Brief Definitive Report

Lysis of Ovarian Cancer Cells by Human Lymphocytes Redirected with a Chimeric Gene Composed of an Antibody Variable Region and the Fc Receptor γ Chain

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Summary

To expand the spectrum of recognition of effector lymphocytes and to redirect them towards predefined targets, we have altered the specificity of human tumor-infiltrating lymphocytes (TIL) through stable modification with chimeric receptor genes consisting of single-chain antibody variable regions linked to the γ subunit common to the immunoglobulin (Ig)G and IgE Fc receptors. Using either hapten or ovarian carcinoma-specific monoclonal antibodies, we constructed chimeric receptor genes and retrovirally introduced them into $CD8^+$ TIL. Redirected TIL specifically lysed trinitrophenyl-labeled Daudi or a human ovarian carcinoma cell line (IGROV-1), and secreted granulocyte/macrophage colony-stimulating factor upon stimulation with the appropriate antigen. This strategy may allow new approaches towards the adoptive immunotherapy of cancer in humans.

A limitation in the use of adoptive cellular immunotherapy for cancer lies in the difficulty in obtaining specific tumor-infiltrating lymphocytes (TIL) for many histologic types of cancer. In contrast, many mAbs have been described that bind tumor-associated antigens shared by tumors of the same histology. Two approaches have been attempted to combine the effector function of T cells with the antitumor specificity of antibodies. One is destruction of tumor by bispecific antibodies possessing dual specificity for both the target and the immune cell (1, 2). However, the use of bispecific antibodies for therapy is limited by the inaccessibility of many solid tumors to antibody penetration (3) and dissociation of antibody from the lymphocyte membrane in a relatively short period of time (4). A second approach involves the construction of chimeric receptors containing the V regions of antibodies joined to the C regions of the TCR (5–9). Because antigen binding by most antibodies requires both light and heavy V region chains (V_H and V_L), and TCR function requires both α and β (or γ/δ) chains, this approach necessitates the coexpression of two chimeric genes. While the stable expression of multiple genes has been possible in transformed T cells and hybridomas, it has been difficult in primary T cells. To overcome these problems, we have constructed chimeric receptor genes containing V_L/V_H single-chain V domains (scFv) from mAbs linked to the Fc receptor–associated γ chain (10). The scFv, which consists of V_L bridged to V_H via a flexible linker, has been demonstrated to have similar binding affinities and specificities compared to natural Fab fragments (11). The γ chain is the signal transducing subunit of both the high affinity IgE receptor (FcεRI) of mast cells and basophils, and of the low affinity receptor for IgG (FcγRIII), expressed primarily by macrophages and NK cells (12). The γ subunit is very similar in structure and function to the CD3ζ chain and in fact can form heterodimers with it in some T and NK cells (13). The scFv-γ design that we have used for the present studies combines antibody recognition and T cell signaling in one continuous gene, and has been successfully used to endow murine CTL hybridoma cells with non-MHC-restricted, antibody-type specificity (10).

Materials and Methods

Establishment and Maintenance of TIL. TIL were derived from enzymatically digested tumor biopsies as previously described (14). Briefly, melanoma tumor biopsies were digested overnight in collagenase type IV (1 μg/ml), hyaluronidase (0.1 μg/ml), and DNAse (30 U/ml) (Sigma Chemical Co., St. Louis, MO). After digestion, the single-cell suspensions were passed through a sterile wire screen grid, and subjected to Ficoll-Hypaque separation to remove dead cells and red blood cells. TIL cell cultures were established at 5.0 × 10^5 cells/ml in 24-well tissue culture plates in RPMI 1640 supplemented with 10% human A serum (BioWhittaker, Inc., Walkersville, MD). This was mixed 1:1 (vol/vol) with AIM V serum-free medium (Gibco Laboratories, Grand Island, NY) and was further supplemented with gentamicin-sulfate (10 μg/ml), penicillin G so-

dium (10,000 U/ml), glutamine (200 mM) (all from Gibco Laboratories), IL-2 (7,200 IU/ml) (Chiron Corp., Emeryville, CA) and 10% (vol/vol) lymphokine-activated killer (LAK) cell-conditioned supernatant. Since TIL can double every 2–4 d, TIL densities were maintained at 5×10^5 by splitting cultures every 3–5 d with fresh medium containing IL-2 and passaging to larger cell culture plates (six well) when required. When TIL reached $> 2 \times 10^8$ in number, they were cultured in AIM V serum-free medium alone containing 6,000 IU/ml IL-2. Large-scale TIL expansion was performed in gas-permeable PL732 3-liter plastic bags (Fenwal, Deerfield, IL).

Construction of Chimeric Genes. The Sp6 anti-2,4,6 TNP antibody (15, 16) and MOv18 anti–ovarian carcinoma antibody (17, 18) V_H and V_L genes were derived by PCR amplifications using oligodeoxynucleotide primers corresponding to the 5′ and 3′ consensus amino acid sequences of Ig V regions, and joined together using a flexible linker, as described (10), introducing a BstEII site at the 3′ end of the scFv. Sp6 genomic DNA clones was kindly provided by Dr. Georges Köhler (Max Planck Institute for Immunobiology, Freiburg, Germany), and MOv18 genomic DNA was kindly provided by Dr. Leslie Coney (Centocor, Malvern, PA). The γ chain DNA was amplified from a human cDNA clone (19) using primers introducing BstE II and Xho I at the 5′ and 3′ ends, and was then ligated to the scFv via BstE II, to form plasmid pRSVscFvRγ (10).

Retroviral Vectors and Gene Transfer. The retroviral LXSN vector containing the chimeric receptor ScFv-γ construct (see Fig. 1) was introduced by $CaPO_4$ transfection into the GP+E 86 ecotropic packaging cell line (21). 48 h later, supernatant from these cells was used to transduce the PA317 amphotropic packaging cell line (22). High-titer G418-resistant PA317 clones were then selected, and used for 72-h cocultivation with human melanoma CD8$^+$ TIL in the presence of protamine sulfate (5 μg/ml). TIL, which grow in suspension, were then separated by careful pipetting from the adherent PA317 cell line. 24–48 h later, TIL were selected for 5 d in 0.5 mg/ml of the neomycin analogue G418 (Geneticin; Gibco Laboratories), followed by expansion in AIM V, 10% FCS, and IL-2 (6,000 IU/ml) (23). After transduction and G418 selection of TIL, Southern and Northern analyses using V_H genomic DNA as a probe were performed to confirm successful gene insertion and transcriptional expression.

^{51}Cr Release Cytotoxicity Assay. TIL were evaluated for their ability to lyse specific targets using a standard ^{51}Cr release assay as previously described (24). After ^{51}Cr labeling of targets, TNP labeling of cells was performed by addition of a freshly prepared 10-mM solution of 2,4,6-trinitrobenzenesulfonic acid (Fluka Chemika-BioChemika, Buchs, Switzerland) in a 1:1 (vol/vol) ratio followed by a 10-min incubation at 37°C. Cells were then washed three times in complete medium before use in the cytolysis assay. Effectors and targets were cocultured for 4–16 h. The percent spontaneous release for all studies was <30%. For the lysis inhibition studies, a constant E/T ratio of 90:1 was used.

GM-CSF Assay. 10^6 TIL and 10^6 stimulator cells were cocultured for 24 h at 37°C in a final volume of 1 ml AIMV/IL-2. Supernatants were then aspirated, centrifuged at 2,000 rpm to remove cells, decanted, and frozen at −70°C. Thawed aliquots were tested in an ELISA for human GM-CSF (Genzyme Corp., Cambridge, MA). The ELISA used a solid-phase murine mAb specific for human GM-CSF. After addition of either sample or recombinant GM-CSF standard, a rabbit anti-human GM-CSF polyclonal antibody was used. A biotin-labeled goat anti-rabbit polyclonal antibody was then added followed by streptavidin-peroxidase.

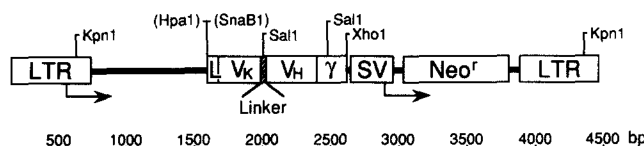

Figure 1. Schematic representation of the scFv-γ chimeric receptor gene within the retroviral LXSN backbone. The continuous V_L-linker-V_H-γ chain chimeric genes were excised from pRSVscFvRγ (10) with SnaBI and XhoI and ligated into the HpaI-XhoI site of LXSN (20), under the transcriptional regulation of the Moloney murine leukemia virus LTR. The neomycin phosphotransferase gene (*neo^r*) is under the transcriptional control of the SV-40 early region promoter.

Results

So far, studies reporting antibody/TCR chimeras have used antihapten antibodies. For our studies using the scFv-γ design, we not only used an anti-TNP mAb (Sp6; 15, 16), but also a mAb (MOv18) against the 38-kD folate binding protein, a surface antigen present on most ovarian carcinomas (17, 18). To obtain stable gene transfer into primary human lymphocytes, we used retroviral transduction, which we have previously shown to be effective in generating stable, gene-modified lymphocytes (25). As an effector cell in our studies, we used a human CD8$^+$ melanoma-derived TIL (24). This TIL is capable of specifically lysing its autologous melanoma target.

Initially, anti-TNP scFv-γ transduced TIL (Sp-γ) were examined to determine if they expressed functional receptors. Sp-γ TIL could lyse TNP labeled Daudi, a Burkitt's lymphoma cell line, but could not lyse unlabeled Daudi, in a standard ^{51}Cr release assay (24) (Fig. 2 *A*). Nontransduced TIL (NV) and TIL transduced with a chimeric receptor of another specificity (MOv-γ) could lyse neither TNP-labeled nor unlabeled Daudi. Similar results were obtained with a TNP-labeled EBV-transformed B cell line as a target. Percent specific lysis against TNP-labeled EBV-B cells was 39 and 9% when using either SP-γ TIL or NV TIL, respectively, at equivalent E/T values in a 4-h standard ^{51}Cr release assay. Under similar conditions, no lysis of unlabeled EBV-B cells was observed by either TIL line. Lysis against TNP-labeled cells by Sp-γ TIL was specific for TNP, and was blocked by soluble TNP fowl gamma globulin (Fig. 2 *B*).

We next examined whether this approach could be used to specifically redirect lymphocytes against tumor. A chimeric receptor (MOv-γ) was constructed using an scFv from MOv18, a mAb that is relatively specific for human ovarian carcinoma. The same TIL were retrovirally transduced with the chimeric MOv-γ receptor and selected in G418. The MOv-γ-modified TIL (MOv-γ TIL) lysed IGROV-1 cells, a human ovarian carcinoma cell line that expresses high levels of the 38-kD folate binding protein recognized by MOv18 (26, 27) (Fig. 3 *A*). However, neither nontransduced TIL nor the TNP-specific Sp-γ TIL could lyse IGROV-1. Lysis of IGROV-1 by MOv-γ TIL was specific for the MOv18-defined protein, and was blocked by anti-MOv18 idiotypic antibody (28) in a dose-dependent fashion (Fig. 3 *B*). In addition, specific lysis

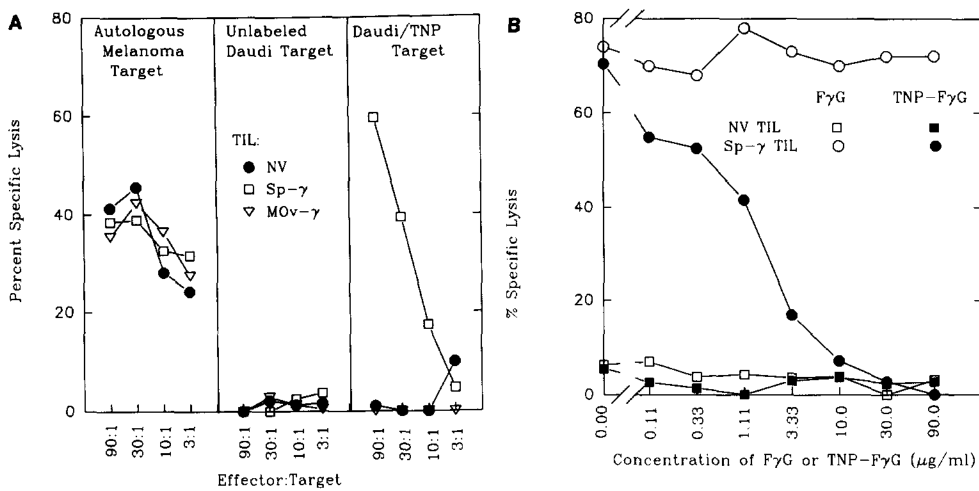

Figure 2. Anti-TNP Sp-γ TIL are redirected to specifically lyse TNP-labeled targets. (*A*) Lysis of autologous melanoma and TNP-unlabeled and labeled Daudi cells. Only the Sp-γ TIL, transduced with the chimeric svFv-γ gene derived from the anti-TNP mAb Sp6, lyse the TNP-labeled Daudi, while nontransduced and MOv-γ TIL do not. (*B*) Lysis of TNP-labeled Daudi by Sp-γ TIL is inhibited by soluble TNP-fowl gamma globulin (FγG) but not by unlabeled FγG in a dose-dependent fashion. TIL were incubated with either FγG or TNP-FγG for 2 h before the addition of ^{51}Cr-labeled targets. Lysis of autologous melanoma was used as a specificity control, and was not affected by either soluble TNP-FγG or unlabeled FγG (not shown).

by Sp-γ and MOv-γ TIL for their respective targets was stable on serial assays over a 33-d period (not shown).

To further study the specificity of MOv-γ and Sp-γ TIL, lysis was tested against multiple targets. The IGROV-1 cell line and the TNP-labeled Daudi cell line were specifically lysed by the MOv-γ and Sp-γ TIL, respectively. Since the TIL used were derived from melanoma, all groups were able to lyse the autologous melanoma, but were unable to lyse allogeneic melanoma, breast, or unmodified Daudi cell lines. FACS® analysis using the MOv18 antibody demonstrated that, indeed, only the IGROV-1 cell line expressed significant levels of the MOv18-defined protein (not shown).

To additionally evaluate the function of the Sp-γ and MOv-γ receptors in TIL, we examined their ability to mediate GM-CSF secretion after receptor activation by cocultivation with different stimulator cells (Table 1). Sp-γ TIL specifically secreted GM-CSF after stimulation by TNP-labeled EBV-transformed B cells and Daudi cells, but not after stimulation by unlabeled cells. Likewise, only IGROV-1 cells stimulated specific GM-CSF secretion by MOv-γ TIL.

Discussion

This study demonstrates that primary CD8$^+$ T cells can be stably modified genetically to be redirected against new antigens, defined by mAbs. Thus far, studies using chimeric antibody/TCRs have used antihapten antibodies, wherein the V_H by itself could account for most of the antigen binding (5–9). Here we report for the first time a chimeric scFv-γ receptor made of a mAb specific for a more complex protein antigen. Apparently, both V_H and V_L of MOv18 are needed to bind antigen, since the MOv18 V_HC_β chimeric receptor did not confer antibody specificity after transduction into TIL (not shown), in contrast to the reactivity conferred by the anti-TNP (Sp6) V_HC_β receptor (8). The present work complements recent studies in which chimeras of the γ or ζ chain joined to extracellular CD4, CD8, IL-2 receptor, and CD16 domains could be functionally expressed in transformed T lymphocytes and basophils (29–31). Here we have extended this work to include single-chain antibody variable fragments as the extracellular ligand binding domains (10). We have

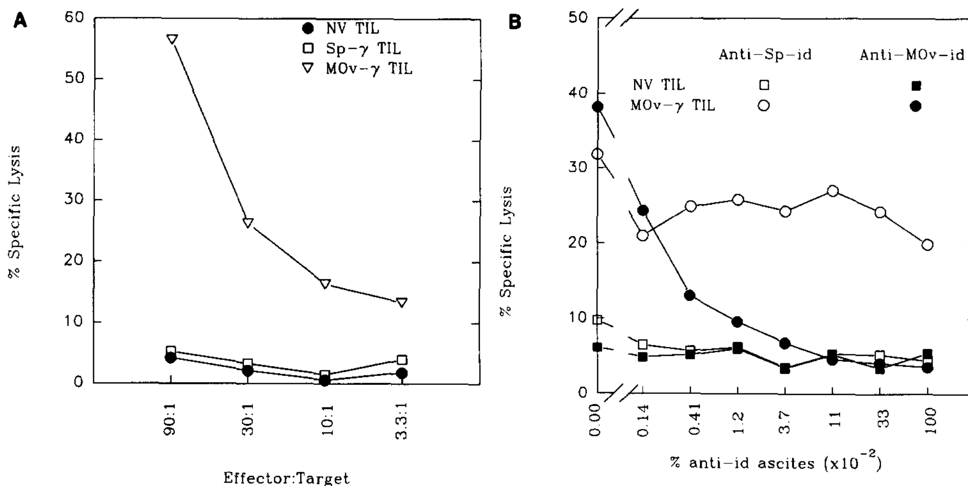

Figure 3. The human ovarian carcinoma cell line IGROV-1 is specifically lysed by MOv-γ TIL. (*A*) Lysis of IGROV-1. Only the MOv-γ TIL, transduced with a chimeric receptor gene derived from the MOv18 antibody, lyse IGROV-1, while nontransduced and Sp-γ TIL do not. (*B*) Lysis of IGROV-1 by MOv-γ TIL is inhibited by anti-MOv18 idiotype antibody in a dose-dependent fashion. TIL were incubated with either anti-MOv18 idiotype antibody or an irrelevant antibody, anti-Sp6 idiotype antibody, for 2 h before the addition of ^{51}Cr-labeled targets. Lysis of autologous melanoma was studied as a control, and was not effected by either anti-idiotype antibody (not shown).

Table 1. *GM-CSF Secretion by SP-γ and MOv-γ TIL after Antigen Stimulation*

Exp.	TIL	GM-CSF production					
			EBV–B cell		Daudi		
		None	No TNP	+ TNP	No TNP	+ TNP	IGROV-1
		pg/ml/10^6 TIL/24 h					
1	Nontransduced	8	27	43	27	28	7
	Sp-γ	13	52	>512	73	>512	4
	MOv-γ	22	38	62	36	40	269
2	Nontransduced	24	63	130	45	32	37
	Sp-γ	17	62	493	38	152	25
	MOv-γ	6	47	81	35	34	165

10^6 TIL were cocultured with 10^6 stimulators in a final volume of 1 ml AIM V medium (containing 6,000 IU/ml IL-2). After a 24-h incubation at 37°C, supernatants were harvested and cells were removed by centrifugation. Supernatants were stored at −70°C before GM-CSF ELISA (Genzyme Corp.).

used the γ subunit of the Fc receptor to transduce the signal from ligand binding as well as to anchor the scFv receptor to the plasma membrane. However, other transmembrane/cytoplasmic signaling molecules that trigger cell activation or proliferation could potentially be used, such as the CD3-associated ζ chain (10), TCR α or β chains, or the IL-2 receptor α, β, or γ chains.

As our effector cell, we have chosen a primary human T lymphocyte that could be used for immunotherapy, rather than transformed cell lines used in previous studies. In these redirected TIL, the chimeric receptor mediated both specific cytolysis against tumor as well as cytokine release, both of which have been implicated in the antitumor effects of TIL (32, 33). Although we have used TIL in this study, alternative effector cells, such as OKT3-stimulated PBL, NK cells, and $CD4^+$ T cells could potentially be used. We have used retroviral-directed gene modification, as opposed to transient methods of lymphocyte gene transfer, such as recombinant vaccinia virus infection. Retroviral transduction has been shown to be capable of stably introducing foreign genes without adversely affecting lymphocyte function over time (25), and indeed the chimeric receptors were functional in TIL by serial studies over a 33-d period. In fact, TIL transduced retrovirally with a neomycin phosphotransferase marker gene have been safely infused into patients receiving immunotherapy for metastatic melanoma (34). Therefore, effector T cells redirected by retroviral modification with the chimeric receptor described here could potentially be used for adoptive immunotherapy in cancer patients.

In this study, we have conferred on TIL antitumor specificity using the MOv18 antibody directed against ovarian carcinoma. However, mAbs directed against many different tumor histologies are available, and can serve to generate chimeric receptors against other histologies, including breast, colon, prostate, and lung cancer. In addition, antiviral mAbs could be used to redirect lymphocytes against virally infected cells. Of note, the adoptive cellular immunotherapy of viral infections was recently demonstrated to be of potential benefit for cytomegalovirus prophylaxis in the setting of bone marrow transplantation (35). Therefore, the stable, genetic modification of primary T cells with chimeric receptors containing single-chain antibody V regions, as described here, has the potential to extend the current scope of antitumor adoptive cellular immunotherapy to include multiple additional cancer histologies, and, in addition, could broaden the immunotherapy of viral infections such as HIV and hepatitis B without the cumbersome process of isolating naturally occurring specific immune T cells.

We acknowledge T. Waks and S. Tel-Or for technical assistance and expertise, J. Mulé for helpful advice, J. Yannelli for cell lines and reagents, and Dr. Maria I. Colnaghi for MOv18 and anti-MOv18 idiotype antibodies.

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Received for publication 15 January 1993 and in revised form 31 March 1993.

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