Functional Inhibitory Receptors Expressed by a Cutaneous T Cell Lymphoma-Specific Cytolytic Clonal T Cell Population

Martine Bagot,[1] Denis Martinvallet,[1] Hamid Echchakir, Fabienne Chabanette-Schirm, Laurence Boumsell, and Armand Bensussan

INSERM U448, Paris XII University, Hôpital Henri Mondor, AP-HP, Créteil, France

Inhibitory receptors on natural killer cells and on a minority of T lymphocytes are major histocompatibility complex class Ia or Ib specific. We have previously reported several tumor-specific cytotoxic T cell clones infiltrating a CD4$^+$ Vβ13$^+$ cutaneous T cell lymphoma. These clones mediated a specific major histocompatibility complex class I-restricted cytotoxic activity toward the uncultured tumor cells and autologous long-term tumor T cell lines. In this study, we cultured with interleukin-2 the peripheral blood lymphocytes of the same patient a few weeks before invasion of the blood by tumor cells. We report the rapid and selective expansion of a CD8$^+$ Vβ13$^+$ lymphoid population. This population was clonal, as it expressed a unique T cell receptor-Vβ junctional region. Vβ13$^+$ tumor cells and Vβ13$^+$ reactive T cells were shown to have different junctional sequences. The CD8$^+$ reactive clone was functional, as it had a specific autologous tumor-specific, human leukocyte antigen-A2 restricted, cytotoxic activity. This clone coexpressed high levels of CD158a, CD158b, p70, and CD94/NKG2A inhibitory receptors. Interestingly, we found that anti-CD158a and anti-CD158b monoclonal antibodies could inhibit anti-CD3 redirected cytotoxicity mediated by the reactive clonal population. Further, an anti-human leukocyte antigen-B/C monoclonal antibody enhanced the specific cytotoxic activity of the clone against autologous tumor cells. These results are the first evidence that inhibitory receptor expression can lead to the inhibition of cutaneous T cell lymphoma-specific T cell responses. ***Key words: cutaneous T cell lymphoma/inhibitory receptors/T cell clone. J Invest Dermatol 115:994–999, 2000***

Inhibitory receptors are major histocompatibility complex (MHC) class Ia/Ib-specific receptors that have recently been reported on natural killer cells (Lanier, 1998). The well-known susceptibility of some cell lines to the so-called natural killer lysis is attributable to the absence of MHC class I expression by these cell lines (Ljunggren and Karre, 1990). There are several natural killer receptors, specific for different MHC class I alleles (Moretta and Moretta, 1997). In humans, inhibitory receptors are subdivided into two structural types of molecules that display a different specificity. One consists of immunoglobulin superfamily inhibitory receptors, which encompass several members, termed killer cell immunoglobulin-like receptors (KIR), characterized by several extracellular immunoglobulin-like domains, that specifically recognize human leukocyte antigen (HLA) -C (p58.1/CD158a and p58.2/CD158b) (Moretta *et al*, 1990, 1993, 1995; Wagtmann *et al*, 1995), HLA-B (p70/NKB-1) (Litwin *et al*, 1993; Lanier *et al*, 1995; Vitale *et al*, 1996), and HLA-A (p140) (Pende *et al*, 1996) alleles. The other type comprises type II membrane molecules that belong to the C-type lectin inhibitory receptors. These receptors are composed of CD94 covalently associated with NKG2A molecules, which display a specificity toward self MHC peptides presented by HLA-E (Braud *et al*, 1998). Recently, inhibitory receptors have also been shown to be expressed on a subset of activated T cells (mainly CD8$^+$) in which they can inhibit T cell receptor mediated functions (Mingari *et al*, 1997). Both families of receptors transduce negative signals to cytotoxic lymphocytes after their ligation with MHC class I molecules. The negative signal is associated with two tyrosine-x-x-leucine (YXXL) sequences in the cytoplasmic tail, termed immunoreceptor tyrosine-based inhibitory motif (Bensussan, 1998).

We have previously reported several tumor-specific cytotoxic T cell clones infiltrating a cutaneous T cell lymphoma (CTCL) (Bagot *et al*, 1998). These clones, although they had a CD4$^+$ phenotype, mediated a specific MHC class I-restricted cytotoxic activity toward the uncultured tumor cells and autologous long-term tumor T cell lines. In this study, we cultured in the presence of interleukin (IL) –2 the peripheral blood lymphocytes of the same patient at an early phase, before the invasion of the blood by tumor cells, and in the absence of tumor cells. We report the rapid selective expansion of a clonal T cell receptor (TCR) -Vβ13$^+$ CD8$^+$ tumor-specific cytotoxic cell population. We show that this reactive Vβ13$^+$ CD8$^+$ T cell clonal population, which was already detectable in the blood, was different from the Vβ13$^+$ tumor cells and expressed several functional inhibitory receptors, including CD158a, and CD158b, that led to the inhibition of its specific anti-tumor cytotoxic activity.

MATERIALS AND METHODS

Patient A 81 y old man presented with disseminated pruriginous infiltrated patches and plaques that had been present for 8 y. There was no palpable lymph node. The skin biopsy revealed an epidermotropic

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Reprint requests to: Dr. Martine Bagot, INSERM U448, Faculté de Médecine, 8 Avenue du Général Sarrail, 94010, Créteil, France. Email: martine.bagot@hmn.ap-hop-paris.fr

Abbreviation: KIR, killer cell immunoglobulin-like receptors.

[1]The first two authors contributed equally to this work.

infiltrate composed of small atypical lymphocytes with a cerebriform nucleus. A diagnosis of mycosis fungoides, or epidermotropic CTCL (stage Ib) was made. The phenotype of skin tumor cells was $CD3^+$ $CD2^-$ $CD5^+$ $CD7^-$ $CD4^+$ $CD8^-$ TCR-α/β^+ TCR-γ/δ^- MHC class I^+ MHC class II^-. Serology for human T cell lymphotropic virus-1 was negative. The patient was treated with topical mechlorethamine, which induced a complete remission. Two years later, identical lesions recurred, and the patient was treated with PUVA therapy, which again induced a complete remission. Three years later, the patient presented with disseminated skin tumors. The skin biopsy showed a nonepidermotropic infiltrate composed of large pleomorphic cells. The phenotype of tumor cells was unchanged. Despite a treatment with polychemotherapy and radiotherapy, the patient died after 6 mo of disseminated infection. Skin and blood samples were taken at different periods after informed consent. Ethical committee approval for the study was obtained.

Tumor cell lines Fresh CTCL tumor cells were obtained from mechanically dispersed tumor fragments. The tumor T cell line Cou-L was cultured as already reported (Bagot *et al*, 1998). Fresh CTCL cells and Cou-L failed to express MHC class II molecules and were HLA-A*0101, A*0201, and HLA-B*3503 and B*5101. The clonal origin of the growing cell line was systematically tested by analyzing the clonal reactivity with an anti-TCR-Vβ13 monoclonal antibody and by TCR-Vβ transcripts analysis (Bagot *et al*, 1998).

Culture of the T cell line DEN-1 To expand the T cell population DEN-1, patient peripheral blood mononuclear cells were isolated using Ficoll-Isopaque density gradient centrifugation (Pharmacia, Piscataway, NJ), and cultured at 10^6 cells per ml in RPMI 1640 supplemented with 2 mmol per l L-glutamine, penicillin (100 U per ml), streptomycin (100 μg per ml), 10% heat-inactivated human serum, and 25 U per ml recombinant IL-2. Cultures were fed every 3 d for 4 wk with recombinant IL-2-containing medium. It should be noted that no irradiated tumor cells were used as stimulator cells for the expansion of DEN-1.

Monoclonal antibodies (MoAb) and phenotypic analysis MoAb such as phycoerythrin (PE) -conjugated or fluorescein isothiocyanate (FITC) -conjugated anti-CD4 and anti-CD8 were purchased from Beckman Coulter (Marseille, France). The anti-TCR-Vβ13 MoAb was obtained from BIOadvance (Emerainville, France). Dr P. Le Bouteiller (INSERM, Toulouse, France) kindly provided: (i) MoAb B1.23.2, which reacts with monomorphic determinants shared by HLA-B, HLA-C, and only a few HLA-A alleles; (ii) MoAb L243, reactive with monomorphic determinants shared by HLA-DR, HLA-DP, and HLA-DQ; and (iii) the anti-HLA-A2 MoAb BB7.2. The anti-inhibitory receptors MoAb, including EB6 (anti-CD158a), GL183 (anti-CD158b), NKB1 (anti-p70), NKH3 (anti-CD94), and Z270 (anti-NKG2A), were obtained through the exchanges of the sixth and seventh international workshops on the human leukocyte differentiation antigens. The anti-CD1a MoAb O10 was produced in our laboratory. These MoAb were used as ascite fluids hybridoma supernatants. Phenotypic analysis was performed, using a single argon flow cytometer analyzer (Epics XL, Coulter, Miami, FL). Indirect immunofluorescence assays were performed, using a FITC-conjugated goat anti-mouse immunoglobulin from Caltag Laboratories (San Francisco, CA) or a PE-labeled goat anti-mouse immunoglobulin from Beckman Coulter. Two-color analyses were performed as already described (Bagot *et al*, 1998).

Cytotoxicity assays Cytotoxicity assays were performed according to a standard ^{51}Cr-release method. Assays at various effector to target cell (E/T) ratios with 5×10^3 ^{51}Cr-labeled target cells per well were carried out in triplicate, using 96-well V-bottomed microtiter plates. For blocking experiments, the target cells were incubated for 20 min with the appropriate MoAb, and subsequently washed before being used in the cytotoxic assay. The final culture volume was 200 μl per well. After 4 h of culture, plates were spun and 100 μl of supernatant was removed from each well and counted in a gamma-counter for the determination of ^{51}Cr release. The percentage of lysis was determined as previously described (Maïza *et al*, 1993). For the antibody-redirected cytolytic assay, the murine mastocytoma cell line P815 was used as source of target cells, as previously described (Echchakir *et al* 2000). Briefly, DEN-1 effector cells were incubated with various concentrations of an anti-CD3 MoAb alone or in combination with an anti-CD158a, an anti-CD158b, or an irrelevant anti-CD1a MoAb (O10) of similar isotype (IgG1), before the addition of ^{51}Cr-labeled P815 target cells at an E/T ratio of 10/1.

Directed sequencing of polymerase chain reaction (PCR) products PCR products were obtained as described elsewhere (Bagot *et al*, 1998), and were purified using Qiagen columns (Qiaquick PCR purification kit, Hilden, Germany), and resuspended in 20 μl of sterile water. The purified products were directly sequenced in both directions with a PRISM ready reactions DyeDeoxy Terminator cycle sequencing kit and a 373-A DNA sequencer (Applied Biosystems, Foster City, CA).

RESULTS AND DISCUSSION

Rapid expansion of a $CD8^+$ $V\beta13^+$ reactive T cell clonal population Flow cytometric analysis performed with peripheral blood lymphocytes of the patient revealed a minor population of $CD8^+$ lymphoid cells, and a major population of $CD4^+$ cells. Looking for the presence of $V\beta13^+$ $CD4^+$ tumor cells in the blood, we found within the $CD8^+$ population a small but significant subset of $CD8^+$ $V\beta13^+$ cells, whereas no $CD4^+$ $V\beta13^+$ tumor cells were detectable at that time (**Fig 1**). Culture with IL-2 alone selectively and rapidly expanded this $CD8^+$ $V\beta13^+$ lymphoid population, that became predominant after 28 d and was named DEN-1 (**Fig 1**). All TCR-$V\beta13^+$ lymphocytes were found within the $CD8^+$ subset, and few $V\beta13^-$ $CD4^+$ and $V\beta13^-$ $CD8^+$ lymphocytes were still present in the culture after 28 d. The TCR-β VDJ sequence of DEN-1 was determined and compared with the TCR-Vβ13 junctional region of the tumor T cell line Cou-L. As shown in **Table I**, DEN-1 was found to be a clonal population, because a unique junctional region sequence was obtained with Vβ13 and Cβ primers. Both Cou-L and DEN-1 expressed Vβ13.2-Jβ2.5 TCR transcripts, but their TCR-Vβ junctional sequences were different. This T cell population was not related to the tumor cells previously reported (Bagot *et al*, 1998), as it failed to contain a trisomy 7, found on fresh tumor cells as well as on long-term tumor T cell lines. Furthermore, we could distinguish tumor cells from the reactive T cell line, as the former failed to express MHC class II molecules, whereas the latter expressed high amounts of HLA-DR, -DP, and -DQ molecules (data not shown).

The cytotoxic activity of the $CD8^+$ $V\beta13^+$ T cell clonal population is restricted by HLA-A2 and enhanced by an anti-HLA-B/C MoAb The cytotoxic activity of the 4 wk cultured clone DEN-1 was measured against different target cells: the autologous tumor T cell line Cou-L (**Fig 2*A***), the MHC class I-negative natural killer-susceptible cell line K562, and the allogeneic tumor T cell line PNO (**Fig 2*B***). The results showed that DEN-1 had both a tumor-specific cytotoxic activity on Cou-L and a natural killer-like cytotoxic activity on K562. The natural killer-like reactivity of DEN-1 against the MHC class I-negative target cell line K562 can be attributable to the clonal $CD8^+$ $V\beta13^+$ autologous tumor-specific population, as we already reported MHC class I-restricted cytotoxic T cell clones exhibiting a natural killer-like reactivity (David *et al*, 1987). In contrast, DEN-1 had no cytotoxic activity against the allogeneic tumor T cell line PNO. The autologous tumor-specific cytotoxic activity was restricted by HLA-A2, as it was inhibited by preincubation of the target cells with the HLA-A2-specific MoAb BB7.2 (**Fig 2*A***), whereas the preincubation with the control MoAb L243 had no effect (data not shown). The autologous tumor-specific reactivity of DEN-1 was attributable to the $CD8^+$ $V\beta13^+$ major cell subset, as removal of $V\beta13^+$ cells with magnetic beads completely abolished the tumor-specific cytotoxic activity (data not shown). Interestingly, we found that the anti-tumoral cytotoxic activity of DEN-1 was strongly enhanced by the preincubation of the target cells with the MoAb B1.23.2, which binds HLA-B/C molecules (**Fig 2*A***). It must be noted that the preincubation of K562 or PNO with B1.23.2 MoAb did not modify the results obtained in **Fig 2(*B*)** (data not shown). In order to further demonstrate the MHC class I-restricted anti-tumor cytotoxic activity of DEN-1, we cultured this clone in the absence of IL-2 for 3 d. These culture conditions allowed us to dissociate the natural killer-like cytotoxicity, which almost disappeared, from the specific cytotoxicity occurring via CD3/TCR engagement, which was maintained (**Fig 2*C***). Thus, the cytotoxic activity mediated against autologous tumor cells by DEN-1 could not be

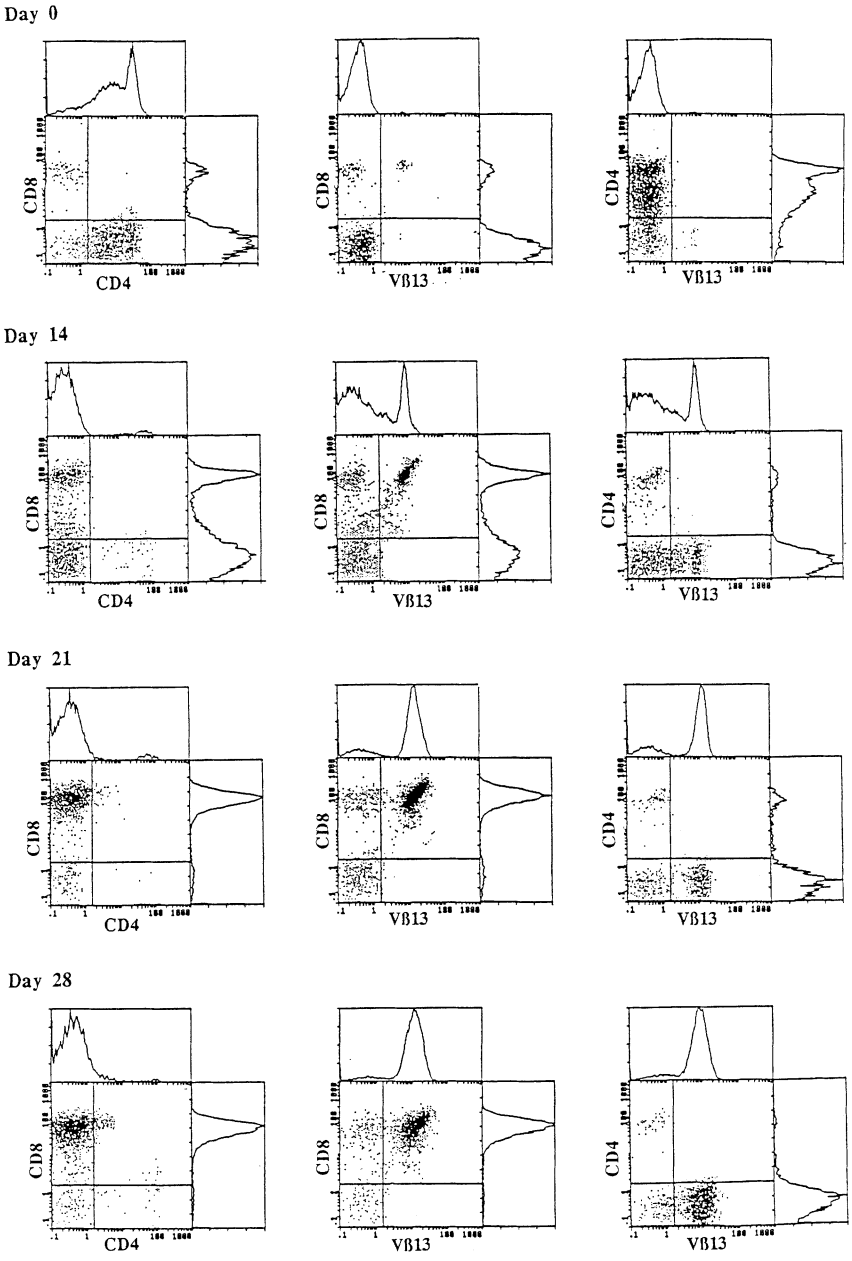

Figure 1. Rapid expansion of a CD8$^+$ Vβ13$^+$ T cell population from the blood of a patient with a CD4$^+$ Vβ13$^+$ cutaneous T cell lymphoma. Double immunostaining flow-cytometric analysis was performed, using a PE-conjugated or an FITC-conjugated anti-CD4 MoAb, a PE-conjugated anti-CD8, and an anti-Vβ13 MoAb plus a FITC-conjugated goat anti-mouse immunoglobulin. Labeling was performed by incubating the cells with the anti-Vβ13 MoAb for 20 min, then the FITC-conjugated goat anti-mouse immunoglobulin was added for 30 min. The cells were washed and incubated with a saturating amount of mouse immunoglobulins before adding the conjugated MoAb.

Table I. VDJ sequences of Cou-L and DEN-1

Cell line	Vβ	Framework	CDR3	Framework	Jβ
Cou-L	13.2	TGT GCC AGC AG	C CCC AGC GGG CGG AAA	CAA GAG ACC	2.5
DEN-1	13.2	TGT GCC AGC AG	T TAC TCT TTA GGT AGC GGG AGT ACC	TAC AAT GAG CAG	2.5

attributed to its natural killer-like activity observed with the presence of high amounts of IL-2 in the culture medium.

The CD8$^+$ Vβ13$^+$ T cell clonal population expresses several inhibitory receptors In order to determine whether the increase of the anti-tumoral cytotoxic activity of DEN-1, induced by the addition of an anti-HLA-B/C MoAb, was due to the blocking of the interaction of KIR with the HLA-B or -C molecules expressed by tumor cells, we studied the expression of several KIR and CD94/NKG2A by the tumor-specific cytotoxic T cell clone DEN-1. As shown in **Fig 3**, DEN-1 expressed high levels of several inhibitory receptors, including CD158a, CD158b, p70, and CD94/NKG2A. It should be noted that an identical staining profile was obtained with anti-CD94 and anti-NKG2A MoAb (data not shown). In contrast, the Vβ13$^+$ tumor T cell line Cou-L did not express any of the inhibitory receptors tested. Both DEN-1 and the tumor cell line were CD56$^-$ (data not shown). The recognition of HLA class I molecules on tumor cells by these inhibitory receptors clearly inhibited the specific cytotoxic activity of DEN-1 on autologous tumor cells, as addition of an anti-HLA-B/C MoAb strongly enhanced this specific cytotoxic activity. This increase of the specific cytotoxic activity of DEN-1 could not be

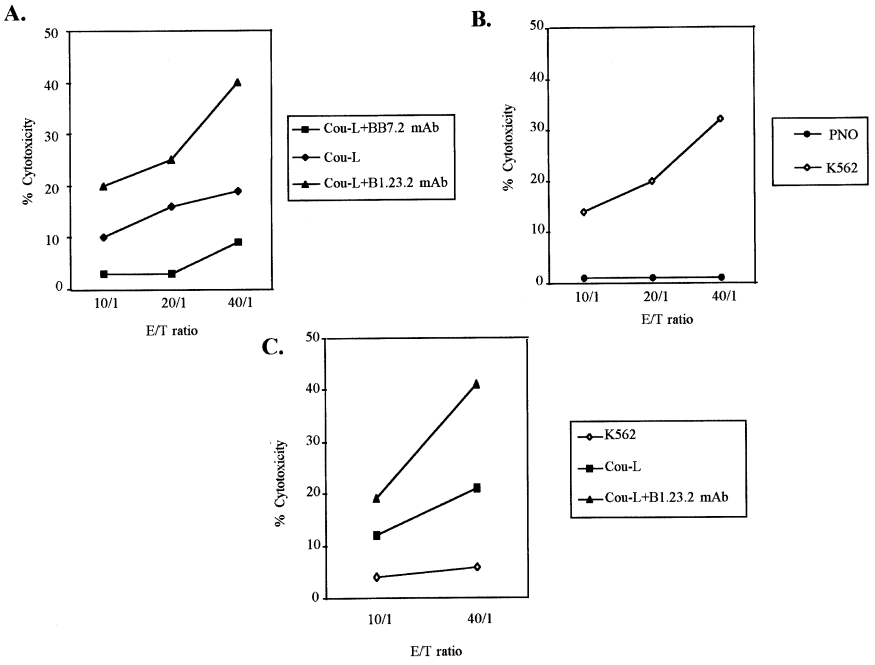

Figure 2. The cytotoxic activity of the CD8$^+$ Vβ13$^+$ T cell clonal population DEN-1 is restricted by HLA-A2 and enhanced by an anti-HLA-B/C MoAb. The cytotoxic activity of DEN-1 was measured against different target cells: the autologous tumor T cell line Cou-L (*A*), the MHC-class I-negative natural killer-susceptible cell line K562, and the allogeneic tumor T cell line PNO (*B*). DEN-1 was cultured in the absence of recombinant IL-2 for 3 d. The cytotoxic activity was then tested both on the autologous tumor cell line Cou-L and K562 (*C*). Results are expressed as percentages of specific lysis.

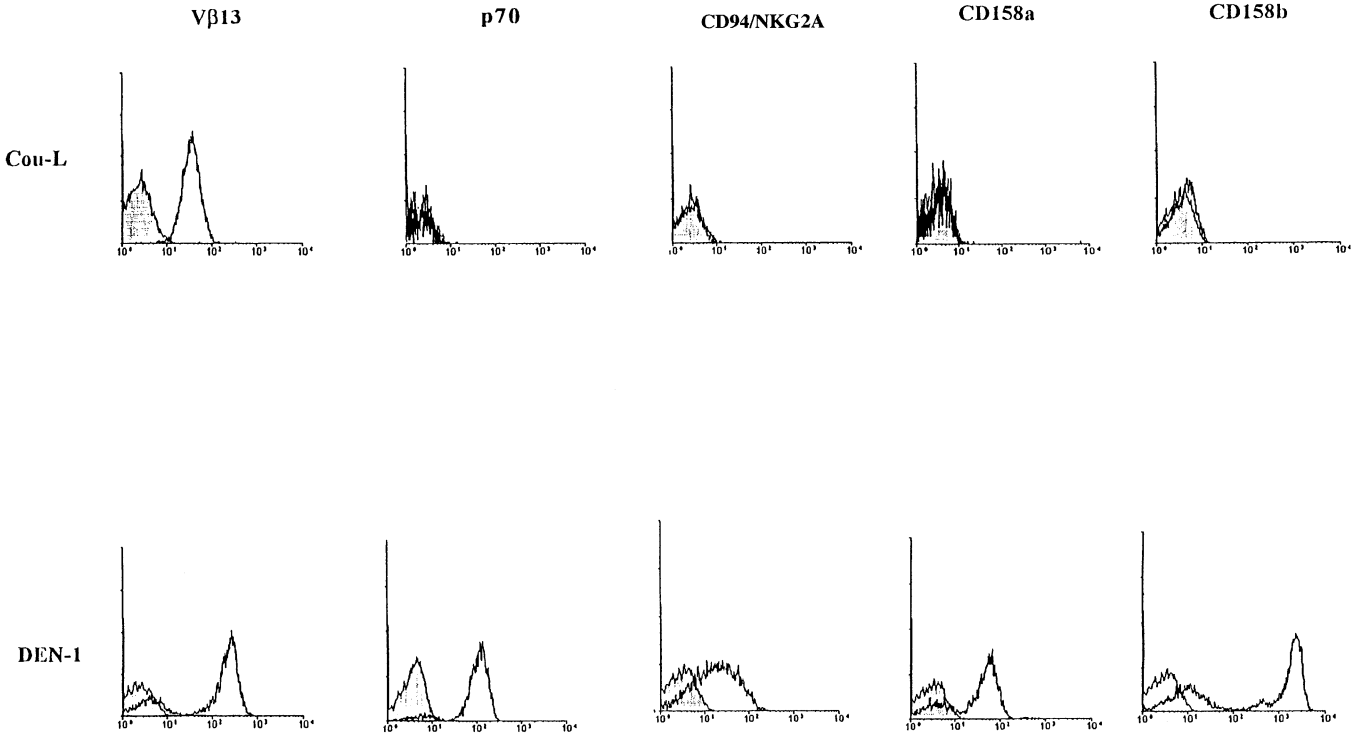

Figure 3. The CD8$^+$ Vβ13$^+$ T cell clone expresses several inhibitory receptors. Flow-cytometric analysis was performed, using diluted ascites of several MoAb directed against inhibitory receptors (anti-CD158a, CD158b, p70, and CD94/NKG2A) or an anti-Vβ13 MoAb, plus a FITC-conjugated goat anti-mouse immunoglobulin.

attributable to antibody-dependent cell cytotoxicity, as an isotype-matched MoAb reacting with the tumor cells failed to increase the lysis (data not shown). Furthermore, as the CD158a and CD158b receptors can be expressed as activatory or inhibitory isoform receptors (Bensussan, 1998; Lanier, 1998), we tested whether the stimulation of these receptors in DEN-1 cells might provide the inhibition of their anti-CD3 MoAb-redirected cytolytic activity. As shown in **Table II**, the results clearly indicate that triggering of CD158a or CD158b in DEN-1 cells induced a significant inhibition of the anti-CD3 MoAb-redirected cytolysis, whereas an isotype-matched control with an irrelevant antibody had no effect. Finally, it remained to determine the exact contribution of the inhibitory signal provided by the interaction of CD94/NKG2A molecules with HLA-E expressed by the tumor target cell.

Inhibitory receptors are MHC class I-specific receptors that have recently been reported on natural killer cells. Some T lymphocytes, including a minority of α/β T cells, and a fraction of γ/δ T cells, also express the MHC class I-binding inhibitory receptors found on

Table II. Triggering CD158a and CD158b on DEN-1 inhibited anti-CD3-redirected cytolysis[a]

Effector cells	P815 cells			
	+ medium	+ anti-CD3 MoAb (μg per ml)		
		0.1	1	10
DEN-1	< 5[b]	8	19	36
DEN-1 + anti-CD158a	< 5	< 5	8	14
DEN-1 + anti-CD158b	< 5	< 5	6	13
DEN-1 + anti-CD1a	< 5	7	15	34

[a]DEN-1 cells were incubated with anti-CD3 MoAb alone at several concentrations with or without anti-CD158a, anti-CD158b, or anti-CD1a MoAb (1/100 final dilutions of ascites). The effector cells were then cocultured with P815 target cells at an effector-to-target ratio of 10/1. The table shows representative data of three experiments.

[b]Results are expressed as percentage of specific cytotoxicity.

natural killer cells (Lanier, 1998; Mingari *et al*, 1998). Inhibitory receptor-expressing T lymphocytes have been found in peripheral lymphoid organs, but not in the thymus. They usually have a memory CD8 phenotype, and an oligoclonal Vβ repertoire. It has been shown that the recognition of the corresponding HLA class I molecule by a KIR can inhibit the cytotoxic activity and the cytokine production by a T cell clone (Mingari *et al*, 1995; D'Andrea *et al*, 1996; Le Dréan *et al*, 1998). The HLA-specific inhibitory receptors on such T cells may allow preferential lysis of cells that have downregulated the expression of the appropriate HLA class I molecules. In one patient with melanoma, the cytotoxic activity of a TCR-α/β^+, HLA-A24-restricted, *in vitro* raised T cell clone specific for the PRAME gene product, was inhibited by the interaction of a p58.2 inhibitory receptor expressed by the tumor reactive clone with the HLA-Cw7 class I molecule present on tumor cells (Ikeda *et al*, 1997). The subsequent loss of HLA-Cw7 unblocked the inhibitory signal, that limited the killing of tumor cells. Such cytotoxic clones, active against tumor cells showing partial HLA loss, may constitute an intermediate line of anti-tumor defense between the cytotoxic lymphocytes, recognizing highly specific tumor antigens, and the broadly reactive natural killer cells, recognizing HLA loss variants (Ikeda *et al*, 1997). Moreover, it has been shown that disrupting interactions of KIR with their ligands on tumor cells may enhance melanoma-specific anti-tumor responses mediated by both innate and adaptive immune effector cells (Bakker *et al*, 1998). Expression of inhibitory receptors by tumor-specific cytotoxic lymphocytes may therefore lead to an altered anti-tumor immune response. In addition, a recent study demonstrated the expression of KIR by human melanoma-specific cytolytic T lymphocytes *in vivo* (Speiser *et al*, 1999). Apoptosis of cytotoxic clones can also be induced by the triggering of KIR molecules (Nakajima *et al*, 1998).

In this study, we show the rapid and selective expansion of a circulating $CD8^+$ CTCL-reactive clonal population. This clonal expansion was obtained *ex vivo* without any stimulation with the tumor cells. In addition, we found that inhibitory receptor expression could be responsible for the inhibition of its specific MHC-restricted cytotoxic activity, and may therefore represent an escape mechanism able to antagonize CTCL-specific cytotoxic responses. As expression of KIR (in contrast to CD94/NKG2A) is not acquired during the *in vitro* culture with IL-2, these findings might have clinical relevance, because this minor specifically cytotoxic population was detected a few weeks before the invasion of the blood by tumoral $CD4^+$ T cells. In addition, we tested the expression of CD94/NKG2A on fresh patient peripheral blood lymphocytes before culture. Results show that the $CD8^+ V\beta13^+$ population present at that time in the blood was stained by the anti-NKG2A MoAb Z270 (data not shown). The expression of inhibitory receptors by this reactive T cell clone may have contributed to the lack of efficacy of the immune response of the patient against his tumor, and to the dissemination of the disease, although the evidence that there is inhibition of cytotoxicity *in vivo* is still weak. These data also suggest that strategies modulating inhibitory receptor expression or function could increase the efficacy of immunotherapy in cutaneous lymphomas.

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