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In Situ Sensory Adaptation of Tumor-Infiltrating T Lymphocytes to Peptide-MHC Levels Elicits Strong Antitumor Reactivity[1]

Guillaume Dorothée,[2]* Isabelle Vergnon,[2]* Faten El Hage,* Béatrice Le Maux Chansac,* Vincent Ferrand,[§] Yann Lécluse,[†] Paule Opolon,[‡] Salem Chouaib,* Georges Bismuth,[§] and Fathia Mami-Chouaib[3]*

We have isolated from tumor-infiltrating lymphocytes (TIL) and PBL of a lung carcinoma patient several tumor-specific T cell clones displaying similar peptide-MHC tetramer staining and expressing a unique TCR. Although these clones elicited identical functional avidity and similar cytolytic potential, only T cell clones derived from TIL efficiently lysed autologous tumor cells. Interestingly, all of these clones expressed the same T cell surface markers except for the TCR inhibitory molecule CD5, which was expressed at much lower levels in TIL than in PBL. Video-imaging recordings demonstrated that, although both T cell clones could form stable conjugates with tumor cells, the Ca^{2+} response occurred in TIL clones only. Significantly, analysis of a panel of circulating clones indicated that antitumor cytolytic activity was inversely proportional to CD5 expression levels. Importantly, CD5 levels in TIL appeared to parallel the signaling intensity of the TCR/peptide-MHC interaction. Thus, in situ regulation of CD5 expression may be a strategy used by CTL to adapt their sensitivity to intratumoral peptide-MHC levels. ***The Journal of Immunology,*** **2005, 174: 6888–6897.**

An increasing number of tumor-associated Ags (TAA)[4] have been identified from various human solid tumors, including melanoma and non-small cell lung carcinoma (NSCLC), using specific autologous CTL (1–3). These Ags opened up new opportunities for cancer immunotherapy, and several vaccination trials, mainly in melanoma, are now underway to induce an efficient and long-lasting CTL response to defined tumor Ag-derived peptides (4, 5). The major concern of immunomonitoring in the setting of a cancer vaccine is to quantify accurately the elicited tumor-reactive T cells. The introduction of peptide-MHC tetramers for enumerating and characterizing phenotypically Ag-specific T lymphocytes (6), which develop spontaneously or after vaccination in cancer patients, has rapidly advanced our understanding of the antitumor immune response (7, 8). These reagents have been used successfully for detecting and isolating various low-frequency tumor-specific T lymphocytes (7). However, we (9) and others (10–13) have reported that peptide-MHC tetramer labeling may not reflect functional avidity of the isolated T cell clones. Therefore, additional parameters have been introduced to more accurately reflect tumor recognition efficacy and the lytic capacity of peptide-MHC tetramer-isolated T cells. Among these parameters, the stability of peptide-HLA multimer binding to TCR, as measured in dissociation kinetic experiments, has been reported to correlate directly with the functional avidity of Ag-specific CTL (12). More recently, a combination of peptide-MHC tetramer staining with surface mobilization of CD107a, a marker for degranulation after tumor stimulation, has been reported as a new strategy to directly correlate Ag specificity and cytolytic efficacy at a single-cell level (14).

We had previously isolated from tumor-infiltrating lymphocytes (TIL) and PBL of a lung carcinoma patient several tumor-specific T cell clones recognizing an HLA-A2-restricted decamer encoded by a mutated α-actinin-4 (*ACTN4*) gene (3). TCR repertoire distribution of mutated α-actinin-4 peptide-HLA-A2 tetramer$^+$ cells indicated that, although T cell clones with high and low functional avidity were amplified selectively within the tumor and in circulating lymphocytes, respectively, those with intermediate avidity were unequally expanded in both compartments (9). In the present study, using peptide-MHC tetramers, we have isolated from patient PBL and TIL several T cell clones displaying the same functional avidity and expressing a unique TCR$\alpha\beta$. We show that the levels of the TCR inhibitory molecule CD5 reflect antitumor CTL responsiveness, and we suggest that CD5 expression is adaptable to signals received within the tumor. Indeed, CD5 levels decrease in T lymphocytes infiltrating low peptide-MHC-expressing tumors, and this phenotype is associated with an increase in Ca^{2+} and antitumor cytotoxic responses. Hence, depending on the strength of the TCR/peptide-MHC interaction, tumor-specific CTL

*Laboratoire Cytokines et Immunologie des Tumeurs Humaines, Institut National de la Santé et de la Recherche Médicale Unité 487, Institut Fédératif de Recherche 54, and [†]Unité Mixte de Recherche 8126, and [‡]Unité Mixte de Recherche 8121, Centre National de la Recherche Scientifique, Institut Gustave Roussy, Villejuif, France; and [§]Institut Cochin, Département de Biologie Cellulaire, Institut National de la Santé et de la Recherche Médicale Unité 567, Centre National de la Recherche Scientifique, Unité Mixte de Recherche 8104, Université René Descartes, Paris, France

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[2] G.D. and I.V. contributed equally to this work.

[3] Address correspondence and reprint requests to Dr. Fathia Mami-Chouaib, Laboratory Cytokines et Immunologie des Tumeurs Humaines, Institut National de la Santé et de la Recherche Médicale Unité 487, Institut Gustave Roussy, 39 rue Camille-Desmoulins, F-94805 Villejuif, France. E-mail address: cfathia@igr.fr

[4] Abbreviations used in this paper: TAA, tumor-associated Ag; NSCLC, non-small cell lung carcinoma; TIL, tumor-infiltrating lymphocyte; *ACTN4*, α-actinin-4 gene; LCC, large cell carcinoma; ADC, adenocarcinoma; SCC, squamous cell carcinoma; MHC-I, MHC class I; MHC-II, MHC class II; KIR, killer inhibitory receptor; KLR, killer cell lectin-like subfamily receptor.

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appear to undergo an intratumoral adaptation process as a means to enhance TCR signaling and to overcome tumor escape related to altered peptide-MHC expression.

Materials and Methods

Derivation and culture of tumor cell lines

The IGR-Heu cell line was derived from patient 1 large cell carcinoma (LCC) biopsy as described previously (3, 15). The NSCLC cell lines IGR-Pub (adenocarcinoma (ADC)), IGR-B1 and IGR-B2 (LCC), LCC-M4 (LCC) (16), ADC-Coco (ADC), squamous cell carcinoma (SCC)-Cher (SCC), and ADC-Tor (ADC) were derived from patient 2, 3, 4, 5, 6, and 7 tumor biopsies, respectively. These cell lines were maintained in culture as described previously (3, 15). Fresh tumor lesions, infiltrated by T lymphocytes, were dissociated mechanically and frozen along with blood samples for additional experiments.

Derivation and culture of T cell clones Heu171 and H32-22

The Heu171 clone was derived from TIL as described previously (9), and the H32-22 T cell clone was derived from PBL stimulated in vitro with the autologous tumor cell line. Briefly, patient 1 PBMC were thawed, and CD8-enriched fraction was stimulated five times, at a 1- or 2-wk interval, with the irradiated autologous IGR-Heu tumor cell line alone or supplemented with the irradiated autologous EBV-transformed B cell line and IL-2 (25 U/ml). Responding cells were then collected, washed, and peptide-MHC tetramer-stained (3). Positive cells were sorted and seeded, using a FACSVantage (BD Biosciences), at 1 cell/well in round-bottom microplates in the presence of irradiated autologous tumor and EBV-transformed B cells in complete medium supplemented with IL-2. The resulting clonal populations were labeled with peptide-MHC tetramers. Some of them, chosen randomly, including clones H32-22, H32-8, and H32-25, were selected and maintained in culture as described previously (3).

mAbs and immunofluorescence analyses

Allophycocyanin-conjugated anti-CD5 and PE-conjugated anti-CD3 mAb were purchased from BD Biosciences and Immunotech, respectively. EB6 (anti-KIR2DL1/p58.1 and KIR2DS1/p50.1), GL183 (anti-KIR2DL2/3/p58.2 and KIR2DS2/p50.2), AZ158 (recognizing both KIR3DL1/p70-NKB1 and KIR3DL2/p140), and anti-NKG2D were generous gifts from Prof. A. Moretta (University of Genoa, Genoa, Italy). Anti-CD94, anti-NKG2A, and anti-TCRVβ8 mAbs were purchased from Immunotech. The δG9 and GB12 mAb were purchased from BD Pharmingen and Tebu-Bio, respectively. W6/32 (anti-HLA class I) and 9-49 (anti-HLA class II) mAb were reported previously (16, 17).

Phenotypic analyses of TIL cell lines and clones were performed by direct or indirect immunofluorescence using a FACSCalibur flow cytometer, and data were processed using the CellQuest program (BD Biosciences). Double-color immunofluorescence analyses were performed using PE-conjugated anti-CD3 and allophycocyanin-conjugated anti-CD5 mAb. Briefly, 3×10^5 cells were stained with mAb, washed, fixed with formaldehyde, and analyzed by flow cytometry. Statistical analyses were performed using Student's *t* test. Intracytoplasmic perforin and granzyme B expressions were determined by immunofluorescence analysis using δG9 and GB12 mAb. Briefly, T cells were fixed using PBS containing 2% formaldehyde, and cell membrane was then permeabilized using PBS supplemented with 0.5% BSA and 0.2% saponin.

For MHC-tetramer labeling, cells were incubated for 1 h at room temperature (9) with PE-labeled mutated α-actinin-4 peptide-HLA-A2 tetramers (3).

MHC class I (MHC-I) molecule expression on uncultured cancer cells was tested by immunohistochemistry on paraffin sections of tumor biopsies as described previously (18). HC10, anti-MHC-I H chain mAb (IgG2, a generous gift from Dr. F. Lemonnier, Institut Pasteur, Paris, France) was used. Power Vision from Microm (Microm Microtech) was used for visualization.

Cytotoxicity and cytokine release assays

The cytotoxic activity of the T cell clones was measured by a conventional 4-h ^{51}Cr release assay using 3000 target cells/well. The autologous IGR-Heu tumor cell line and the autologous EBV-transformed B cell line, Heu-EBV, pulsed (30 min at room temperature) with different concentrations of the antigenic peptide were used as targets in cytotoxicity experiments. FcR-positive P815 murine cells were used as targets in a redirected cytolytic assay. ^{51}Cr-labeled P815 target cells were preincubated for 1 h with OKT3 mAb (1/200,000 diluted ascite) or an isotypic control, and then Heu171 or H32-22 T lymphocytes were added (17).

TNFβ release was detected by measuring the cytotoxicity of the culture supernatants on the TNF-sensitive WEHI-164c13 cells, with an MTT colorimetric assay (9). Heu171 and H32-22 T cells (3×10^3/well) were cultured in the absence or presence of IGR-Heu (3×10^4/well) or in the presence of transfected melanoma cell line (MZ-2-MEL; a generous gift of P. Coulie, Ludwig Institute, Brussels, Belgium) (3) for 24 h. Culture supernatants were then tested for TNFβ production.

Video imaging and single-cell calcium measurements

Time-lapse experiments were conducted using IGR-Heu tumor cells plated on glass coverslips mounted on 30-mm petri dishes and incubated at 37°C for 18 h. Before the experiment, T cells were loaded for 20 min at 37°C with 1 μM fura-2AM (Molecular Probes) in 1 ml of HEPES buffer (10 mM; pH 7.5) containing 140 nM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 mM Na_2HPO_4, and 1 mg/ml glucose. Cells were then washed and added to the tumor cells in a final volume of 200 μl of the same buffer supplemented with 1% FCS. Transmitted light and intracellular calcium measurement images were acquired sequentially every 10 s on an inverted Eclipse TE300 microscope (Nikon) equipped with a $\times$20 objective and the MetaFluor Imaging System (Universal Imaging). Calcium responses to mutated α-actinin-4 peptide-HLA-A2 tetramers were measured with the same imaging system after adding tetramer solution directly to fura 2-loaded T cells allowed to settle for 10 min on glass coverslips. Movies were made with the Adobe Premiere software (Adobe System).

TCR cDNA sequence analysis

Total RNA was extracted from the H32-22 T cell clone, and TCRα and TCRβ cDNA were synthesized and amplified by RT-PCR using TCRVα12 and TCRCα or TCRVβ8 and TCRCβ oligonucleotides. PCR products were then purified and sequenced as described previously (9).

Results

TCRVβ8$^+$ TIL- and PBL-derived clones display similar peptide-MHC tetramer staining and identical functional avidity

From lymphocytes infiltrating a LCC of the lung, we have isolated a T cell clone named Heu171 recognizing an antigenic peptide (FIAS**N**GVKLV) encoded by a mutated *ACTN4* gene and expressing Vβ8-GTD-Jβ1.5/Vα12-NAR-Jα8 rearrangements (9). TCRβ CDR3 size distribution analysis, performed using the Vβ8 oligonucleotide in combination with Jβ1.5 or clonotypic primers, indicated that Heu171 TCR transcripts were oligoclonally expanded in TIL and, to a lesser extent, in PBL (9). The aim of the following experiment was to isolate from patient 1 antimutated α-actinin-4 circulating T lymphocytes bearing the same TCR as Heu171. For this purpose, PBMC, which include $<$0.01% of peptide-MHC tetramer$^+$CD8$^+$ T cells, were stimulated with the autologous tumor cell line, IGR-Heu, in similar conditions as for TIL. After five stimulations, the derived T cell lines were analyzed for MHC tetramer staining, and positive cells were seeded at 1 cell/well. Nineteen T cell clones were randomly selected, 18 of them, including H32-22, expressed TCRVβ8, and one clone expressed TCRVβ13.2 (H32-3) as determined by immunofluorescence analysis or DNA sequencing (Fig. 1*A*, *left panels*, and data not shown). TCRα and TCRβ chain nucleic acid sequences showed that H32-22 expresses exactly the same rearrangements as Heu171, including identical CDR3, indicating that both clones are likely to be derived from the same cell.

To further confirm T cell clone specificity for mutated α-actinin-4 peptide, we performed TNFβ secretion assays following stimulation of both clones with an allogeneic HLA-A2-transfected melanoma cell line (MZ-2-MEL-A2) transfected transiently with a pcDNA3 vector encoding the mutated α-actinin-4 transcript (*ACTN4*) (3). Data depicted in Fig. 1*B* show that both clones secreted TNFβ following stimulation with the transfected cells. We then tested H32-22 and Heu171 T cell clones for peptide-MHC tetramer labeling. As expected, the two clones displayed similar multimer staining (Fig. 1*A*, *right panels*). Functional avidity of

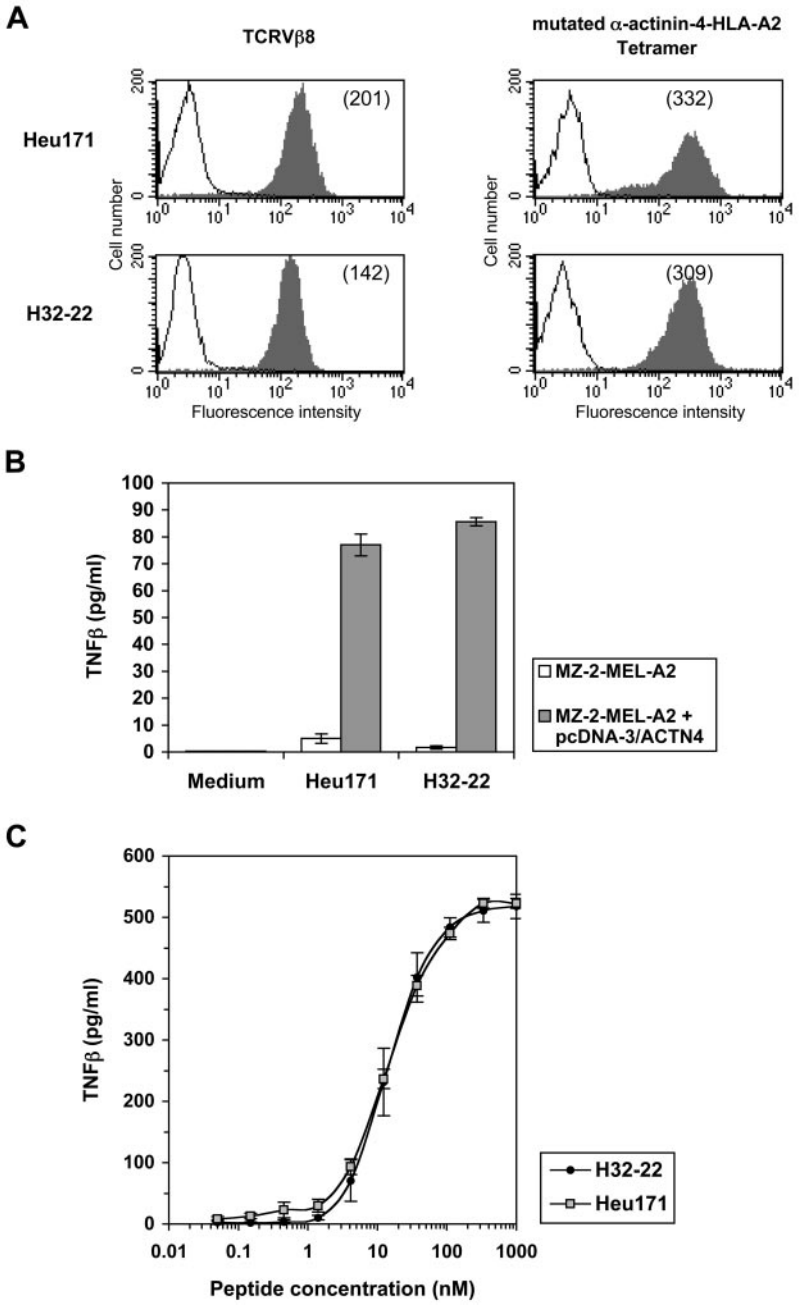

FIGURE 1. *A*, Labeling of antimutated α-actinin-4 T cell clones with anti-TCRVβ8 mAb and HLA tetramers. Negative controls are shown as white histograms. Numbers in parentheses correspond to mean fluorescence intensity. Data shown are representative of three independent experiments. *B*, TNFβ secretion by Heu171 and H32-22 T cell clones. Allogeneic HLA-A2-transfected MZ-2-MEL melanoma cells were transfected transiently with a pcDNA-3 vector encoding the mutated *ACTN4* transcript before addition of the clones. TNFβ produced by the clones was measured by testing the killing activity of the culture supernatant on the TNF-sensitive WEHI-164c13 cells. *C*, TNFβ secretion and functional avidity determination of mutated peptide-reactive T cell clones. Allogeneic HLA-A2-transfected MZ-2-MEL melanoma cells were incubated with the indicated concentrations of α-actinin-4 peptide before addition of Heu171 or H32-22 clones. TNFβ secretion was measured as in *B*. Data shown are representative of three independent experiments.

TIL- and PBL-derived T cell clones was then determined using TNFβ secretion assays performed in the presence of the allogeneic HLA-A2-transfected melanoma cell line loaded with different concentrations of the antigenic peptide. A titration curve, over a wide range of mutated α-actinin-4 peptide concentrations (0.1 nM to 1 μM) was generated, and the functional avidity, defined as the concentration of peptide required to obtain 50% of maximal TNFβ secretion, was found to be identical (~20 nM) for both clones (Fig. 1*C*).

TIL- and PBL-derived T cell clones display differential antitumor reactivity but similar lytic potential

Next, experiments were performed to assess the cytotoxic activity of Heu171 and H32-22 T cell clones toward autologous tumor cells. Chromium release assay indicated that the Heu171 TIL-derived clone mediates strong cytolytic activity toward the IGR-Heu tumor cell line (Fig. 2*A*, *left panel*). This lysis was inhibited partially by anti-MHC-I mAb, supporting previous results indicating that the CTL clone recognizes its target in HLA-A2-restricted fashion (data not shown). Strikingly, the H32-22 PBL-derived T cell clone was unable to kill the autologous tumor cells (Fig. 2*A*, *left panel*). Similarly, the TNFβ (Fig. 2*A*, *right panel*) and IFN-γ (data not shown) secretion assays indicated that, as opposed to Heu171, the H32-22 clone failed to secrete both cytokines following stimulation with IGR-Heu target cells.

To determine whether the H32-22 cytotoxicity defect was associated with an alteration in its lytic potential, we assessed the expression of cytotoxic molecules implicated in the granule exocytosis-mediated pathway. Indeed, we had shown previously that IGR-Heu cells were defective for Fas/CD95, TRAIL-R1/DR4, TRAIL-R2/DR5, and TNF-R1 death receptor surface expression and that autologous CTL use mainly the secretory pathway to lyse specific target cells (19, 20). For this purpose, we analyzed the expression of granzyme B and perforin in Heu171 and H32-22 T

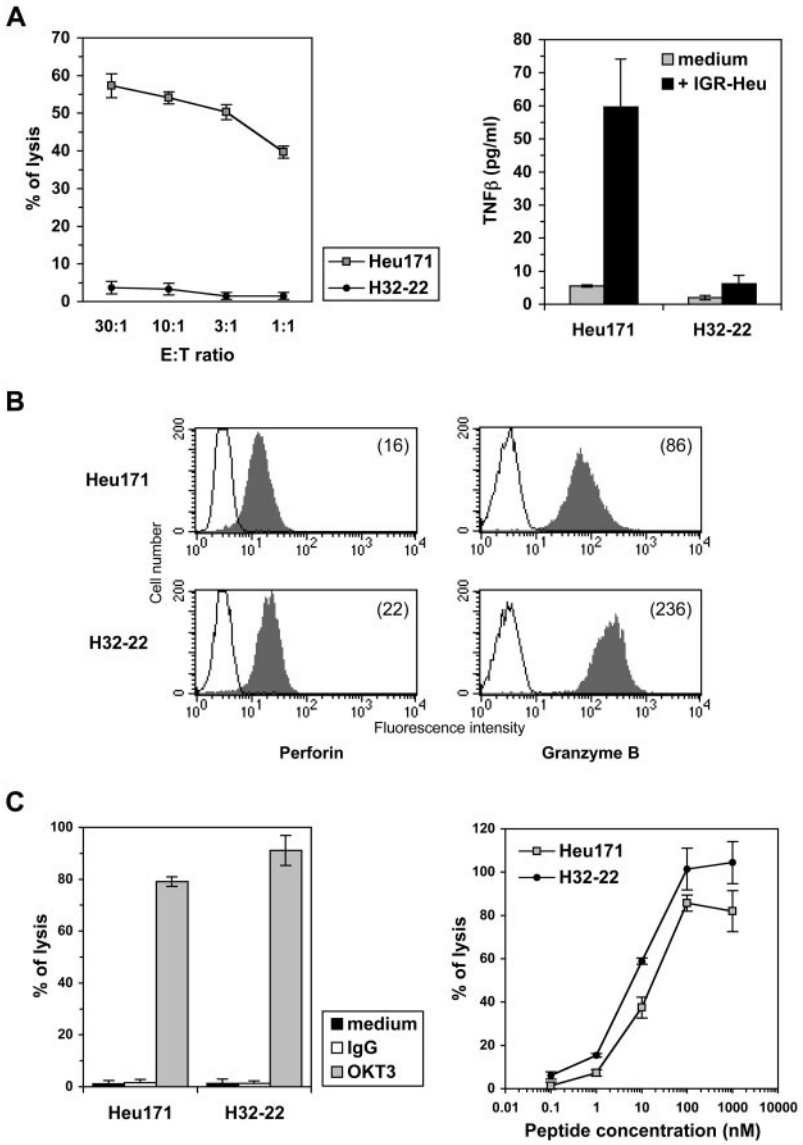

FIGURE 2. *A*, Cytotoxic activity of Heu171 and H32-22 T cell clones (*left panel*). Cytotoxicity was determined by a conventional 4-h ^{51}Cr release assay at indicated E:T ratios. The IGR-Heu autologous LCC cell line was used as target. Recognition of the IGR-Heu tumor cell line by autologous T cell clones (*right panel*). IGR-Heu cells were incubated with Heu171 or H32-22 T cells, and the amount of TNFβ produced by the clones was measured as in Fig. 1*B*. Data shown are representative of five independent experiments. *B*, Intracytoplasmic expression of perforin and granzyme B in Heu171 and H32-22 CTL clones as determined by intracytoplasmic immunofluorescence analysis (gray). Isotypic control mAb was included (white). The T cell membrane was permeabilized as described in *Materials and Methods*, and then mAb were added. Percentages of positive cells are indicated. Numbers in parentheses correspond to mean fluorescence intensity. Data shown are representative of three independent experiments. *C*, Redirected cytotoxic activity of Heu171 and H32-22 CTL clones (*left panel*). The FcR-positive P815 murine cells were used as target. OKT3 mAb or an isotypic control were used. Cytotoxic activity of Heu171 and H32-22 T cell clones toward peptide-pulsed autologous EBV-transformed B cells (*right panel*). Heu-EBV B cell line was incubated with the indicated concentrations of α-actinin-4 peptide before addition of the clones. Cytotoxicity was determined by a conventional 4-h ^{51}Cr release assay at E:T ratio of 10:1. Data shown are representative of two independent experiments.

cells by intracytoplasmic immunofluorescence analysis. Fig. 2*B* shows that both clones express similar amounts of perforin and granzyme B. An even higher granzyme B level is observed in the H32-22 clone (Fig. 2*B*). We then tested whether H32-22 was able to kill the FcR-positive P815 target in a redirected cytotoxicity assay. As shown in Fig. 2*C*, anti-CD3 mAb, even at very low concentrations, triggered a very strong cytotoxic activity in both T cell clones toward the murine target P815. A higher lytic potential was often observed with clone H32-22 (Fig. 2*C*, *left panel*). Moreover, H32-22 was able to lyse at least as efficiently as Heu171 the autologous EBV-transformed B cells pulsed with different concentrations of the antigenic peptide (Fig. 2*C*, *right panel*). These results indicate that PBL- and TIL-derived clones display similar cytolytic potential and suggest that the H32-22 defect is restricted to autologous tumor cells.

TIL- and PBL-derived clones express different levels of CD5 molecule

To investigate the molecular basis for the differential antitumor reactivity of TIL- and PBL-derived clones, we analyzed the cell surface expression of a large panel of molecules involved in T cell recognition and/or costimulation. Initial phenotypic studies revealed that both clones have a CD28$^-$CD27$^-$CD45RA$^+$CD62L$^-$CCR7$^-$ phenotype, indicating that they belong to terminally differentiated effector T cells (data not shown). We then tested the expression of transmembrane proteins involved in T cell activation and adhesion. Flow cytometry analysis indicated that H32-22 and Heu171 T cell clones express similar levels of TCR$\alpha\beta$, CD3, CD8, CD2, CD56, CD69, LFA-1 (CD11a), and NKG2D, excluding their implication in the H32-22 activation defect (data not shown).

Next, experiments were performed to assess the expression of receptors known for their capacity to inhibit TCR signaling. As shown in Fig. 3, both clones lacked the expression of Ig-like killer inhibitory receptors (KIR), p58.1 (KIR2DL1), p58.2 (KIR2DL2/3), p70 (KIR3DL1), and p140 (KIR3DL2). In contrast, expression of the lectin-like receptors CD94 and NKG2A was more intense on the Heu171 cell surface than on H32-22, ruling out their involvement in the inhibition seen with H32-22 (Fig. 3). Interestingly, data depicted in Fig. 3 indicate clearly that the TCR inhibitory molecule CD5 is much more highly expressed in H32-22 cells, suggesting that their poor responsiveness might be the consequence of these high CD5 expression levels. Because the distribution of CD5 was bimodal in Heu171 (Fig. 3), CD5^{low} and CD5$^-$ subpopulations were sorted, and cytotoxicity experiments indicated that the latter

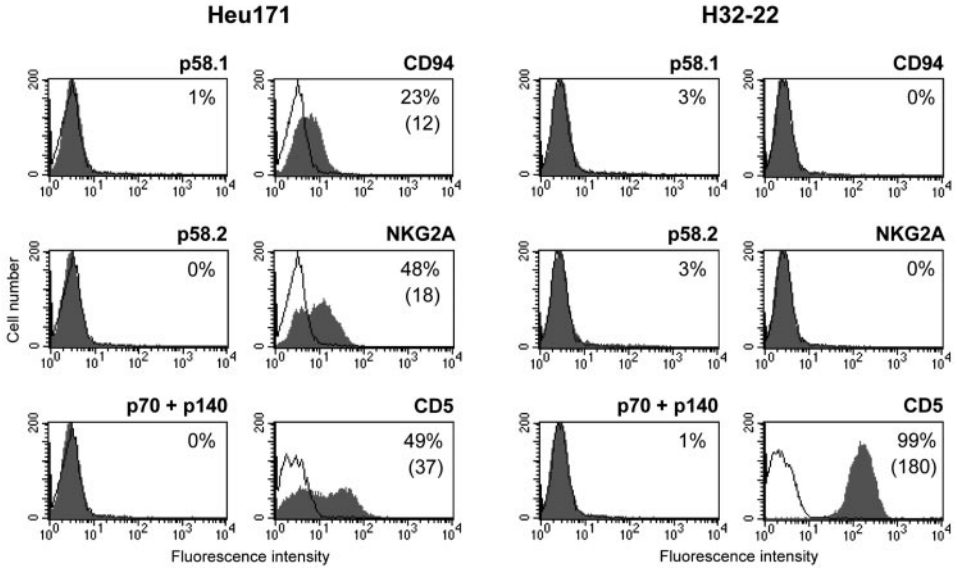

FIGURE 3. Flow cytometry analysis of Heu171 and H32-22 T cell clones. Cells were stained with the indicated receptor-specific mAb (gray) or an isotype-matched control (white). Percentages of positive cells are indicated. Numbers in parentheses correspond to mean fluorescence intensity. Data shown are representative of three independent experiments.

subset lysed the autologous tumor target more efficiently than the $CD5^{low}$ subset (Table I). To further assess the relationship between CD5 expression and tumor cell recognition, two additional tetramer$^+$TCRVβ8$^+$ T cell clones, H32-8 and H32-25, were isolated from patient 1 circulating T lymphocytes and tested for CD5 expression and autologous tumor cell lysis. Table II shows that the two clones express lower CD5 levels than H32-22 and display significant cytotoxic activity toward autologous IGR-Heu tumor cells. Significantly, CD5 levels in PBL-derived T cell clones were inversely proportional to their lytic efficacy and never reached those of Heu171 (Table II). It should be noted that TIL- and PBL-derived T cell clones were maintained in the same culture conditions for comparable follow-up period and that extensive proliferation did not induce change in CD5 levels, excluding that difference in CD5 expression may be the reflection of the replicative history of the cells.

Efficient conjugate formation but impaired signaling in $CD5^{high}$ antitumor T cells

Recent reports demonstrated that CD5 impairs TCR signaling during cell-to-cell contact between T cells and APC, without affecting conjugate formation and stability (21). Therefore, we analyzed the interaction between Heu171 or H32-22 T cells and autologous IGR-Heu tumor cells using video-imaging recordings at the single-cell level. Imaging of intracellular calcium increase, a powerful means of monitoring the dynamics of T cell activation induced by Ag recognition, was followed in parallel by loading T cells with the calcium indicator fura 2. Fig. 4*A* (also see online supplementary material)[5] shows a sequence of images representing a typical interaction between a tumor cell and Heu171 ($CD5^{low}$) or H32-22 ($CD5^{high}$) T cells. Results indicate that both TIL-derived and PBL-derived T cells can form conjugates and also that the interaction remains stable throughout the experiment (20 min). The number of conjugates formed was not significantly different between the two cell types (data not shown). However, much higher Ca^{2+} responses were observed with Heu171 cells as illustrated in Fig. 4, *A* and *B*, where Ca^{2+} elevation of three individual cells and mean responses ($n = 40$) are shown for each T cell clone. Oscillating Ca^{2+} responses were usually observed, but with very small peaks for H32-22 cells, a result further exemplified in the averaged traces because of the asynchronous character of the response at the individual cell level. In contrast, peptide-MHC tetramers triggered similar Ca^{2+} responses in both T cell clones over a wide range of concentrations (0.2–200 nM) (Fig. 4*C*). These results further emphasize the involvement of CD5 expression levels in regulating TCR-mediated cytotoxic activity.

Table I. *CD5 expression levels reflect Heu171 antitumor cytotoxicity*[a]

	CD5 Expression (mean fluorescence)	% of Lysis (E:T = 3:1)
Heu171		
$CD5^{low}$	72	22
$CD5^-$		33

[a] Mean fluorescence and cytotoxic activity of $CD5^{low}$ and $CD5^-$ subsets of Heu171 TIL clone towards autologous IGR-Heu tumor cell line.

CD5 expression levels in TIL parallel the intensity of intratumoral TCR/peptide-MHC signals

To further assess the relevance of intratumoral TCR signaling optimization in TAA-specific TIL, CD5 expression was analyzed in a large panel of uncultured PBL and TIL collected from 10 healthy donors and 14 NSCLC patients. Results indicated that the $CD3^+CD5^+$ fraction corresponded to virtually all healthy donor circulating T lymphocyte populations. Only a small fraction ($1.21 \pm 0.73\%$) of $CD3^+$ cells was defective for CD5 expression (Fig. 5*A*, *left panel*). Very slightly higher percentages ($1.57 \pm 1.11\%$) of $CD3^+CD5^-$ lymphocytes were obtained for NSCLC PBL. In contrast, this subpopulation of $CD3^+CD5^-$ lymphocytes was increased frequently ($3.57 \pm 2.02\%$) in uncultured TIL collected from 14 lung cancer patients (Fig. 5*A*, *left panel*). However, its very small size made it difficult to conclude that the tumor environment promotes the generation of $CD5^-$ T cells. Moreover, these cells may also include subpopulations of individual clones expressing low and heterogeneous levels of CD5, such as Heu171.

[5] The online version of this article contains supplemental material.

Table II. *CD5 levels reflect T cell clone antitumor cytolytic activity*[a]

	CD5 Expression (mean fluorescence)	% of Lysis (E:T = 10:1)
PBL		
H32–22	270	8
H32–8	186	23
H32–25	143	48
TIL		
Heu171	63	84

[a] Mean fluorescence and cytotoxic activity of T cell clones towards autologous IGR-Heu tumor cell line. Data shown are representative of four independent experiments.

More significantly, we found that CD5 expression levels were reduced in the $CD5^+$ T cells infiltrating the tumors (mean fluorescence intensity of 294) as compared with healthy donors (mean fluorescence intensity of 410) and NSCLC patient (mean fluorescence intensity of 389) peripheral T cells (Fig. 5*A*, *right panel*). These results further support a CD5 down-regulation within the tumor lesions.

It has been reported previously that CD5 levels on mature thymocytes and T cells paralleled the avidity of the TCR/peptide-MHC interaction (22). Therefore, we analyzed MHC molecule expression on the surface of several NSCLC tumor cell lines (Table III) and the corresponding tumor biopsies (Fig. 6) together with CD5 expression in autologous TIL-derived T cell lines and clones (Fig. 5*B*). Regarding patient 1, Fig. 5*B* shows that all T cell lines and clones isolated from TIL and exhibiting distinct Ag specificities displayed either low CD5 levels or were defective for CD5 expression. All of these $CD8^+$ T cell clones mediated high MHC-I-restricted cytotoxic activity toward autologous IGR-Heu cell line (Refs. 3, 15, and 17, and data not shown). Importantly, the IGR-Heu tumor cells expressed very low levels of MHC-I molecules as compared with allogeneic MZ2-MEL-A2 melanoma cell line and autologous EBV-transformed B cells used as controls (Table III). Low MHC-I expression was also detected by immunohistochemistry in the corresponding tumor biopsy (Fig. 6).

With regard to TIL from patient 2, low CD5 expression levels were detected on short-term-cultured TIL and on the $CD4^+$ CTL clone (Fig. 5*B*) displaying an efficient HLA-DR-restricted cytotoxic activity toward the autologous ADC cell line, IGR-Pub (16). Significantly, the IGR-Pub cells expressed very low levels of MHC class II (MHC-II), as compared with the allogeneic EBV-transformed B cell line (Table III). Low CD5 levels were also observed on TIL cell lines and T cell clones infiltrating LCC-M4 tumor of patient 4. As shown in Table III and Fig. 6, LCC-M4 tumor cell line and biopsy expressed low levels of MHC-I molecules. Similarly, low CD5 expression levels were observed on a TIL cell line and a tumor-specific $CD8^+$ T cell clone infiltrating a β_2-microglobin-deficient ADC isolated from patient 5 (Table III and Fig. 6). The ADC-Coco cell line, derived from the latter tumor biopsy, was extremely sensitive to CTL clone-mediated lysis following its transfection with β_2-microglobin, which restored MHC-I expression (data not shown). Only TIL from patient 3 were found to express more diverse CD5 levels, and some clones displayed a $CD5^{high}$ phenotype (Fig. 5*B*). These clones were able to kill the autologous IGR-B2 tumor cell line in a MHC-I-restricted manner (Ref. 16 and data not shown). Interestingly, the IGR-B2 LCC cell line expressed high levels of MHC-I molecules (Table III). The IGR-B1 cell line, established from the same tumor biopsy as IGR-B2, displayed lower MHC-I levels and was less susceptible to autologous CTL clone lysis (data not shown). Heterogeneous MHC-I expression was also detected in the corresponding tumor biopsy by immunohistochemical analysis (Fig. 6 and data not shown).

Regarding PBL, patient 1 T cell lines, stimulated under the same conditions as TIL and selected or not with peptide-MHC tetramers, and $tetramer^+TCRV\beta13.2^+$ T cell clones, isolated by tetramers following stimulation of autologous PBL with mutated α-actinin-4 peptide (3, 9), expressed high levels of CD5 comparable to clone H32-22 (Fig. 5*B*). Similarly, high CD5 expression levels were observed on all T cell lines established from PBL of patients 2, 3, 4, and 5 (Fig. 5*B*). These results suggest that CD5 expression levels in TIL may parallel MHC expression by the autologous tumor cells and that an intratumoral decrease in CD5 expression on these specific CTL may represent a flexible process for enhancing TCR signaling intensity.

Discussion

We had identified previously a tumor-specific epitope encoded by a mutated *ACTN4* gene and presented by HLA-A2 molecules on a LCC cell line generated from a patient with long survival (3). Peptide-MHC $tetramer^+$ T cell clones isolated from TIL and PBL displayed similar multimer staining but distinct functional avidities, demonstrating that no correlation between the two parameters could be made (9). Furthermore, TCRβ chain gene usage analysis indicated that only Ag-specific clones with high functional avidity ($tetramer^+TCRV\beta22^+$) were expanded at the tumor site, whereas low avidity clones ($tetramer^+TCRV\beta13.2^+$) were amplified in patient peripheral blood (9). These results argue for selective in situ expansion of tumor-specific T cell clones exhibiting high functional avidity and mediating antitumor cytotoxic activity. In addition, we demonstrated that T cell clones with intermediate functional avidity ($tetramer^+TCRV\beta8^+$) were expanded in TIL and, to a lesser extent, in patient PBL. In the present study, we have characterized the tumor reactivity of the $tetramer^+TCRV\beta8^+$ T cell clones isolated from both compartments. Strikingly, although these clones expressed a unique TCR and displayed similar tetramer labeling and identical functional avidity, only T cell clones isolated from TIL efficiently lysed the autologous tumor cells and secreted cytokines following specific stimulation. These results indicate that no correlation between functional avidity, as currently measured, and efficacy for reacting with target cells can be made. Furthermore, taking into account that both T cell clones display similar lytic potentials, as measured by granzyme B and perforin intracellular expression and redirected cell killing, our results suggest a tumor-restricted TCR signaling defect in PBL-derived CTL.

The T cell surface phenotype indicated that PBL- and TIL-derived clones belong to terminally differentiated effector T cells and that they express the same T cell surface markers, except for the TCR inhibitory receptors CD94/NKG2A and CD5. NK receptors (KIR and CD94/NKG2A) were found previously on subpopulations of cancer patient TIL (17, 23–25) and peripheral blood CTL (26), and the receptors have been shown to inhibit TCR stimulatory signals, thereby inhibiting autologous tumor cell lysis. However, our data indicate that the killer cell lectin-like subfamily receptors (KLR), CD94 (KLRD1), and NKG2A (KLRC1), are more highly expressed in TIL- than in PBL-derived T cell clones, excluding their implication in the latter clone activation defect. KLR intratumoral up-regulation may be associated with a local inflammation and/or a continuous and persistent T cell stimulation. With regard to CD5, TIL-derived T lymphocytes were found to express much lower levels than circulating T cells.

Human CD5 is a 67-kDa type I transmembrane glycoprotein expressed on thymocytes, mature T cells, and a subpopulation of B cells (B1a) (27). CD5 belongs to the family of receptors bearing extracellular domains of the scavenger receptor cysteine-rich type

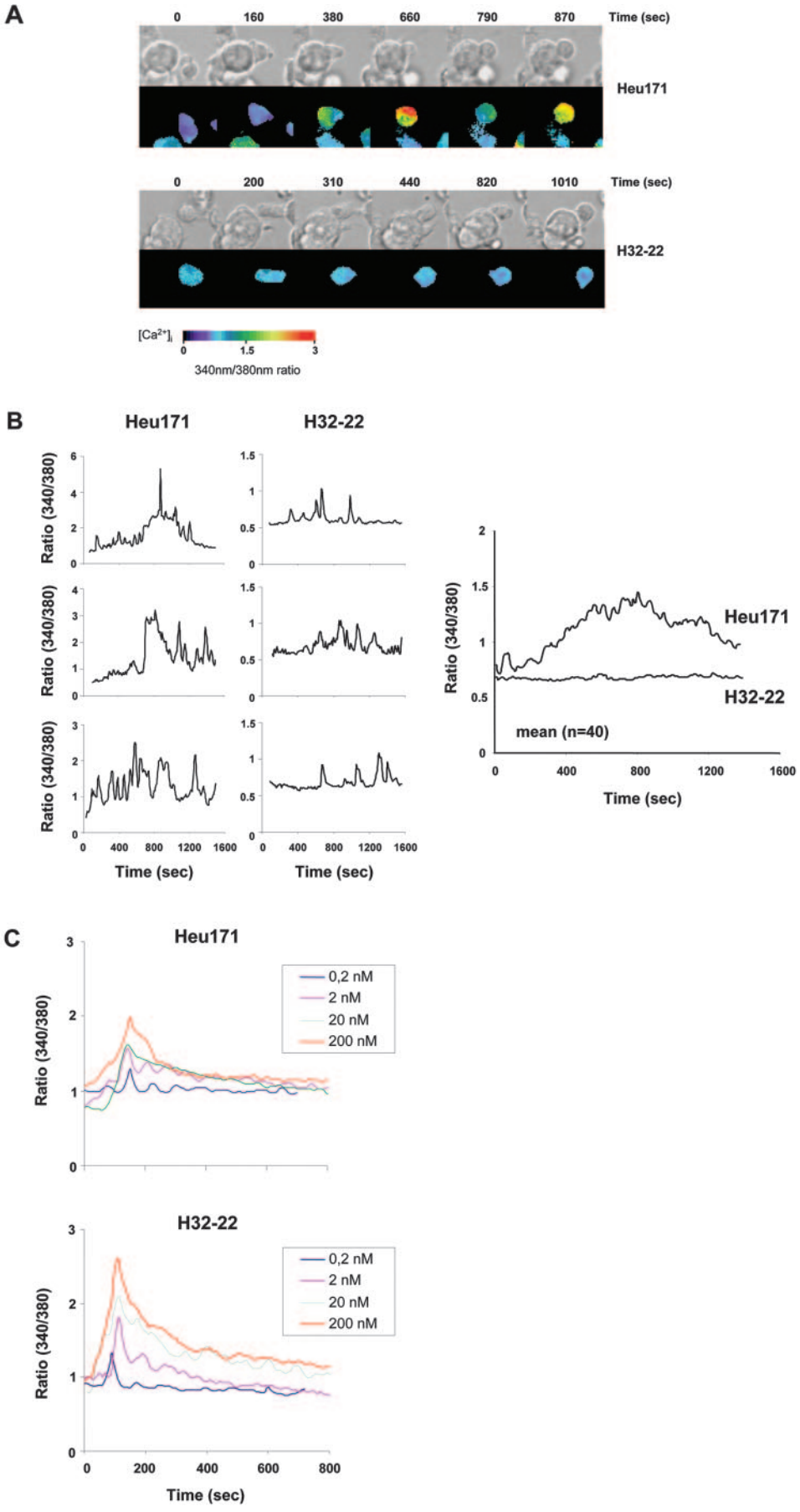

FIGURE 4. *A*, The Heu171 ($CD5^{low}$) or H32-22 ($CD5^{high}$) T lymphocytes were loaded with fura 2, and the Ca^{2+} response in individual cells was measured during conjugate formation with the IGR-Heu autologous tumor cells in a time-lapse experiment. The Ca^{2+} levels were analyzed by single-cell imaging, and a color-coded scale represents intracellular Ca^{2+} ($[Ca^{2+}]$). Corresponding differential interference contrast images are shown. *B*, Three single-cell Ca^{2+} responses induced by IGR-Heu cells (*left panel*). Averaged Ca^{2+} responses of 40 individual Heu171 and H32-22 cells forming conjugates with IGR-Heu were calculated (*right panel*). *C*, Ca^{2+} responses induced in Heu171 and H32-22 cells by different concentrations of mutated-α-actinin-4 peptide-HLA-A2 tetramers were measured by single cell imaging. Mean values for 50 individual cells are shown. Data shown are representative of three independent experiments.

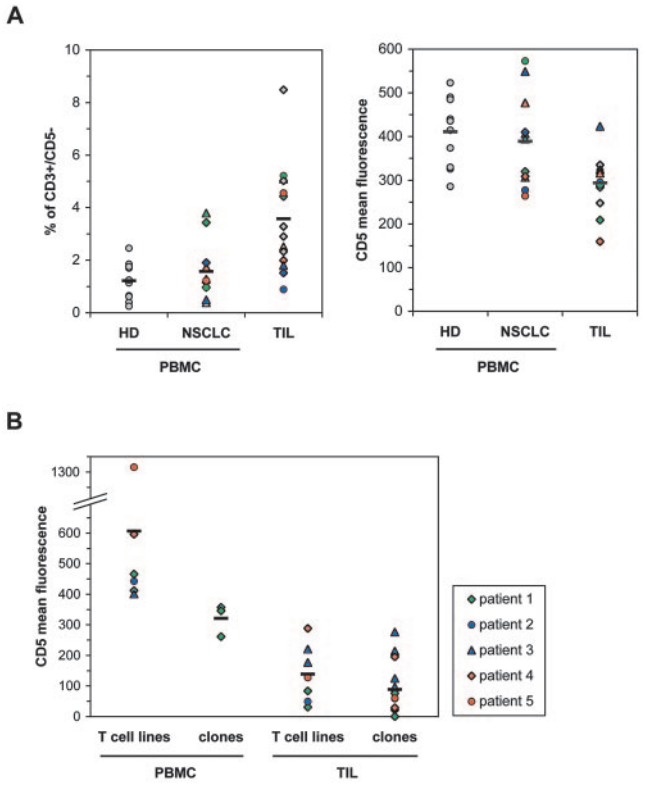

FIGURE 5. *A*, Flow cytometry analysis of uncultured PBMC and TIL collected from healthy donors (HD) or NSCLC patients. Percentage of $CD3^+CD5^-$ fraction in PBMC and TIL (*left panel*). Mean percentage of $CD3^+CD5^-$ cells was significantly higher in TIL than in patient PBMC ($p < 0.005$) and HD PBMC ($p < 0.001$). CD5 mean fluorescence intensity in $CD5^+$ T cell fraction (*right panel*). Mean fluorescence intensity was significantly lower in TIL than in patient PBMC ($p < 0.02$) and HD PBMC ($p < 0.002$). *B*, Flow cytometry analysis of T cell lines and CTL clones generated from NSCLC patients TIL and PBL. CD5 mean fluorescence intensity is represented. Means of the analyzed samples are represented by a dash.

(28) and is associated both physically and functionally with TCR/CD3 and BCRs. Accumulating evidence indicates that T lymphocyte activation and selection are sensitive to variations in levels of cell surface CD5. Based on data from CD5-deficient mice, it has been reported that CD5 exerts a negative effect on TCR signaling (29). Indeed, immature T cells in $CD5^{-/-}$ mice are hyperresponsive to TCR stimulation and exhibit altered positive and negative selection (29). CD5 also inhibits peripheral blood T cell signaling, and $CD5^-$ T cells show enhanced proliferation upon TCR triggering (29, 30). Furthermore, it has been reported recently that immunological unresponsiveness of peripheral T cells is characterized by increased expression of CD5 induced by dendritic cells in vivo (31). Various potential ligands for CD5 have been described previously (32–35), but the true identity of the physiologically relevant ligand(s) remains to be established. Nevertheless, it has been reported that CD5-mediated inhibition of TCR signaling does not require the CD5 extracellular domain (36), but only requires its cytoplasmic tail (30, 37), where a pseudo-ITAM is likely to play an essential role (21). The latter study also reports that CD5 is recruited and colocalized tightly with CD3 at the immunological synapse so as to inhibit TCR signaling in T cells interacting with APC, but without influencing conjugate formation (21) as opposed to KIR (23). Likewise, we found that although CD5 levels in TIL and PBL correlate with signaling inhibition, both clones interact similarly with tumor cells. In contrast, these CD5 levels had no

Table III. *MHC molecule expression on NSCLC tumor cell lines*[a]

Tumor Cell Lines	MHC-I	MHC-II
IGR-Heu (patient 1)	85[b] (99%)	(1%)
IGR-Pub (patient 2)	608 (97%)	43 (68%)
IGR-B2 (patient 3)	499 (97%)	(0%)
LCC-M4 (patient 4)	82 (90%)	ND
ADC-Coco (patient 5)	(0%)	(2%)
SCC-Cher (patient 6)	32 (47%)	(0%)
MZ-2-MEL-A2	999 (100%)	310 (100%)
Heu-EBV (patient 1)	993 (97%)	277 (97%)

[a] Numbers in parentheses correspond to percentages of positive cells. The EBV-transformed B cell line, Heu-EBV, and the allogeneic HLA-A2-transfected melanoma cell line, MZ2-MEL-A2, were included as controls. ND, Not done. Data shown are representative of three independent experiments.
[b] Mean fluorescence intensities are indicated.

inhibitory effect on the lysis of peptide-pulsed APC, such as the autologous EBV-transformed B cells and the allogeneic HLA-A2-transfected melanoma cells, expressing much higher MHC-I levels than autologous tumor cells (Table III). Indeed, in the setting of higher effector cell/target cell interaction strength, CD5 may not exert its negative effect. All of these results fit very well with the assumed functions of CD5 as a fine modulator of the immune response downstream of TCR signaling. It should be noted that CD6, the CD5 closely related lymphocyte surface receptor (38), is as highly expressed in PBL- as in TIL-derived clones (data not shown), suggesting differential expression regulation of the two molecules.

MHC-I molecules play a major role in Ag presentation to $CD8^+$ CTL. Alteration in MHC-I expression has been observed frequently in human neoplastic cells, including lung carcinoma, and represents a common tumor escape mechanism from the immune response. Indeed, four of eight NSCLC target cell lines and corresponding tumor biopsies used in the present study display low or defective MHC-I expression (Table III, Fig. 6, and data not shown). Low MHC-II expression is also observed on tumor cells recognized by autologous $CD4^+$ CTL. Significantly, low MHC expression on the tumor cell surface appeared

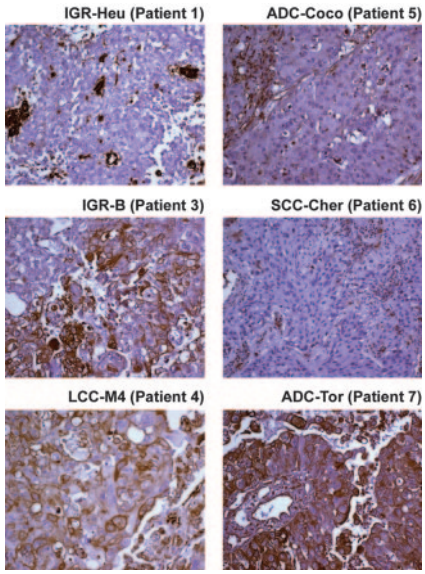

FIGURE 6. Immunohistochemical analysis of IGR-Heu, IGR-B, LCC-M4, ADC-Coco, and SCC-Cher tumor biopsies. ADC-Tor tumor lesion was used as a positive control. Anti-MHC-I H chain (HC10) mAb was used. Magnification was ×200.

to correlate with a decrease in CD5 levels in tumor-specific $CD8^+$ and $CD4^+$ TIL. These results suggest an influence of the intensity of the TCR/peptide-MHC interaction on CD5 expression levels in TIL and argue for the hypothesis that intratumoral CD5 down-regulation enhances TCR signaling and subsequent triggering of cancer cell lysis. This hypothesis is supported by previous findings demonstrating that, in vivo, CD5 expression on mature, single-positive thymocytes and T cells directly parallels the avidity or signaling intensity of the positively selecting TCR/MHC-ligand interaction (22).

With respect to mature T cells, it has been shown that peptide-MHC contact modulates the expression of CD5 by naive $CD4^+$ T cells and that CD5 levels decrease rapidly in T cells deprived of TCR/peptide-MHC interactions, thus forecasting an increased response to TCR engagement (39). Moreover, it has been reported recently that CD5 levels reflect T cell avidity for the self-peptide-MHC complex and that the homeostatic behavior of naive T cells can be deduced from the density of TCR and CD5 (40). Together, these data indicate that CD5 levels may be adaptable to signals received in the periphery and that the adjustment of TCR sensitivity by CD5 molecule may be a continuous process throughout the T cell lifetime (39, 41). A decrease in CD5 levels has also been observed in PBL of HIV-infected patients (42); inversely, an increase has been observed in peripheral anergic $CD8^+$ T cells (43) and in autoimmune B cells (44). Our results suggest that CD5 expression in TAA-specific CTL is adjusted so as to reflect the in situ intensity of interactions between TCR and the peptide-MHC complex. Indeed, the expression level of CD5 by the antitumoral T cells might drop when the intensity of the interaction between CTL and tumor cells is weak, thereby enhancing the TCR signaling response. Interestingly, intratumoral transfer of $CD5^-$ tumor-specific CTL induced tumor regression in vivo in SCID/NOD mice transplanted with autologous tumor cells (16, 45). However, the precise mechanism that governs CD5 expression regulation in situ remains to be established. Regarding PBL-derived T cell clones, high CD5 levels are associated with defective lysis of autologous tumor cells displaying low peptide-MHC-I levels. Circulating T cells expressing intermediate CD5 levels and displaying significant cytotoxic activity toward autologous tumor target may have transited during their lifetime within the tumor. The quiescent status of PBL-derived tumor-specific CTL may have a potential impact on cancer immunotherapy and may, at least in part, elucidate the paradoxical lack of correlation between the frequency of MHC-tetramer$^+$ circulating T lymphocytes induced in vaccination trials and tumor regression. Furthermore, our results favor an immunotherapy strategy based on peptide-MHC tetramer selection of tumor-specific T lymphocytes from patient TIL, which were adapted previously to the tumor microenvironment, rather than from PBL for use in cancer clinical trials.

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Disclosures

The authors have no financial conflict of interest.

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