CD158 Receptor Controls Cytotoxic T-Lymphocyte Susceptibility to Tumor-Mediated Activation-Induced Cell Death by Interfering with Fas Signaling

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ABSTRACT

Renal cell carcinoma-infiltrating lymphocytes express killer cell immunoglobulin-like receptors (KIRs) that inhibit antitumor CD8+ T-cell functions and may contribute to local self-tolerance. In the present study, to better examine the functional consequences of KIR engagement on CTL–tumor interactions, we investigated the influence of KIR2DL1/CD158a on CTL survival. We show that both KIR+ and KIR− antigen-specific CTLs express Fas and Fas ligand and were susceptible to activation-induced cell death (AICD) triggered by coated anti-CD3 monoclonal antibodies. In KIR+ CTLs, anti-CD158a monoclonal antibodies partially inhibited anti-CD3-induced AICD. Interestingly, T-cell receptor activation by cognate tumor cells induced apoptosis in KIR+ CTLs but not in KIR− CTLs. In addition, co-engagement of T-cell receptors and KIRs by tumor cells decreased tumor-mediated CTL apoptosis. Blocking the interaction of KIR/HLA-Cw4 resulted in the restoration of tumor-induced AICD. Most importantly, our data indicate that KIR engagement affected two proximal events of Fas signaling pathway, a sustained c-FLIP-L induction and a decrease in caspase 8 activity. These studies provide evidence that tumor cells selectively favor the local persistence of nonfunctional KIR+ CTLs by promoting their survival.

INTRODUCTION

RCC represents a unique model of human immunogenic tumors. The clinical responses in metastatic patients treated with cytokines and the rare but documented spontaneous regressions indicate that an immune response may at least partly control tumor growth (1). However, these tumors are also characterized by several immune defects. RCC infiltrating lymphocytes display poor proliferative and lytic capacities, leading to a global functional anergy. In this regard, several mechanisms associated with alteration of TCR signaling and immunosuppressive factors secretion by tumor cells have been reported (2, 3). We recently provided evidence in support of a potential role of inhibitory NK receptors (KIRs) in the alteration of TIL cytolytic function (4, 5).

We have previously shown that inhibitory NK receptors belonging to the Killer immunoglobulin-like family KIR, expressed by 5–40% of CD8+ TILs, contribute to the altered cytotoxic activity of tumor-reactive CTLs (4, 5). Among inhibitory NK receptors for HLA-I molecules, KIRs are potent inhibitory receptors. They recognize specific polymorphisms on the classical HLA-A,-B,-C molecules. All of these inhibitory receptors have in common one or more ITIMs in their cytoplasmic tails. On tyrosine phosphorylation, ITIMs recruit the tyrosine phosphatase SHP-1, which can dephosphorylate molecules involved in immunoreceptor tyrosine-based activation motif-induced signaling pathways (6, 7). KIR2DL (CD158a and b) receptors possessing two immunoglobulin domains are recognized by specific mAbs and distinguish HLA-C alleles (8).

Inhibitory KIR receptors are also expressed by a small subset of peripheral T cells in healthy individuals and counterbalance TCR-mediated activation (9–11). These T cells express a memory phenotype (CD28−, IL-2Rβ), have a restricted TCR repertoire (9, 12), and lack CCR7. These KIR+ T cells may expand in response to chronic antigenic stimulation to self-antigen and most likely represent a precise subset of the memory T-cell pool (13, 14). Expression of inhibitory KIRs appears late in T-cell maturation on antigen-experienced T cells, and their expression probably involves modalities different from those on NK cells.

The presence of tolerant antigen-specific T cells has been documented in experimental models and, more rarely, in human tumors (15, 16). In renal tumors, the possibility to select clonal cytotoxic effectors from TILs by means of KIR expression allowed us to study the mechanism of peripheral tolerance in these tumor-associated specific T cells.

Apoptotic death of lymphocytes is an important homeostatic mechanism of peripheral tolerance to self-antigens (17, 18). The induction of apoptosis in mature T cells after antigenic stimulation, referred as AICD, is an important process for terminating and controlling the expansion of activated T cells. The death of mature T cells involves either suicide or fratricide and in most cases depends on the Fas/FasL pathway. Ligation of Fas by homotrimeric FasL results in clustering of Fas and recruitment of the adaptor protein FADD to the clustered Fas through their mutual death domain. FADD also contains two death effector domains through which it can recruit pro-caspase 8, leading to activation of the caspase cascade (19). The cellular homologue of the virus-encoded molecule FLIP that can inhibit Fas-L induced-cell death in vitro is suggested to play a central role in the regulation of T-cell homeostasis in vivo (20, 21).

In the present studies, we investigated the role of the KIR engagement on survival of antigen-specific KIR+ CTLs in response to stimulation by autologous tumor cells. We demonstrate that KIR engagement on tumor-specific CTLs favors their survival as a consequence of inhibition of AICD. The present studies emphasize that KIR, in addition to its inhibition of CTL lytic function, also plays a role in the control of T-cell homeostasis by interfering with Fas signaling.

MATERIALS AND METHODS

Culture of Tumors Cells and CTLs. Tumor cells derived from RCC were maintained in DMEM/Ham F12 medium supplemented with 10% FCS and 1% Ultrosen G (Life Technologies, Inc., Cergy Pontoise, France). Tumor cells

Received 3/21/03; revised 8/6/03; accepted 8/13/03.

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1 This work was supported by INSERM, the Association pour la Recherche sur le Cancer (Grants 2038 and 5253 to A. C.). A. G. and N. G. were recipients of grants from the Association pour la Recherche sur le Cancer and Ligue Nationale Contre le Cancer, respectively.

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3 The abbreviations used are: RCC, renal cell carcinoma; TCR, T-cell receptor; NK, natural killer; KIR, killer immunoglobulin-like receptor; TIL, tumor-infiltrating lymphocyte; ITIM, immunoreceptor tyrosine-based inhibition motif; SHP-1, Src homology 2 domain-containing protein 1; mAb, monoclonal antibody; IL, interleukin; AICD, activation-induced cell death; FLIP, Fas-ligand; FADD, Fas-associated death domain; DISC, death inducing signaling complex.

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were HLA genotyped RCC7 [HLA-A2, A29, B51, B44, Cw14, and Cw16 (Cw4 supertype)], RCC6 [HLA-A1, A2, B51, B8, Cw7 and Cw14 (Cw3 supertype)], and RCC5 [HLA-A1, A32, B7, B49, and Cw7]). CTLs were obtained after in vitro stimulation of TILs derived from RCC7 for 2–6 weeks and subsequent cloning by limiting dilution. CTL clones were expanded (3 × 10^6 cells/well on irradiated feeder cells (7 × 10^6 cells/well of allogeneic peripheral blood mononucleated cells) and 10^6 cells/well of an allogeneic EBV-transformed cell line (LAZ2509) in the presence of IL-2 (100 IU/ml)] and allowed to proliferate for 10 days. At the end of expansion, CTLs were frozen and used for the different experiments.

**Cytotoxicity Assay and CTL Survival Rescue Assay.** The cytolytic activity of T-cell clones against tumor cells was measured in a 4-h ^51^Cr-release assay. Tumor cells were used in amounts of 2 × 10^5 cells/well, and the E:T ratio ranged from 10:1 to 2:1. In some experiments, mAbs EB6 (anti-CD158a, IgG1), GL183 (anti-CD158b, IgG1), B1.23.2 (anti-HLA-B or -C, IgG2a), or W6.32 (anti-HLA-A, IgG1) or control murine IgG were added at saturating concentrations at the beginning of the cytolytic assay. The cytolytic activity of T-cell clones was also assessed in a CD3-directed lysis assay using P815 mastocytoma mouse cells. Briefly, ^3^Cr-labeled P815 (3 × 10^5) cells coated with decreasing doses of anti-CD3 (10 μg/ml–1 ng/ml) were incubated with T cells at a 5:1 ratio. Data were expressed as the percentages of specific lysis at the indicated E:T ratio. The percentage of specific ^3^Cr release was calculated as: (experimental release – spontaneous release)/total release – spontaneous release) × 100.

**Flow Cytometric Detection of Cell Surface Molecules.** The phenotypes of CTLs and tumor cells were determined by direct fluorescence. Briefly, 2 × 10^5 cells were incubated for 30 min at 4°C with conjugated mAbs: EB6-PE, CD3-FITC, CD56-PeCy^5^, CD6-FITC, and UB2-PE (anti-Fas, IgG1), purchased from Immunotech (Marseille, France), and B-R17-PE (anti-Fasl, IgG1), purchased from Becton Dickinson (San Jose, CA, France). CTL Stimulation and Cell Death Analysis. Early apoptotic events were evaluated by an annexin V labeling method using the Vybrant apoptosis assay kit (Molecular Probes, Interchim, Montluçon, France). AICD was evaluated after triggering of TCRs on IL-2-starved CTLs by coated anti-CD3 (UCHT1) mAb (1 μg/ml), alone or in combination with control mouse IgG (1 μg/ml), or with the anti-KIR mAb anti-CD158a (EB6, 1 μg/ml) for 6 h. Cells (2 × 10^6 cells) from each sample were then washed twice with cold PBS and resuspended in a binding buffer [50 mM HEPES, 700 mM NaCl, 12.5 mM CaCl_2 (pH 7.4)] at a concentration of 1 × 10^6 cells/ml. One hundred μl of this suspension were reacted with 5 μl of annexin V-FITC and 1 μl of 100 μg/ml PI. The mixture was gently vortexed and then incubated for 15 min at room temperature. After incubation, 400 μl of 1× binding buffer was added to each tube. Analysis by flow cytometry was conducted within 1 h of assay completion for optimal results. PI-stained cells, CD3-unstained cells, and annexin V-stained cells were run first to optimize the settings. Viable apoptotic cells were differentiated from necrotic cells by flow cytometry after PI staining of nonpermeabilized cells. Apoptotic cells were defined as annexin V+/PI-. Results were plotted as the percentage of annexin V+ cells and PI- cells. In some experiments, anti-Fasl (4H9, IgG1) purchased from Immunotech was used to block Fas/Fasl interaction.

Alternatively, CTLs were stimulated by tumor cells for 24 h in the absence of IL-2. After coulture, CTLs were removed gently and stained with Allophycocyanin-conjugated anti-CD8 mAb, and apoptosis was measured in the annexin V+/PI-/CD8+ population. In some experiments, anti-HLA-B/C mAbs were added in saturating concentrations to block KIR/HLA-C interactions. T-cell apoptosis was induced by incubation with 1–0.5 μM staurosporine (Sigma, Saint Quentin Fallavier, France) for 2 h or with anti-Fas mAb 7C11 (IgG1; purchased from Immunotech, Marseille, France), which triggers aggre-
Fig. 1. KIR engagement modulates the lytic activity of KIR+ CTLs. A and B, lysis of autologous RCC7 tumor cells by KIR+ 4D4 CTLs (A) and KIR− 22G7 CTLs (B) in the presence of saturating concentrations of anti-HLA mAbs or isotype-matched control immunoglobulin (clgG). C, production of IFNγ by KIR+ 4D4 CTLs in response to RCC7 A2Cw4 is increased when KIR/HLA-C interaction is blocked. D, dose-response curves for CD3-directed lysis of P815 cells showing CD3-directed lysis of the KIR+ and KIR− CTL clones.

KIR Receptor Enhances CTL Survival

KIR Engagement Induced Down-Regulation of AICD by Interfering with Fas Signaling. Because the above experiments revealed that KIR affects the Fas/FasL-mediated AICD pathway, we therefore asked whether this receptor interferes with Fas signaling. For this purpose, 4D4 KIR+ CTLs were simultaneously stimulated with anti-Fas (7C11) and anti-CD158a mAbs, and T-cell death was evaluated by annexin V/PI. The data shown in Fig. 5A revealed that KIR2DL1 engagement resulted in a significant inhibition (>50%) of anti-Fas-induced apoptosis. Dose–response experiments revealed that supraoptimal Fas stimulation (>0.75 μg/ml) was not inhibited by KIR triggering (Fig. 5A). It is well established that Fas/FasL can be proximally blocked by the expression of c-FLIP, a caspase 8 homologue that binds FADD without transducing a death signal (23). The long form of c-FLIP-L shares extensive homology with procaspase 8 and contains two death effector domains that interact with FADD, but has a mutation in the caspase-like domain that renders it inactive. In targets for 24 h, T-cell death was measured by triple staining with anti-CD8 and annexin V/PI. As clearly shown in Fig. 4A, RCC6 A2Cw3 cells, which optimally activate TCRs on 4D4 KIR+ CTLs, induce significant AICD, whereas RCC5 A1Cw3 cells did not, confirming that cognate tumor cell-induced AICD was antigen dependent. Interestingly, apoptosis of 4D4 KIR+ CTLs was clearly reduced in response to autologous RCC7 A2Cw4 cells, indicating that engagement of the KIRs by tumor cells decreased T-cell death and favored KIR+ CTL survival. In addition, the presence of blocking anti-HLA-B/C mAbs during the stimulation with RCC7 restored 4D4 apoptosis to the level obtained in response to RCC6 A2Cw3 tumor cells (Fig. 4B). Furthermore, increasing the tumor:CTL ratio to 2:1 led to apoptosis in up to 90% of 4D4 after TCR stimulation by cognate RCC6 tumor cells (Fig. 4C). Tumor cells did not stimulate nonmemory CD28+/22G7KIR− CTLs to AICD (data not shown).

KIR Engagement by Tumor Cells Reduced Tumor-Induced-AICD in KIR+ CTLs. The above experiments indicate that TCR activation resulted in AICD in tumor-specific 4D4 KIR+ and 22G7 KIR− CTLs and that KIRs partially control this response in KIR+ CTLs. We then asked whether TCR activation by tumors controls AICD in our model and whether KIR engagement influences AICD. For this purpose, CTLs were incubated with tumor cells that engaged either TCRs or KIRs or both. After CTL stimulation with tumor targets for 24 h, T-cell death was measured by triple staining with anti-CD8 and annexin V/PI. As clearly shown in Fig. 4A, RCC6 A2Cw3 cells, which optimally activate TCRs on 4D4 KIR+ CTLs, induce significant AICD, whereas RCC5 A1Cw3 cells did not, confirming that cognate tumor cell-induced AICD was antigen dependent. Interestingly, apoptosis of 4D4 KIR+ CTLs was clearly reduced in response to autologous RCC7 A2Cw4 cells, indicating that engagement of the KIRs by tumor cells decreased T-cell death and favored KIR+ CTL survival. In addition, the presence of blocking anti-HLA-B/C mAbs during the stimulation with RCC7 restored 4D4 apoptosis to the level obtained in response to RCC6 A2Cw3 tumor cells (Fig. 4B). Furthermore, increasing the tumor:CTL ratio to 2:1 led to apoptosis in up to 90% of 4D4 after TCR stimulation by cognate RCC6 tumor cells (Fig. 4C). Tumor cells did not stimulate nonmemory CD28+/22G7KIR− CTLs to AICD (data not shown).

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to anti-CD3-induced AICD measured by annexin V/PI staining, and low concentrations of coated anti-CD3 mAbs (up to 0.25 μg/ml) produced 50–60% apoptosis in the two CTLs. However, in response to higher concentrations of coated anti-CD3 mAbs (>0.5 μg/ml), 85–90% of 22G7 KIR− CTLs became apoptotic, whereas the percentages of apoptosis remained unchanged in 4D4 KIR+ CTLs (Fig. 2B). As evidence of the involvement of Fas/FasL in AICD in our experimental model, we showed that anti-FasL mAb completely abrogates AICD induced by anti-CD3 in both 4D4 KIR+ CTLs (3% versus 49%) and 22G7 KIR− CTLs (14% versus 72%), as shown in Fig. 2C.

Triggering of KIRs by Specific mAbs Reduced Anti-CD3-Induced Apoptosis in KIR+ CTLs. The data depicted in Fig. 3A show that simultaneous triggering of TCRs and KIRs on 4D4 KIR+ CTLs with coated anti-CD3 and anti-CD158a mAbs resulted in a significant decrease of apoptosis compared with anti-CD3 and isotype-matched control (clgG). Triggering of KIR receptor alone did not induce CTL apoptosis. The decrease in apoptosis varied from 35% to 60% in four independent experiments. In addition, TCR activation-independent apoptosis induced by staurosporine, an inhibitor of the protein kinase C pathway, led to death of the two CTLs, and the triggering of KIRs had no effect on 4D4 KIR+ CTLs (Fig. 3). These results indicate that the influence of KIRs on AICD is presumably mediated through a modulation of TCR-driven signaling events.

KIR Engagement by Tumor Cells Reduced Tumor-Induced-AICD in KIR+ CTLs. The above experiments indicate that TCR activation resulted in AICD in tumor-specific 4D4 KIR+ and 22G7 KIR− CTLs and that KIRs partially control this response in KIR+ CTLs. We then asked whether TCR activation by tumors controls AICD in our model and whether KIR engagement influences AICD. For this purpose, CTLs were incubated with tumor cells that engaged either TCRs or KIRs or both. After CTL stimulation with tumor targets for 24 h, T-cell death was measured by triple staining with anti-CD8 and annexin V/PI. As clearly shown in Fig. 4A, RCC6 A2Cw3 cells, which optimally activate TCRs on 4D4 KIR+ CTLs, induce significant AICD, whereas RCC5 A1Cw3 cells did not, confirming that cognate tumor cell-induced AICD was antigen dependent. Interestingly, apoptosis of 4D4 KIR+ CTLs was clearly reduced in response to autologous RCC7 A2Cw4 cells, indicating that engagement of the KIRs by tumor cells decreased T-cell death and favored KIR+ CTL survival. In addition, the presence of blocking anti-HLA-B/C mAbs during the stimulation with RCC7 restored 4D4 apoptosis to the level obtained in response to RCC6 A2Cw3 tumor cells (Fig. 4B). Furthermore, increasing the tumor:CTL ratio to 2:1 led to apoptosis in up to 90% of 4D4 after TCR stimulation by cognate RCC6 tumor cells (Fig. 4C). Tumor cells did not stimulate nonmemory CD28+/22G7KIR− CTLs to AICD (data not shown).
of four independent experiments, after triggering of TCRs and KIRs by anti-CD3 + anti-CD158a mAbs, the cellular c-FLIP-L level was significantly increased 3 min poststimulation compared with the level of FLIP detected in anti-CD3 + cIgG-stimulated CTLs. KIR engagement alone induced c-FLIP-L, but cotriggering of TCRs led to higher induction. Interestingly, in the presence of anti-CD158a mAbs, the c-FLIP-L level was sustained after 3 h, rendering the cells resistant to AICD (Fig. 5B).

In addition, anti-CD3-induced AICD correlated with pro-caspase 8 activation, which was inhibited by KIR engagement (data not shown). Co-engagement of TCRs and KIRs on 4D4 by KIR ligand-positive RCC7 for 16 h also resulted in low caspase 8 activity compared with RCC6, which engages only TCRs (Fig. 5C, upper panels). When KIR/HLA-Cw4 interaction was blocked during stimulation of RCC7 cells, the caspase 8 activity in 4D4 was comparable to that of RCC6 cells (Fig. 5C, lower panels). Finally, specific caspase 8 inhibitor clearly reduced anti-CD3-triggered AICD as well as anti-Fas-induced apoptosis, indicating that AICD is caspase dependent (Fig. 5D). However, it exerted no clear effect in experiments using tumor-stimulated 4D4 KIR+ CTLs. Stimulation by tumor cells required 16 h, and the inhibitor, by acting on tumor cells, may increase their resistance to CTL lysis. Taken together, our data indicate an essential role of KIRs in the control of AICD by a mechanism involving, at least in part, positive modulation of c-FLIP-L and a subsequent alteration of pro-caspase 8 activation.

**KIR Engagement by Tumor Cells Maintains T-Cell Survival.** 4D4 KIR+ CTLs were incubated for 3 days in cytokine-free medium in the absence or presence of different RCC targets. CTL survival was measured by triple staining as described above, and T cells were counted every 24 h during the time of culturing (Fig. 6). In accordance with the influence of KIRs on AICD, 4D4 KIR+ CTL survival was higher in response to RCC7 A2Cw4 targets compared with RCC6 A2Cw3 targets, which do not engage the KIRs. In addition, a CTL rescue assay indicated that by controlling the CTL activation level, KIRs may protect them from functional exhaustion and preserve their lytic potential toward KIR ligand-lacking tumor cells (data not shown).

**DISCUSSION**

It is well established that the immune system maintains an optimal number of lymphocytes and prevents autoimmune responses by eliminating any excess of nonfunctional and autoreactive T cells. It also supports the survival of antigen-specific T cells, thus promoting protective immune responses and immunological memory (24, 25). This balance is achieved by a complex network of lymphocyte survival and death signals, in which the Fas/FasL system plays a significant role to maintain T-cell homeostasis (26, 27).

In response to repeated antigen challenges, the elimination of high-affinity CD8+ T cells by clonal downsizing occurs during viral......
infections. It has been proposed that AICD serves a role in limiting expansion of an immune response, in that few effector T cells may survive as memory T cells. It may be assumed that in response to overexpressed self-antigens (28), tumor-specific CTLs may behave similarly. Although numerous reports support the existence of a tumor attack phenomenon, tumor-induced AICD of CTLs has rarely been investigated (29, 30). We therefore took advantage of our experimental model to further investigate whether the engagement of TCRs by tumor targets results in CTL AICD and whether KIRs are involved in the regulation of CTL survival. Our studies demonstrate that AICD in response to tumor cells was high in KIR+ CTLs and minimal in KIR− CTLs. The different behaviors of the two CTL clones may reflect the nature of the recognized antigen or the level of its expression. KIR− 22G7 CTLs recognize a private tumor-specific antigen, and the cognate HLA-A2 restricted peptide may be sparse on targets cells compared with self-antigens recognized by KIR+ CTLs. In this regard, whereas only a few TCR molecules are necessary to induce cytolysis in activated CTLs (31), the triggering of AICD may require higher numbers of TCR-MHC-peptide complexes. Both CTLs derived from blood (22G7 KIR−) and tumor (4D4 KIR+) may display different TCR avidities (32–34). The importance of high-avidity CTLs as potent cytotoxic effectors and their regulation by AICD were recently demonstrated in patients with chronic myeloid leukemia, indicating the likelihood of immune tolerance in patients with persistent disease by elimination of high-avidity specific T cells (30).

A role for KIRs in the survival of memory T cells and inhibition of AICD was suggested recently (13, 14, 35). In our experimental model, sensitivity to AICD of KIR+ CTLs in response to tumor-induced reactivation is in accordance with their memory effector phenotype and suggests that they correspond to preactivated, potentially autoreactive T cells. Furthermore, the present studies indicate, for the first time, a role of KIRs in the control of AICD in a tumor-specific CTL. Triggering experiments using anti-CD158 mAbs indicated that KIR engagement indeed resulted in a significant enhancement of CTL survival by inhibiting AICD. Nevertheless, KIR triggering had no effect on staurosporine-induced apoptosis, further confirming that triggering of KIRs results in the modulation of TCR signaling events. With respect to the mechanisms involved in the inhibition of AICD by KIRs, few data are available at present. The inhibitory effect of KIR3DL on T-cell apoptosis was reported previously in transfected Jurkat T cells. In this model, KIR inhibited AICD by blocking FasL induction in a KIR ligation-independent process involving inhibition of protein kinase C recruitment (36). In our model, triggering of KIR further decreased anti-CD3-induced AICD by affecting Fas signaling. The dependence on KIR ligation may likely reflect the nature of the T cells studied, i.e., antigen-specific CTLs versus KIR3DL-transfected Jurkat cells, and may be related to the levels of KIRs expressed by these cells (36).

Recently we obtained evidence indicating that KIR engagement resulted in the attenuation of early signaling and the subsequent T-cell activation (22). In the present studies, we showed that the down-regulation of T-cell activation by KIRs interferes with the Fas pathway, decreasing AICD. Exploring the mechanisms by which KIR engagement interferes with Fas-induced cell death, we showed by confocal microscopy that the formation of CD95 clusters in response to anti-CD3 was not decreased by engagement of KIRs (data not shown). We next investigated the proximal events of the Fas signaling pathway, focusing on the c-FLIP protein, which acts as a dominant negative of caspase 8. It is known that the affinity of FLIP/caspase 8 heterodimer for FADD is much higher than that of the caspase 8 homodimer and is critical in determining the fate of Fas ligation (21). KIR engagement was accompanied by an increase in c-FLIP-L levels in CTLs that was sustained after TCR and KIR co-engagement. The role of c-FLIP in the regulation of TCR-induced Fas-mediated apoptosis in T cells has been documented in vitro (37, 38) and in vivo (39). Fas-resistant T cells express high levels of c-FLIP compared with sensitive T cells (40, 41). In addition, it has been demonstrated that antigen concentration and costimulation signals are critical parameters in regulating AICD in memory T cells and that TCR-mediated higher neosynthesis of c-FLIP mRNA levels in these T cells than in naive T cells (32). Furthermore, our data indicate that in addition to higher c-FLIP-L levels, KIR engagement correlated with a decrease in pro-caspase 8 activation in KIR+ CTLs. The critical role of pro-caspase 8 in Fas signaling was demonstrated in mice with targeted caspase 8 or FADD mutations restricted to T-cell lineage that resulted in complete blockade of Fas-induced apoptosis (42, 43). In humans, a homozygous caspase 8 deficiency results in defective T-cell apoptosis and activation (44). In peripheral T cells resistant to Fas, pro-caspase

Fig. 4. Tumor-induced AICD in KIR+ CTLs is inhibited by KIR triggering. A, tumor-induced AICD in 4D4 KIR+ CTLs by cognate but not noncognate tumor cells. CTLs were stimulated for 24 h with RCC targets, and CTL cell death was measured by annexin V/PI staining. Numbers correspond to percentages of apoptotic T cells (one representative experiments of four). B, tumor-induced AICD in 4D4 KIR+ CTLs is restored by blocking HLA-C/KIR interactions. RCC7 targets were incubated with anti-HLA-B/C mAb for 10 min before the addition of CTL. C, tumor-induced AICD of KIR+ CTLs is dependent on CTL-tumor ratios.
Fig. 5. KIR engagement interferes with the Fas signaling pathway. 
A, KIRs decrease Fas-induced apoptosis. 4D4 KIR+ CTLs, incubated with anti-CD158a (1 μg/ml) or cIgG for 10 min, were treated with increasing doses of anti-Fas mAbs (7C11) for 16 h, and apoptosis was measured by annexin V/PI staining. Numbers are percentages of apoptotic cells (percentages of annexin V/PI were <10%). B, analysis of c-FLIP by Western blotting in 4D4 KIR+ CTLs stimulated by anti-CD158a, cIgG, anti-CD3+cIgG, or anti-CD3+anti-CD158a. Membrane was reprobed with antibody for actin to control for protein amounts. C, detection of pro-caspase 8 activity by flow cytometry in 4D4 CTLs stimulated for 16 h by RCC7, RCC6, and RCC5 tumor cells with use of the caspase Tag kit. 4D4 KIR+ CTLs were stimulated by RCC7 in the presence of anti-HLA-BC or cIgG. Results are expressed as percentages of cells with caspase 8 activity. D, specific caspase inhibitor Z-IETD-FMK (25 μM) inhibits anti-CD3- and anti-Fas-triggered T-cell apoptosis.
8, and FADD and caspase 8 were recruited in lipid rafts in Fas-treated human T cells, and the kinetics and affinity of KIRs for their ligands are compatible with their recruitment along with Fas to the DISC and their role in Fas signaling. The present studies show that in tumor-specific KIR+ CTLs, co-engagement of TCRs and KIRs by the cognate tumor cells was efficient in promoting tumor-specific CTL survival by attenuation of AICD. On stimulation by FasL+ RCC that did not present the appropriate MHC/peptide, KIR+ CTL survival was not affected. Whereas solid tumors may trigger Fas apoptotic pathway and cell death in interacting lymphocytes by functional FasL expressed on tumor cells (49–51), our results favor the hypothesis that tumor-specific CTL elimination at the tumor site is governed mainly by fratricidal or suicidal AICD and minimizes the involvement of FasL present on tumors in CTL destruction. However, tumor cells may indirectly be involved in the control of AICD by maintaining the inhibitory function of KIRs (5).

Recent studies further report a functional role of clonal KIR+ CTLs present in most healthy donors that were expanded in vitro after allogeneic stimulation. These CTLs were lytic for tumor cells from various origins, including NK targets, and were restricted by HLA-E molecules, nonclassical HLA-I molecules (52, 53). Although their role is not yet understood, they may correspond to virus-induced CTLs (54) maintained as memory effectors in the peripheral blood. RCC-infiltrating KIRs are probably different CTL subtypes, restricted by classical HLA-I, potentially autoactive, and present mainly at the tumor site. However, both types of CTLs have a effector memory phenotype, and their function is strictly controlled by the inhibitory NK receptor.

Altogether, our data suggest that in RCC, KIRs expressed by antigen-specific CTLs exert a dual effect, leading to the control of the lytic potential as well as the survival of the CTLs, and represent a powerful mechanism for maintaining a local self-tolerance at the tumor site. Recently, Molldrem et al. (30) reported that host immune response in patients with active leukemia was effective in deleting high-avidity cytotoxic CTLs. Our data further indicate that tumors, by locally controlling KIR function on CTLs, increase the T-cell activation threshold, resulting in decreased lytic activity and improved survival. Intratumoral rescued KIR+ CTLs with maintained lytic capacities do not represent a threat for tumor cells because the KIRs interrupt their activation, preventing the completion of the lytic process. Because there is growing knowledge of the antitumoral immune response, it is becoming evident that the challenge to improve cancer immunotherapy will be to maintain T-cell activation while preventing T-cell apoptosis and eliminating the effect of negative regulatory factors.

ACKNOWLEDGMENTS

We thank Dr. Guido Kroemer for helpful discussions.

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