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 (IgG) 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\(_L\) and V\(_H\)), and TCR function requires both \(\alpha\) and \(\beta\) (or \(\gamma/\delta\)) 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 (FceRI) of mast cells and basophils, and of the low affinity receptor for IgG (Fc\(\gamma\)RIII), expressed primarily by macrophages and NK cells (12). The γ subunit is very similar in structure and function to the CD3\(\gamma\) 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 × 10^6 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-
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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 passing to larger cell culture plates (six well) when required. When TIL reached > 2 × 10^6 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) Vα and Vγ 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 BstEII and XhoI at the 5' and 3' ends, and was then ligated to the scFv via BstEII, to form plasmid pRSVscFvR3' (10).

Retroviral Vectors and Gene Transfer. The retroviral LXSN vector containing the chimeric receptor ScFv-γ construct (see Fig. 1) was introduced by CaPO4 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 proamine sulfamate (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 Vγ genomic DNA as a probe were performed to confirm successful gene insertion and transcriptional expression.

11Cr Release Cytotoxicity Assay. TIL were evaluated for their ability to lyse specific targets using a standard 11Cr release assay as previously described (24). After 11Cr 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 cytotoxicity assay. Effector 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 Vγ-linker-Vα chain chimeric genes were excised from pRSVscFvR3' (10) with SnBl 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) 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 51Cr 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 51Cr 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-kDa 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

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by Sp-γ and M0v-γ TIL for their respective targets was stable on serial assays over a 33-d period (not shown).

To further study the specificity of M0v-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 M0v-γ and Sp-γ TIL, respectively. Since the TIL 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 M0v18 antibody demonstrated that, in fact, only the IGROV-1 cell line expressed significant levels of the M0v18-defined protein (not shown).

To additionally evaluate the function of the Sp-γ and M0v-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 M0v-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 Vγ 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 Vγ and Vδ of MOv18 are needed to bind antigen, since the MOv18 VγCδ chimeric receptor did not confer antibody specificity after transduction into TIL (not shown), in contrast to the reactivity conferred by the anti-TNP (Sp6) VγCδ receptor (8). The present work complements recent studies in which chimeras of the γ or δ 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

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**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 M0v-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 3HCr-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).

**Figure 3.** The human ovarian carcinoma cell line IGROV-1 is specifically lysed by M0v-γ TIL. (A) Lysis of IGROV-1. Only the M0v-γ TIL, transduced with a chimeric receptor gene derived from the M0v18 antibody, lyse IGROV-1, while nontransduced and Sp-γ TIL do not. (B) Lysis of IGROV-1 by M0v-γ TIL is inhibited by anti-M0v18 idiotype antibody in a dose-dependent fashion. TIL were incubated with either anti-M0v18 idiotype antibody or an irrelevant antibody, anti-Sp6 idiotype antibody, for 2 h before the addition of 3HCr-labeled targets. Lysis of autologous melanoma was used as a control, and was not affected by either anti-idiotype antibody (not shown).
Table 1. GM-CSF Secretion by SP-γ and MOv-γ TIL after Antigen Stimulation

| Exp. | TIL           | EBV-B cell | Daudi  |
|------|---------------|------------|--------|
|      |               | None | No TNP | + TNP | No TNP | + TNP | IGROV-I |
| 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     |

pg/ml/10^6 TIL/24 h

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.).

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