Two CMV-specific clones from an RA patient (see Figs. 3, A and D, and 4, A–H), a CD4⁺KIR2DL3⁺ T cell clone (A, C, and E), and a CD4⁺KIR⁻ clone (B, D, and F), were stimulated with plate-bound anti-CD3 in combination with either a KIR-specific (NKVSVF1) or an isotype control mAb, also plate-bound. TNF-α (C and D) and IL-4 (E and F) concentrations were measured in supernatants (pooled triplicates) after 48 h, and [³H]thymidine incorporation (A and B) was measured during the following 18 h. One experiment is shown, representative of three.
Discussion

We previously showed that, although very few CD4+ T cells express KIR, CD4+ T cells with a late memory surface phenotype (CD28-; CD27-; and/or CD57-) do frequently express KIR (7). Here, we demonstrate for the first time that KIR expression on CD4+ T cells is also associated with memory T cell function, and that KIR can inhibit the Ag-specific response of memory CD4+ T cells.

In marked contrast with reports on CD8+ T cells (8, 9), the frequency of CMV-specific cells among CD4+ KIR+ T cells was higher than among CD4+ KIR- T cells. This discrepancy was unexpected, as in both T cell subsets KIR are found predominantly on T cells with a memory phenotype. What’s more, in both subsets the presence of KIR is strongly associated with a late memory phenotype characterized by the absence of CD28, which in turn is associated almost exclusively with CMV-specificity. To reconcile these data, it is conceivable that KIR+ and KIR- T cells recognize distinct sets of CMV proteins. CMV-specific CD8+ T cells were identified by tetramer staining, using a single HLA-A2-restricted CMV peptide derived from pp65, one of the immunodominant CMV proteins (8, 9). In contrast, in our assays CMV-specific CD4+ T cells were identified after stimulation with a CMV lysate, which contains a much larger number of potential CD4+ T cell epitopes. It will therefore be of interest to test whether CD8+ T cells specific for other CMV Ags express KIR. Alternatively, it is possible that functional memory CD4+ but not memory CD8+ T cells can express KIR.

A recent report (38) suggested that a sizeable fraction of CMV-specific CD4+ T cells expresses KIR (and NK2G2D). In these experiments, PBMC were stimulated with IL-2 in the presence or absence of CMV, and KIR (and NK2G2D) expression on CD4+ T cells was measured after 10 days. In the presence of CMV, the CD4+ KIR+ T cell subset was greatly expanded. It was, however, unclear whether this was a direct or indirect consequence of the presence of CMV. In addition, the apparent expansion of CMV-specific CD4+ KIR- T cells might have been due to a higher proliferative capacity of CD4+ KIR+ T cells rather than a high frequency of CMV-specific cells among the CD4+ KIR- T cells at the beginning of the culture. In our hands, culturing PBMC in the presence of a CMV lysate (without IL-2) or an a-specific stimulus (irradiated allogeneic PBMC, PHA, IL-2) invariably led to a clear reduction rather than an increase of the proportion of CD4+ KIR- T cells (data not shown). In the type of short-term experiments presented here, T cells do not proliferate, giving a more accurate measurement of the relative frequencies of CMV-specific cells within different CD4+ T cell subsets. Our experiments therefore provide definitive evidence that in the CD4+ T cell compartment KIR expression is associated with memory function, at least in the case of CMV.

When the interaction was blocked between an inhibitory KIR on a CMV-specific CD4+ T cell and its HLA-C ligand on APCs, the proliferative response of this CD4+ T cell was greatly increased. This is probably relevant to CMV infection, as CMV-encoded proteins US2, US6, and US11 down-regulate the expression of HLA class I molecules on host cells, including HLA-C (39). Thus, evasion of the HLA class I-restricted CD8+ T cell responses by CMV may selectively enhance proliferation of HLA class II-restricted CD4+ KIR+ T cells.

Although ligation of an inhibitory KIR drastically reduced TCR/CD3-induced proliferation, no effect on cytokine production was observed. Using CD4+ KIR+ clones of unknown specificity, others have shown that activating KIR are also able to selectively influence T cell functions. Triggering of KIR2DS1, KIR2DS2, or KIR2DS4 on these clones costimulated TCR-induced proliferation and IFN-γ production but had no effect on cytotoxicity or IL-4 production (40–43). Therefore, signaling via inhibitory and activating KIR may intersect some but not other signaling pathways linked to the TCR. In support of this idea, binding of HLA-C by the inhibitory KIR2DL2 affects late but not early TCR-induced intracellular signals and did not inhibit cytotoxicity of CD4+ CD28+ T cell clones from RA patients in multiple clones and over a wide range of stimulatory signals (44). The down-regulation of HLA class I by CMV is therefore likely to have quantitative and qualitative effects on the response of CD4+ KIR+ T cells.

A series of studies has indicated that an expanded CD4+ CD28− T cell subset contributes to progression to extra-articular disease in RA (reviewed in Ref. 35), but the Ag specificity of these cells has remained obscure. We now provide evidence that a large fraction of CD4+ CD28− T cells in RA patients is CMV specific. This conclusion is based on two observations. First, in line with a previous report (36), CD4+ CD28− T cells were detected only in CMV-seropositive patients. Second, in these patients CMV-specific T cells were far more frequent among CD4+ CD28− T cells relative to CD4+ CD28+ T cells. While this manuscript was in preparation, two other groups also observed that CD4+ CD28− T cells were found only in CMV-seropositive RA patients (45) and responded to CMV (45, 46). In fact, the response of CD4+ CD28− T cells to CMV was equal to the response to plate-bound anti-CD3 (46). Furthermore, the TCR repertoires of CD4+ CD28− T cells expanded in vitro by stimulation with CMV or anti-CD3 were virtually identical (46). Finally, patient CD4+ CD28− T cells did not respond to human collagen type II, a potential autoantigen in RA (46). Taken together, it is plausible that most—if not all—CD4+ CD28− T cells in RA patients are CMV specific. Hence, the role of CMV in the pathogenesis of RA complications deserves more attention.

In summary, we have shown that KIR expression on CD4+ T cells is associated with memory T cell function and that KIR can selectively impair a subset of CD4+ T cell functions. This is a surprising finding, given previous reports that CMV specificity is inversely correlated with KIR expression in the CD8+ T cell compartment (8, 9). In vivo, these CMV-specific CD4+ KIR+ effecter memory T cells may have a critical role in the response to HLA class II-positive CMV-infected cells, such as monocytes and endothelial cells.

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Disclosures

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References


