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Killer Cell Activating Receptors Function as Costimulatory Molecules on CD4^{+}CD28[null] T Cells Clonally Expanded in Rheumatoid Arthritis[1]

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Expansion of CD4^{+}CD28[null] T cells is a characteristic finding in patients with rheumatoid arthritis. Despite lacking CD28 molecules, these unusual CD4 T cells undergo clonal proliferation and form large and long-lived clonal populations. They produce high levels of IFN-γ, exhibit autoreactivity, and have cytolytic function. The mechanisms facilitating the expansion and longevity of CD4^{+}CD28[null] T cell clones in vivo are unknown. Here, we report that CD4^{+}CD28[null], but not CD4^{+}CD28^{+}, T cells express MHC class I-recognizing receptors normally found on NK cells. CD4^{+}CD28[null] T cells preferentially expressed killer cell activating receptors (KAR), often in the absence of killer cell inhibitory receptors. Cross-linking of KAR molecules enhanced the proliferative response to TCR-mediated stimulation, but not the cytolytic function of CD4^{+}CD28[null] T cells, suggesting different signaling pathways in CD4 T cells and NK cells. Triggering of KAR signaling led to the phosphorylation of several cellular targets, although the pattern of phosphorylation differed from that induced by the TCR. Aberrant expression of KAR molecules in the absence of inhibitory receptors and in the appropriate HLA setting may lead to the clonal outgrowth of autoreactive CD4^{+}CD28[null] T cells commonly seen in rheumatoid arthritis. *The Journal of Immunology,* 2000, 165: 1138–1145.

T he majority of CD4 T cells constitutively expresses the CD28 molecule, a key player in providing costimulatory signals to induce T cell activation and to prevent T cell apoptosis. CD4 T cells lacking the CD28 molecule are distinctly infrequent in most normal individuals (1, 2). However, in patients with rheumatoid arthritis (RA)[4] or insulin-dependent diabetes mellitus (IDDM), CD4^{+}CD28[null] cells are expanded. Studies on the functional characteristics of CD4^{+}CD28[null] cells have demonstrated that compared with their CD4^{+}CD28^{+} counterparts, these unusual T cells display unique features, such as longevity and clonal expansion in vivo (3, 4). There is evidence in RA and IDDM that CD4^{+}CD28[null] T cells are autoreactive (3, 5, 6). Ag-driven selection has been suggested by the finding that CD4^{+}CD28[null] T cell clones derived from different RA patients expressed TCR β chains with identical amino acid sequences (7). The emergence of CD4^{+}CD28[null] T cells in two human autoimmune diseases and their functional characteristics, including the preferential production of IFN-γ, have given rise to the hypothesis that these unusual T cells have a role in pathogenic immune responses (8–10).

To understand the contribution of CD4^{+}CD28[null] T cells to disease, it is important to know how these cells proliferate and function despite a deficiency of CD28. Previous studies have shown that CD4^{+}CD28[null] cells require costimulatory signals for optimal proliferative responses, but not cytokine production (10). The molecular nature of the implicated costimulatory pathway has not been defined, except that it requires cell-cell contact and does not involve CD80 or CD86. Identification of the costimulatory molecules involved in optimizing CD28[null] T cell proliferation would be critical in the attempt to understand the longevity and clonal outgrowth of these cells.

CD4^{+}CD28[null] cells express perforin and granzyme B and exhibit TCR-mediated cytotoxicity (11). These cells can express CD11b and CD57 (3, 6), cell surface markers that are generally not found on CD4 T cells but are found on NK cells. In contrast to NK cells, they lack the CD16 molecule. To explore how the activation of CD4^{+}CD28[null] T cells is regulated, we have now examined CD4 T cells in RA patients for the expression of a new class of MHC class I-recognizing receptors that are preferentially found on NK cells and that deliver either inhibitory or stimulatory signals (12–16). Stimulatory and inhibitory NK receptors possess similar extracellular domains but differ in their transmembrane and cytoplasmic sequences (13, 14, 17). Two distinct types of MHC-recognizing human receptors have been identified. Killer cell inhibitory receptors (KIR) and killer cell activating receptors (KAR) belong to the Ig family (18, 19), and C-type lectin molecules (NKG2 polypeptides) are expressed as heterodimers with the CD94 molecule (20, 21). CD94/NKG2 receptors recognize HLA-E molecules (22–24), whereas the KIR/KAR family includes multiple receptors with specificities for different HLA-A, -B, and -C alleles and the HLA-G molecule (13, 14, 17–19). KIR receptors have also been described on T cells, where they are usually restricted to a subset of CD8 memory T cells (25–27). In this paper, we report that in RA patients, KIR/KAR receptors are expressed on CD4^{+}CD28[null], but not CD4^{+}CD28^{+}, T cells. More importantly, CD4^{+}CD28[null] T cell

clones from RA patients were characterized by the preferential and sometimes exclusive expression of KARs and the absence of inhibitory CD94/NKG2 receptors and KIRs. These stimulatory receptors functioned as costimulatory molecules in proliferative responses but did not enhance the cytotoxic effector function of these cells. Therefore, KAR-mediated signals may contribute to the oligoclonal expansion of $CD4^+CD28^{null}$ cells in vivo and may have a role in the dysregulated immune responses of RA patients.

Materials and Methods

Study population

PBMC were obtained from 10 patients with seropositive RA according to the 1988 American College of Rheumatology criteria (28). This protocol was approved by the Mayo Clinic internal review board, and all patients gave written, informed consent. All patients had an expanded subset of $CD4^+CD28^{null}$ T cells. Patients were typed for their HLA-C alleles by PCR and oligonucleotide hybridization (Dynal, Lake Success, NY). $CD4^+CD28^{null}$ T cell clones were generated from PBMC of three patients by limiting dilution cloning in IL-2-containing medium as recently described (3). TCR β chains were sequenced, and clones were selected that expressed a sequence clonally expanded in the peripheral blood in vivo. T cell clones from RA patient H have been described (29). Human NK cells were cloned and passaged as previously described (30).

Flow cytometry

PBMC and CD4 T cell clones were incubated with anti-CD94 (HP3B1, gift from Miguel Lopez-Botet, Hospital de la Princesa, Universidad Autonoma de Madrid, Madrid, Spain), DX9 (anti-KIR3DL1, gift from Lewis Lanier, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA), EB6 (anti-KIR2DL1/KIR2DS1, Beckman Coulter, Miami, FL), GL183 (anti-KIR2DL2/KIR2DL3/KIR2DS2, Beckman Coulter), or control mouse IgG1 (Zymed, South San Francisco, CA). After washing, the cells were stained with FITC-conjugated goat anti-mouse Ig (Becton Dickinson, San Jose, CA) and peridin chlorophyll protein-conjugated anti-CD4 and PE-conjugated anti-CD28 or PE-conjugated anti-CD16 mAb (Becton Dickinson).

PCR amplification and sequencing

Total RNA from T cell clones was extracted using TRIzol reagent (Life Technologies, Grand Island, NY). cDNA was amplified with a primer set amplifying all known KIR receptor sequences (GGAGCTCCTATGA CATGTACC and GACAGGGCTGTTGTCTCCCTA). Amplified products were cloned using the TA cloning kit (Invitrogen, San Diego, CA) and sequenced. Sequences were matched with known KIR and KAR sequences (19, 31, 32). In addition, cDNA was amplified with the KIR family member-specific primer sets as described by Uhrberg et al., and the amplified product was directly sequenced (31). The nomenclature suggested by E. Long, L. Lanier, and M. Colonna was used as reported by Uhrberg et al. (31). According to this nomenclature, all KIR and KAR sequences have the KIR prefix; inhibitory members with a long cytoplasmic tail are designated KIR DL, and stimulatory members with a short cytoplasmic domain are designated KIR DS. The following primer sets were used to amplify DAP12 and β-actin sequences: DAP12, CAGTTGCTCTACGGTGAGC and TGTGTGTTGAGG TCGCTG; and β-actin, ATGGCCACGGCT GCTTCCAGC and CAGGAGGAGCAATGATCTTGAT. To determine whether T cell clones express the AV24/BV11 TCR commonly expressed in CD1-restricted NK T cells, the following primers were used: TCR AV24, GATATACAGCAACTCTGGATGCA and GGCAGACAGACTT GTCACTGGAT; and TCR BV11, TCAACAGTCTCCAGAATAAG GACG and GTGGGAGATCTCTGCTTCTG.

Cytotoxicity assay

T cell clones were stimulated with immobilized anti-CD3 (OKT3, CRL 8001, American Type Culture Collection, Manassas, VA) in the presence of accessory cells and were examined after 5 days for cytotoxic activity in a ^{51}Cr release assay. Serial dilutions of T cell clones were incubated with 1×10^4 Fc receptor-expressing P815 target cells in the presence of 0.5 μg/ml anti-CD3, 0.5 μg/ml anti-CD3 plus 10 μg/ml GL183, or 10 μg/ml GL183 only. Supernatants were harvested after 4 h. NK clones expressing either the DX9-reactive inhibitory KIR3DL1 or the GL183-reactive stimulatory KIR2DS2 receptor were assayed for cytotoxic activity using anti-CD16 instead of anti-CD3. Results were given as the mean percent specific lysis of assay triplicates. Spontaneous lysis was <15% of maximal lysis for all experiments.

Proliferation assay

Anti-CD3 (10 μg/ml) together with either control IgG1 or GL183 (each 20 μg/ml) were adsorbed on 96-well flat-bottom plates. In selected experiments, plates were coated with rabbit anti-mouse IgG and then incubated with serial dilutions of anti-CD3 (5–50 ng/ml) followed by incubation with GL183, L368 (anti-β_2-microglobulin, HB 149, American Type Culture Collection), or control IgG (each 200 ng/ml). T cell clones (2×10^4/well) were stimulated on the plastic-immobilized Abs in the presence of 2 U/ml IL-2. Microcultures were pulsed with 1 μCi of [^{3}H]thymidine for the last 16 h of a 72-h culture. All results were expressed as the mean $\pm$ SD of triplicate cultures.

Vaccinia virus infection

$CD4^+CD28^{null}$ T cells were infected for 5 h at a ratio of 20:1 (viral PFU:T cell ratio) with recombinant vaccinia virus containing the KIR2DS2 cDNA sequence or with control virus containing the vector sequence only. Cell surface expression of KIR2DS2 was confirmed using flow cytometry.

Phosphotyrosine assay

The signaling activity of KIR2DS2 receptor was analyzed by phosphotyrosine Western blotting. T cell clones were incubated with 5 μg/ml anti-CD3 or 5 μg/ml GL183 on ice for 15 min. The Abs were cross-linked using rabbit anti-mouse IgG for 10 min at 37°C. The cells were lysed in 50 mM Tris (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholic acid, and 0.1 mM orthovanadate on ice for 10 min. Clarified lysates were collected and analyzed by SDS-PAGE, and then transferred to a polyvinylidene difluoride membrane. The membrane was blotted with phosphotyrosine Ab 4G10 (Upstate Biotechnology, Lake Placid, NY). The blot was detected using HRP-conjugated goat anti-mouse IgG and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Arlington Heights, IL).

Results

MHC class I-recognizing receptors on $CD4^+CD28^{null}$ T cells

To assess KIR and CD94/NKG2 expression on $CD4^+CD28^+$ and $CD4^+CD28^{null}$ T cells, PBMC from 10 patients with RA who had expanded numbers of $CD4^+CD28^{null}$ T cells (2.3–19.4%) were analyzed. Two p50/p58 KIRs (GL183, EB6) and one p70 KIR (DX9) were examined. The expression of C-type lectin receptors was assessed using anti-CD94. All 10 patients expressed at least one of the three KIR members on CD4 T cells. Three individuals also expressed CD94; KIR and CD94 expression were exclusively found on $CD4^+CD28^{null}$, but not on $CD4^+CD28^+$, T cells (Fig. 1). Healthy individuals ($n = 8$) who had <1% $CD4^+CD28^{null}$ T cells did not express either CD94 or any of the KIR members on CD4 T cells (data not shown). We also analyzed two normal individuals who were known to have an expanded subset of $CD4^+CD28^{null}$ T cells. KIR expression in these two normal individuals was comparable to that in RA patients.

The patterns of MHC class I-recognizing receptors on $CD4^+CD28^{null}$ T cells appeared to differ from the patterns seen on NK cells. The frequency of $CD4^+CD28^{null}$ T cells expressing at least one of the KIR molecules was lower than the corresponding frequency in the NK cell population. KIR receptors recognized by GL183 appeared to be preferentially selected in $CD4^+CD28^{null}$ T cells, while EB6 receptors were not found. The most striking difference was seen for CD94 expression. In all patients, $CD94^+$ cells were represented among the NK cell population, and in some patients the majority of NK cells expressed CD94. In contrast, seven of the patients did not have detectable levels of CD94 on their $CD4^+CD28^{null}$ T cells. In the three individuals who had $CD94^+CD4^+$ T cells, the cell surface density of CD94 was different on $CD4^+CD28^{null}$ T cells and NK cells. Expression of CD94 on NK cells displayed a bimodal distribution, with the majority of NK cells expressing CD94 at a high cell surface density. $CD4^+CD28^{null}$ T cells expressed CD94 only at a low cell surface
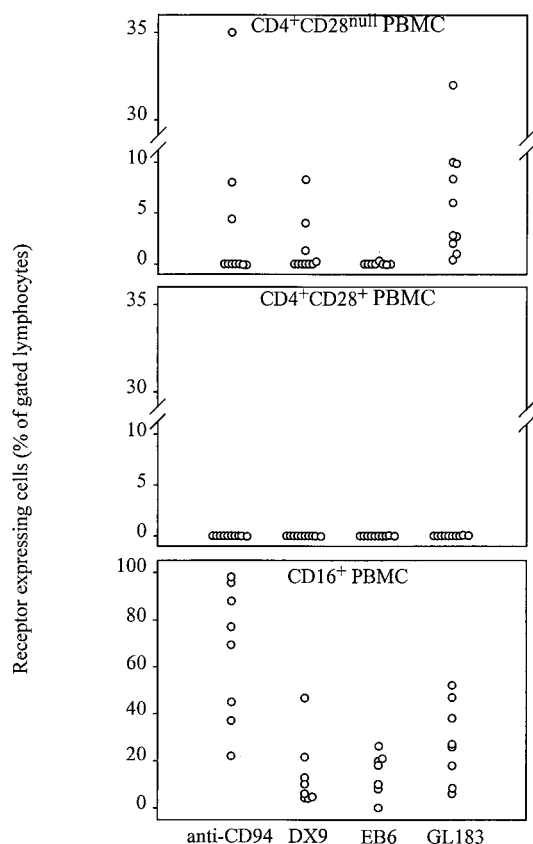
FIGURE 1. NK receptor expression on CD4$^+$CD28$^+$ and CD4$^+$CD28^{null} T cells and on CD16$^+$ NK cells in patients with RA. PBMC of 10 patients with RA were analyzed by three-color flow cytometry. The frequencies of CD94$^+$ (anti-CD94) and KIR$^+$ (DX, EB6, and GL183) cells are shown after gating for CD4$^+$CD28^{null} T cells (*upper panel*), CD4$^+$CD28$^+$ T cells (*middle panel*), and CD16$^+$ NK cells (*lower panel*).

FIGURE 2. Cell surface expression of GL183 on CD4$^+$CD28^{null} T cell clones. PBMC from patients were analyzed for expression of CD158b; representative results for gated CD4$^+$CD28^{null} T cells (*upper panel*), CD16$^+$ NK cells (*middle panel*), and a CD4$^+$CD28^{null} T cell clone (*lower panel*) are shown for the RA patient HD. The black line indicates the staining by GL183, whereas the shaded area represents the staining by an isotype-matched control IgG. GL183 staining was equally bright on CD16$^+$ as well as on CD4$^+$CD28^{null} cells and the CD4$^+$CD28^{null} T cell clone.
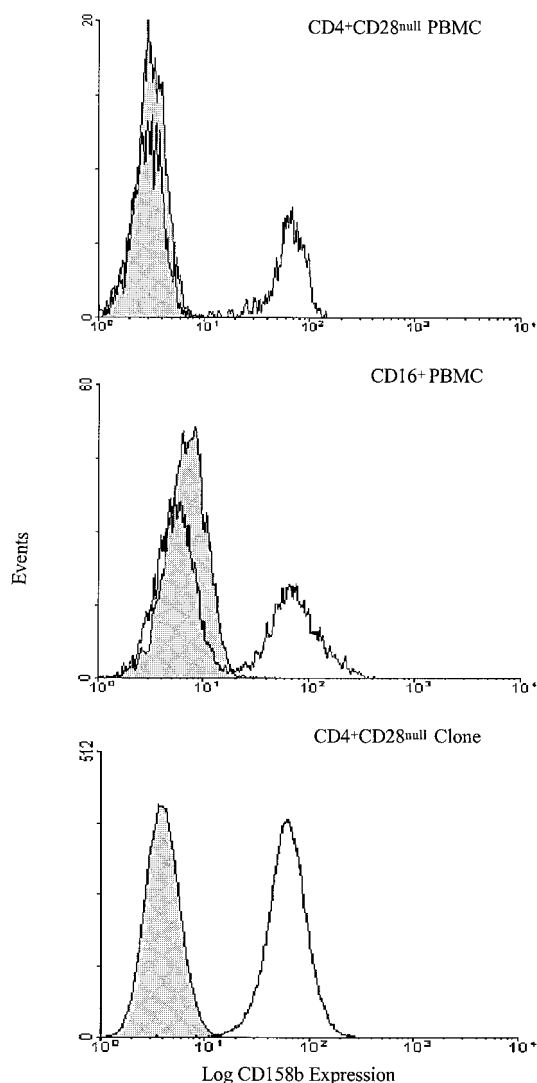
concentration (data not shown). Low expression of CD94 has recently been correlated with the presence of stimulatory NKG2 variants on NK clones (33).

In contrast to CD94, cell surface expression of KIR/KARs on CD4$^+$CD28^{null} T cells was at a similar level as that on CD16$^+$ NK cells. A representative flow cytometry experiment is shown in Fig. 2. In this particular patient, ~30% of the CD4$^+$CD28^{null} T cells expressed KIRs or KARs that are recognized by GL183. CD4$^+$CD28^{null} T cell clones established from this patient also expressed the KIRs and KARs recognized by GL183 at similar levels. This indicates that the KIRs and KARs found on CD4$^+$CD28^{null} T cells are expressed at levels that may be functional.

Spectrum of KIR/KAR expression on CD4$^+$CD28^{null} T cell clones

To determine the profile of receptor expression on individual clones, CD4$^+$CD28^{null} T cell clones were established from three of the RA patients. T cell clones were selected that expressed a TCR sequence also found in unseparated peripheral blood CD4$^+$ T cells, suggesting that these clones were expanded in vivo. All T cell clones analyzed were positive for CD3 and CD4 and lacked CD16 and CD28; none of the clones expressed CD94.

The available KIR-specific Abs are only specific for certain subfamilies. To obtain more complete information on the spectrum of KIRs and KARs expressed by these T cell clones, PCR was used to amplify a fragment of the known p50/58 and p70 KIR receptor sequences. The PCR products were cloned, sequenced, and

matched with known KIR sequences (34). Expression of the respective KIR was confirmed using subfamily-specific primers as described by Uhrberg et al. (31). Results are shown in Table I. Each clone expressed between one and three different NK receptors. At least one stimulatory receptor was found on seven of the eight T cell clones. Five T cell clones exclusively expressed stimulatory receptors, and clone H1.10 expressed three KARs. All seven clones had a receptor encoded by the KIR2DS2 gene. This gene product has been shown to recognize HLA-C allotypes characterized by a Ser in position 77 and an Asn in position 80 (C1 allotype) (35). Only one T cell clone, H1.67, concomitantly expressed an inhibitory receptor that recognizes the same C1 allotype as KIR2DS2. Three clones expressed the KIR2DS4 receptor. None of the clones expressed an inhibitory or stimulatory receptor for the C2 allotype. All three patients from whom the T cell clones were derived expressed a C1 allotype (Table I), suggesting that the KIR2DS2 receptors could be functional in vivo.

Table I. *Repertoire of MHC class I-recognizing receptors on CD4+CD28null T cell clones*

RA Patient	HLA-Cw*	CD4+CD28null T Cell Clone	CD94	KAR/KIR	ITIM Motifs	KAR/KIR Specificity
HD	501/07	HD1.1	−	2DS2	0	C1 allotype
		HD2	−	2DS2	0	C1 allotype
		HD3	−	2DS2	0	C1 allotype
				2DS4	0	Unknown
H	02/07	H1.16	−	2DS2	0	C1 allotype
				2DL4 (103 AST)	1	HLA-G
				2DL4 (103 ALP)	1	HLA-G
		H1.67	−	2DS2	0	C1 allotype
				2DL3	2	C1 allotype
				2DL4 (103 ALP)	1	HLA-G
		H1.10	−	2DS2	0	C1 allotype
				2DS3	0	Unknown
				2DS4	0	Unknown
K	0303/0501	KP5	−	3DL2	2	HLA-A?
		KV1	−	2DS2	0	C1 allotype
				2DS4	0	Unknown

Lack of modulation of cytotoxic activity by KAR stimulation

All CD4+CD28null T cell clones displayed TCR-triggered cyto-toxic activity in a redirected cytotoxicity assay. A representative example is shown in Fig. 3 for clones HD1.1 and HD2, both of which effectively killed P815 target cells by anti-CD3-mediated killing. Surprisingly, although both clones expressed a stimulatory KIR2DS2 receptor recognized by Ab GL183 and no inhibitory KIRs (Fig. 2 and Table I), they were not able to kill target cells bearing the GL183 Ab (Fig. 3). Also, the GL183 Ab was not able to enhance the anti-CD3-mediated killing. These findings were in contrast to the results obtained with NK clones expressing KAR receptors. NK clones expressing a KAR (i.e., GL183-reactive KIR2DS2), but not clones expressing a KIR (i.e., DX9-reactive KIR3DL1), exhibited cytotoxicity for GL183-labeled P815 target cells. These results suggest that the functional consequences of trig-gering KARs are different in CD4+CD28null T cells and NK cells.

Expression of DAP12 in CD4+CD28null T cell clones

The failure of KIR2DS2 cross-linking to induce cytotoxicity raised the question of whether this receptor is functional in

CD4+CD28null T cells. In NK cells, the KIR2DS2 protein has been shown to non-covalently associate with the disulfide-bonded ho-modimer DAP12 (36). DAP12 contains an immunoreceptor ty-rosine-based activation motif in its cytoplasmic domain. Phosphor-ylated DAP12 binds Syk protein tyrosine kinases, suggesting that it is intimately involved in the cellular activation cascade (37). To analyze whether CD4+CD28null T cell clones express DAP12, cDNA from the eight CD4+CD28null T cell clones and from five CD4+CD28+ control clones were amplified with DAP12-specific primers. All CD28null clones and two of the five CD28+ clones expressed DAP12 mRNA (Fig. 4). These two CD28+ clones were negative for KIRs and CD94.

Stimulation of KIR2DS2 induces tyrosine phosphorylation in CD4+CD28null T cells

To address the question of whether CD4+CD28null T cells have the ma-chinery to transmit KIR2DS2 signals, a CD4+CD28nullKIR2DS2+ T cell clone from patient HD, HD4−12, was stimulated with anti-CD3 or GL183 followed by analysis of the tyrosine phosphoryla-tion patterns. Similar to the HD clones shown in Fig. 3, HD4−12

FIGURE 3. Cytotoxic activity of CD4+CD28null T cell clones is not modulated by KAR stimula-tion. CD4+CD28null T cell clones (*upper panels*) were tested for cytotoxic activity in a redirected cytotoxicity assay using anti-CD3 and Fc recep-tor-positive P815 targets. Anti-CD16-mediated killing by NK clones expressing either a stimu-latory (GL183-reactive KIR2DS2) or an inhibi-tory (DX9-reactive KIR3DL1) receptor was used as a control (*lower panels*). A representa-tive experiment with clones HD1.1 and HD2 is shown. Anti-CD3 and anti-CD16 induced cyto-toxic responses in T cells and NK cells, respec-tively (○). Cross-linking of GL183 did not in-duce cytotoxic activity (△), nor did it modulate cytotoxic activity in the presence of anti-CD3 (▲) in the T cell clones, although they expressed KIR2DS2 in the absence of inhibitory receptors (see Fig. 2 and Table I). GL183 induced the cy-totoxic activity of the KIR2DS2-expressing NK clone (△), while KIR triggering suppressed anti-CD16-mediated killing of the KIR3DL1-ex-pressing NK clone (▲, *lower panels*). Back-ground cytotoxicity is shown (●).
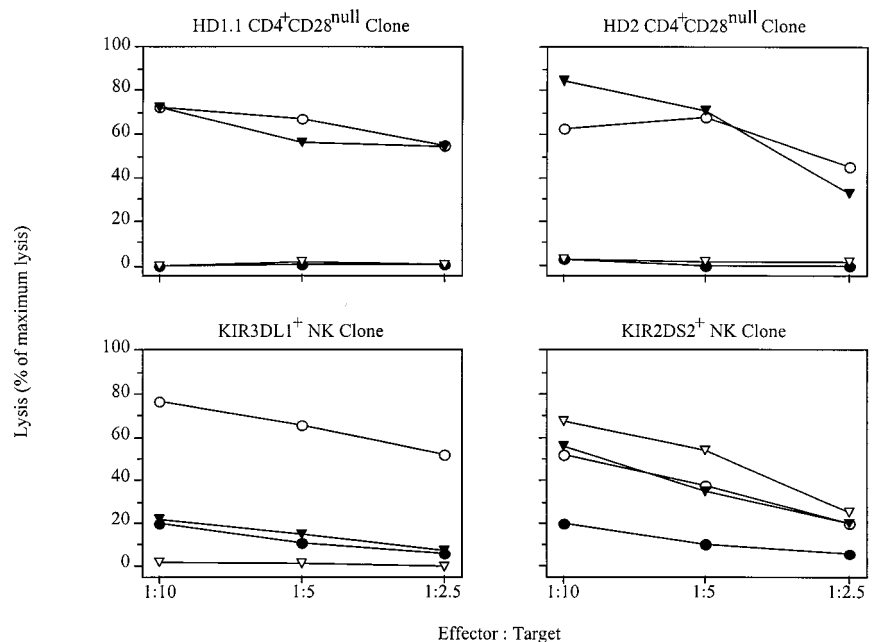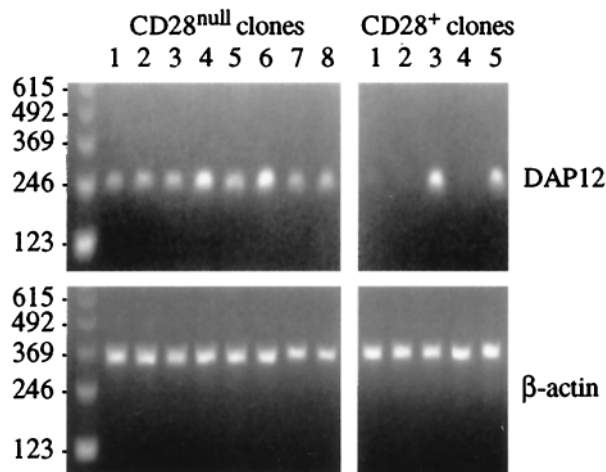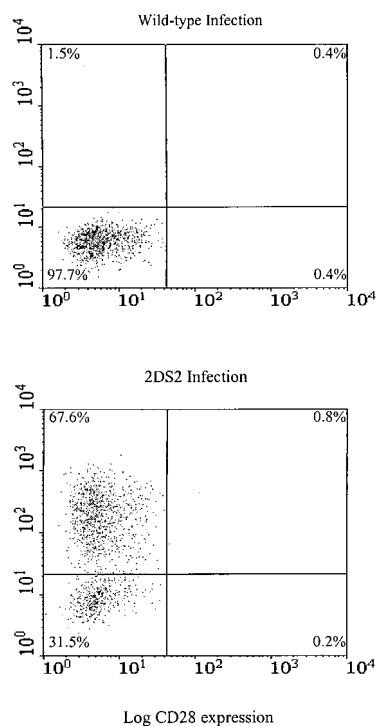
FIGURE 4. Transcription of the DAP12 gene in CD4$^+$CD28^{null} T cell clones. cDNA from eight CD4$^+$CD28^{null} T cell clones and from five CD4$^+$CD28$^+$ control clones were PCR amplified with DAP12-specific (*upper panels*) and β-actin-specific primers (*lower panels*). The DAP12-amplified products were confirmed by sequencing. All eight CD4$^+$CD28^{null} T cell clones and two CD4$^+$CD28$^+$ T cell clones contained mRNA for DAP12.

FIGURE 5. Cell surface expression of GL183 in CD4$^+$CD28^{null} T cells transfected with KIR2DS2. A CD4$^+$CD28^{null}KIR2DS2^{null} T cell line was infected with wild-type vaccinia virus (*upper panel*) or 2DS2$^+$ vaccinia virus (*lower panel*). The lines were analyzed by flow cytometry for CD28 and KIR2DS2 expression after a 5-h incubation with the virus. CD28^{null} T cells possessed the cellular components required to express 2DS2 on the cell surface.

expressed a GL183 Ag that was not functional in Ab-mediated cytolysis. In repeated experiments, cross-linking with GL183 induced phosphorylation of several cellular substrates (data not shown); however, the clone exhibited a high background tyrosine phosphorylation, suggesting continual activation even in the absence of anti-CD3 or GL183. We therefore transfected KIR2DS2 into a CD4$^+$CD28^{null} T cell clone from the same patient that lacked the cell surface expression of receptors recognized by GL183. Transfection with KIR2DS2 was sufficient to obtain cell surface expression that was detectable by GL183 (Fig. 5). Transfected clones were stimulated by cross-linking with either anti-CD3 or GL183, and lysates were analyzed using phosphotyrosine Abs (Fig. 6). Both stimulation conditions yielded an increase in the phosphotyrosine content of several cellular substrates. Affected substrates after anti-CD3 stimulation had apparent masses of 150, 110, 80, 55, 40, and 20 kDa. Stimulation with GL183 resulted in tyrosine phosphorylation of a more restricted set of substrates, particularly a protein with an apparent mass of 55 kDa.

Costimulatory function of KARs

To analyze whether the stimulatory receptors regulate cell activities distinct from cytotoxicity, T cells were stimulated with suboptimal concentrations of immobilized anti-CD3, and proliferative responses in the presence of immobilized control IgG or GL183 were determined (Fig. 7). Stimulation of the CD4$^+$CD28^{null} or CD4$^+$CD28$^+$ T cell clones by GL183 in the absence of anti-CD3 did not elicit any proliferation above the background activity. GL183 in the presence of anti-CD3 increased the proliferative response of all three CD4$^+$CD28^{null} T cell clones compared with that in the control cultures. It did not influence the proliferation of the two control clones that were negative for KIR2DS2. Costimulatory activity of GL183 was observed at concentrations of anti-CD3 that alone were not stimulatory, as shown for clone HD4–12 in Fig. 7. This increase in proliferation was not observed when HD4–12 was incubated with immobilized anti-CD3 and L368, an anti-β$_2$-microglobulin Ab (data not shown). This indicates that the increased proliferation was specific and was not due to increased trapping of the cells to the Ab-coated plates. These results dem-

onstrated that stimulatory KARs are functional on CD4$^+$CD28^{null} T cells. They promoted proliferation, although they did not enhance their cytotoxic activity.

Discussion

T cells that undergo clonal expansion in patients with RA contribute to the inflammatory lesions in the synovial membrane, but are also present in the peripheral blood and are characterized by the unusual phenotype CD4$^+$CD28^{null} (3, 38). Multiple lines of evidence support a role for CD28-deficient CD4 T cells in the systemic manifestations of this autoimmune disease as opposed to the localized inflammation in affected joints (2–4). The association of these unusual CD4 T cells with the disease predicts that unique stimulatory conditions exist in RA patients that favor the expansion and oligoclonality of the CD4$^+$CD28^{null} compartment. The present study found that CD4$^+$CD28^{null} T cells, in contrast to CD4$^+$CD28$^+$ T cells, can express MHC class I-recognizing receptors. The preference for KARs on CD4$^+$CD28^{null} T cells together with the relative decrease in KIRs and CD94/NKG2 receptors should shift the balance toward the initiation and amplification of immune responses instead of inhibition. Stimulatory function was confirmed for the proliferative response of CD4$^+$CD28^{null} T cells. We propose that the aberrant and unbalanced expression of KARs on CD4 T cells has a role in the expansion of potentially autoreactive T cells in RA.
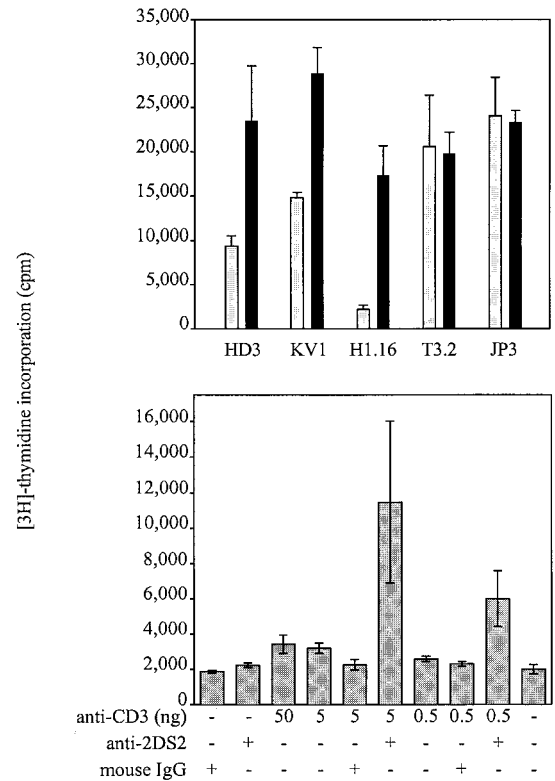
KIRs, as well as CD94/NKG2 receptors, were first described as receptors on NK cells that transduced negative signals to block NK cell-mediated killing and have been generally accepted as the molecular basis of the "missing self" hypothesis (39). NK cells are
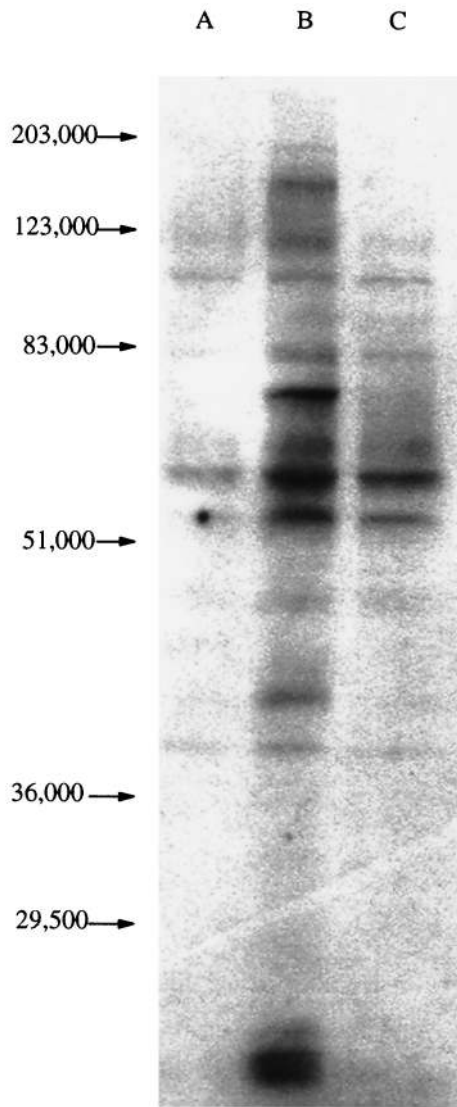
FIGURE 6. GL183-induced tyrosine phosphorylation in CD4$^+$CD28^{null} T cells transfected with KIR2DS2. CD4$^+$CD28^{null}KIR2DS2^{null} T cells were infected with KIR2DS2$^+$ vaccinia virus. The cells were cross-linked with 5 μg/ml IgG (*lane A*), 5 μg/ml anti-CD3 (*lane B*), or 5 μg/ml GL183 (*lane C*). The cells were lysed, the proteins were resolved, and the gels were transferred to a polyvinylidene difluoride membrane and blotted with anti-phosphotyrosine Ab. After blotting, the membrane was stained to confirm equal protein loading (data not shown). The gel is representative of four experiments.

FIGURE 7. Costimulatory activity of KIR on proliferative responses of CD4$^+$CD28^{null} T cell clones. CD4$^+$CD28^{null} T cell clones and CD4$^+$CD28$^+$ control clones were stimulated by cross-linking CD3 in the presence of immobilized GL183 (■) or isotype-matched control Ig (▨) and suboptimal concentrations of IL-2 (2 U/ml; *upper panel*). GL183 enhanced the anti-CD3-induced proliferative response of the three CD4$^+$CD28^{null} clones (HD3, KV1, and H1.16), but not that of the CD4$^+$CD28$^+$ control clones (T3.2 and JP3). Cross-linking of GL183 in the absence of anti-CD3 was not sufficient to induce T cell proliferation above background (<1000 cpm). Costimulatory activity of GL183 was observed at suboptimal anti-CD3 concentrations that were 100- to 1000-fold lower than the amount of Ab required for stimulation with anti-CD3 alone. A representative titration experiment is shown for clone HD4–12 in the *lower panel*. An increase in proliferation was not observed when the clone was stimulated with a combination of L368 and anti-CD3 (data not shown).

believed to survey the tissue for the presence of MHC class I molecules that are normally ubiquitously expressed. If MHC class I molecules are mutated or down-regulated, NK cells have the potential to be released from the inhibitory function of the receptors and to kill. Subsequent studies have shown that some receptor homologues promote rather than inhibit killing (13, 17). These receptors signal by associating with the disulfide-linked homodimer DAP12 (36), leading to the phosphorylation of immunoreceptor tyrosine-based activation motif in the cytoplasmic domain of DAP12 and the subsequent binding and activation of Syk (37).

Stimulatory as well as inhibitory MHC class I-recognizing receptors can be found on T cells. However, in most individuals, expression is limited to CD8 T cells (25, 26). Several authors have demonstrated that inhibitory receptors modulate the TCR-mediated function of CD8 T cells (27). Interestingly, CD94/NKG2A, but not KIRs, could be induced on CD8 T cells by IL-15, IL-10, and TGF-β, with subsequent inhibition of cytotoxic activity (40). MHC class I-recognizing receptors on CD8 T cells may therefore serve an immunosuppressive function that is regulated by cytokines in the microenvironment (41). In contrast, Mandelboim et al. (42) have demonstrated that KARs on CD4 T cells provide costimulatory signals. These authors speculated that the ubiquitous presence of MHC class I molecules triggering costimulatory signals in these CD4 T cells could have an important role in initiating and sustaining immune responses, including autoimmune responses.

The data presented here demonstrate that the expression of MHC class I-binding receptors on T cells is limited to individuals who have an expanded subset of CD4$^+$CD28^{null} T cells. CD4$^+$CD28^{null} cells are virtually absent in the majority of healthy individuals (2), suggesting that KAR-mediated costimulatory functions are unlikely to be of general relevance for T cell-dependent immune responses. Rather, expansion of the CD4$^+$CD28^{null} T cells is a characteristic finding in patients with RA, particularly in

patients with systemic complications. In these patients, the expression of KARs on CD4 T cells in the setting of the appropriate MHC class I Ag could facilitate the evolution of an autoimmune response by providing costimulatory signals, thereby lowering the threshold for low affinity recognition of autoantigens. In support of this concept, CD4$^+$CD28^{null} T cell clones isolated from RA patients as well as from IDDM patients have been found to display autoreactivity to autologous monocytes (3, 5). Because KIRs/KARs are polymorphic and bind to a family of highly polymorphic MHC class I molecules, the question arises of whether appropriate receptor-ligand pairs are available in RA patients. T cell clones presented here preferentially expressed KIR2DS2. This receptor is predicted to interact with a polymorphic site on HLA-Cw*03 and *07 molecules that were expressed in the RA patients from whom these clones were isolated.

The relationship between CD4$^+$CD28^{null} T cells and classical CD4 T cells and NK T cells is not understood. NK T cells have been best characterized in murine systems where they represent a separate lineage of T cells with unique specificity, selection mechanisms, and function (43). In humans, these cells express an invariant TCR α-chain (AV24-AJQ or AJ281), frequently use the TCR BV11 element, and recognize Ag in restriction to CD1 (44). We determined TCRAV and BV gene segment usage in 20 CD4$^+$CD28^{null} T cell clones, including the clones presented here, and none of them expressed the TCR characteristic for NK T cells (data not shown). Alternatively, CD4$^+$CD28^{null} T cells may derive from CD4$^+$CD28$^+$ T cells. Indeed, we have previously found the same clone in both the CD28$^+$ and CD28^{null} fractions (3). Moreover, CD28 expression is down-regulated with replicative senescence (45).

The repertoire of MHC class I-recognizing receptors on CD4$^+$CD28^{null} T cells was different from that on NK cells, suggesting different selection mechanisms. Valiante et al. have characterized ~100 NK clones each from two healthy individuals and have found that individual clones expressed between two and nine different receptors (32). By far, the most frequent receptor was the inhibitory lectin receptor CD94/NKG2A. In contrast, all CD4$^+$CD28^{null} T cell clones lacked this receptor. CD94/NKG2 receptors recognize the HLA-E molecule (22–24), which has limited sequence polymorphism. The CD94/NKG2A receptor has been postulated to provide a dominant negative signal and to control the effects of stimulatory receptors coexpressed on the same cell (32). The lack of expression of this important inhibitory receptor on CD4$^+$CD28^{null} T cells adds additional support to the idea that they are biased toward the expression of stimulatory receptors. Moreover, inhibitory KIRs were distinctly infrequent, and five of the eight T cell clones exclusively expressed a stimulatory KAR. Only H1.67 coexpressed the stimulatory KIR2DS2 and the inhibitory KIR2DL3, both of which putatively recognize the same HLA-C allotype. One additional clone expressed the KIR2DL4 receptor that, in contrast to other receptors, has only one YxxL motif in its cytoplasmic domain and recognizes HLA-G. In contrast, in the study by Valiante et al., none of the NK clones expressed stimulatory receptors in the absence of inhibitory receptors (32).

One of the striking findings of this study was that cross-linking of the KIR2DS2 receptor on CD4$^+$CD28^{null} T cell clones with GL183 Ab did not induce cytotoxic activity or enhance TCR-mediated cytotoxicity. The T cell clones lacked inhibitory receptors that could also be recognized by GL183, such as KIR2DL3, excluding the possibility that a stimulatory signal was neutralized. Stimulatory receptors have been shown to signal through DAP12, which has been reported to not be transcribed in CD4 T cells (36). To rule out the possibility that CD4$^+$CD28^{null} T cells lacked this important signaling molecule, we amplified DAP12-specific se-

quences. All the CD4$^+$CD28^{null} T cell clones and even two of the CD4$^+$CD28$^+$ T cell clones expressed DAP12 mRNA. The presence of DAP12 transcripts as demonstrated by PCR is, however, not sufficient to allow conclusions about the amount of functional DAP12 protein. Signaling of KIR2DS2 was confirmed in experiments demonstrating that cross-linking of the receptor induced tyrosine phosphorylation of particular cellular substrates. This suggests that KIR2DS2 is functional and that the increased proliferation with KIR2DS2 triggering is the result of stimulatory signals. However, the phosphorylation pattern was clearly different compared with anti-CD3 stimulation, consistent with the interpretation that KIR2DS2 stimulation does not amplify or mimic TCR stimulation and therefore does not enhance cytotoxic activity. Of particular interest, no lower m.w. phosphorylation product that might represent phosphorylated DAP12 was detected after KIR2DS2 stimulation. Indeed, preliminary data have indicated that KIR2DS2 triggering in CD4 T cells is not sufficient to induce calcium mobilization (data not shown). Signaling events downstream of DAP12, or perhaps even an alternate adaptor molecule, may explain the apparent functional differences between NK cells and CD4 T cells. Based upon these data, it could be speculated that KIR2DS2-mediated signals do not influence the TCR-dependent signaling pathway leading to cytotoxic responses, but interfere with cellular events regulating proliferation.

In summary, expression of KIR/KAR receptors is a characteristic finding for CD4 T cells that are deficient for the CD28 molecule. The functional consequences of MHC class I-recognizing receptors on CD4$^+$CD28^{null} T cells in RA patients appear to be biased toward stimulatory events. Whereas the coexpression of inhibitory and stimulatory receptors on NK cells results in the dominance of immunosuppressive signals, the unopposed expression of stimulatory receptors on CD4$^+$CD28^{null} cells should result in immunostimulation. Cross-linking of KARs enhanced the proliferation of CD4$^+$CD28^{null} cells, providing a possible explanation for the propensity of these cells to form large clonal populations in vivo. The over-representation of KARs on the expanded clonotypes of RA patients may be the result of peripheral selection with such KAR-expressing cells having a survival advantage. Because the emergence of CD4$^+$CD28^{null}-expanded clonotypes has been characteristically found in patients with autoimmune disorders, it is possible that the combination of KAR-mediated signals and suboptimal TCR-mediated stimulation is sufficient to facilitate the expansion of autoreactive T cells.

Acknowledgments

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