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Characterization of a Novel Nonclassical T Cell Clone with Broad Reactivity against Human Renal Cell Carcinomas

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A CD4+ T cell clone (HC/2G-1) was established by stimulating peripheral blood T cells from a patient with renal cell carcinoma (RCC) with dendritic cells preincubated with the autologous apoptotic renal tumor line in the presence of IFN-α. It recognizes the autologous RCC and most allogeneic RCC lines by IFN-γ release (10 of 11 lines) and lysis (9 of 10 lines), but does not recognize multiple EBV B cells or fibroblasts. It shows little or no recognition of a panel of melanomas, breast cancers and non-small–cell lung cancers. Phenotypically, HC/2G-1 is CD3+CD4+ TCR αβ+, but CD161−CD16−NKG2D−. Tumor recognition by clone HC/2G-1 was not blocked by Abs to HLA class I or class II, but was significantly reduced by anti-TCR αβ Ab. Furthermore, tumor recognition was β2-microglobulin-independent. HC/2G-1 does not use a Vα or Vβ described for classical NKT cells, but rather Vα14 and Vβ2.1. Allogeneic T cells cotransfected with mRNAs encoding the α and β chains of the HC/2G-1 TCR recognized renal tumor lines, demonstrating that tumor recognition is TCR-mediated. Interestingly, TRAIL appears to play a role in tumor recognition by HC/2G-1 in that reactivity was blocked by anti-TRAIL Ab, and soluble TRAIL could enhance IFN-γ secretion by HC/2G-1 in response to renal tumors. Our findings suggest that clone HC/2G-1 represents a novel type of CD4+ cell that has broad TCR-mediated recognition of a determinant widely expressed by RCC. The Journal of Immunology, 2008, 181: 3769–3776.

The antitumor immune response is a complex event that can involve many different types of cells. Classical T cells, such as MHC class I-restricted CD8+ CTL and MHC class II-restricted CD4+ T cells, have always been at the center of this response. CD8+ CTL can directly kill tumor cells by recognizing epitopes presented by MHC molecules on the tumor surface, and from murine models, they are thought to play a major role in antitumor immunity (1). CD4+ T cells, in particular Th cells, in contrast, are thought to be important in modulating the antitumor response. CD4 Th cells can activate APCs to present Ag to CD8 CTL, maintain CD8 T cell memory and survival, and secrete cytokines such as IL-2, IFN-γ, and TNF-α, which can also affect CTL, APC, and tumor cells (2–4). Other types of cells with tumor reactivity, such as nonclassical CD8 and CD4 T cells, NK cells, and NKT cells, have also been reported and characterized (5–9).

Among them, type I NKT cells, typically expressing an invariant TCR α-chain (Vα24 in human; Vα14 in mice) and β-chain (Vβ11 in human; varying in mice) and recognizing CD1d-restricted glycolipid Ags, such as α-galactosylceramide, iGb3 and diacylglycerol, have been shown to inhibit tumor growth in vivo (10, 11).

Clear cell renal cell carcinoma (RCC) has been shown to respond to immunotherapy with a 15–20% response rate to IL-2 and a 5–7% cure rate seen in selected patients with metastatic disease (12). However, there has been little further progress in treating metastatic RCC patients with immunotherapy in contrast with another immunoresponsive cancer, melanoma. This is mainly due to a disparity in identifying tumor-reactive T cells recognizing these two cancers. Previously, we developed a method to generate RCC-reactive T cells by stimulating patient PBL with dendritic cells (DCs) engulfing and presenting apoptotic tumor cells in a completely autologous setting (13). Because DCs express both MHC class I and class II molecules on their surface, we were able to generate both MHC class I- and class II-restricted RCC-specific CD8 and CD4 T cells. The ability to generate such T cells might be exploited by identifying tumor-associated Ags, developing vaccines, and administering cultured T cells for therapy in patients with RCC. However, there are no constraints on the nature of the tumor reactivity generated by this in vitro autologous tumor stimulation and there is also the potential for generating nonclassical immune cells that recognize tumor. In this study, we report the generation of such an RCC-reactive CD4 T cell clone (HC/2G-1) that recognizes multiple renal tumors in an MHC-independent fashion with IFN-γ secretion and tumor lysis. This appears to be a novel immune reactivity by function and phenotype, which is largely specific for renal cancer. Tumor recognition is TCR αβ-mediated, but MHC-independent and β2-microglobulin (β2m)-independent. Introducing the cloned TCR α and β chains of this clone into allogeneic lymphocytes stimulated with anti-CD3 confers a pattern of tumor reactivity similar to the parental clone. Furthermore, TNF-related apoptosis-inducing ligand (TRAIL) plays a role in tumor recognition by HC/2G-1 reactivity, as shown by blocking with anti-TRAIL Ab, and enhanced recognition when renal tumors were pretreated with soluble TRAIL. It is not clear what role this type of T cell plays in the immune response to human RCC, but it may be the source of novel immunotherapeutic strategies for treating this malignancy.
Materials and Methods

Cell lines

Tumor lines from RCC patients and EBV-transformed B cells (EBV-B cells) were established as previously described. RCC lines were maintained in DMEM (Invitrogen) including 10% FBS (Invitrogen), and EBV-B cells were maintained in RPMI 1640 (Invitrogen) including 10% FBS. Tumor lines used as controls in experiments were obtained from Surgery Branch Laboratories (National Cancer Institute, Bethesda, MD), and maintained in RPMI 1640 including 10% FBS. Human primary renal epithelial cells were a gift from Drs. S. Garrett and D. Sens (University of North Dakota, Grand Forks, ND), or purchased from Cambrex.

Reagents

For immunophenotyping, mAbs including FITC-labeled anti-human IgG isotypes, CD3, CD4, CD8, CD16, CD57, CD94, CD244, and TCR γδ, PE-labeled anti-human IgG isotypes, CD3, CD4, CD8, TCR αβ, CD56, CD161, NKG2D, and TRAIL, and allophycocyanin-labeled anti-human IgG isotypes, CD3, CD4, and CD8 were purchased from BD Pharmingen. PE-labeled anti-Vβ2 Ab was purchased from Beckman Coulter.

For blocking experiments, W6/32 (pan-anti-MHC class I) and IVA12 (pan-anti-MHC class II) were gifts of Dr. P. Robbins (National Cancer Institute, Bethesda, MD). Purified anti-human TCR αβ (clone T10B9.1A-31), anti-human CD4, and anti-human TRAIL Abs were purchased from BD Pharmingen.

Granzyme B ELISA kit was purchased from Abcam. Soluble TRAIL (Apo2 ligand; aa 114–281) was purchased from Biomol. To establish RCC-specific T cells, CD8- or CD4-enriched T cells (Miltenyi Biotec) were stimulated with day 6 DCs cocultured with UV-irradiated autologous tumor cells, as previously described with some modifications (13, 14). Briefly, CD14− were isolated from patient PBMC using CD14 microbeads according to the manufacturer’s instructions (Miltenyi Biotec), and cultured in RPMI 1640, supplemented with 10% human serum (Valley Biomedical), GM-CSF (1000 U/ml; PeproTech), and IL-4 (1000 U/ml; PeproTech) for 6 days to generate monocyte-derived DCs. To stimulate T cells, day 6 DC were cocultured with UV-irradiated tumor cells (312 mm; 37°C with 51Cr (200 Ci; GE Healthcare Life Sciences). Labeled target cell lysis assay

A standard 4-h 51Cr release assay was performed to test the cytotoxicity of T cells against tumors. Briefly, target cells were labeled for 1 h at 37°C with 51Cr (200 μCi; GE Healthcare Life Sciences). Labeled target cells were then washed three times and plated in triplicate at a concentration of 1 × 104 per well in 96-well round-bottom plates. Effector cells were prepared and added to target cells at various E:T ratio. After 4-h incubation, supernatants were harvested and counted on a Wallac 1470 Wizard automatic gamma counter (PerkinElmer). Maximum or
FIGURE 2. The reactivity of HC/2G-1 is TCR-dependent, but MHCI-, CD4-, and β2m-independent. A, Immunophenotype of HC/2G-1. HC/2G-1 was stained with FITC-, PE-, and allophycocyanin-labeled mAbs, and analyzed by FACS Calibur. B, Anti-TCR Ab reduces the reactivity of HC/2G-1. HC/2G-1 was cocultured with autologous renal tumor line (RCC 1) in the presence of anti-HLA class I, HLA class II, TCR αβ, and CD4 Abs overnight and tested for IFN-γ secretion. Control T cells include a RCC-reactive HLA-B44-restricted CTL clone (MW/5H-5) and an HLA-class II-restricted CD4 T cell clone (HC/10C-3) cocultured with their autologous tumors. C, The reactivity of HC/2G-1 is β2m-independent. HLA class I and class II expression on RCC 12, RCC 12 transduced with CIITA, and RCC 12 transduced with both CIITA and β2m (left). Tumor cells were stained with FITC-labeled anti-HLA class I (W6/32) or PE-labeled anti-HLA-DR mAb and analyzed by FACS Calibur. Isotype control (open histogram) and anti-HLA class I or HLA-DR (gray-shaded histogram) are shown. Right: HC/2G-1 was cocultured with RCC 12, RCC 12 transduced with CIITA, and RCC 12 transduced with both CIITA and β2m overnight, and tested for IFN-γ secretion. RCC 8, 6, and 11 were included as controls. D, CD1d expression on RCC tumor lines. RCC tumor lines were stained with PE-labeled anti-CD1d Ab or isotype control and analyzed by FACS Calibur. Isotype control (open histogram) and CD1d (gray-shaded histogram) are shown. MOLT4 was used as a positive control cell line.
FIGURE 3. Introduction of α and β chains of the HC2G-1 TCR into anti-CD3-stimulated allogeneic T cells. A, Vβ2 Expression on anti-CD3-stimulated allogeneic T cells electroporated with HC2G-1 TCR mRNAs. PBL from allogeneic donors were stimulated with OKT-3 (50 ng/ml) and IL-2 (300 IU/ml) for 2 days, and electroporated with HC2G-1 TCR α and β mRNAs (2–8 μg/10⁶ cells). Electroporated cells were incubated overnight in the presence of IL-2, and Vβ2 expression was analyzed by flow cytometry. The percentage of CD3⁺ Vβ2⁺ cells in total PBL was shown in the top right quadrant. B, Both TCR α and β chains are required for TCR recognition. OKT-3-stimulated T cells were electroporated with mRNAs of either TCR α-chain, TCR β-chain, or both (4 μg each) and cocultured with RCC 6 overnight and tested for IFN-γ secretion. RCC 11 served as a negative control. C, Allogeneic T cells electroporated with HC2G-1 TCR mRNAs recognize renal tumors. T cells (1 × 10⁵) electroporated with mRNAs (2–8 μg/10⁶ cells) were cocultured with renal tumors overnight, and supernatant was harvested and tested for IFN-γ secretion. HC2G-1 was included in the same assay. D, HC2G-1 TCR transfection of CD8- and CD4-purified T cell populations. PBL were separated into CD8-enriched and CD4-enriched cells, and then stimulated by OKT-3 and IL-2. They were then electroporated with HC2G-1.
spontaneous release was determined by adding 2% SDS or medium to target cells, respectively. The percentage of specific lysis was calculated with the following: (experimental cpm − spontaneous cpm)/maximum cpm × 100.

Blocking assay
RCC cells (5 × 10^4 cells in 100 μl of culture medium) were incubated with each blocking mAb at a concentration of 10 μg/ml for 30 min at 37°C in a 96-well flat-bottom plate. T cells (1 × 10^5 cells/well) were then added and incubated with target cells overnight at 37°C. The supernatants were harvested and assayed for IFN-γ secretion by ELISA.

Isolation of TCR α and β chains and vector construction
Total RNA from T cell clone HC/2G-1 was purified from T cells using an RNaseasy mini kit (Qiagen). Primers that are complimentary to the 3′ end of the coding sequences were synthesized (Operon Technologies) to make full-length cDNAs of TCR α and β chains. These primers were Co (TCAGCTGACACAGCCAGGACG), Cβ1 (TCAGAAATCCTTCTCTTGACCATG), and Cβ2 (CTAGCCTCTGGAATCCTTCTC TTG). A 5′ RACE reaction was performed by SMART RACE cDNA amplification kit (Clontech Laboratories) following the manufacturer’s instructions. The RACE cDNAs (~1 kb) were obtained with Co and Cβ1 primers and then inserted into the pCR2.1 vector by TA cloning (Invitrogen). The sequences of HC/2G-1 TCR α and β chains (GenBank accession numbers EF101779 and EF101778, respectively) can be retrieved (http://www.ncbi.nlm.nih.gov/GenBank/).

In vitro transcription and electroporation
In vitro transcription of TCR α and β chains was performed using the mMESSAGE mMACHINE ULTRA according to the manufacturer’s recommendations (Applied Biosystem). The RNA was purified using the RNAeasy mini kit (Qiagen). Electroporation of mRNAs encoding the TCR α and β chains were synthesized (Operon Technologies) to end of the coding sequences were synthesized (Operon Technologies) to make full-length cDNAs of TCR α and β chains. These primers were Co (TCAGCTGACACAGCCAGGACG), Cβ1 (TCAGAAATCCTTCTCTTGACCATG), and Cβ2 (CTAGCCTCTGGAATCCTTCTC TTG). A 5′ RACE reaction was performed by SMART RACE cDNA amplification kit (Clontech Laboratories) following the manufacturer’s instructions. The RACE cDNAs (~1 kb) were obtained with Co and Cβ1 primers and then inserted into the pCR2.1 vector by TA cloning (Invitrogen). The sequences of HC/2G-1 TCR α and β chains (GenBank accession numbers EF101779 and EF101778, respectively) can be retrieved (http://www.ncbi.nlm.nih.gov/GenBank/).

Characteristics of clone HC/2G-1
Phenotypically, clone HC/2G-1 expressed CD3 and CD4, but did not express CD16, CD161, CD94, NKG2D, or CD244 on its surface (Fig. 2A). Clone HC/2G-1 did not express CD8. Approximately 5% and 9% of HC/2G-1 expressed some degree of CD56 and CD57, respectively, compared with isotype controls. The reactivity of HC/2G-1 was not due to a CD56+ contaminant because HC/2G-1 cells depleted of CD56+ cells with microbeads showed the same reactivity against renal tumor cells as unseparated HC/2G-1 (data not shown).

Blocking characterization of HC/2G-1 TCR expression by surface staining with fluorescence-labeled mAbs showed surface expression of TCR αβ but not TCR γδ (Fig. 2A). Blocking mAb against HLA class I, HLA class II, TCR αβ, and CD4 were evaluated in the recognition of autologous tumor by HC/2G-1. Anti-TCR αβ mAb blocked reactivity, and a small effect compared with positive controls was seen with anti-HLA class I mAb, but no effect was seen with anti-HLA class II and anti-CD4 mAbs (Fig. 2B). This result suggests that HC/2G-1 recognition of renal tumor is mediated by the αβ TCR, but is not classically MHC class I- or class II-restricted (as supported by its broad alloreactivity vs RCCs). The minor effect of the anti-HLA class I mAb suggested that a related presenting molecule may have a role, as is seen for some NKT cells. Therefore, we investigated whether HC/2G-1 recognition was dependent on β2m by testing HC/2G-1 against a renal tumor cell line that is naturally β2m-deficient and does not express HLA class I on its surface unless β2m is retrovirally introduced (Fig. 2C, RCC 12). The β2m-deficient RCC line was well recognized by HC/2G-1, and induction of HLA class I or class II expression by

TCR mRNAs (4 μg/ml) and cocultured with renal tumors overnight, and supernatant was harvested and tested for IFN-γ secretion. RCC 11, HEK293, and 624 mel were served as negative controls in the assay. Vβ2 expression of OKT-3-stimulated PBL, CD8-enriched cells, and CD4-enriched cells were also shown. Results were representative of more than three allogeneic PBL in independent experiments that gave similar results.
retroviral transduction of β2m or CIITA, respectively, did not affect this recognition (Fig. 2C). This observation was also confirmed by testing HC/2G-1 reactivity against two well-recognized renal tumors after silencing β2m gene expression by introducing two β2m-specific short-hairpin RNAs. There were no differences in recognition between the two native RCC lines and their β2m-
suppressed variants (data not shown). Furthermore, no recognized RCC tumor expressed CD1d or other CD1 molecules on their surface by flow analysis (Fig. 2D and data not shown), which excludes the possible involvement of CD1 molecules in tumor recognition by HC/2G-1.

**Tumor recognition of HC/2G-1 is mediated by TCR**
To further delineate the role of TCR αβ in tumor recognition by HC/2G-1, we isolated total RNA from HC/2G-1, amplified cDNAs encoding the TCR α and β chains by 5’ RACE, and cloned these cDNAs into the pCR2.1 vector. Only one pair of TCR α and β chains were identified, and these were Vα14 and Vβ2.1 (GenBank accession numbers EF101778, EF101779). This finding supports the clonal nature of the HC/2G-1 population. To confirm that tumor recognition by HC/2G-1 is mediated by its αβ TCR, we synthesized mRNAs from the TCR α and β chains by in vitro transcription and electroporated them into allogeneic PBL stimulated with anti-CD3 and IL-2. The expression of the HC/2G-1 TCR after transfection was determined by Vβ2 staining when varying amounts of mRNAs were used for electroporation (Fig. 3A). Vβ2 expression increased in T cells transfected with increasing amounts of mRNAs encoding both α and β chains. As shown in Fig. 3R, introduction of only the α or β chains alone into stimulated allogeneic T cells did not result in recognition of RCC 6. Reactivity after transfection with both TCR chains was seen against RCC 6 but not RCC 11 as was also seen with HC/2G-1. We further measured the reactivity of T cells transfected with different amounts of HC/2G-1 TCR α and β mRNAs (Fig. 3C). Using 8 μg of mRNA of each TCR chain per 10^6 cells resulted in the highest percentage of Vβ2 expression (Fig. 3A), and those TCR-transfected T cells recognized multiple renal tumors including RCC 1, 5, 6, 7, 8, and 10. Again, RCC 11, a negative target for HC/2G-1, was not recognized by TCR-transfected T cells. Furthermore, we tested CD8- and CD4-enriched T cell populations independently after transfection with HC/2G-1 TCR mRNAs (Fig. 3D). Although TCR-transfected CD8 T cells showed slightly less overall reactivity against renal tumors than TCR-transfected PBL and CD4 T cells, the pattern of induced recognition was similar. The fact that both CD8 and CD4 T cells recognize renal tumors when transfected with HC/2G-1 TCR is in accord with anti-CD4 blocking experiments that showed HC/2G-1 reactivity is CD4 independent.

**HC/2G-1 reactivity is “modulated” by TRAIL**
To delineate the effector molecules involved in tumor recognition, we first assessed CD107a mobilization by HC/2G-1. As shown in Fig. 3R, introduction of only the α or β chains alone into stimulated allogeneic T cells did not result in recognition of RCC 6. Reactivity after transfection with both TCR chains was seen against RCC 6 but not RCC 11 as was also seen with HC/2G-1. We further measured the reactivity of T cells transfected with different amounts of HC/2G-1 TCR α and β mRNAs (Fig. 3C). Using 8 μg of mRNA of each TCR chain per 10^6 cells resulted in the highest percentage of Vβ2 expression (Fig. 3A), and those TCR-transfected T cells recognized multiple renal tumors including RCC 1, 5, 6, 7, 8, and 10. Again, RCC 11, a negative target for HC/2G-1, was not recognized by TCR-transfected T cells. Furthermore, we tested CD8- and CD4-enriched T cell populations independently after transfection with HC/2G-1 TCR mRNAs (Fig. 3D). Although TCR-transfected CD8 T cells showed slightly less overall reactivity against renal tumors than TCR-transfected PBL and CD4 T cells, the pattern of induced recognition was similar. The fact that both CD8 and CD4 T cells recognize renal tumors when transfected with HC/2G-1 TCR is in accord with anti-CD4 blocking experiments that showed HC/2G-1 reactivity is CD4 independent.

In the present study, we have identified a novel CD4+ T cell clone that is distinct from these relatively well-characterized populations in its phenotype and function. This CD3+CD4+ T cell clone possesses broad reactivity against RCCs with cytokine release, tumor lysis, CD107a mobilization, and granzyme/perforin release. However, this clone differs from classical T cells in that tumor recognition is not restricted by MHC class I or class II. It is not an NK cell, as this clone expresses CD3 and TCR αβ, does not express any NK cell markers such as CD16, CD94, CD244, and NKG2D (22, 23), and does not lyse NK-sensitive target cells, such as K562 (21). It also differs from type I NKT cells because it does not express CD161, and its TCR usage is Vα14 and Vβ2.1, whereas type I NKT cells use an invariant TCR Vα24 and Vβ11 in humans (21). Furthermore, no sensitive RCC target line expresses CD1d on its surface (Fig. 2D). Perhaps its most striking characteristic is the strong bias of HC/2G-1 toward recognition of RCC as opposed to other tumors. In addition, HC/2G-1 appears to discriminate well between RCC and normal tissues. Although two unvalidated commercial renal epithelial lines showed weak recognition, four other lines procured from NCI nephrectomy specimens and generated by well-documented methods (17), were not recognized.

Our data prove that tumor recognition by HC/2G-1 is TCR αβ-mediated. First, anti-TCR αβ mAb blocking reduced HC/2G-1 reactivity against autologous tumor. Second, when allogeneic T cells were transfected with TCR mRNAs encoding the TCR α and β chains from HC/2G-1, these T cells acquired the RCC recognition pattern of parental HC/2G-1. This response was true for CD4+ or CD8+ recipient populations. Neither MHC class II on tumor nor CD4 on T cells seems to participate in tumor recognition by HC/2G-1. Another interesting observation is that HC/2G-1 reactivity is β2m-independent. It recognizes RCC 12 despite β2m deficiency, and β2m gene expression silencing with short-hairpin RNA in other RCC lines did not affect tumor recognition (data not shown).

Our finding argues against the possibility of MHC class Ib molecules such as HLA-E, HLA-F, HLA-G, or HLA-H participating as potential restriction elements because β2m is required for stability of these molecules on the cell surface (24). Other β2m-dependent molecules, such as CD1d are also not seen on recognized RCC lines and cannot be restriction elements for HC/2G-1. Broad recognition of RCC lines via an αβ TCR suggests an Ag or epitope presented by a relatively nonpolymorphic MHC-like presenting entity, but so far, the basis of HC/2G-1 recognition remains unidentified. We are currently making modifications in the CD2R2 and CD3R regions of the HC/2G-1 TCR to determine whether these structures are involved in TCR-Ag interaction.

Our data also suggest that HC/2G-1 recognition is modulated by TRAIL. It constitutively expresses surface TRAIL, and blocking Ab to TRAIL resulted in reduced tumor recognition. Previous studies have demonstrated that CD4+ T cells can express TRAIL (18, 19, 20, 21).
25). These studies also suggest that CD4+ T cells can exhibit cytotoxicity to renal tumors through both TRAIL and granzyme/perforin pathways. Yet for HC/2G-1, TRAIL augments both target lysis and cytokine release. Target cell apoptosis and destruction might be expected to reduce cytokine release by removing antigenic stimulation. Furthermore, we see no positive or negative relationship between RCC susceptibility to soluble TRAIL and the ability to stimulate HC/2G-1 T cells by DR4. These findings imply that TRAIL on HC/2G-1 may not simply be an effector molecule in addition to granzyme/perforin. One possible explanation is that TRAIL is a costimulatory molecule, as demonstrated by Chou et al. (26), wherein cross-linking TRAIL on CD4+ T cells with immobilized death receptor DR4 enhanced proliferation and IFN-γ production by CD4 T cells. However, in their study, soluble TRAIL reduced T cell stimulation by competing for DR4, whereas we found the opposite. Another possibility is that TRAIL may induce changes in renal tumors such as up-regulating the unidentified restriction element or its presenting molecule, but knowing its restriction element and Ag is needed to study these possibilities. Nonetheless, the involvement of TRAIL may give us a tool to identify its restriction element and Ag is needed to study these possibilities.

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Disclosures

The authors have no financial conflict of interest.

References


