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# A Fully Human Chimeric Immune Receptor for Retargeting T-cells to CEA-expressing Tumor Cells

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**Abstract.** *Background:* Recombinant chimeric immune receptors (CIRs) with anti-CEA specificity can retarget grafted T-cells to CEA-expressing tumors in an HLA-independent manner. To reduce the immunogenicity of conventional CIR in humans, an attempt was made to generate a CIR encoded by all human genes. *Materials and Methods:* A single-chain variable fragmented (scFv) antibody gene was prepared from variable region genes of the C2-45 human mAb clone specific for CEA. The scFv gene was connected to a gene construct comprised of the cDNAs for the human CD8 $\alpha$  hinge region, the human CD28 transmembrane and cytoplasmic domains, and the human CD3 $\zeta$  intracellular domain. The resulting human CIR gene, designated L45scFv-CIR, was inserted into the pcDNA3.1 expression vector and transfected into human primary T-cells. *Results:* Flow cytometric analysis using allophycocyanin-labeled CEA demonstrated the expression of the L45scFv-CIR protein on the T-cells and its specific antigen binding activity. *Conclusion:* This L45scFv-CIR gene, consisting of four human genes, may be a useful tool for eradication of CEA-expressing but HLA-downregulated tumor cells.

Most immunotherapeutic strategies targeting tumor cells aim to induce and enhance the number of tumor-specific T-lymphocytes in patients by peptide vaccination. Transfer of such tumor specific T-lymphocytes into patients can have clinical significance (1). However, the isolation of tumor-specific cytotoxic T-lymphocytes (CTLs) and their expansion to significant numbers for clinical application on an

individual basis is cumbersome and the outcome unpredictable (2-4). Furthermore, HLA class I molecules on tumor cells, essential for target recognition of CTL, are frequently down-regulated and/or lost during the development of malignancies (5, 6).

The clinical application of the chimeric immune receptor (CIR), as a mean to redirect the specificity of T-cells toward the cellular target of interest, holds great promise for the adoptive immunotherapy of cancer and infectious disease (7-9). The CIR is based on an artificial immune receptor composed of an extracellular antigen-binding domain linked through a transmembrane motif to a cytoplasmic lymphocyte-signaling moiety. The CIR approach using antibody-derived single-chain variable fragment (scFv) as a recognition unit, is especially suited to immunotherapy of cancer, being able to bypass many of the mechanisms by which tumors avoid immunorecognition, such as HLA down-regulation, lack of expression of co-stimulatory molecules, CTL resistance and induction of T-cell suppression, and where the use of both CD8<sup>+</sup> CTL and CD4<sup>+</sup> T-cells are best combined for optimum anti-tumor efficacy (6, 10, 11).

To date, the majority of CIR genes are reconstructed with murine scFv specific for the tumor antigen (12). However, murine scFv is immunogenic in humans (13). Therefore, we have developed a novel CIR with a human scFv portion that recognizes CEA, one of the most important tumor-associated antigens (TAAs). Recently, we generated fully human monoclonal antibodies (mAbs) specific for CEA using KM mouse<sup>TM</sup> that lacks the endogenous genes for immunoglobulin and instead carries human immunoglobulin genes (14). In a recent study, we also generated a human scFv antibody, designated 45kHscFv, after cloning the variable region cDNAs of the C2-45 human mAb (15).

In the present study, the scFv gene was fused to a construct containing the human CD8 $\alpha$  hinge region, human CD28 transmembrane and cytoplasmic domain and human CD3 $\zeta$  intracellular domain genes. After transfection of this

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**Key Words:** Chimeric immune receptor, scFv, T-lymphocytes, electroporation, antibody-based therapy, carcinoembryonic antigen, CEA.

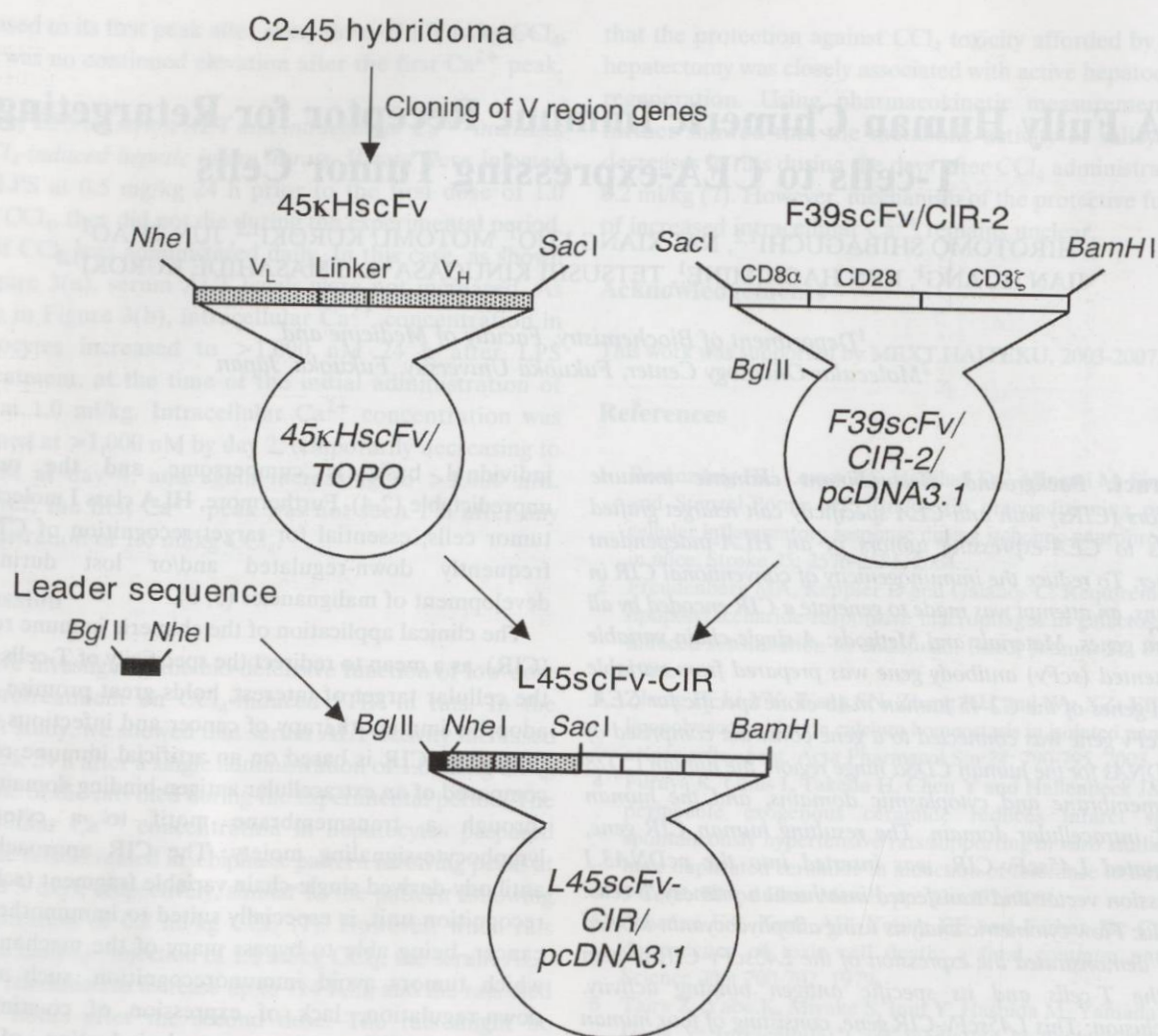


Figure 1. Schematic illustration of the construction of the L45scFv-CIR expression vector.

fully human gene into primary human T-lymphocytes, their expression of the resulting CIR and binding to CEA-positive cells was investigated.

## Materials and Methods

**Cells.** A CEA-expressing gastric cancer cell line, MKN-45, was maintained in DMEM supplemented with 10% FBS, 100 U/ml of penicillin and 100 µg/ml of streptomycin. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers using LSM® lymphocyte separation medium according to the manufacturer's protocol. After 1 h incubation in 10-cm diameter culture dishes, non-adherent PBMCs were collected and cultured in AIM-V® medium (Invitrogen, Carlsbad, CA, USA).

**Chimeric immune receptor construction and expression vector production.** cDNA encoding the anti-CEA chimeric immune receptor,

designated *L45scFv-CIR*, was generated with specific primers including unique restriction enzyme sites using the human 45κHscFv gene and another mouse/human CIR, *F39scFv/CIR-2*, with mouse scFv (14-16). The specific primers were as follows: *L45scFv-CIR* forward, 5'-aatgagctccagctccaggcaccctgtcttct-3'; *L45scFv* reverse, 5'-ccatggctcgagaaatgaggcccaaccggcca-3'; CIR forward, 5'-agcggccgcctgagcaatacatcatg-3' and *L45scFv-CIR* reverse, 5'-taagcttgatccgcggctaagggggcaggcctgcatgt-3'. As schematically represented in Figure 1, the *L45scFv-CIR* with a leader sequence was inserted into a pcDNA3.1 expression vector at the restriction enzyme sites, *Bgl*II and *Bam*HI with T4 ligase, and transformed into TOP10 competent cells. The plasmid DNA was cloned and sequenced with a DNA sequencer using BigDye™ terminator (PE Applied Biosystems, Foster City, CA, USA) as described previously (15).

**Gene transfection.** The plasmid DNA was purified using the PureLink™ HQ Mini Plasmid Purification kit (Invitrogen) for transfection. The *L45scFv-CIR* or *pmaxGFP* (Amara Biosystems,

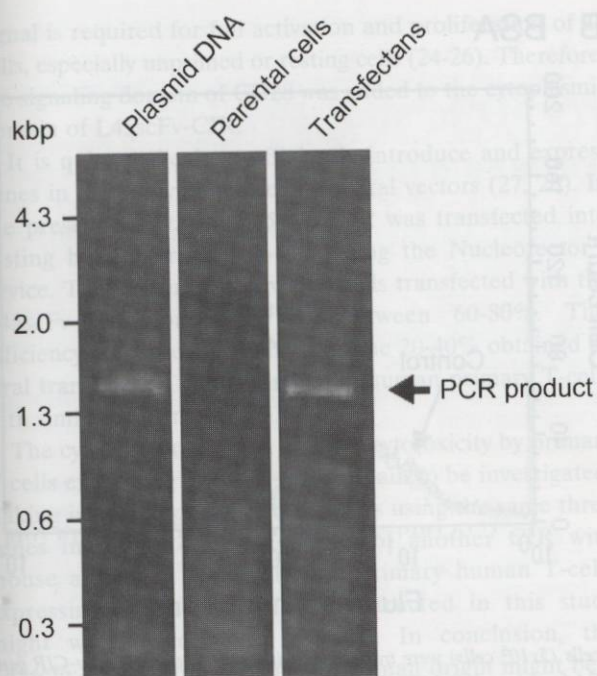


Figure 2. Detection of *L45scFv-CIR* gene mRNA on transfected primary T-cells by RT-PCR with specific primers. Total RNA (1 µg) obtained from the parental cells and transfectants was used as template RNA. The plasmid DNA containing *L45scFv-CIR* was used as a positive control.

Cologne, Germany) genes were transfected into freshly isolated PBMCs using Nucleofector™ (Amaxa Biosystems) with the human T-cell Nucleofector® kit according to the manufacturer's protocol with minor modification. Briefly, 3 µg of DNA was added to 5x10<sup>6</sup> PBMCs resuspended in 100 µl of human T-cell Nucleofector® Solution, electroporated using the U-14 program of Nucleofector™ device, and immediately transferred into the prewarmed AIM-V medium.

**RT-PCR.** Total RNA was extracted from the transfectants 24 h after transfection with ISOGEN (Nippon Gene, Toyama, Japan) according to the manufacture's protocol. The RT-PCR was performed using the AccessQuick™ RT-PCR system (Promega, Madison, WI, USA) with *L45scFv-CIR* specific primers described above (*L45scFv-CIR* forward and *L45scFv-CIR* reverse). The final products were analyzed by electrophoresis on 1.5% agarose gel followed by staining with ethidium bromide.

**Flow cytometry.** CEA and BSA were labeled with the Allophycocyanin Labeling kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan). T-cells expressing *L45scFv-CIR* were identified by immunofluorescence with allophycocyanin (APC)-labeled CEA or BSA 24 h after transfection. Briefly, 1x10<sup>5</sup> transfectants were incubated with 5 µg/ml of APC-CEA or -BSA for 1 h at 4°C. After washing twice with PBS, cells were analyzed by a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA, USA) using CellQuest software (BD Biosciences). The electroporated cells without gene were used as a control.

**Rosetting test.** MKN-45 cells were cultured to about 50% confluence in a 4-well LAB-TEK® Chamber Slide™ (Nalge Nunc International, Naperville, IL, USA), and were incubated with transfected or non-transfected T-cells (1x10<sup>6</sup> cells/ml) at 37°C for 1 h. Cells were then washed three times with PBS to remove nonspecific binding T-cells.

## Results

**Detection of *L45scFv-CIR* gene in T-cells.** Twenty-four h after transfection of the *L45scFv-CIR* gene into primary T-cells, total RNA was extracted from the transfectants and non-transfectants, and analyzed by RT-PCR using specific primers described above to detect the gene transcription. As shown in Figure 2, the RT-PCR products from the transfectants and the plasmid DNA including *L45scFv-CIR* showed a major band of about 1.4 kbp, but no specific band was detected in the non-transfectants, indicating that the mRNA for *L45scFv-CIR* gene was transcribed in the transfectants.

**Expression of *L45scFv-CIR* in T-cells.** Flow cytometric analysis was employed to detect the functional expression of *L45scFv-CIR* on the surface of transfectants. CEA molecules labeled with APC were specifically bound to transfectants, but not to non-transfectants (Figure 3A). In contrast, APC-BSA was bound to neither *L45scFv-CIR* transfectants nor non-transfectants, as shown in Figure 3B. The efficacy of *L45scFv-CIR* gene transfection to primary human T-cells was about 80%.

**Targeting of T-cells expressing *L45scFv-CIR* to CEA-positive tumor cells.** The binding capability of *L45scFv-CIR* transfected T-cells was tested by rosette formation. MKN-45 target cells were incubated with transfectants or non-transfectants. Nonspecifically bound T-cells were removed by washing. The *L45scFv-CIR* transfected T-cells were bound to MKN-45 cells, whereas the non-transfected T-cells did not react with MKN-45 cells (Figure 4).

## Discussion

In the present study, a novel CIR gene, *L45scFv-CIR*, with four human genes was successfully generated. Primary human T-cells expressing *L45scFv-CIR* on their surface specifically recognized APC-labeled CEA and bound to MKN-45 cells *in vitro*.

CIRs expressed on T-cells have usually been generated using variable region genes of mouse mAbs or humanized mAbs (12). The reports of phase I/II trials using mAbs of mouse origin, however, indicated that the production of human anti-mouse antibody (HAMA) was still a considerable problem (17-19), suggesting that CIRs containing variable regions of mouse origin are possibly

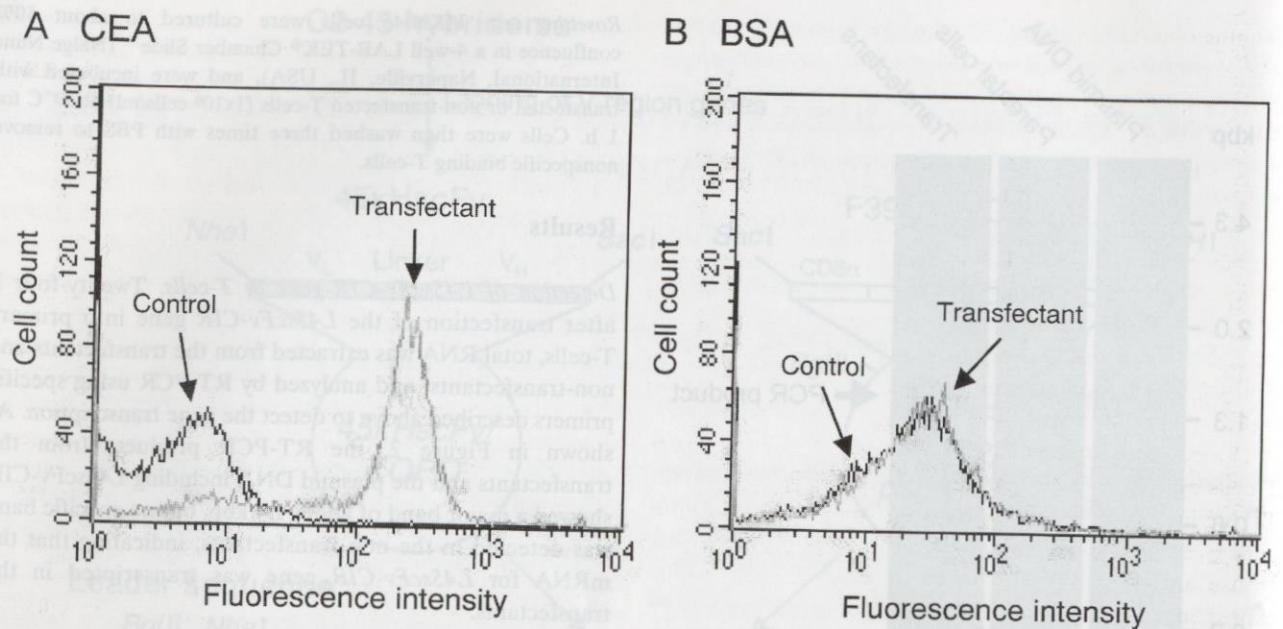


Figure 3. The L45scFv-CIR expression on human T-cells. Primary human T-cells ( $5 \times 10^6$  cells) were transfected with  $2 \mu\text{g}$  of the L45scFv-CIR gene. Transfectants ( $1 \times 10^6$  cells) were stained with  $1 \mu\text{g}/\text{ml}$  of APC-labeled BSA or CEA for 1 h at  $4^\circ\text{C}$  24 hrs after nucleofection. The expression of L45scFv-CIR on human T-cells was then determined by flow cytometry. Non-transfected T-cells were also stained as control

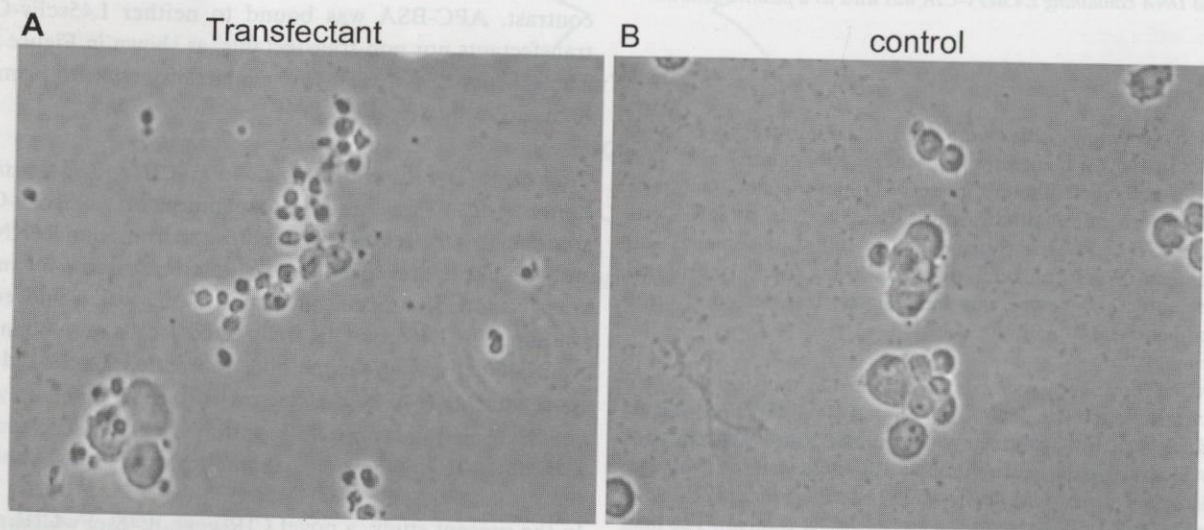


Figure 4. Binding of L45scFv-CIR transfected primary human T-cells to CEA-expressing tumor cells. The CEA expressing MKN-45 cells were mixed with parental T-cells or transfectants at the ratio of 1:5 and incubated for rosette formation for 1 h at  $37^\circ\text{C}$ .

immunogenic. Therefore, our L45scFv-CIR has the advantage of avoiding the HAMA problem because of its construction design with all genes of human origin.

To date, it has been widely accepted that CD28 is one of the most important co-stimulatory molecules on T-lymphocytes. An early study showed that the

simultaneous expression of recombinant scFv-CD3 $\zeta$  and scFv-CD28 receptors in a T-cell line resulted in signaling via both receptors and enhanced cellular activation (20). Signaling via CD28 is required for optimum IL-2 production, cell cycle progression and survival (21-23). In addition to signaling through the TCR/CD3 complex, a co-stimulatory

signal is required for full activation and proliferation of T-cells, especially unprimed or resting cells (24-26). Therefore, the signaling domain of CD28 was added to the cytoplasmic domain of L45scFv-CIR.

It is quite difficult to efficiently introduce and express genes in resting lymphocytes with viral vectors (27, 28). In the present study, the L45scFv-CIR was transfected into resting human primary T-cells using the Nucleofector™ device. The percentage of viable cells transfected with the L45scFv-CIR constructs was between 60-80%. This efficiency compares favorably with the 20-40% obtained by viral transduction of prestimulated human primary T-cells with similar constructs (27-30).

The cytokine production and cell cytotoxicity by primary T-cells expressing L45scFv-CIR remain to be investigated. Taking into consideration the results using the same three genes in the cytoplasmic region of another CIR with mouse anti-CEA scFv (31), the primary human T-cells expressing the L45scFv-CIR constructed in this study might work functionally as well. In conclusion, the L45scFv-CIR with four genes of human origin might be a useful tool to enhance effector function to resting human T-cells and to eradicate CEA-positive tumor cells in an HLA-independent manner.

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