

# Functional and molecular characterization of a KIR3DL2/p140 expressing tumor-specific cytotoxic T lymphocyte clone infiltrating a human lung carcinoma

Guillaume Dorothée<sup>1</sup>, Hamid Echchakir<sup>1</sup>, Béatrice Le Maux Chansac<sup>1</sup>, Isabelle Vergnon<sup>1</sup>, Faten El Hage<sup>1</sup>, Alessandro Moretta<sup>2</sup>, Armand Bensussan<sup>3</sup>, Salem Chouaib<sup>1</sup> and Fathia Mami-Chouaib<sup>\*1</sup>

<sup>1</sup>Laboratoire Cytokines et Immunologie des tumeurs Humaines, INSERM U487, Institut Gustave Roussy, F-94805 Villejuif, Cedex, France; <sup>2</sup>Dipartimento di Medicina Sperimentale, Sezione di Istologia, and Centro di Eccellenza per la Ricerca Biomedica, Università di Genova, 16132 Genova, Italy; <sup>3</sup>INSERM U448, Faculté de Médecine de Créteil, 94010 Créteil, Cedex, France

T lymphocytes infiltrating a human lung carcinoma stimulated *in vitro* with autologous tumor cell line showed a TCRV $\beta$ 13.6<sup>+</sup> T-cell expansion. This subset was isolated using TCRV $\beta$ -specific antibody and several T-cell clones were generated. All these clones expressed a unique V $\beta$ 13.6-J $\beta$ 2.7 TCR with the same junctional region strongly suggesting that they derived from the same cell. They were CD8<sup>+</sup>/CD28<sup>-</sup> and expressed the MHC class I binding killer cell Ig-like receptor (KIR)3DL2/p140, but not KIR3DL1/p70, KIR2DL1/p58.1 and KIR2DL2/3/p58.2. Sequence analysis indicated that KIR3DL2/p140 cDNA was identical to the previously reported 3DL2\*002 allele except for two nucleic acid substitutions. Functional studies showed that KIR3DL2/p140<sup>+</sup> CTL secrete a significant level of IFN $\gamma$  and mediate an HLA-A2-restricted cytotoxicity against the autologous and some allogeneic tumor cells but not towards the autologous EBV-B cells. Strikingly, both the lytic and the cytokine secretion activities induced upon specific cell interactions were unaffected by anti-KIR3DL2/p140 antibody. In addition, crosslinking KIR3DL2/p140 molecules on CTL did not result into the modification of cytotoxicity and cytokine production triggered by anti-CD3 antibody. These results strongly suggest that, as opposed to distinct KIR expressed by CTL, the *in vitro* KIR3DL2/p140 engagement does not result into inhibitory (nor activatory) effects on tumor-specific CTL.

Oncogene (2003) 22, 7192–7198. doi:10.1038/sj.onc.1206627

**Keywords:** TIL; KIR3DL2; CTL; TCR

## Introduction

The function of effector lymphocytes is regulated by a balance between signals transmitted by activating

receptors, which recognize ligands on target cells, and inhibitory receptors specific for MHC class I molecules. Ag/MHC complexes serve as activating signals for T cells and are recognized by TCR/CD3 complex (Zinkernagel and Doherty, 1974; Townsend *et al.*, 1986). Reciprocally, inhibitory signals can be provided by engagement of a variety of cell surface inhibitory receptors. These include the Ig-like killer inhibitory receptors (KIRs) specific for HLA-A, -B, -C, the CD94/NKG2A heterodimer, specific for HLA-E and the leukocyte Ig-like receptor 1/Ig-like transcript 2 (LIR1/ILT2), characterized by a broad specificity for different HLA class I molecules (Moretta *et al.*, 1994; Moretta *et al.*, 1995; Colonna *et al.*, 1997). KIRs are expressed on a subset of human CD8<sup>+</sup> T cells and their interaction with their specific ligands induces inhibition of cell-mediated lysis of target cells bearing the appropriate HLA class I allotype (Ferrini *et al.*, 1994; Phillips *et al.*, 1995; Ikeda *et al.*, 1997; Bakker *et al.*, 1998). Ligation of KIRs results in tyrosine phosphorylation of the immunoreceptor tyrosine-based inhibition motifs (ITIM) in their cytoplasmic domain, leading to the recruitment of SH2 domain-containing tyrosine phosphatase 1 (SHP-1) and inhibition of cell-mediated cytotoxicity and cytokine production (Burshtyn *et al.*, 1996; Fry *et al.*, 1996).

It has been previously reported that CD94/NKG2A heterodimer (Le Drian *et al.*, 1998; Noppen *et al.*, 1998; Speiser *et al.*, 1999) and KIRs, in particular KIR2DL1/p58.1 and KIR2DL2/3/p58.2 inhibitory receptors for HLA-C (Ikeda *et al.*, 1997; Guerra *et al.*, 2000; Gati *et al.*, 2001), were expressed by tumor-specific CTL clones and were able to inhibit their lytic activity towards the autologous tumor cells. Regarding KIR3DL2/p140 inhibitory receptor for HLA-A, no data related to CTLs are available and its expression and function on tumor infiltrating T lymphocytes (TILs) has never been reported. Solid tumors, including non-small cell lung carcinomas (NSCLC), are often infiltrated by TILs, most of which are TCR $\alpha/\beta$ <sup>+</sup>, CD8<sup>+</sup>, CD28<sup>-</sup> (Echchakir *et al.*, 2000 and unpublished data).

\*Correspondence: F Mami-Chouaib, U487 INSERM, Institut Gustave Roussy, 39 rue Camille-Desmoulins, F-94805 Villejuif, France; E-mail: cfathia@igr.fr.

We have previously demonstrated a T-cell response in NSCLC and isolated several tumor-specific CTL clones from TILs infiltrating a human lung carcinoma where they appeared clonally expanded (Echchakir *et al.*, 2000, 2001). In the present study we have isolated from the same patient TILs, several tumor-specific CTLs expressing KIR3DL2/p140 receptor and a unique V $\beta$ 13.6-J $\beta$ 2.7 TCR rearrangement. The functional effects of the engagement of KIR3DL2/p140 on cytokine release and cytotoxic functions of these effector T cells were investigated.

## Results

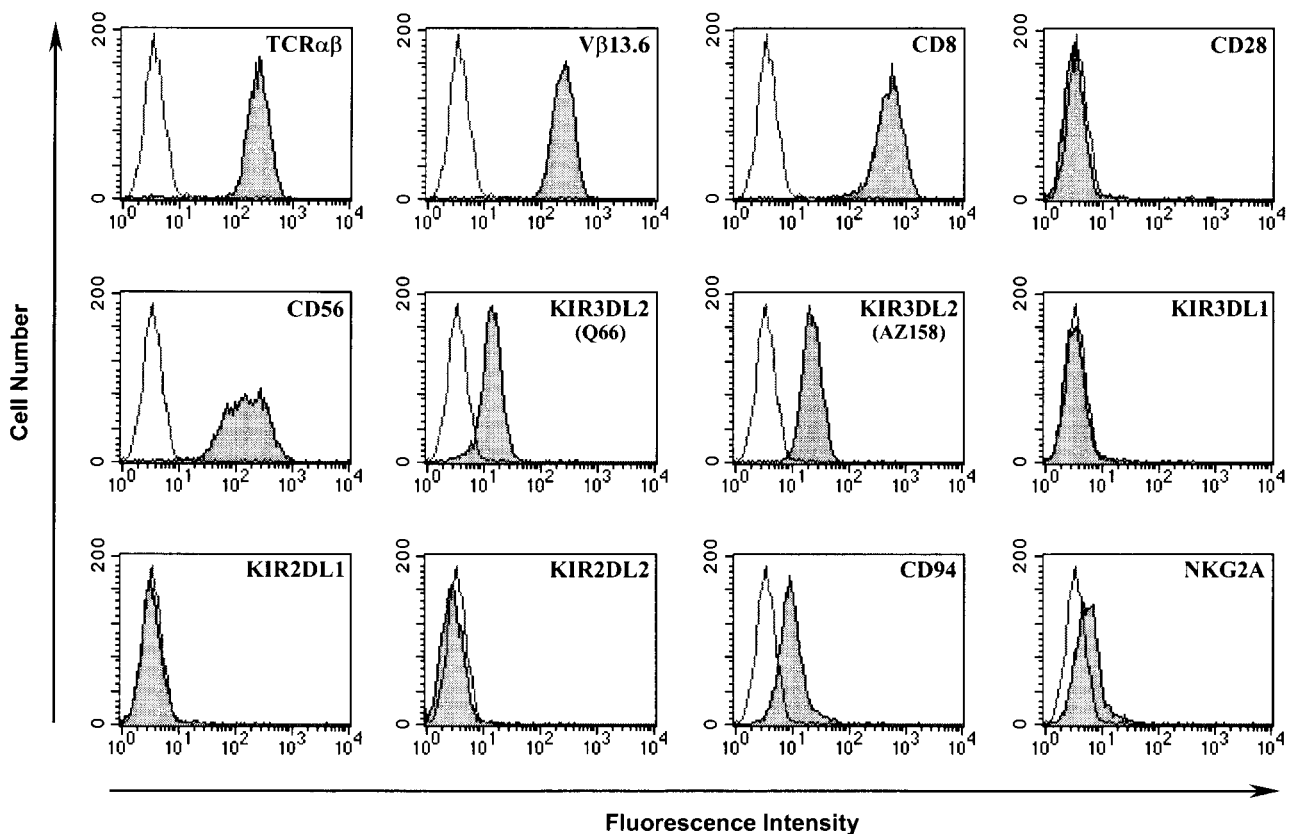
### Isolation and characterization of Heu33 CTL clone

The IGR-Heu tumor cell line was derived from a NSCLC tumor biopsy (Asselin-Paturel *et al.*, 1998). Mononuclear cells that infiltrated the primary tumor were isolated by Ficoll-Hypaque density gradient centrifugation and stimulated with irradiated IGR-Heu cells, irradiated autologous EBV-transformed B cells and IL-2. On day 15, the responding lymphocytes were analysed by flow cytometry and were shown to include 97% CD3<sup>+</sup> and 49% TCRV $\beta$ 13.6<sup>+</sup> cells. The TCRV $\beta$ 13.6<sup>+</sup> TILs were immunoselected using specific monoclonal antibody (mAb), and a cell line (H4C)

including 99% TCRV $\beta$ 13.6<sup>+</sup> was generated. This cell line was cloned by limiting dilution and a panel of T-cell clones, expressing a unique V $\beta$ 13.6-GGA-J $\beta$ 2.7 sequence, was isolated (data not shown). This result strongly suggests that these clones are derived from the same cell. Immunofluorescence analysis of one selected clone, Heu33, indicated that it expressed CD3 complex (data not shown) associated to a TCR $\alpha/\beta$  (Figure 1). Furthermore, this clone was TCR $\beta$ 13.6<sup>+</sup>/CD8<sup>+</sup>/CD56<sup>+</sup>/CD28<sup>-</sup> and consistently expressed significant level of p140-KIR3DL2 determined with both Q66 and AZ158 mAbs (Figure 1). In contrast, expression of CD94 and NKG2A was low and KIR3DL1/p70, KIR2DL1 (p58.1/CD158a) and KIR2DL2/3 (p58.2/CD158b) inhibitory receptors were not expressed (Figure 1). The same phenotype was obtained with the parental H4C TIL cell line. In contrast, the TCR $\beta$ 13.6<sup>-</sup> fraction did not express KIR3DL2/p140 (data not shown).

### Molecular characterization of KIR3DL2/p140 receptor expressed by Heu33 CTL clone

To determine whether the cDNA encoding KIR3DL2/p140 molecule expressed by Heu33 CTL clone corresponds to one of the 12 already described alleles (Shilling *et al.*, 2002), reverse transcription-polymerase chain reaction (RT-PCR) was performed and the

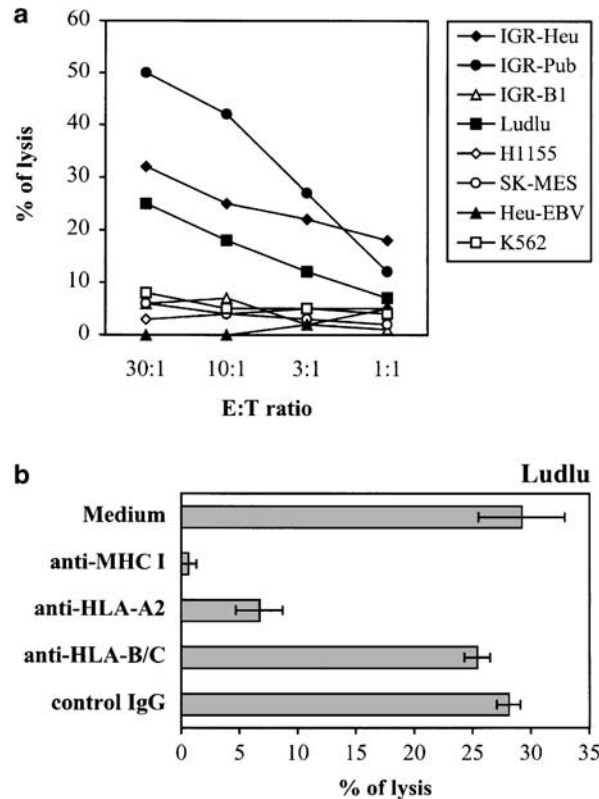


**Figure 1** Flow cytometry analysis of Heu33 CTL clone. Briefly,  $3 \times 10^5$  cells were stained with the indicated receptor-specific mAbs (gray fill) or isotype-matched control (without fill). For KIR3DL2/p140 staining, two mAbs Q66 (anti-KIR3DL2/p140) and AZ158 (recognizing both KIR3DL2/p140 and KIR3DL1/p70) were used and were indicated between parentheses

resulting product was cloned into pcDNA3.1 plasmid and sequenced. RT-PCR performed on RNA derived from the T-cell clone, using a set of primers able to amplify the entire cDNA encoding KIR3DL2 (Bagot *et al.*, 2001), showed a 1.4 kb size product (data not shown), similar to that previously described (Pende *et al.*, 1996; Bagot *et al.*, 2001; Gardiner *et al.*, 2001). Furthermore, nucleic acid sequence analysis indicated that it was identical to one of the previously reported alleles, the 3DL2\*002 allele (Pende *et al.*, 1996; Shilling *et al.*, 2002), except for two nucleotide replacements in exon 1 which may represent an additional polymorphism in the KIR3DL2/p140 gene. Figure 2 shows the amino-acid sequence alignment of Heu33 KIR3DL2 together with the 3DL2\*001 (Colonna and Samaridis, 1995) and the 3DL2\*002 (Pende *et al.*, 1996) encoded proteins. The two amino-acid substitutions in the leader peptide sequence are shown (Figure 2).

*Heu33 CTL clone recognizes autologous and allogeneic lung tumor cell lines*

Heu33 T-cell clone was assessed for its cytotoxic activity against IGR-Heu autologous tumor and EBV-B cells as well as a panel of allogeneic tumor cell lines including K562 and Daudi in conventional <sup>51</sup>Cr release assay. This clone displayed a high cytotoxic activity towards the autologous IGR-Heu tumor cell line as well as some allogeneic cells, including IGR-Pub and Ludlu HLA-A2<sup>+</sup> NSCLC cell lines, but not against the autologous EBV-B cells, Daudi (data not shown) and K562 (Figure 3a). It should however be mentioned that the cytotoxicity towards K562 varied from one experiment to another (0–30% of lysis at 30:1 E/T ratio) and depended upon the effector cell status for IL-2-induced activation. Note that the IGR-B1, allogeneic HLA-A2<sup>+</sup> NSCLC cell line was not lysed by the Heu33 CTL clone suggesting a broad but restricted expression of the recognized tumor Ag.



**Figure 3** Analysis of specificity and HLA restriction element of Heu33 CTL clone (a) Cytotoxic activity of Heu33 T-cell clone against IGR-Heu autologous tumor cell line, IGR-Pub, IGR-B1, Ludlu, H1155 and SK-MES selected allogeneic NSCLC tumor cell lines, Heu-EBV autologous lymphoblastoid cell line and K562 targets. E/T ratios were as indicated. One representative experiment out of three is shown (b) Cytotoxic activity of Heu33 T-cell clone against Ludlu allogeneic tumor cell line. E/T ratio was 10:1. Cytolytic experiments were performed either in medium or in the presence of the indicated mAbs. Target cells were preincubated for 2 h with saturating concentrations of anti-class I (W6/32, B1.23.2 or BB7.2) or control mAbs, and then effector cells were added. Data represent mean ± s.d. of triplicates

|            |            |            |                            |                   |            |            |            |            |
|------------|------------|------------|----------------------------|-------------------|------------|------------|------------|------------|
| 3DL2*001   | msltvsmac  | vgffllqgaw | pLMGGQDKPF                 | LSARPSTVVP        | RGGHVALQCH | YRRGFNNFML | YKEDRSVPI  | FHGRIFQESF |
| 3DL2*002   | .....      | .....      | .....                      | .....             | .....      | .....      | .....      | .....      |
| 3DL2*Heu33 | .l.m.....  | .....      | .....                      | .....             | .....      | .....      | .....      | .....      |
| 3DL2*001   | IMGPVTPAHA | GTYRCGRSRP | HSLTGWASAPS                | NPLVIMVTGN        | HRKPSLLAHP | GPLLKSGETV | ILQCWSDVMF | EHFFLHREGI |
| 3DL2*002   | .....      | .....      | .....                      | .....             | .....      | .....      | .....      | .....D..   |
| 3DL2*Heu33 | .....      | .....      | .....                      | .....             | .....      | .....      | .....      | .....D..   |
| 3DL2*001   | SEDPSRLVGQ | IHDGVSKANF | SIGPLMPVLA                 | GTYRCYGSVP        | HSPYQLSAPS | DPLDIVITGL | YEKPSLSAQP | GPTVQAGENV |
| 3DL2*002   | .....      | .....      | .....                      | .....             | .....      | .....      | .....      | .....      |
| 3DL2*Heu33 | .....      | .....      | .....                      | .....             | .....      | .....      | .....      | .....      |
| 3DL2*001   | TLSCSSWSSY | DIYHLSREGE | AHERRLRAPV                 | KVNRTFQADF        | PLGPATHGGT | YRCFGSFRAL | PCVWSNSSDP | LLVSVTGNPS |
| 3DL2*002   | .....      | .....      | .....                      | .....             | .....      | .....      | .....      | .....      |
| 3DL2*Heu33 | .....      | .....      | .....                      | .....             | .....      | .....      | .....      | .....      |
| 3DL2*001   | SSWPSPEPS  | SKSGICRHLH | <u>V</u> LIGTSVVI <u>F</u> | <u>L</u> FILLFFLL | YRWCSNKKNA | AVMDQEPAGD | RTVNRQDSDE | QDPQEVTYAQ |
| 3DL2*002   | .....      | .....      | .....                      | .....             | .....      | .....      | .....      | .....      |
| 3DL2*Heu33 | .....      | .....      | .....                      | .....             | .....      | .....      | .....      | .....      |
| 3DL2*001   | LDHCVFQQRK | ISRPSQRPKT | PLTDTSVYTE                 | LPNAEPRSKV        | VSCPRAPQSG | LEGVF      |            |            |
| 3DL2*002   | .....      | .....      | .....                      | .....             | .....      | .....      |            |            |
| 3DL2*Heu33 | .....      | .....      | .....                      | .....             | .....      | .....      |            |            |

**Figure 2** Amino-acid sequence alignment of Heu33 KIR3DL2/p140 and 3DL2\*001 (Colonna and Samaridis, 1995) and 3DL2\*002 (Pende *et al.*, 1996) alleles encoded proteins. The transmembrane region is underlined in the 3DL2\*001 encoded sequence. Amino acids identical to the 3DL2\*001 encoded sequence are indicated by dots. Amino acids corresponding to the signal peptide are in lower case letters

The cytotoxic activity of Heu33 T-cell clone against the autologous (IGR-Heu) and the allogeneic IGR-Pub and Ludlu tumor cell lines was inhibited by anti-MHC class I (W6/32) and anti-HLA-A2 (BB7.2) mAb, but not by anti-HLA-B and -C mAb (data not shown and Figure 3b). Figure 3b shows the blocking effect of anti-HLA class I mAbs on the cytotoxic activity mediated by Heu33 CTL towards the HLA-A2<sup>+</sup> Ludlu tumor cell line. These results indicate that Heu33 T-cell clone exhibits a strong cytotoxic reactivity and recognizes its specific target in an HLA-A2-restricted manner.

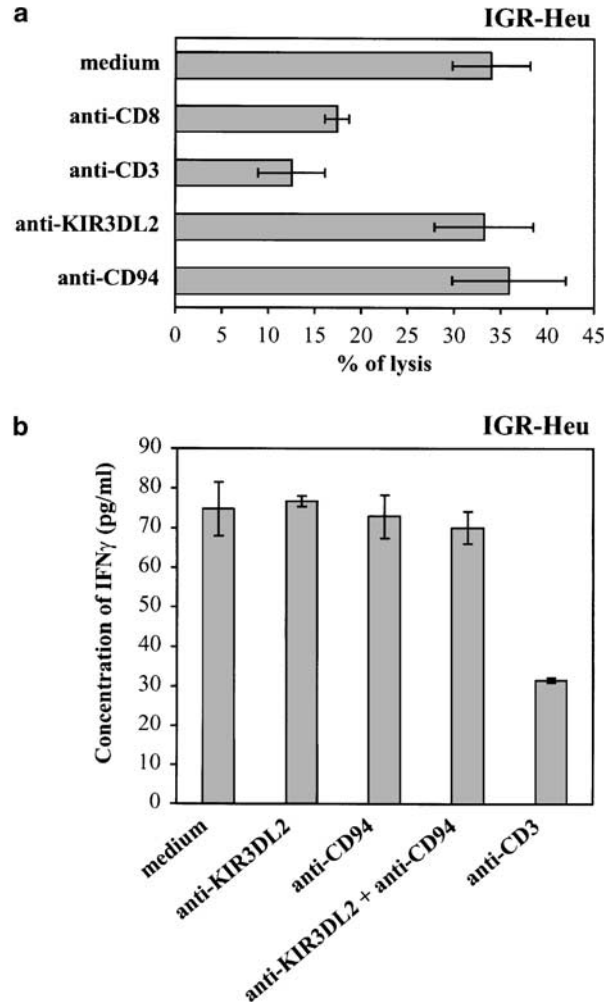
*Role of KIR3DL2/p140 receptor in the interaction of Heu33 CTL clone with its specific tumor target*

To determine the role of KIR3DL2/p140 in the interaction of Heu33 with IGR-Heu autologous tumor cells, cytotoxic experiments were performed following incubation of the effector cells with anti-KIR3DL2/p140. Anti-CD94 or anti-NKG2A mAb were also included during the assay. Neither of these mAbs, including anti-KIR3DL2/p140 used at various concentrations, had any effect on the lytic activity of the T-cell clone towards its specific target (Figure 4a and data not shown). The lack of cytotoxicity modulation obtained with anti-CD94 and anti-NKG2A mAbs was much likely due to the low expression level of the corresponding molecules on the CTL clone cell surface. In contrast and as expected, anti-CD3 and anti-CD8 mAbs inhibited the cytotoxicity of Heu33 T-cell clone against IGR-Heu. These results strongly suggest that KIR3DL2/p140 is not triggered during the interaction of this CTL clone with its specific target. Since KIR3DL2/p140 was described to interact with HLA-A3 and HLA-A11 (Dohring *et al.*, 1996; Pende *et al.*, 1996), we used K562 transfected with HLA-A3 cDNA in cytotoxic assays. It is noteworthy that this allele had no effect on Heu33 ‘NK-like’ activity and that this lysis was not affected by anti-class I mAb (data not shown).

In further experiment, we assayed IFN- $\gamma$  release of Heu33 CTL clone stimulated with autologous tumor cells. An IFN- $\gamma$  production was observed when the T-cell clone was incubated with autologous tumor cells (Figure 4b). However, while this production was inhibited by anti-CD3 mAb, it was unaffected by anti-KIR3DL2/p140 mAb (Figure 4b). Anti-CD94 mAb, added alone or in combination with anti-KIR3DL2/p140, had also no effect on cytokine release (Figure 4b). These results further suggest that KIR3DL2/p140 is unable to modulate cytokine production by the Heu33 clone.

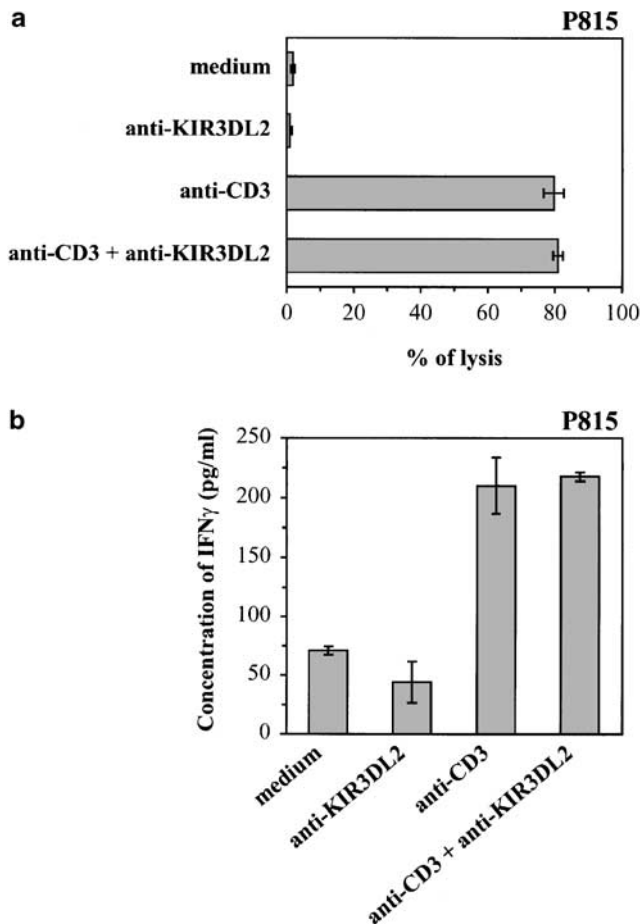
*KIR3DL2/p140 crosslinking does not modulate cytotoxicity or cytokine release by Heu33 CTL clone*

It is noteworthy to precise that neither the autologous tumor cells nor the allogeneic IGR-Pub NSCLC target expressed HLA-A3 or HLA-A11 molecules described to interact with KIR3DL2/p140. Therefore, to further analyse whether KIR3DL2/p140 engagement may modulate the lysis mediated by Heu33 CTL clone, the



**Figure 4** Role of KIR3DL2 in Heu33 interaction with autologous target (a) Cytotoxic activity of Heu33 T-cell clone against IGR-Heu autologous tumor cell line. E/T ratio was 10:1. Cytolytic experiments were performed either in medium or in the presence of the indicated mAbs. CTL clones were preincubated for 2 h with saturating concentrations of anti-CD8 (OKT8), anti-CD3 (OKT3), anti-KIR3DL2 (AZ158) or anti-CD94 mAbs, and then chromium-labeled IGR-Heu target cells were added. (b) IFN- $\gamma$  release by Heu33 T-cell clone cocultured with IGR-Heu autologous tumor cell line. Experiments were performed either in medium or in the presence of anti-KIR3DL2 (AZ158), anti-CD94, anti-KIR3DL2 plus anti-CD94 or anti-CD3 (OKT3), mAbs. Concentrations of IFN- $\gamma$  secretion were determined on 24 h supernatants using ELISA. Data represent mean  $\pm$  s.d. of triplicates

FcR-positive P815 cells were used as target in redirected cytotoxicity assay. As shown in Figure 5a, anti-KIR3DL2/p140 (AZ158 IgG2a mAb), associated or not with anti-CD94, was unable to induce a cytotoxic activity by Heu33 CTL clone towards P815 target. In contrast, a very strong cytotoxic activity towards P815 murine target was induced by anti-CD3 OKT3 mAb even when used at very low concentrations. Furthermore, lysis of P815 induced by anti-CD3 mAb was unaffected by various concentrations of AZ158 (data not shown and Figure 5a for saturating concentration of AZ158 mAb).



**Figure 5** Redirected functional activities of KIR3DL2-expressing Heu33 CTL clone. (a) Redirected cytotoxic activity of Heu33 CTL clone. Heu33 CTLs were preincubated for 1 h with the indicated mAbs (used at saturating concentration) and then  $^{51}\text{Cr}$ -labeled P815 target cells were added (E:T ratio of 10:1). (b) Redirected IFN- $\gamma$  release of Heu33 TIL clone. Heu33 T-cell clone was preincubated for 1 h in the presence of receptor-specific mAb and then cocultured for 24 h with P815 cells. IFN- $\gamma$  secreted in culture supernatants was then measured by ELISA. Data represent mean  $\pm$  s.d. of triplicates

We then measured IFN- $\gamma$  secretion by Heu33 clone after coculture with P815 cells in the absence or presence of mAbs to KIR3DL2/p140 and/or CD3 molecules. KIR3DL2 was unable, within the limit of sensitivity of the assay, to induce IFN- $\gamma$  secretion by Heu33 T-cell clone (Figure 5b). On the contrary, mAb-mediated ligation of CD3 induced high cytokine response. Furthermore and as seen in cytotoxicity assay (Figure 5a), coligation of KIR3DL2/p140 with CD3 did not result in inhibition of IFN- $\gamma$  production (Figure 5b). These results further indicate that KIR3DL2 expressed by Heu33 CTL clone has no inhibitory (nor triggering) properties on the signaling provided by TCR engagement.

*Discussion*

Ag-specific CTLs play an important role in cellular antitumor immune responses. Expression of MHC

class I molecules is essential for CD8 $^{+}$  T-cell-mediated tumor cell recognition. However, it has been described that a small percentage of CD8 $^{+}$  TILs express receptors for HLA class I that are inhibitory for TCR-mediated functions. Various KIR molecules, including KIR2DL1/p58.1 and KIR2DL2/3/p58.2, have been shown to be expressed by T-cell clones infiltrating human solid tumors and were able to inhibit their tumor-specific lysis (Ikeda *et al.*, 1997; Guerra *et al.*, 2000; Gati *et al.*, 2001). So far no data are available on KIR3DL2/p140 expression by human CTL clones and on the role of this receptor in TCR-mediated lysis and cytokine production.

KIR3DL2/p140 displays three extracellular Ig-like domains. As opposed to other KIRs, this receptor is expressed as a disulfide-linked dimer of 140 kDa with two cysteines in the extracellular portion proximal to the transmembrane region. The function and the exact HLA-A ligand of this receptor have not so far been precisely determined (Bakker *et al.*, 1998). An inhibitory form of KIR3DL2/p140 has been identified on NK cells and has been demonstrated to interact with HLA-A3 or -A11 (Pende *et al.*, 1996). However, the level of inhibition determined by HLA-A3 following interaction with KIR3DL2/p140 is lower than that induced by HLA-B and -C following interaction with KIR3DL1/p70 and KIR2DL1/p58.1 or KIR2DL2/3/p58.2, respectively (Dohring *et al.*, 1996). With regard to T cells, a unique study demonstrated that KIR3DL2/p140 receptor was expressed on CD4 $^{+}$  cutaneous T-cell lymphoma cells and suggested that it may correspond to a prognosis marker (Bagot *et al.*, 2001). However, no functional study was performed on the KIR3DL2/p140 receptor expressed by these tumoral T cells.

In the present report, we have isolated from human lung carcinoma TIL several TCRV $\beta$ 13.6 $^{+}$ /KIR3DL2/p140 $^{+}$  T-cell clones. These clones were able to lyse the autologous tumor cell line, but not the autologous EBV-B cells, in an HLA-A2-restricted manner. The cytotoxic activity of Heu33 CTL clone towards its specific target is unaffected by different concentrations of anti-KIR3DL2/p140 mAb. In addition, crosslinking of KIR3DL2/p140 in Heu33 does not lead to an inhibition of anti-CD3 mAb-induced cytotoxicity. These results strongly suggested that, as opposed to KIR3DL1/p70, KIR2DL1/p58.1 and KIR2DL2/3/p58.2 (Ferrini *et al.*, 1994; Mingari *et al.*, 1995; Phillips *et al.*, 1995), KIR3DL2/p140 receptor has no inhibitory effect on TCR-mediated T-cell activation. Furthermore, as opposed to KIR2DL4 (CD158d), recently described to display both inhibitory (Faure and Long, 2002) and activating (Rajagopalan *et al.*, 2001) potentials in human NK cells, crosslinking of KIR3DL2/p140, expressed by Heu33, does not induce an activatory effect on the cytotoxicity and IFN- $\gamma$  or IL-2 (data not shown) production by the CTL clone.

KIR expression has been reported to occur in a minor proportion of expanded clones, which survives activation-induced cell death to become long-term memory T cells. The expression of KIR appeared to correlate with this resistance to activation-induced cell death in these

cell populations, although the requirement for KIR interaction with self-HLA class I in this process is still unknown (Young *et al.*, 2001). Interestingly, we have found that the engagement of the KIR3DL2/p140 in Heu33 cells did not result in the inhibition of the cell death induced by soluble anti-CD3 mAb (data not shown). Heu33 KIR3DL2/p140-expressing T cells belong to memory T cells with a CD8<sup>+</sup>, CD28<sup>-</sup>, CD56<sup>+</sup>, CD45RO<sup>+</sup> and CD45RA<sup>+</sup> phenotype. However, as opposed to previously reported CD8<sup>+</sup>/CD28<sup>-</sup> T cells shown to proliferate poorly *in vitro* (Azuma *et al.*, 1993), Heu33 has a very high *in vitro* proliferative activity. It is possible that KIR3DL2/p140 expressed by these CTLs may be involved in regulatory mechanisms leading to the proliferation of these tumor-reactive TILs. Indeed, one may suggest that even though no effect of this receptor was observed *in vitro*, it may modulate T-cell activities *in vivo*. Along the same line, Heu33 tumor-specific CTL did not appear to be clonally expanded *in situ* (data not shown), as opposed to previously described KIR3DL2<sup>-</sup> tumor-reactive CTL clones isolated from the same cancer biopsy (Echchakir *et al.*, 2000, 2002).

At least 12 different allelic forms of KIR3DL2/p140 have been described so far (Gardiner *et al.*, 2001; Shilling *et al.*, 2002). Sequence analysis of KIR3DL2/p140 expressed by Heu33 clone indicated that it was identical to 3DL2\*002 allele encoded protein (Pende *et al.*, 1996), except for two amino-acid substitutions in the leader peptide sequence with likely no functional consequence, including two ITIM motifs in its intracytoplasmic tail. Since KIR3DL2/p140 had no inhibitory function, one may suggest either that some peptides presented by HLA class I molecules might contribute to trigger the KIR3DL2/p140 inhibitory function and some not, or that the ITIM-based inhibitory pathway may not function, at least *in vitro*, in Heu33 TIL clone. Indeed, our preliminary results appeared to indicate that the KIR3DL2/p140, expressed by the Heu33 clone stimulated or not with pervanadate, immunoprecipitated with AZ158 mAb does not recruit the protein tyrosine phosphatase SHP-1 (data not shown).

Previous studies indicated that KIR3DL2/p140 receptor was able to generate inhibitory signals upon recognition of HLA-A3 and HLA-A11 alleles (Dohring *et al.*, 1996; Pende *et al.*, 1996). It should, however, be noted that the CTL clone analysed in the present report was derived from an HLA-A2/-A68 patient. Furthermore, it has been previously described that KIR3DL2/p140 molecule was expressed by CD4<sup>+</sup> cutaneous TILs of various patients apparently independently of their HLA class I haplotype. It is possible that the different KIR3DL2/p140 allele products described may recognize HLA-A allele products distinct from those reacting with the first described receptors (Dohring *et al.*, 1996; Pende *et al.*, 1996). One plausible candidate may correspond to HLA-A68, reported to share with HLA-A3 an Asp at position 74 of the  $\alpha$ 1-helix, described to provide an NK-resistant phenotype as opposed to the nonpermissive residue His-74 present in HLA-A2 (Storkus *et al.*, 1991). Additional studies will however be required to further

support this hypothesis. Together, the present results strongly suggest that KIR3DL2/p140 expressed by Heu33 had no inhibitory (nor activatory) potential on cytotoxicity and IFN- $\gamma$  production by the CTL clone. Nevertheless, the actual role and kinetics of expression induction of KIR3DL2/p140 on a CD8<sup>+</sup> lung carcinoma infiltrating CTL remain to be determined. Future studies will allow the identification of the potential role of this Ig-like receptor in the long-term survival of memory T cells.

## Materials and methods

### *Derivation and culture of Heu33 CTL clone*

The NSCLC cell line, IGR-Heu, was derived from a biopsy of patient Heu (HLA-A2, A68, B7, B35, C4, C7) as described (Asselin-Paturel *et al.*, 1998). Fresh tumor sample was dissociated and the resulting cell suspension was frozen. After thawing, viable TILs were isolated using Ficoll-Hypaque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient centrifugation. The lymphocytes were then seeded at 10<sup>4</sup> cells/microwell and stimulated for 2 weeks by the addition of irradiated (10 000 rad) autologous tumor cells (3  $\times$  10<sup>3</sup>/well) and irradiated autologous Epstein-Barr virus (EBV)-transformed B cells (Heu-EBV, 4  $\times$  10<sup>4</sup>/well) in RPMI-1640 medium supplemented with 10% human AB serum (Institut Jacques Boy, Reims, France) and rIL-2 (20 U/ml, Roussel-Uclaf, Romainville, France). Cells were fed every 3 days with medium and IL-2, and then analysed by flow cytometry for TCRV $\beta$  expression using available specific mAbs (mAbs; Beckman-Coulter, Marseille, France). Responding TCRV $\beta$ 13.6<sup>+</sup> cells were immunoselected three times using specific mAb and dynabeads (DynaL AS, Oslo, Norway). The resulting T-cell line (H4C, 99% TCRV $\beta$ 13.6<sup>+</sup>) was cloned by limiting dilution, and several T-cell clones, including Heu33, were isolated and restimulated with the same protocol.

### *mAbs and immunofluorescence analysis*

Anti-CD3, -CD4, -CD8, -CD28, -CD56 and -TCR $\alpha/\beta$  mAbs were previously described (Echchakir *et al.*, 2000). W6/32 (anti-HLA class I), BB7.2 (anti-HLA-A2) and B1.23.2 (anti-HLA-B and -C) were used in functional assays (Echchakir *et al.*, 2000). Anti-TCR mAbs were purchased from Beckman-Coulter and Z27 (IgG1, anti-KIR3DL1/p70-NKB1), EB6 (IgG1, anti-KIR2DL1/p58.1 and KIR2DS1/p50.1), GL183 (IgG1, anti-KIR2DL2/3/p58.2 and KIR2DS2/p50.2), Y9 (IgM, anti-CD94) and Z199 (IgG2b, anti-NKG2A) were purchased from Immunotech (Marseille, France). Q66 (IgM, anti-KIR3DL2/p140), AZ158 (IgG2a, recognizing both KIR3DL1/p70-NKB1 and KIR3DL2/p140) were produced in one of our laboratories.

Phenotypic analyses of the TIL cell line and clones were performed by indirect immunofluorescence using a FACScalibur flow cytometer and data were processed using the Cell Quest program (Becton Dickinson, San Jose, CA, USA).

### *Cytotoxicity and cytokine release assays*

The cytotoxic activity of the T-cell clone was measured by a conventional 4-h <sup>51</sup>Cr-release assay. K562 and allogeneic NSCLC cell lines, including Ludlu (squamous cell carcinoma, SCC; HLA-A2<sup>+</sup>), SK-MES (SCC; HLA-A3/30), H1155 (LCC, HLA-A2<sup>-</sup>), IGR-Pub (adenocarcinoma, ADC; HLA-A2/A68)

and IGR-B1 (LCC, A2/68) were used as targets in cytotoxicity assays (Echchakir *et al.*, 2000). The FcR-positive P815 murine cells were used as target in redirected cytotoxicity assay. Functional effects of the mAbs W6/32, BB7.2, B1.23.2, OKT3, OKT8, anti-CD94, Q66 and AZ158 on target or effector cells were tested by incubating each of them for 2 h at 37°C before the assay at the predetermined saturating concentration.

For cytokine production, Heu33 T cells ( $5 \times 10^5$ /well) were cocultured in the absence or presence of IGR-Heu or P815 cells ( $1 \times 10^5$ /well) for 24 h with mAbs as indicated. Culture supernatants were tested for interferon (IFN)- $\gamma$  production by ELISA (Biosource International, Camarillo, CA, USA). The level of sensitivity of the assay was 1.6 pg/ml.

#### KIR3DL2/p140 and TCR $\beta$ RT-PCR and sequence analyses

Total RNA was extracted from Heu33 CTL clone and cDNA was synthesized and amplified by RT-PCR. Primers used for cDNA amplification of the complete open-reading frame of KIR3DL2/p140 (1395 bp) were the following: 5'-

CATGTCTGCTCACTGGTCGTC-3' and 5'-GGTTTT-GA-GACAGGGCTG-3' (Bagot *et al.*, 2001). PCR products were then purified, cloned in pcDNA3.1 plasmid and sequenced. TCR $\beta$  cDNA expressed by Heu33 CTL clone was amplified by RT-PCR, and PCR products were purified and sequenced as previously described (Echchakir *et al.*, 2000).

#### Acknowledgements

We thank S Megherat, Y Lecluse for FACS analyses, R Tamouza and D Charron for HLA typing and D Grunenwald from the Thoracic Surgery Department of Institut Mutualiste Montsouris, Paris, France. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (INSERM), the Institut Gustave Roussy, the Association pour la Recherche sur le Cancer (Grants 9307, 5253, 2129), the Fondation de France and GEFLUC and the associazione Italiana per la Ricerca sul cancro (AIRC). HE was supported by a fellowship from the Fondation de France (Comité tumeurs solides).

#### References

- Asselin-Paturel C, Echchakir H, Carayol G, Gay F, Opolon P, Grunenwald D, Chouaib S and Mami-Chouaib F. (1998). *Int. J. Cancer*, **77**, 7–12.
- Azuma M, Phillips JH and Lanier LL. (1993). *J. Immunol.*, **150**, 1147–1159.
- Bagot M, Moretta A, Sivori S, Biassoni R, Cantoni C, Bottino C, Boumsell L and Bensussan A. (2001). *Blood*, **97**, 1388–1391.
- Bakker AB, Phillips JH, Figdor CG and Lanier LL. (1998). *J. Immunol.*, **160**, 5239–5245.
- Burshtyn DN, Scharenberg AM, Wagtmann N, Rajagopalan S, Berrada K, Yi T, Kinet JP and Long EO. (1996). *Immunity*, **4**, 77–85.
- Colonna M, Navarro F, Bellon T, Llano M, Garcia P, Samaridis J, Angman L, Cella M and Lopez-Botet M. (1997). *J. Exp. Med.*, **186**, 1809–1818.
- Colonna M and Samaridis J. (1995). *Science*, **268**, 405–408.
- Dohring C, Scheidegger D, Samaridis J, Cella M and Colonna M. (1996). *J. Immunol.*, **156**, 3098–3101.
- Echchakir H, Dorothée G, Vergnon I, Menez J, Chouaib S and Mami-Chouaib F. (2002). *Proc. Natl. Acad. Sci. USA*, **99**, 9358–9363.
- Echchakir H, Mami-Chouaib F, Vergnon I, Baurain JF, Karanikas V, Chouaib S and Coulie PG. (2001). *Cancer Res.*, **61**, 4078–4083.
- Echchakir H, Vergnon I, Dorothée G, Grunenwald D, Chouaib S and Mami-Chouaib F. (2000). *Int. Immunol.*, **12**, 537–546.
- Faure M and Long EO. (2002). *J. Immunol.*, **168**, 6208–6214.
- Ferrini S, Cambiaggi A, Meazza R, Sforzini S, Marciano S, Mingari MC and Moretta L. (1994). *Eur. J. Immunol.*, **24**, 2294–2298.
- Fry AM, Lanier LL and Weiss A. (1996). *J. Exp. Med.*, **184**, 295–300.
- Gardiner CM, Guethlein LA, Shilling HG, Pando M, Carr WH, Rajalingam R, Vilches C and Parham P. (2001). *J. Immunol.*, **166**, 2992–3001.
- Gati A, Guerra N, Giron-Michel J, Azzarone B, Angevin E, Moretta A, Chouaib S and Caignard A. (2001). *Cancer Res.*, **61**, 3240–3244.
- Guerra N, Guillard M, Angevin E, Echchakir H, Escudier B, Moretta A, Chouaib S and Caignard A. (2000). *Blood*, **95**, 2883–2889.
- Ikeda H, Lethe B, Lehmann F, van Baren N, Baurain JF, de Smet C, Chambost H, Vitale M, Moretta A, Boon T and Coulie PG. (1997). *Immunity*, **6**, 199–208.
- Le Drian E, Vely F, Olcese L, Cambiaggi A, Guida S, Krystal G, Gervois N, Moretta A, Jotereau F and Vivier E. (1998). *Eur. J. Immunol.*, **28**, 264–276.
- Mingari MC, Vitale C, Cambiaggi A, Schiavetti F, Melioli G, Ferrini S and Poggi A. (1995). *Int. Immunol.*, **7**, 697–703.
- Moretta A, Sivori S, Vitale M, Pende D, Morelli L, Augugliaro R, Bottino C and Moretta L. (1995). *J. Exp. Med.*, **182**, 875–884.
- Moretta A, Vitale M, Sivori S, Bottino C, Morelli L, Augugliaro R, Barbaresi M, Pende D, Ciccone E and Lopez-Botet M. (1994). *J. Exp. Med.*, **180**, 545–555.
- Noppen C, Schaefer C, Zajac P, Schutz A, Kocher T, Kloth J, Heberer M, Colonna M, De Libero G and Spagnoli GC. (1998). *Eur. J. Immunol.*, **28**, 1134–1142.
- Pende D, Biassoni R, Cantoni C, Verdiani S, Falco M, di Donato C, Accame L, Bottino C, Moretta A and Moretta L. (1996). *J. Exp. Med.*, **184**, 505–518.
- Phillips JH, Gumperz JE, Parham P and Lanier LL. (1995). *Science*, **268**, 403–405.
- Rajagopalan S, Fu J and Long EO. (2001). *J. Immunol.*, **167**, 1877–1881.
- Shilling HG, Guethlein LA, Cheng NW, Gardiner CM, Rodriguez R, Tyan D and Parham P. (2002). *J. Immunol.*, **168**, 2307–2315.
- Speiser DE, Pittet MJ, Valmori D, Dunbar R, Rimoldi D, Lienard D, MacDonald HR, Cerottini JC, Cerundolo V and Romero P. (1999). *J. Exp. Med.*, **190**, 775–782.
- Storkus WJ, Salter RD, Alexander J, Ward FE, Ruiz RE, Cresswell P and Dawson JR. (1991). *Proc. Natl. Acad. Sci. USA*, **88**, 5989–5992.
- Townsend AR, Rothbard J, Gotch FM, Bahadur G, Wraith D and McMichael AJ. (1986). *Cell*, **44**, 959–968.
- Young NT, Uhrberg M, Phillips JH, Lanier LL and Parham P. (2001). *J. Immunol.*, **166**, 3933–3941.
- Zinkernagel RM and Doherty PC. (1974). *Nature*, **248**, 701–702.