Human Intestinal Vδ1$^+$ T Lymphocytes Recognize Tumor Cells of Epithelial Origin

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Summary

$\gamma\delta$ T cells can be grouped into discrete subsets based upon their expression of T cell receptor (TCR) variable (V) region families, their tissue distribution, and their specificity. Vδ2$^+$ T cells constitute the majority of $\gamma\delta$ T cells in peripheral blood whereas Vδ1$^+$ T cells reside preferentially in skin epithelium and in the intestine. $\gamma\delta$ T cells are envisioned as first line host defense mechanisms capable of providing a source of immune effector T cells and immunomodulating cytokines such as interleukin (IL) 4 or interferon (IFN) γ. We describe here the fine specificity of three distinct $\gamma\delta^+$ tumor-infiltrating lymphocytes (TIL) obtained from patients with primary or metastatic colorectal cancer, that could be readily expanded in vitro in the presence of IL-1β and IL-7. Irrespective of donor, these individual $\gamma\delta$ T cells exhibited a similar pattern of reactivity defined by recognition of autologous and allogeneic colorectal cancer cells, renal cell cancer, pancreatic cancer, and a freshly isolated explant from human intestine as measured by cytolytic T cell responses and by IFN-γ release. In contrast, tumors of alternate histologies were not lysed, including lung cancer, squamous cell cancer, as well as the natural/lymphocyte-activated killer cell–sensitive hematopoietic cell lines T2, C1R, or Daudi. The cell line K562 was only poorly lysed when compared with colorectal cancer targets. Target cell reactivity mediated by Vδ1$^+$ T cells was partially blocked with Abs directed against the TCR, the β2 or β7 integrin chains, or fibronectin receptor. Marker analysis using flow cytometry revealed that all three $\gamma\delta$ T cell lines exhibit a similar phenotype. Analysis of the $\gamma\delta$ TCR junctional suggested exclusive usage of the Vδ1/Dδ3/Jδ1 TCR segments with extensive ($\leq$29 bp) N/P region diversity. T cell recognition of target cells did not appear to be major histocompatibility complex restricted or to be correlated with target cell expression of heat–shock proteins. Based on the ability of some epithelial tumors, including colorectal, pancreatic, and renal cell cancers to effectively cold target inhibit the lysis of colorectal cancer cell lines by these Vδ1$^+$ T cell lines, we suggest that intestinal Vδ1$^+$ T cells are capable of recognizing cell surface Ag(s) shared by tumors of epithelial origin.

Two distinct populations of $\gamma\delta^+$ TCR-bearing cells have been previously characterized based on their expression of distinct TCR V gene segments and their unique tissue distribution. The dominant $\gamma\delta^+$ T cell population in the peripheral circulation is defined by coexpression of the Vδ2 and the Vγ9 TCR (1, 2) segments, constituting 70–90% of the $\gamma\delta^+$ T subsets in PBL, and representing 5–10% of all TCR$^+$ peripheral T lymphocytes. A different, far less frequent, $\gamma\delta^+$ T cell population in PBL is detected by the mAb δTCS-1 (3) recognizing the Vδ1/Jδ1/Jδ2 TCR (4). This particular subpopulation constitutes only 5–10% of the $\gamma\delta$ T cell population in human PBL, but it represents the majority of $\gamma\delta$ T lymphocytes found in the oral and intestinal epithelia (5–9). The TCR Vδ2/Vγ9 predominance in PBL is thought to reflect an Ag-driven expansion potentially involving the superantigen staphylococcal enterotoxin A (10), isopentyl pyrophosphate, and related prenyl pyrophosphate derivates, components of mycobacterial Ags (11), or cell-derived proteins expressed upon viral infection (12). Individual $\gamma\delta$ T cell clones have been previously reported to recognize MHC class I and II, CD1c Ags, nonclassical minor MHC Ags, or heat–shock

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proteins (HSPs[1]; 13–20). Extensive analysis of the CDR3 region of the γδ TCR (21) suggests that the topology of the interaction of the γδ TCR with MHC Ags is clearly different from that observed for αβ$^+$ T cells, suggesting that the γδ TCR may be able to recognize native (i.e., non-MHC presented) protein Ags (12). This hypothesis implies that Ag recognition by γδ$^+$ T cells may more closely resemble soluble Ag recognition by Igs, rather than recognition of short peptide fragments presented by MHC molecules to αβ$^+$ TCR cells (12, 19, 20). Whereas some targets have been identified for Vδ2$^+$ T cells, the Ags recognized by the Vδ1$^+$ TCR subset are unknown. Exclusive expansion of this particular subset has been observed in a variety of different diseases: Vδ1$^+$ T cells are selectively expanded in vivo in the peripheral blood of patients with EBV or HIV infections (22–24). Vδ1$^+$ T cells are also preferentially expanded in the lungs of patients with pulmonary sarcoidosis and in lesions from patients infected with *Mycobacterium leprae* (25), in synovial fluid from patients with rheumatoid arthritis (26, 27), in cerebospinal fluid from patients with acute multiple sclerosis (28, 29), in intestinal lesions from patients with coelical disease (30, 31), and in acute or chronic graft rejections in heart transplant patients (32). Preferential expansion and cytotoxic antitumor responses of the Vδ1$^+$ T cell subset have been demonstrated in patients with tumors of epithelial origin, including adenocarcinomas of the lung (33, 34) and, most recently, in renal cell cancer (35). Such Vδ1 TCR$^+$ T cells exhibit a characteristic recognition pattern, lysing the autologous and allogeneic cell lines with similar histologies (35). Vδ1$^+$ T cells lyse, albeit to a lesser extent, the NK-sensitive cell line K562, but not other NK/LAK–sensitive cell lines, and are apparently not MHC restricted (33–35). We report the in vitro expansion of three Vδ1$^+$ T cell lines cultured in the presence of IL-1β and IL-7 from tumor-infiltrating lymphocytes (TIL) derived from patients with primary or metastatic colorectal cancer. These γδ T cell lines appear to recognize common Ag(s) shared by colorectal, renal cell, and pancreatic cancers defined by both cytolytic T cell reactivity and by IFN-γ release. These data suggest that cytotoxic Vδ1$^+$ T cells may be critically involved in immune responses against cancer of epithelial origin in situ.

Materials and Methods

Cell Lines. Target cell lines used in cytotoxicity or cytokine release assays were maintained in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (GIBCO BRL, Gaithersburg, MD; complete medium [CM]) unless otherwise indicated. Short-term cultures of freshly harvested tumor single cell suspensions from colorectal cancer patients were also grown in CM. C1R or human EBV-transformed B cell lines (S-EBV) expressing the muc 1 molecules have been described previously (36) and were grown in CM supplemented with 600 μg/ml G418 (GIBCO BRL). The breast cancer cell line BT20 and the cell line U937 (MHC class II negative), its MHC class II–positive subclone I 937, the colorectal cancer cell line DLD-1, the pancreatic cancer cell lines pancreas89 and pancreasQGP9 were kind gifts from Dr. O. Finn (University of Pittsburgh Medical School). The colorectal cancer cell lines LS174 and SW480, the Wilms tumor cell line SKNEP-1, and the explant culture (passage 1) from normal human intestine (CRL7136, HS186.INT) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). The colorectal cancer cell lines HCT116 and HT29, the squamous cell cancer cell lines PCI-13 and PCI-50 (head and neck cancer), and target cell lines Daudi, Molt-4, and K562 were kindly provided by Dr. Theresa Whiteside, University of Pittsburgh Cancer Institute. The lung cancer cell lines lung201 and lung89 were obtained from Dr. Steven Dobrowolski (University of Pittsburgh Medical School). The melanoma cell line 526 was kindly provided by Dr. S.A. Rosenberg, (National Cancer Institute). The renal cell cancer cell lines NT1973 and NT1257 and the melanoma cell line Mz18 were kindly provided from Dr. Alex Knuth (Krankenhaus Nordwest, Frankfurt, Germany).

TIL. TIL from patients 1881 (colon adenocarcinoma), 3481 (colon adenocarcinoma) and 7179 (colon adenocarcinoma metastatic to liver) were generated as described previously (37) by mincing freshly harvested tumor, by digestion with collagenase (Sigma Chemical Co., St. Louis, MO), and by centrifugation over a Ficoll gradient. The gradient interface was collected, washed twice, resuspended in AIM-V medium (GIBCO BRL), supplemented with 1,000 IU/ml rhIL-1β and 1,000 IU/ml rhIL-7 (kindly provided by Dr. Michael Widmer, Immunex, Seattle, WA) and seeded at 5 × 10^5 cells/ml in 24-well tissue culture plates. TIL have never been restimulated in vitro with tumor. They were split if the cell number exceeded 1.5 × 10^6 cells/ml and fed with IL-1β/IL-7 containing AIM-V medium. TIL were tested at various time points after in vitro propagation for the presence of γδ$^+$ T lymphocytes by flow cytometry. γδ$^+$ TIL were purified by flow cytometric cell sorting using the anti–pan-γδ TCR mAb TCRδ1 (clone 5A6.E9[3]; T-Cell Sciences, Cambridge, MA; see Table 1) and the FACStar® (Becton Dickinson & Co., Mountain View, CA) and were then expanded in IL-1β/IL-7–containing AIM-V medium over a period of 3 mo. Highly purified γδ$^+$ TIL were used at various time points during extended culture as effector T cells in cytotoxicity or cytokine release assays, as indicated. The purity of the respective γδ T cell lines was determined by flow cytometric analysis before each experiment. γδ$^+$-sorted TIL lines exhibited a stable γδ$^+$ TCR phenotype (purity >98%) over the 3-mo period tested.

Cytolytic Assays. A standard 4-h chromium release assay was used to assess cytolytic recognition of tumor cells by γδ TIL lines 1881, 3481, and 7279 using an E/T ratio of 5:1 unless otherwise indicated. LAK cells were generated using 7-d 6,000 IU IL-2–stimulated PBL obtained from donor leupacks as control effector cells. ^{51}Cr-labeled target cell suspensions were adjusted to 0.5 × 10^5 cells/ml and 100 μl of this cell suspension was added to individual assay wells in triplicate determinations. 100 μl of TIL effector cells (E/T ratio, 5:1) were then added to experimental wells and plates incubated for 4 h at 37°C. Spontaneous release wells received 100 μl of TIL medium (AIM-V) and maximum release wells received 100 μl of Triton X–100 (10% vol/vol in water). For blocking studies, anti–T cell reagents (mAbs against TCR, anti -β2 or -β7 integrin chains, or Abs against the human vitronectin receptor αvβ3 (HVR), or the human fibronectin receptor α5β1 (HFR; see Table 1) were used to pretreat effector

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[1] *Abbreviations used in this paper:* CM, complete medium; HFR, human fibronectin receptor; HVR, human vitronectin receptor; RT, reverse transcriptase; TIL, tumor-infiltrating lymphocyte(s).

cells. Cells were seeded in individual assay wells and incubated for 45 min at 4°C with the respective Ab (10 μg/10^4 cells) and the appropriate isotype controls, followed by two washing steps using AIM-V medium. After careful removal of the supernatant, TIL were resuspended in 100 μl AIM-V medium supplemented with IL-1β and IL-7 and the appropriate target cell suspensions were added as indicated in 100 μl. Target single cell suspensions were treated as indicated in blocking experiments using anti–MHC class I and II, anti-CD1, or anti- HSP Abs (see Table 1). For cold target inhibition assays, 100 μl of the ^{51}Cr-labeled target cells was added to individual assay wells along with 100 μl of the respective cold target single cell suspension at various dilutions as indicated. T lymphocytes were then added (effector TIL/^{51}Cr target cells = 5:1) in a volume of 20 μl per assay well and a standard ^{51}Cr-release assay was performed twice. 100 μl aliquots was harvested from each well and counted in a gamma counter (Pharmacia LKB). Results are reported as percent specific chromium release calculated as: [(Experimental cpm − spontaneous cpm)/(Maximum cpm − spontaneous cpm)] × 100.

Cytokine Release Assays. Stimulator cells were irradiated (3,000 Gy) and adjusted to 10^5 cells/ml in CM. 100 μl of this single cell suspension were added to individual wells and incubated with 100 μl single cell suspension containing individual γδ TIL lines (5×10^5 cells/ml) in AIM-V. Control wells included stimulator cells alone or T cells alone. After incubation at 37°C for 16 h, supernatants were harvested and stored at −20°C until assayed for IFNγ (R&D Systems Inc., Minneapolis, MN) by ELISA according to the manufacturer's instructions. Sensitivity for IFN-γ is reported as 5 pg/ml in cell culture supernatants.

Abs and Flow Cytometry. The specificity of mAbs or polyclonal antisera used in this study is listed in Table 1. The distributors were T-cell Sciences, Becton Dickinson & Co., Coulter Corp. (Hialeah, FL), Amac, Inc. (Westbrook, ME), Upstate Biotechnology, Inc. (Lake Placid, NY), Telios (now GIBCO BRL): GIBCO BRL, Chemicon (Temecula, CA), Mizzen, (Victoria, Canada), and Dako (Carpinteria, CA). The mAb w6/32 (anti–MHC class I) and mAb L243 (MHC class II, DR) were obtained from the ATCC and were derived as hybridoma culture supernatants. Briefly, cells were incubated for 30 min at 4°C with directly labeled FITC- or PE- conjugated Ab. For some unlabeled Abs, a secondary reagent FITC or PE F(ab′)$_2$ anti–mouse or anti–rabbit IgG was used. Controls included normal mouse or rabbit IgG obtained from Becton Dickinson & Co. prelabeled with FITC or PE. In each flow cytometric sample, 5,000 events were measured and gates were set to exclude nonviable cells. Individual samples were analyzed using a FACScan® (Becton Dickinson & Co.) flow cytometer using the software program Lysis II, and results are reported as percent positive-staining cells evaluated in two-color flow cytometric analysis. Using single-color (FITC or PE)–based detection systems, results are reported as percent positive-staining cells and are depicted as fluorescence intensity versus cell size (FSC) histograms in order to allow for the identification of potential T cell subsets demonstrating differentially dim or bright Ab staining.

Template cDNA Ppreparation. Total RNA from 5×10^6 to 5×10^7 cells was extracted from γδ TIL lines, from freshly harvested biopsies obtained from colon cancer lesions, or from tumor-free mucosa (adjacent to tumor) obtained from patients undergoing surgery for resection of colorectal cancer at the University of Pittsburgh Medical Center or at the Veterans Administration Hospital (Pittsburgh, PA) using RNAzol (Biotecx, Inc., Houston, TX) according to the method of Chomczynski and Sacchi (38). First strand cDNA synthesis was perfomed by heating the reaction at 37°C for 1 h, followed by 5 min at 95°C using a PCR thermal cycler (Perkin-Elmer Corp., Norwalk, CT). The 40-μl reaction volume contained 8 μg of RNA in 16 μl H_2O, 8 μl 5× reaction buffer (GIBCO BRL), 4 μl dithiothreitol (final concentration, 10 mM), 2 μl dNTP (dATP, dCTP, dGTP, dTTP, final concentration, 1 mM each), 3 μl RNase inhibitor (final concentration, 120 U), 1 μl actinomycin D (final concentration, 2 μg), 4 μl Oligo-dT random primers (final concentration, 0.8 μg), and 2 μl Moloney murine leukemia virus reverse transcriptase (RT) (Perkin-Elmer Corp., final concentration, 400 IU/ml).

Amplification of TCR-specific Templates. 150 ng of cDNA was used in each individual PCR reaction throughout all experiments and the integrity of individual cDNA samples was analyzed using human β-actin–specific primers (5–3′; forward: ATTTGCGGTGGACGATGGAGGGGC, backward: GGCATCGTCACCAACTGGGACGAC) using the temperature profile 95°C for denaturation, annealing for 1 min at 60°C, followed by 1 min at 72°C for extension (30 cycles). The protocol for amplification of Vδ- or Vγ-specific mRNA transcripts was 95°C (2 min for denaturation) followed by 33 cycles of 94°C (1 min), 59°C (1 min, 30 s), and 72°C (1 min). The primers sequences for specific Vγ or Vδ transcripts have been previously reported elsewhere (28). Generally, each reaction mixture (50 μl vol) contained 25 μl H_2O, 5 μl PCR 10X reaction buffer (Perkin-Elmer Co.), 8 μl dNTP (concentration, 1.25 mM each), 5 μl of each primer (final concentration, 50 pmol each primer), 1.5 μl cDNA (150 ng), and 1 μl Taq-polymerase (Perkin-Elmer Corp.). The respective PCR reactions were run on 1.5% ethidium bromide–stained agarose gels. In each PCR run, a positive control (cDNA from heat-killed *Mycobacterium tuberculosis*–stimulated PBL) and a negative control (PCR mastermix without template cDNA) were included. Primers were synthesized at the DNA Synthesis Facility, University of Pittsburgh. The identity of individual PCR products was confirmed by Southern blot using internal Vγ- or Vδ-specific primers (data not shown).

Sequencing of Vδ1$^+$ PCR Products. Vδ1$^+$ PCR products generated from in vitro–cultured (>98% pure) γδ$^+$ T cell lines were purified from agarose gels using microfilters obtained from Millipore Corp. (Bedford, MA) and subcloned into the TA cloning vector obtained from Invitrogen (San Diego, CA). Positive clones were randomly selected from each individual γδ TIL line and sequenced using the ABI system (Applied Biosystems, Foster City, CA) at the University of Pittsburgh DNA sequencing facility.

Results

Colorectal Cancer–derived γδ T Cells Expanded in IL-1β and IL-7 Exclusively Express the Vδ1 TCR Segment and Preferentially Coexpress the Vγ2 TCR. Tumor-infiltrating T lymphocytes were obtained from three patients with colorectal cancer (patients 1881: primary colon adenocarcinoma; 3481: primary colon adenocarcinoma; 7279: colon adenocarcinoma metastatic to the liver) and were cultured for 6 wk in IL-1β and IL-7 (1,000 IU/ml each) containing AIM-V medium. Phenotypic analysis of individual (bulk) cultures performed at day 54 revealed 10–48% CD3$^+$/γδ$^+$/Vδ1$^+$ positive-staining T cells (data not shown). γδ$^+$ T cells were purified from TIL cultures by flow cytometric cell sorting at day 54 using a mAb recognizing a framework epitope present on all γδ TCR. Positively sorted γδ T cells were expanded in CM containing IL-1β and IL-7 for at least

Table 1. *List of Antibodies Employed for Flow Cytometry or Blocking Experiments*

Antibody designation	Specificity	Distributor
WT-31	Framework (pan) αβ TCR	Becton Dickinson
SA6.E9^3	Framework (pan) γδ TCR	T-cell Diagnostics
δTCS-1	Vδ1/Jδ1/Jδ2 TCR segments	T-cell Diagnostics
15D	Vδ2 TCR segment	T-cell Diagnostics
7A5	Vγ2 (V-γ9) TCR segment	T-cell Diagnostics
5T-022	CD1a	CD workshop Ab
5T-024	CD1c	CD workshop Ab
S5.2	LFA-3, CD58 ligand	Becton Dickinson
SK3	CD4	Becton Dickinson
SK1	CD8	Becton Dickinson
G25.2*	CD11a (a^L/β2), LFA-1	Becton Dickinson
D12*	CD11b (a^M/β2). C3bi receptor	Becton Dickinson
SHCL-3*	CD11c (a^X/β2) C3bi,dg receptor	Becton Dickinson
BL5	CD18, β2 chain of integrins	Amac
2A3	CD25, IL-2 (TAC) receptor	Becton Dickinson
K20	CD29, β1 chain of integrins	Amac
Ber-H2	CD30, Ki-I Ag	Dako
L-178	CD44, HCAM, homing receptor	Becton Dickinson
2H4	CD45-RA	Coulter
UCHL-1	CD45-RO	Becton Dickinson
1.2B6	CD62E, ELAM-1, E-selectin	Immunotech (Marseille, France)
Dreg 56	CD62L, MEL14-Ag, L-selectin	Immunotech
L78	CD69, gp34/28	Becton Dickinson
LB-2	CD54, ICAM-1	Chemicon
133	CD80, B7.1 (CD28 ligand)	CD workshop Ab
1331, Fun-1	CD86, B7.2 (CD28 ligand)	Pharmingen
3B1.18,KP43	CD94, αβ,γδ, and NK subset	CD workshop Ab
MA-ICAM2-F	CD102, ICAM-2	Endogen, Inc. (Boston, MA)
P1	VLA-1, α1/β1, laminin$^+$ collagen receptor	Telios
P1E6	VLA-2, α2/β1, laminin$^+$ collagen receptor	Telios
P1E5	VLA-3, α3/β1, collagen, fibronectin receptor	Telios
B-5G10	VLA-4, α4/β1, VCAM-1, fibronectin receptor	Upstate Biotechnology
P1-D6	VLA-5, α5/β1, fibronectin receptor	Telios
GoH3	VLA-6, α5/β1, laminin receptor	Amac
LM142	αv (CD51), vitronectin receptor chain	Chemicon
LM609	αvβ3 (CD51/CD61) vitronectin receptor	Chemicon
P1F6	αvβ5, vitronectin receptor	Chemicon
Polyclonal antiserum, absorbed with α5β1	αvb3/β5, vitronectin receptor	GIBCO BRL
Polyclonal antiserum, absorbed with αvβ3/β5	α5β1, fibronectin receptor	GIBCO BRL
HML-1	CD103, β7 integrin chain on IEL	Amac and Immunotech
SPA804	HSP 60	Mizzen
SPA826-2, polyclonal antiserum	HSP 68	Mizzen
SPA810	HSP 70	Mizzen
SPA825	HSP 75	Mizzen
SPA811	HSP 75	Mizzen
SPA820	HSP 73	Mizzen

three additional months and those cells displayed a stable phenotype, which was documented on a weekly basis by flow cytometric analysis. Fig. 1 shows a characteristic flow cytometric analysis of sorted $\gamma\delta^+$ TIL after 2 mo of in vitro expansion. 96–98% of the T cells stained positive with an Ab recognizing all $\gamma\delta^+$ T cells, and 99% of T cells from three different TIL lines stained positive with a mAb defining the Vδ1/Jδ1/J$\delta 2^+$ subset (mAb δTCS1). TIL stained negative for reactivity with a mAb detecting the V$\delta 2^+$ T cell subset, which is the most frequent $\gamma\delta$ T cell population in PBL. The majority of the Vδ1/J$\delta 1^+$ TIL population also coordinately stained positive with an Ab recognizing the V$\gamma 2^+$ subset (or according to an alternate nomenclature, Vγ9) staining 89% of cells in TIL 1881, 76% of cells in TIL 3481, and 55% of cells in TIL 7279. The $\alpha\beta^+$ T cell population represented a minor contaminant of 0.1–2% in individual TIL cultures. To obtain additional characterization of specific Vγ usage in such V$\delta 1^+$ TIL, we evaluated Vγ usage by RT-PCR using a primer panel specific for Vδ/γ subset families. All three $\gamma\delta$ T cell lines exhibited Vδ1-specific transcripts by RT-PCR and common usage of the Vγ1.2, γ1.8, and γ2 chains of the TCR (data not shown).

In Vitro-cultured $\gamma\delta^+$ T Lymphocytes Obtained from Different Patients Express a Similar Pattern of Expression of Adhesion Molecules. Positively selected $\gamma\delta$ T cells were analyzed for expression of activation markers or adhesion molecules by flow cytometry using a panel of mAbs listed in Table 1. Characteristically, examination of cell surface expression of CD8 and CD4 in these TIL lines indicated the presence of more than one discrete $\gamma\delta^+$ T cell population showing high and intermediate level of CD8 expression, as well as a population reproducibly characterized by intermediate level of CD8 concomitant with CD4 expression (Fig. 2). These $\gamma\delta^+$ TIL lines expressed a reproducibly similar pat-

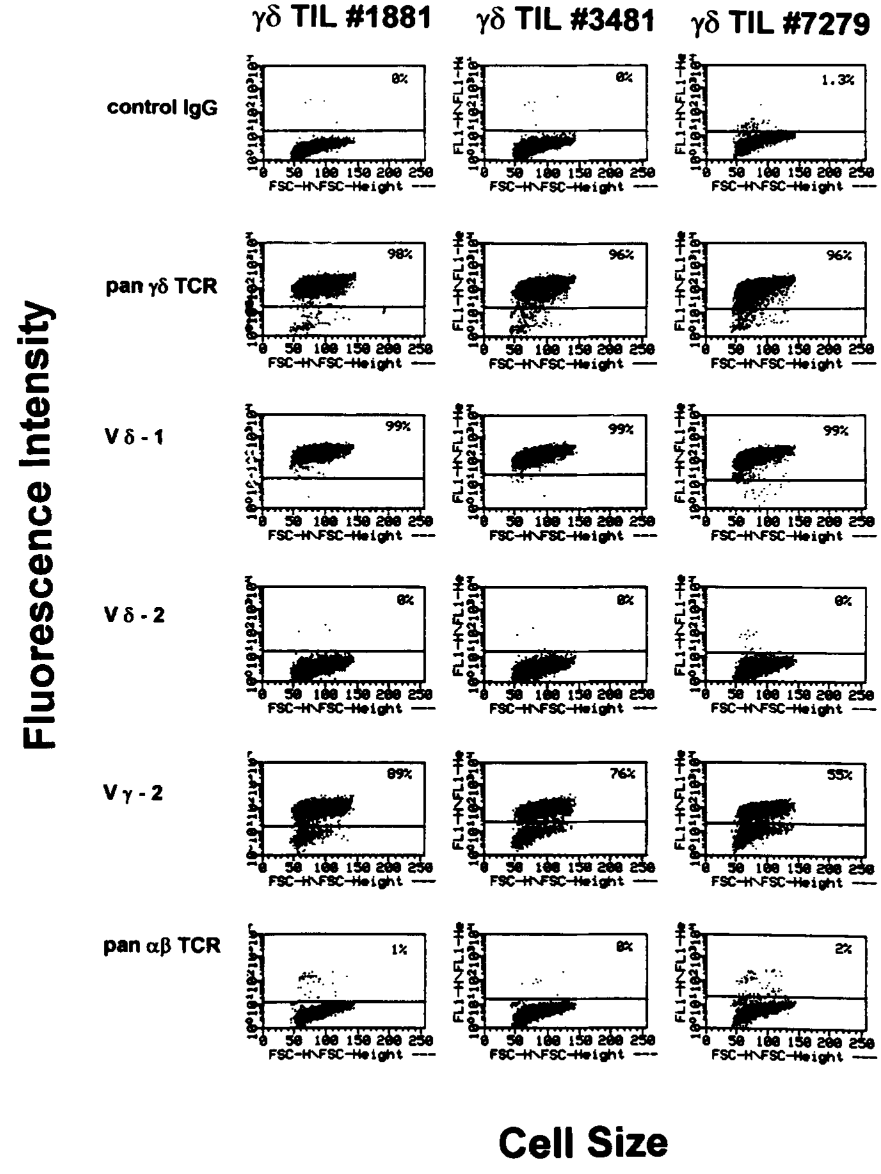

Figure 1. Colorectal cancer–derived $\gamma\delta$ TIL exclusively use the Vδ1 chain and preferentially coexpress the Vγ2 chain of the TCR. $\gamma\delta^+$ T cells (obtained from patients 1881, 3481, and 7279) were isolated by cell sorting using the anti–pan-$\gamma\delta$ mAb5A6.E9[3], and cultured for 2 wk in AIM-V medium containing IL1β/IL-7 (1,000 IU/ml) before phenotypic analysis by flow cytometry using mAbs recognizing $\alpha\beta$ or $\gamma\delta$ T cell subsets. Positively sorted TIL stained for 98% of the cell population positive with an anti–pan-$\gamma\delta$ TCR mAb or using the Vδ1/Jδ1/Jδ2–specific mAb δTCS-1. The majority of cells expressed the Vγ2 chain and stained negative for expression of the Vδ2 chain or the $\alpha\beta$ TCR.

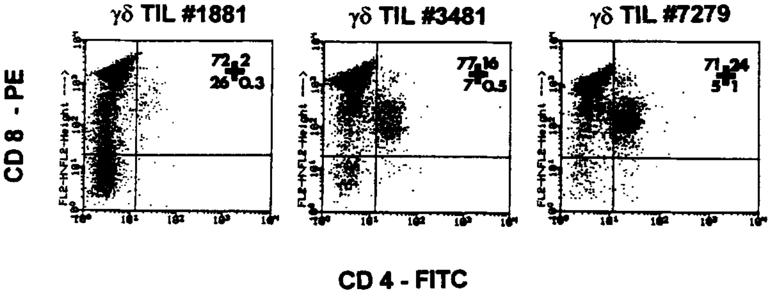

Figure 2. CD4 and CD8 expression in colorectal cancer $\gamma\delta^+$ TIL. γδ positively-sorted TIL (see Fig. 1) were stained for CD4 and CD8 expression by flow cytometry. The majority of $\gamma\delta^+$ TIL exhibited the $CD8^+CD4^-$ phenotype. $CD4^+CD8^+$ (double positive) γδ TIL were repetitively observed in γδ TIL lines 3481 and 7279.

tern of reactivity using mAb specific for activation or adhesion molecules (summarized in Table 2), exhibiting a CD11a–c^+, $CD30^+$, $CD44^+$, CD45RA high, CD45RO low, MHC class II low, IL-2 receptor (TAC) low, $CD69^+$, very late Ag (VLA) 1–6^+, vitronectin $receptor^-$, and fibronectin $receptor^+$ phenotype.

Colorectal Cancer–derived $V\delta 1^+$ TIL Exhibit a Similar Pattern of Cellular Reactivity against Colorectal, Pancreatic, and Renal Cell Cancers Defined by Cytolysis and IFN-γ Release. $\gamma\delta^+$ TIL lines were evaluated at three different times during in vitro culture for cytolytic reactivity against a panel of target cancer cell lines of diverse histological origin (compiled in Fig. 3). TIL release of IFN-γ in response to each target was also evaluated for the three γδ TIL lines. 7-d LAK cells (PBL cultured in 1,000 IU/ml IL-2) served as control effector cells and efficiently lysed each target cell line (>45% lysis at an E/T of 30:1) with the exception of the colorectal cancer cell line HT29 (showing 10% specific lysis). γδ TIL lines lysed the NK target cell line K562 poorly (in the range of 8–14% specific lysis) and failed to lyse a variety of the LAK/NK–sensitive target cell lines including Daudi, C1R, U937, Molt-4, and T2. EBV-transformed B cell targets, as well as mucin-expressing EBV^+ B cell lines (C1R-Muc1, S-EBV-Muc1) were not lysed by γδ TIL. Examination of IFN-γ release by individual γδ TIL lines in response to these target cells revealed no significant increase in IFN-γ secretion compared with the constitutive release of IFN-γ in the range of 37–40 pg/ml/16 h (Fig. 3).

Of interest, γδ TIL lines 1881, 3481, and 7279 lysed the colorectal cancer cell line 3481 (obtained from patient 3481) in addition to four different allogeneic colorectal cancer cell lines, with the exception of HT29. Examination of TIL release of IFN-γ in response to autologous (i.e., in the case of the matched γδ TIL line 3481 and tumor line 3481) and allogeneic colorectal cancer cell lines revealed that all three γδ TIL lines secreted IFN-γ in response to autologous or allogeneic tumor cell lines (≤273 pg/ml/16 h), even in the absence of a significant cytolytic response (i.e., in the case of the target cell line HT29). These γδ TIL lines did not lyse or secrete significant levels of IFN-γ, in response to diverse target cell lines, i.e., melanoma, one breast cancer, lung cancer, or autologous (obtained from patient 3481) or allogeneic fibroblasts. In contrast, γδ TIL lines 1881, 3481, and 7279 efficiently lysed two pancreatic cancer cell lines (pancreas89 and pancreasQGP-1), two renal cell cancer cell lines (RC1257 and RCC1973), the normal human intestine explant culture line (passage 1, CRL7136; ATCC), and to a lesser extent, the Wilms tumor cell line SKNEP-1. However, these γδ TIL did not lyse two additional squamous cell cancer cell lines (PCI-13 and PCI-50, data not shown). Target-specific release of IFN-γ release by $V\delta 1^+$ T cells in response to these targets was not significant. Of note, despite some minimal cytotoxic γδ TIL responses against K562, we could not detect significantly elevated IFN-γ secretion by γδ TIL stimulated by K562 (in the range of 40 pg/ml/16 h compared with ≤260 pg/ml IFN-γ release in response to colorectal cancer stimulator cells). γδ TIL did not proliferate specifically in response to in vitro stimulation with colorectal cancer cells or tumors of alternate histologies (data not shown).

The γδ TCR, the β2 and β7 Integrin Chains and the Fibronectin Receptor Are Involved in $\gamma\delta^+$ T Cell–mediated Cytolysis of Tumor Targets. To evaluate the functional impact of cell surface molecules on cytolytic γδ T cell (colorectal cancer cell line 3481) recognition of target cells, we incubated each γδ TIL line with Abs directed against the (pan-γδ) TCR, $V\delta 1^+$ TCR, $V\delta 2^+$ TCR, the HFR, the HVR, or the β2 or β7 integrin chains before addition of ^{51}Cr-labeled target cells in 4-h cytotoxicity assays. The cytolytic T cell recognition of colorectal cancer cell line 3481 was inhibited by ~30% by the anti–pan-γδ TCR or the Vδ1/Jδ1/Jδ2–specific mAbs (Fig. 4). TIL 1881 and 3481 (cytotoxic) recognition of tumor 3481 was similarly inhibited by 35%. Addition of mAb directed against Vδ2 did not affect T cell–mediated lysis. All three γδ TIL lines showed a diminished cytotoxic T cell response if preincubated with antiserum specific for the fibronectin receptor (≤59% inhibition of specific lysis) but not with antiserum specific for the vitronectin receptor. γδ T cell–mediated lysis was inhibited by 80% by an Ab directed against the β2 integrin chain, and by ≤50% by a mAb directed against the β7 integrin chain, a component of the intraepithelial lymphocyte (IEL) marker. This was, however, not a uniform trend since the γδ TIL line 3481 was only inhibited by ~10% by an Ab directed against CD103 (i.e., β7 chain).

Colorectal Cancer–derived $V\delta 1^+$ T Lymphocytes Recognize Cell Surface Ags Shared by Colorectal, Pancreatic, and Renal Cell Cancer Cell Lines. $\gamma\delta^+$ TIL lines 1881, 3481, and 7279 appear to recognize both autologous and allogeneic col-

Table 2. *Phenotypic Analysis of $\gamma\delta^+$ TIL: Activation Markers and Adhesion Molecules*

Percent positive-staining cells for:	γδ TIL 1881	γδ TIL 3481	γδ TIL 7279
		%	
CD2	98	97	98
CD3	99	99	99
CD11a	99	99	99
CD11b	97	97	98
CD11c	84	80	74
CD25 (TAC)	2	2	2
CD30	92	82	77
CD44	90	86	96
CD45RA	91	94	69
CD45RO	14	43	36
HLA-DR	15	6	6
CD69	95	96	95
CD94	0	23	7
ICAM-1	95	90	95
ICAM-2	99	95	96
CD80	4	8	4
CD86	24	34	36
VLA-1	96	95	97
VLA-2	95	93	93
VLA-3	31	19	14
VLA-4	70	98	98
VLA-5	99	93	93
VLA-6	43	60	65
E-Selectin	0	0	0
L-Selectin	0	0	0
β1-integrin chain	99	99	99
β2-integrin chain	99	99	99
β3-integrin chain	2	2	1
β7-integrin chain	95	92	99
αv	10	8	2
αvβ3	2	2	1
αvβ3/β5 (HVR)	1	1	1
αvβ5	2	2	1
α5β1 (HFR)	98	95	99

Positive sorted $\gamma\delta^+$ T cells, analyzed by flow cytometry for expression of activation markers, exhibited a similar phenotype in three different experiments at various time points. Viable lymphocytes were gated based on size (FSC) and granularity (SSC) criteria, and data were collected for 5,000 cells as determined by forward light scatter intensity using Lysis II software on a FACScan®. Results are reported as percent of cells staining positive for a given marker.

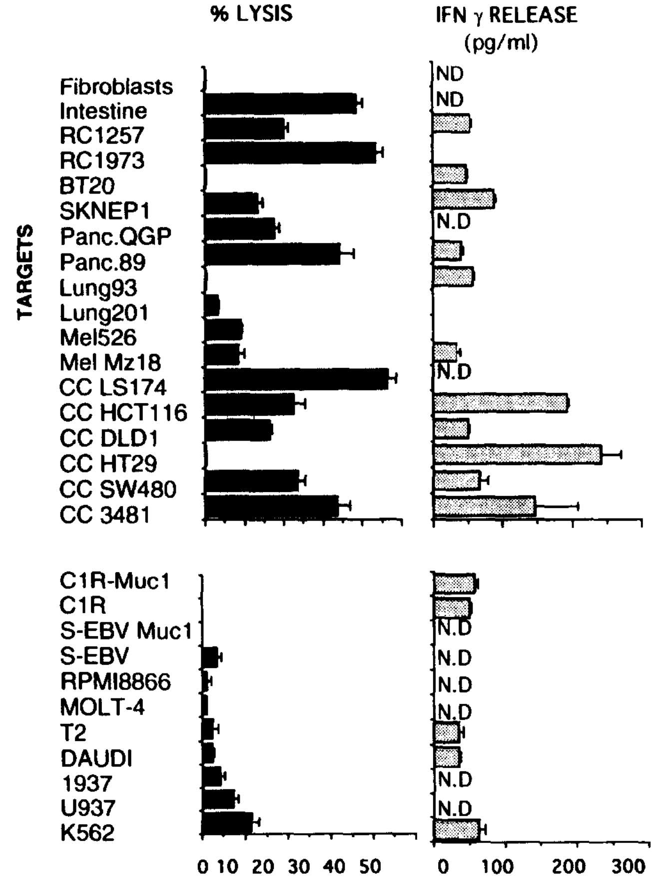

Figure 3. Similar target cell recognition pattern of $V\delta1^+$ TIL as measured by cytotoxicity and IFN-γ release. Colorectal cancer target cell lines and cell lines of alternate histology were tested for γδ T cell recognition using a standard 4-h ^{51}Cr-release assay or using a 16-h IFN-γ release assay as described in Materials and Methods. Results obtained from three individual γδ TIL lines (1881, 3481, and 7279) are expressed as the mean ± SD of percent specific lysis or IFN-γ release in response to individual targets. These γδ TIL lines preferentially lyse colorectal cancer (CC) cell lines, the human intestinal cell explant CRL7186, pancreatic (*Panc.*), and renal cell cancer (*RC*) cell lines. Tumors of alternate origin were not lysed significantly, including two head and neck squamous cell cancer cell lines, allogeneic fibroblasts (data not shown), or autologous fibroblasts (obtained from patient 3481). IFN-γ release by γδ TIL is reported as pg/ml/10^6 cells/16 h. E/T = 5:1.

orectal cancer cell lines, pancreatic cancer, and renal cell cancer (Fig. 3). However, these γδ T cell effector cells also recognize (to comparably lesser extent) the erythroleukemia cell line K562 and early passage cells derived from "normal intestine" (explant culture CRL7136, HS186.INT; ATCC). We performed cold target inhibition assays using the ^{51}Cr-labeled allogeneic colorectal cancer cell line SW480 and a panel of nonlabeled target cell lines as competitive inhibitors in order to evaluate the commonality of ligands expressed on these target cells. Cold targets were admixed with ^{51}Cr-labeled SW480 targets at 30:1, 10:1, and 3:1 (cold/hot target ratios) as indicated (Fig. 5) with two γδ TIL lines (1881 and 7279) evaluated as effector cells. Both γδ TIL lines efficiently lysed the target cell line SW480 in the absence of cold target inhibitors. Recognition of SW480 targets by TIL effector cells was effectively inhibited by cold targets including the colorectal cancer cell lines SW480 and DLD-1, and pancreatic cancer (pancreas89), renal cell cancer (RC1257),

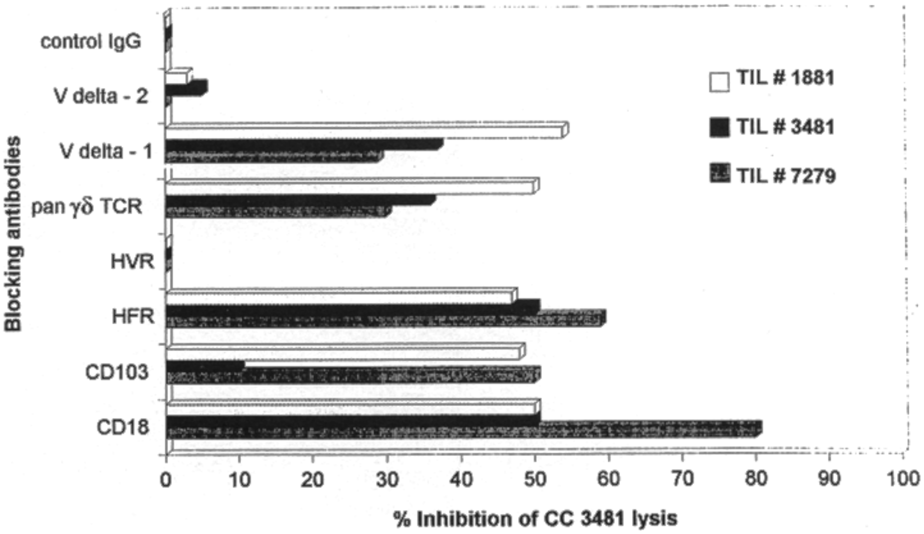

Figure 4. γδ TIL cell recognition can be blocked by Abs directed against the β2 or β7 integrin chains, or with Abs directed against the fibronectin receptor. γδ TIL lines were incubated at 4°C for 30 min with Abs against the TCR (anti–*pan γδ*, δTCS1-*Vδ1* and *Vδ2*), anti-β2 (*CD18*), or -β7 (*CD103*) integrin chains, or with Abs directed against the human vitronectin $\alpha v\beta 3$(*HVR*) or fibronectin $\alpha_5\beta 1$ (*HFR*) receptor, or in medium alone. After washing these $\gamma\delta^+$ effector T cells in AIM-V, ^{51}Cr-labeled colorectal cancer cells (CC *3481*, autologous to γδ *TIL 3481*) were added to effector cells and a standard 4-h ^{51}Cr-release assay was performed. Evaluation of T cell reactivity without blocking Abs (i.e., incubation in medium alone) revealed that γδ TIL line 1881 lysed the CC 3481 line to a level of 35%, 3481 γδ TIL to a level of 30%, and γδ 7279 exhibited 43% specific lysis of CC 3481 at an E/T ratio of 10:1. Results are reported as percent inhibition of specific lysis of control CC 3481 (no Ab) by γδ TIL. Preincubation of γδ TIL with relevant anti-TCR Abs reduced CC 3481–specific recognition in the range of 30-35%. Killing of CC 3481 by γδ TIL could also be inhibited using Abs directed against the β2 and the β7 integrin chains, and with Abs directed against the fibronectin receptor (*HFR*). In contrast, Abs against the vitronectin receptor (*HVR*), or nonrelevant control IgG, did not efficiently block γδ T cell–mediated lysis. Incubation of target cells with these mAbs or with mAbs directed against MHC class I or II molecules did not result in significant inhibition of target cell cytolysis (data not shown).

and the erythroleukemia K562 cell lines. In contrast, cytolytic γδ T cell recognition of ^{51}Cr-labeled SW480 cells was only marginally inhibited by cold targets such as the Burkitt lymphoma cell line Daudi, or cells derived from normal intestine (CRL7136, HS186.INT; ATCC).

TCR Vδ1 Transcripts in γδ TIL Lines 1881, 3481, and 7279. To assess potential preferential usage of Vδ1 transcripts by individual γδ T cell lines (which apparently recognize shared tumor Ags), we extracted mRNA from each TIL line, reverse transcribed it into cDNA, and used Vδ1-specific PCR primers (spanning the Vδ1 segment to the constant region of the TCR) to amplify Vδ1-specific products; we subcloned the PCR product and sequenced 8–10 individual clones from each TIL line (Fig. 6). Each subcloned PCR product showed the TCR Vδ1 region paired exclusively with Jδ1. 72% (20/28) of TCR transcripts were in frame and showed high diversity in the CDR3 region. TCR usage of the Dδ3 segment (20/20) compared with

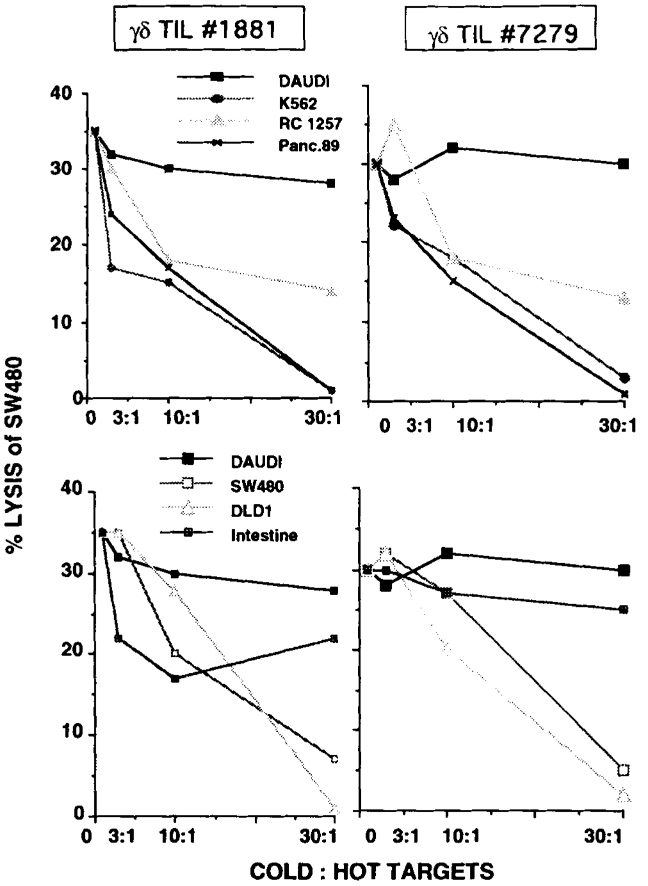

Figure 5. $V\delta 1^+$ TIL lines recognize common target antigens expressed by tumor cells of epithelial origin. ^{51}Cr labeled SW480 cells (*HOT TARGETS*) were seeded in 96-well plates and (non–^{51}Cr-labeled, *COLD TARGETS*) single cell suspension of the cell lines K562, Daudi, the renal cell cancer cell line RC1257, the pancreatic cancer cell line panc89, the colorectal cancer cell lines SW480 and DLD-1, or the human intestinal cell explant culture CRL7186 were added concomitantly at various concentrations resulting in 3:1, 10:1, and 30:1 cold/hot target cell ratios. γδ TIL lines 1881 or 7279 were then added to individual assay wells. The effector/(hot) target ratio was 10:1 and a 4-h ^{51}Cr-release assay was performed. γδ TIL 1881 exhibited 35%, and γδ TIL 7279 30% specific lysis of CC 3481 target cells in the absence of cold targets. γδ TIL recognition of (hot) SW480 cells could be inhibited by cold CC SW480, CC DLD1 targets, by the renal cell cancer line RC1257, the pancreatic cancer cell line panc89, and by K562. In contrast, unlabeled Daudi cell targets did not interfere significantly with CC SW480 target cell recognition by γδ TIL lines.

CLONE	IN FRAME	V delta 1	N/P	D1	N/P	D2	N/P	D3	N/P	J delta 1
germ line	-	ctcttggggaact		gaaatagt		ccttcctac		actgggggatacg		acaccgataaactc
1881-1	+	ctcttggg	aaaaactta	aaat		cctac	gg	ggggg	ctccgt	ccgataaactc
1881-3	+	ctcttggggaa	ttgtacgtggatgag					ctgggggatac	a	gataaactc
1881-5	+	ctcttggggaa	cacaggg			c ttcctac	agataaa	ggggg	tgggcgatactgtaaggggcgtcaggt	cgataaactc
1881-7	+	ctcttggggaact	agacaagcccccggctgggttt					actgg	tac	taaactc
1881-8	+	ctcttggggaact	agacggggtaagg			tac	gtca	gggga	caat	ataaactc
1881-10	+	ctcttg	tcc	aaata	tcttca	ccttcc	aagtctcaccggcgcagcccacgaaccct	gggggа	gggaaccac	cgataaactc
1881-11	+	ctcttgggg					g	ctgggggatacg	tt	acaccgataaactc
3481-2	+	ctcttggg	atcctcttctcacg					ctgggg	agcgt	acaccgataaactc
3481-4	+	ctcttgggga				ccttc	tacgtg	ctgggggata	tggtggct	acaccgataaactc
3481-7	+	ctcttgggga	gctattcc					actgggggа	ccaaccggtgg	accgataaactc
3481-AB	+	ctcttgggga	taatccagccttccatgtgg					tgggg	ccaagatc	actc
3481-AE	+	ctcttggggaact	ttttcgatccc					ggg	tgc	ccgataaactc
7279-2	+	ctcttgggga	ct			tcctac	gttc	tggggg	aagaggg	aaactc
7279-3	+	ctcttggggaac	cgtggagag			cta	gg	tggggggat	cctaac	caccgataaactc
7279-4	+	ctcttggg	aacccc			ccttcctac	ccttac	tgggg	tt	caccgataaactc
7279-5	+	ctcttggtgaac	gccaggcgacgaggttga					ggggat	acgcag	acaccgataaactc
7279-10	+	ctcttggggaac	acagg			ccttcctac	aac	tggggg	ctcaagt	acaccgataaactc
7279-11	+	ctcttgggg	ggttccggt					actgggggata	gctggtgc	taaactc
7279-AA	+	ctcttggg	ccgacagacttacg					tggggg	gccgcttt	ataaactc
7279-AC	+	ctcttggggaac	cccctgaacccc					gggggata	accccctggtgt	cgataaactc

Figure 6. Junctional TCR sequences from IL-1β/IL-7–expanded antitumor-reactive $\gamma\delta^+$ TIL. Vδ1-specific transcripts were generated by RT-PCR as described in Materials and Methods using primers specific for the Vδ1 familiy and the constant region of the δ TCR followed by subcloning of individual Vδ1 transcripts. Numbers indicate the γδ TIL lines (γδ TIL *1881*, *3481*, and *7279*) and the respective designation of individual cDNA clones. Note the preferential use of Jδ1 and Dδ3 with Vδ1 and extensive rearrangement of the TCR junctional regions. These sequence data are available from Genbank/EMBL/DDBJ under accession numbers U30189-U30208.

the Dδ2 segment (9/20); 2/20 cDNA clones used the Dδ1 segment. Nucleotide sequences assigned to the Dδ1 or the Dδ2 regions were typically shorter, compared with segments used by Dδ3. N/P region nucleotides were remarkably long ($\leq$29 bp in cDNA clone 1881-10). Fig. 7 depicts the predicted amino acid sequences encoded by each individual cDNA clone. We noted that in 6/20 TCR sequences (3′ to the Dδ3 segment, NP region), a hydrophobic aromatic tyrosine (Y), and in (different) 8/20 TCR sequences, a hydrophilic, aliphatic leucine (L) residue were encoded. The amino acid residue was in some cDNA clones generated by basepairs provided by the V region and the N/P region, and in other cDNA clones from basepairs in the N/P region alone, 3′ to the Vδ1 chain of the TCR.

TCR Repertoire in Colorectal Cancer. The Vδ1$^+$ T cell population has been previously shown to represent the predominant T cell subset observed in normal human intestine (5–8). Since we did not have normal mucosa or PBL available from patients 1881, 3481, and 7279, we could not directly address whether the mRNA transcripts detected in γδ TIL were also present in normal colonic mucosa adjacent to colon cancer or in PBL from these patients. The three γδ TIL lines showed a preferential usage of the Vγ2, Vγ1.2, and Vγ1.8 TCR segments (as determined by RT-PCR), which were also exclusively identified in the freshly collected tumor samples obtained from these patients, thereby exluding potential in vitro artefacts associated with preferential T cell subset expansion (data not shown). To test for TCR Vδ/Vγ usage in matched (primary) colon adenocarcinoma samples and in tumor-free (distal or proximal to the tumor) adjacent mucosa, we extracted RNA from freshly collected tumor/normal mucosa samples obtained from seven additional patients with primary colorectal cancer. mRNA was reverse transcribed into cDNA and tested for TCR γ or δ transcripts by RT-PCR as described in Materials and Methods. Specific PCR products were validated by Southern blot analysis using internal primers for individual TCR γ or δ transcripts (data not shown). 5/7 adenocarcinoma samples exclusively expressed Vδ1 transcripts, whereas 2/7 adenocarcinoma samples showed additional Vδ2 transcripts, which were also found in 7/7 tumor proximal/distal normal mucosa samples (Table 3). 2/7 adenocarcinoma samples exclusively expressed the TCR γ1.2 and γ2 transcripts, and 2/7 cancer samples expressed additionally Vγ1.8 transcripts (identical to the TCR γ usage pattern in γδ TIL lines described above). 3/7 cancer specimens expressed the same Vγ mRNA expression pattern and additionally expressed Vγ3 mRNA. All seven normal (matched) mucosa specimens showed typical Vδ1 and Vδ2 mRNA transcripts and predominantly Vγ1.2, γ1.8, γ2, γ3, and, in two specimens, Vγ1.4 mRNA, expression. In contrast, the matched adjacent tumor tissue revealed exclusive expression of Vδ1 and Vγ1.2, Vγ1.8, and Vγ2 mRNA (Table 3). Restricted Vδ1/Vγ1.2, Vγ1.8, Vγ2 mRNA expression patterns were also obtained upon examination of 25 primary colorectal cancer specimens and 15 colon adenocarcinomas metastatic to liver (our unpublished observations).

CLONE	V delta 1	N/P; D1; N/P; D2; N/P; D3; N/P	J delta 1
1881-1	L G	K N L K S Y G G A P S	D K L
1881-3	L G E	**L** Y V D E L G D T	D K L
1881-5	L G E	H R A F L Q I K G V G D T V K G R Q V	D K L
1881-7	L G E	**L** D K P P A G F T G T	K L
1881-8	L G E	**L** D G V R Y V R G Q **Y**	K L
1881-10	L	V Q I S S P S K S H R R S P R T L G E G T T	D K L
1881-11	L G	G W G I R **Y**	T D K L
3481-2	L G	I **L** F S R W G A **Y**	T D K L
3481-4	L G	D **L** L R A G G Y G G **Y**	T D K L
3481-7	L G E	**L** F H W G T N R W	T D K L
3481-AB	L G	D N P A F H V V G P R S	L
3481-AE	L G E	**L** F R S R V P	D K L
7279-2	L G	D S L R S G G R G	K L
7279-3	L G E	P W R A R W G I L T	T D K L
7279-4	L G	N P P F L P L L G F	T D K L
7279-5	L G E	R Q A T R L R G Y A D	T D K L
7279-10	L G E	H R P S Y N W G L K **Y**	T D K L
7279-11	L G	G F R Y W G I A G A	K L
7279-AA	L G	P T D **L** R G G P L **Y**	K L
7279-AC	L G E	P P E P R G I T P G V	D K L

Figure 7. Predicted junctional amino acid sequences encoded by Vδ1 transcripts from antitumor-reactive γδ$^+$TIL. Individual cDNA clone designations correspond to those in Fig. 6. Amino acid sequences are reported using the single letter code. Note that $^{8}/_{20}$ cDNA clones exhibit the amino acid L in the N/P region 3′ to the Vδ1 region and 6/20 cDNA clones exhibit the amino acid Y generated by the N/P region 3′ to the Dδ3 segment. Sequence data are available from Genbank/EMBL/DDBJ under accession numbers U30189–U30208.

Discussion

We have succcessfully expanded three individual colorectal cancer–derived $\gamma\delta^+$ T cell lines by in vitro culture using IL-1β and IL-7. This strategy was based on previous observations (39) that a combination of IL-1β and IL-7 promotes the preferential outgrowth of peritoneal γδ T cells from *Listeria monocytogenes*–infected mice. IL-1β may act directly on $\gamma\delta^+$ TCR-responsive T cells (39, 40) or IL-1β–mediated effects may be attributed to initial IL-1β–induced activation of dendritic cells, which have recently been shown to be involved in γδ T cell activation and expansion (41). The IL-1β/IL-7–expanded TIL populations exhibited stable phenotype and functional characteristics for 4 mo in culture. This phenotypic stability may reflect an inherent characteristic of the γδ T cell subset examined, or may be due to IL-7–mediated effects that have been reported to sustain Ag-specific (CD4$^+$ αβ T cells) in the absence of antigenic restimulation for ≤22 mo (42). Cytokine-mediated effects on cell surface expression of adhesion molecules has been well documented for human NK cells that exhibit differential expression of CD11a–c, and VLA1–6 cell upon IL-2 stimulation (43). Alternatively, the similar pattern of adhesion molecule expression in γδ T cell lines derived from three individual patients may reflect a tissue-specific (i.e., intestinal) phenomenon. For example, γδ T cells isolated from intestinal lymph nodes and Peyer's patches appear to express varied levels of CD44 or B7 molecules that impact their tissue-biased homing pattern (44). Synovial fluid Vδ1$^+$ T cells from patients with juvenile rheumatoid arthritis express predominantly the early activation marker CD69 and the high molecular isoform of the CD45 (CD45RA) (45), a pattern that we similarly observed for IL-7–expanded γδ T cells in this report.

The bulk γδ TIL population obtained from three individual patients showed exclusive expression of the Vδ1 TCR and preferential coexpression of the Vγ2 TCR. Additional TCR γ transcripts, including those for the Vγ1.2 and the Vγ1.8 chains, were detected by RT-PCR. Of note, a similar restricted pattern of such Vγ/Vδ TCR transcripts was identified in the majority of colon adenocarcinomas as compared with the adjacent tumor-free mucosa (showing additional Vδ2, and Vγ3, and Vγ1.4 transcripts), indicating that this particular γδ T cell population may be preferentially enriched at the tumor site.

$\gamma\delta^+$ T cells appear to represent effector cells capable of specifically recognizing cancer cells (33–35, 46). All previous reports of tumor-reactive $\gamma\delta^+$ T lymphocytes support the exclusive expression of the Vδ1$^+$ TCR. Vδ1$^+$ T cells specifically recognize pancreatic cancer in a MHC-unrestricted fashion and proliferate in response to pancreatic cancer cells in vitro in sufficient numbers that have prompted some authors to suggested the clinical implementation of this T cell subset in the adoptive immunotherapy of pancreatic cancer. Additional reports have demonstrated (non-MHC–restricted and γδ TCR–mediated, tumor-reactive) cytotoxic γδ T cell responses in TIL derived from lung cancer patients. This T cell subset also exclusively used the Vδ1$^+$

Table 3. *Vγ/Vδ TCR mRNA Transcripts Differ in Colorectal Cancer Lesions Compared with Adjacent Tumor-free Mucosa and Exhibit a Preferential Usage of Vγ 1.2, 1.8, 2, and Vδ1$^+$ TCR*

		TCR Vγ regions						TCR Vδ regions				
Sample no.	Histological diagnosis	1.2	1.4	1.8	2	3	4	1	2	3	4	5
22	Normal mucosa	1.2	1.4	1.8	2	3		1	2			
21	Adenocarcinoma colon	1.2		1.8	2			1				
84	Normal mucosa	1.2		1.8	2	3		1	2			
82	Adenocarcinoma colon			1.8	2			1				
207	Normal mucosa		1.4	1.8	2	3		1	2			
206	Adenocarcinoma colon			1.8	2			1				
213	Normal mucosa	1.2		1.8	2	3		1	2			
212	Adenocarcinoma colon	1.2		1.8	2	3		1	2			
256	Normal mucosa	1.2		1.8	2	3		1	2			
255	Adenocarcinoma colon	1.2		1.8	2			1				
271	Normal mucosa	1.2		1.8	2	3		1				
270	Adenocarcinoma colon	1.2		1.8	2			1				
285	Normal mucosa	1.2		1.8	2	3		1	2			
284	Adenocarcinoma colon			1.8	2			1				

mRNA was extracted from freshly resected (primary) colorectal cancer specimens and from tumor-free adjacent mucosa harvested 3 cm proximal or distal to the tumor lesions. RNA was reverse transcribed into cDNA and tested for Vγ/Vδ TCR–specific mRNA transcripts in individual samples by RT-PCR using a primer panel as described in Materials and Methods. Identity of PCR products was confirmed by using Vγ- or Vδ-specific V-region TCR internal oligonucleotides by Southern blot analysis (data not shown).

TCR (33, 34), a finding that has been corroborated for γδ T cell clones obtained from a patient with renal cell cancer (35). In this latter study, the majority of Vδ1$^+$ T cell clones preferentially recognized the autologous renal cell cancer, but additional Vδ1$^+$ T cell clones from this patient also lysed allogeneic renal cell cancer lines (35). Vδ1$^+$ T cells have also been identified in a variety of human diseases that are thought to have an "autoimmune" etiology (26–31), and have also been identified in increased numbers in Wilms tumors (47) and in the liver of tumor-bearing hosts (48). The Vδ1$^+$ TIL lines generated in our study lyse colorectal, renal cell, and pancreatic cancer cell lines, an early passage line of human normal intestine (CRL7136, HS186.INT; ATCC), and to a lesser extent the erythroleukemia cell line K562. Our results confirm previous observations that Vδ1$^+$/Vγ2 (or γ9)$^+$ T cells typically do not lyse Daudi targets. Whereas such (Vδ1$^+$Vγ2$^+$) T cells may recognize an Ag expressed on Burkitt's lymphoma cell lines (49), this specificity does not appear to be relevant in our study. Based on cold target inhibition assays, our γδ TIL lines appear to exhibit similar specificities and to recognize common target structures expressed by epithelial target cell lines and freshly explanted/cultured intestinal tissue (Fig. 3). However, γδ TIL–mediated lysis of ^{51}Cr-labeled colorectal cancer cells could not be efficiently inhibited by excess unlabeled normal intestinal cells (Fig. 5) suggesting that different, distinct target structures expressed by colon cancer and intestinal epithelium may be recognized by γδ T cells. The observation that a single cell explant culture from normal human intestine is also recognized by such γδ T cells may alternatively suggest that the potential target structures for γδ T cell recognition may represent normal "self" (i.e., not associated with malignant transformation) Ags with differential TIL reactivity due to differential Ag expression levels on tumors versus normal mucosa. In any case, it is not yet clear, based on the available data, whether potential autoreactive γδ$^+$ TIL are functional in vivo (50, 51), or whether such effects are due to the in vitro expansion of effector immune cells in the presence of IL-1β and IL-7. Common (αβ$^+$ TCR CTL–defined) Ags shared by tumors of different histologies have recently been reported in murine (52) and in human (53) tumors. One may speculate that such γδ CTL–defined crossreactivity may reflect similar cellular target stuctures associated with cells of epithelial origin. Of note, antigenically defined (αβ$^+$) T cell reactivity observed in human cancer (melanoma [54] and renal cell cancer [53]) supports the concept that antitumor cellular immunity, in fact, represents an autoimmune response, a finding that may be equally applicable to antitumor-reactive γδ T cells.

CD1 has been reported to be expressed by intestinal cells (14, 55) and may serve as a potential target structure for certain γδ T cells. In mice, the nonclassical Tla Ag, also expressed in the intestine (15), may be recognized by CD8$^+$ intestinal Vγ5$^+$ T cells (15, 16). mAbs directed against MHC class I (w6/32) or II DR (mAb L243), or mAbs recognizing CD1a or CD1c were not able to block γδ T cell–mediated cytotoxicity of target cell lines (data not shown). Whereas this suggests that γδ$^+$ T cell recognition of autologous or allogeneic cancer cell lines is not restricted by MHC class I or II, the inability of anti-CD1 mAbs to block γδ T cell reactivity may not be informative because of inefficient blocking of relevant determinants. Examination of target cell surface expression of CD1 Ags revealed that only the cell line Molt-4, not recognized by Vδ1$^+$ T cells, stained positive for CD1 (data not shown).

Murine Vγ3$^+$ T cells (dendritic epidermal T cells) specifically recognize keratinocytes (56). It is possible that human Vδ1$^+$ T cells recognize cells of epithelial origin in a tissue-specific fashion and react against target structures induced by stress (i.e., in vitro culture) and/or by malignant transformation. Potential candidates for target Ags may represent HSPs (13, 19, 20, 57). Anti-breast–cancer directed γδ T cell responses are augmented by transfecting breast tumor cells with the 27-kD HSP protein (58), suggesting a role for HSP expression in γδ T cell recognition of solid tumors. A strong correlation between cell surface expression of HSP 72 and the 67-kD high affinity laminin receptor in human lung cancer and the outgrowth of antitumor–directed cytotoxic γδ T cells (59) has also been noted. grp 75 has also been suggested to present tumor-specific Ags (presumably in the form of peptides) to γδ T cells (57). However, we were unable to inhibit T cell reactivity with a panel of anti-HSP Abs, a finding that either supports the lack of involvement of the specific Ag in γδ T cell–mediated reactivity or reflects the inability of a given mAb to block a functional epitope present on the indicated HSP (data not shown). To correlate HSP expression on individual target cells with cytolytic γδ$^+$ T cell reactivity or specific IFN-γ release, we examined target cell surface expresssion of several well-characterized HSP subsets (including HSP 60, 68, 70, 72, 73, and 75; see Table 1) by flow cytometry. In summary, all target cell lines tested negative for HSP 60, 68, 70, and 75 expression and showed variable cell surface staining using mAbs directed against HSP 72 or 73 comparably high expression of HSP 72 or 73 (our unpublished observations). Target cell lines exhibiting comparably high expression of HSP 72 or 73 (e.g., Daudi, or I 937) were not recognized by γδ T cells (Fig. 3). In contrast, the human explant culture CRL7136, HS185.INT from normal human intestine lacked HSP 72/73 cell surface expression, but provided target structures recognized by γδ T cells. Based on these data, we could not identify a positive correlation of HSP 72 and 73 cell surface expression and γδ T cell recognition. However, the cell surface density of individual HSP subsets relevant for γδ T cell recognition, as well as potential peptides presented by such alternate Ag-presenting molecules, are poorly defined. A more detailed examination of individual intracellular and cell surface family HSP protein expression in targets recognized by human intestinal γδ T cells may aid clarification of the role of HSPs as candidate target structures.

Of note, four mAbs were able to partially block γδ T cell recognition of sensitive target cells: Abs directed against the TCR, against the β2 or β7 integrin chains, or an Ab against the HFR receptor. Cellular interactions with fibronectin have been shown to induce phosphorylation of a 120-kD protein on T cells and HFR-mediated "costimulation" may augment T cell cytotoxicity induced by TCR stimulation (60). Fibronectin-associated T cell stimulation may therefore facilitate TCR restricted effector cell functions (e.g., granzyme release), or it may lower the signal threshold required for optimal T cell stimulation (60, 61). Blockade of the fibronectin receptor on γδ T cells may therefore interfere with γδ T cell stimulation. Fibronectin also appears to be involved in α4/β7–mediated adhesion of T cells to the vascular cell adhesion molecule 1 (VCAM-1), or to mucosal addressin cell adhesion molecule 1 (MADCAM-1) (62). Interaction of β7 with fibronectin, MADCAM-1, and VCAM-1 involves different, functionally distinct determinants on the β7 integrin molecule (62). Inhibition of cytotoxic γδ T cell responses with an Ab directed against the β7 integrin chain may reflect the importance of β7 integrin ligands (i.e., MADCAM, fibronectin, etc.) expressed by tumor cells of epithelial origin.

It should be noted that differences in γδ T cell responses to individual targets as measured by cytotoxicity and IFN-γ release may be attributed to the presence of more than one discrete effector T cell population as defined by differential cell surface expression of CD8 and CD4 (Fig. 2). CD8 appears to be preferentially expressed on Vδ1$^+$ T cells, predominantly in the form of a CD8 α chain homodimer, which has been shown to be inducible by various cytokines (e.g., IL-4; 63–65). The effects of IL-1β or IL-7 cell surface expression on CD8 chains has not been evaluated to this end and awaits further clarification. CD8 acts as an adhesion molecule for αβ TCR$^+$ T cells and as a signal-transducing molecule. The function of CD8 on intestinal γδ T cells remains elusive, since: (*a*) CD8 expression appears to be rapidly induced after tissue homing, suggesting that CD8 expression may not be correlated with conventional Ag-driven activation occurring in MHC class I–restricted αβ$^+$ T cells (66); and (*b*) target cell recognition by Vδ1$^+$ T cell clones appears not to be affected by CD8 blockade (67). Additionally, direct cloning of freshly isolated CD4$^+$ TCR γδ$^+$ T cells indicated that Vδ1$^+$ T cells may also express CD4 in vivo. It is interesting to note that such CD4$^+$ γδ T cells have been characterized to lack cytolytic functions, but to secrete high levels of cytokines (65). These data may suggest that variations in γδ T cells responses (e.g., different levels of inhibition of target cell lysis using mAbs directed against the γδ TCR; Fig. 4) may reflect the presence of such distinct γδ T cell subpopulations defined by CD8 and CD4 expression, correlating with different (i.e., cytotoxicity and cytokine release) T cell effector functions.

Partial inhibition of target cell recognition by Abs directed against the γδ TCR, indicates that cytolytic Vδ1$^+$ T cell responses are mediated by specific target cell recognition involving the TCR. Potential ligands for γδ TCR recognition may represent native target cell surface structures (11, 12, 17, 19, 20, 21, 68), consolidating the notion that the γδ T cell population may use a fundamentally different method of target cell recognition as compared with that of the MHC-restricted αβ T cell population. Significant IFN-γ release by three γδ TIL lines derived from colon cancer patients in response to autologous or allogeneic colon cancer cell lines suggests that these γδ T cells may be therapeutically important. γδ T cells may therefore not only be useful as effector T cell subsets capable of directly eradicating cancer cells when adoptively transferred into cancer patients, but they also may be useful in creating a "Th1-like" cytokine milieu within the tumor lesion microenvironment. IFN-γ may act directly on tumor cells e.g., by, for example, increasing cellularly expressed MHC products, which serve as restricting molecules for MHC-presented peptides capable of being recognized by αβ$^+$ T cells. IFN-γ release in response to cancer may be particularly important in the case where tumor cells display resistance to perforin-mediated lysis (e.g., HT29; Fig. 3), which may reflect tumor cell overexpression of CD59, a molecule capable of preventing perforin complex formation into lytic structures on the target cell surface (69). The intimate interaction of γδ and αβ T cells has previously been documented and it is envisioned that γδ$^+$ T cells may impact significantly upon the quality and magnitude of αβ T cell–mediated immune responses (70–74).

In summary, our results demonstrate that γδ TIL lines obtained from colon cancer recognize target structures expressed by colon, pancreatic, and by renal cell cancer cells in a MHC-unrestricted manner. This particular recognition pattern is shared by γδ TIL lines obtained from three different patients, indicating that the prevalent Vδ1$^+$ TCR subpopulation may play a role in anti-tumor-directed immunity. Examination of a limited number of TCR transcripts from colon cancer–derived γδ T cell populations expanded in vitro supports the preferential usage of the Vδ1/Dδ3/Jδ1 TCR segments that have been reported to represent common γδ TCR transcripts observed in colon biopsies harvested from normal healthy individuals (5–8). The junctional TCR sequences in γδ TIL appear to be diverse, although there was some common usage of certain amino acid residues in the N/P regions shared by individual cDNA clones. We believe that the exclusive use of TCR Vδ1 does not reflect a preferential homing receptor for human intestinal γδ T cells, but rather reflects the inherent useful nature of this particular VDJ recombination in TCR reactive against certain Ags expressed by stressed intestinal cells or by epithelial cancer cells.

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