



CELL-BASED THERAPY

RESEARCH ARTICLE

T cell activation by recombinant FcεRI γ-chain immune receptors: an extracellular spacer domain impairs antigen-dependent T cell activation but not antigen recognition

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T cells can be endowed with antigen specificity by grafting with a chimeric receptor consisting of an extracellular antigen binding moiety (scFv) derived from an antibody and an intracellular signaling domain. Conflicting data exist on the impact of an extracellular spacer domain between the antigen binding and the signaling domain with respect to cellular activation. Here, we recorded conjugate formation and antigen-driven cellular activation of T cells grafted with receptor molecules that contain the same antigen binding site (anti-CD30 HRS3-scFv) and signaling domain (FcεRI γ-chain), however, with and without an IgG1 CH2CH3 (Fc) spacer domain between the scFv and transmembrane moiety. Receptors of both configurations mediate equally efficient

conjugate formation between receptor grafted T cells and antigen-positive target cells. Specific signaling by the spacer containing receptor, however, is blocked by five- to 10-fold lower concentrations of soluble antigen than by the spacer-less receptor indicating a higher avidity of the spacer containing receptor to soluble antigen. In contrast, cellular activation upon binding to antigen-positive cells is mediated more efficiently by the spacer-less receptor. This demonstrates that the extracellular spacer domain impairs antigen-dependent cellular activation by the chimeric immune receptor, but not intercellular conjugate formation. Gene Therapy (2000) 7, 1067–1075.

Keywords: recombinant immune receptor; FcεRI γ-chain; scFv; Hodgkin's disease; CD30

Introduction

The structural prerequisites of a receptor molecule for efficient signal transduction after binding to its ligand have become increasingly interesting because of the technical practicability of recombining a chimeric receptor with pre-defined properties by fusing an extracellular ligand binding domain with a transmembrane and intracellular signalling domain. This technology has led to the development of a new immunotherapeutic strategy utilizing cytotoxic T cells that are permanently grafted with a chimeric receptor. The recombinant receptor consists of an antigen binding domain derived from an antibody and an intracellular signaling domain for cellular activation.^{1–3} The intention of the strategy is to enable the generation of specific effector cells against any antigen for which an antibody exists and combines the advantages of T cells for target cell lysis with the MHC-unrestricted antigen recognition by the antibody-derived binding domain. Based on recombinant receptor methodology, a number of receptor molecules have been generated and successfully used for antigen-specific targeting and acti-

vation of T cells towards tumor and virus infected cells *in vitro* and *in vivo*^{3,4} (for review see Ref. 5).

The minimal recombinant receptor molecule, that allows specific signaling after antigen binding, consists of an extracellular single chain fragment (scFv) antibody and a transmembrane and intracellular signalling chain derived from the FcεRI γ chain or, alternatively, from the CD3 ζ chain. Conflicting data exist, however, on the requirements of an extracellular constant domain between the antigen binding domain and the transmembrane moiety to retain stable receptor expression and function. Whereas an extracellular spacer domain turned out to be a prerequisite for stable expression of receptors with CD3 ζ chain signalling receptors,⁶ receptors with the FcεRI γ signaling chain are stable and functional without an extracellular spacer domain.⁷ Due to the lack of a rationale design and systematic determination of an optimal receptor configuration, it is still a matter of controversy whether modification of the basic receptor configuration by insertion of an heterologous protein spacer domain affects binding to ligand and/or transduction of the cell activation signal. Recently, Patel *et al*⁸ reported that different extracellular spacer domains of a recombinant receptor harboring a CD3 ζ signaling domain impair receptor-mediated target cell lysis. The stability of ζ chain receptor expression itself, however, depends substantially on an appropriate spacer domain. Therefore, and

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because γ chain signaling receptors do not require a spacer domain for stable expression and function, we used a γ chain receptor to investigate whether an extracellular spacer protein between antigen binding and signaling domain affects antigen binding and/or antigen-dependent cellular activation. In a comparative analysis, we recorded T cell activation by two recombinant receptors that contain the same antigen binding site (anti-CD30 HRS3-scFv) and signaling domain (Fc ϵ RI γ -chain), however, with and without an IgG1 CH2CH3 (Fc) spacer domain between the scFv and transmembrane moiety.^{7,9} Murine T cells and human peripheral blood T cells were grafted with both receptors and compared with respect to: (1) their ability to mediate intercellular conjugate formation; (2) their signaling capacity in the presence of soluble receptor ligand; and (3) their antigen driven cellular activation, ie, proliferation and cytokine secretion, of grafted cells. We demonstrate here that, in the situation of this system of recombinant immune receptors, antigen-dependent conjugate formation between receptor grafted cells and antigen-positive target cells is not affected by the spacer domain, the efficiency in receptor-mediated signaling, however, is substantially impaired by the extracellular constant domain. These studies have implications for the rationale design of recombinant receptors and for the understanding of the potential function of spacer domains in natural receptor molecules.

Results

Characterization of the chimeric HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor

We generated two chimeric membrane receptors, HRS3-scFv- γ and HRS3-scFv-Fc- γ , with identical extracellular binding (HRS3-scFv) domains specific for the CD30 antigen and with identical intracellular signaling domains (γ chain of the Fc ϵ RI receptor).⁷ The antigen binding domain HRS3-scFv, derived from the anti-CD30 mAb HRS3, is joined either directly or via the hinge and CH2CH3 region (Fc) of human IgG1 to the transmembrane and intracellular γ chain (Figure 1). After transfection into MD45 cells, we isolated cell clones (HRS3-scFv- γ F8; HRS3-scFv-Fc- γ 2C11) that stably express the recombinant HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor, respectively. Titration experiments and FACS analysis utilizing the internal image anti-HRS3 idiotype mAb 9G10 demonstrate that both cell lines express similar amounts of the recombinant receptor on the cell surface (Figure 2a). However, at non-saturating antibody concentrations the HRS3-scFv-Fc- γ receptor reacts more strongly with the anti-idiotype mAb than the HRS3-scFv- γ receptor indicating a slightly higher avidity of the HRS3-scFv-Fc- γ receptor (Figure 2a). We recently determined the idiotype profile of the HRS3 scFv domain¹⁰ that was found to be the same as that of the parental mAb HRS3. To test whether the receptor configuration alters the idiotope of the antigen binding domain, we analyzed the idiotype profile of the HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor, respectively, utilizing a panel of four anti-idiotype mAbs (9G10, 12D3, 14B12, 14G9) that define at least three different idiotypes of the HRS3 mAb binding domain.^{10–12} Figure 2b summarizes binding of the four anti-idiotype mAbs to HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor grafted MD45 cells as monitored by FACS

a expression cassette for HRS3-scFv- γ



b expression cassette for HRS3-scFv-Fc- γ

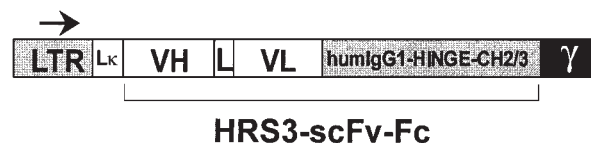


Figure 1 Design of the chimeric anti-CD30 receptors. (a) The anti-CD30 receptor HRS3-scFv- γ consists of the CD30 antigen binding domain HRS3-scFv, derived from the mAb HRS3, and the γ transmembrane and signaling domain, derived from the Fc ϵ RI receptor. The scFv and the γ chain are fused together via a nine amino acid linker. (b) The anti-CD30 receptor HRS3-scFv-Fc- γ harbors the same scFv antigen binding and γ chain transmembrane and signaling domain but contains a human IgG1 CH2CH3 (Fc) domain inserted in between.

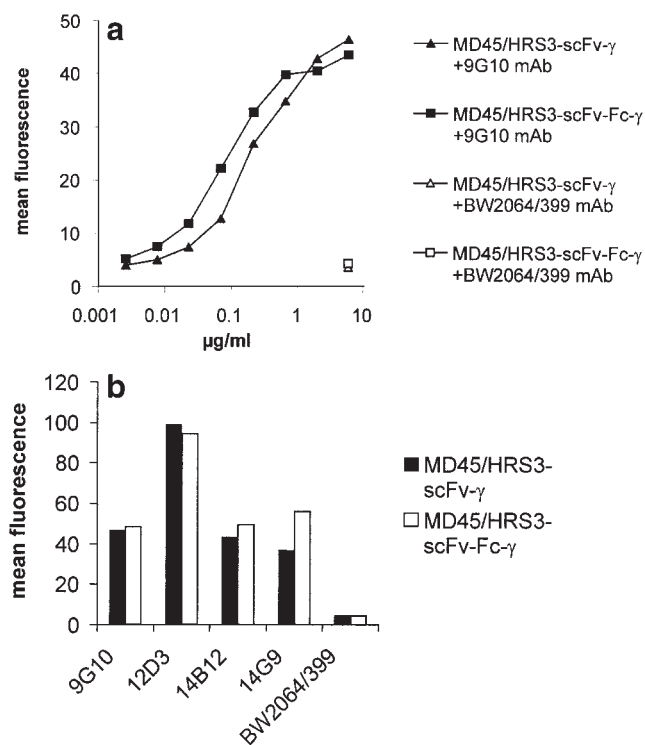


Figure 2 FACS analysis of receptor grafted MD45 T cells. (a) HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor grafted MD45 T cells (5×10^5) were stained with different amounts (0.002–6 μ g/ml) of the anti-HRS3 idiotype mAb 9G10 (solid symbols) or, for control, an anti-idiotype mAb with specificity for the mAb BW431/26 (open symbols). (b) HRS3-scFv- γ (black bars) and HRS3-scFv-Fc- γ (white bars) receptor grafted MD45 T cells, respectively, were incubated with the anti-HRS3 idiotype mAbs 9G10, 12D3, 14B12, 14G9, and, for control, the BW2064/399 mAb with irrelevant specificity (each 6 μ g mAb/ml). Bound mAbs were detected by a FITC-conjugated F(ab')₂-anti-mouse IgG1 antibody (1 μ g/ml) and the mean fluorescence was determined.

analysis utilizing saturating antibody concentrations (6 $\mu\text{g}/\text{ml}$). The HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor reacted in a similar pattern to the tested anti-idiotypic antibodies indicating that the idiotype profile is unlikely to be influenced by the receptor configurations.

Immunoprecipitation with the anti-HRS3 idiotype mAb 9G10 and Western blot analysis under non-reducing conditions with an anti- γ chain-specific antibody revealed a protein band with an apparent molecular mass of about 160 kDa and 80 kDa, respectively, corresponding to the predicted sizes of homodimers of the HRS3-scFv-Fc- γ and HRS3-scFv- γ receptor, respectively (data not shown). Accordingly, the protein band corresponding to the HRS3-scFv-Fc- γ receptor dimer reacts with an anti-human IgG1 antibody whereas the protein band corresponding to the HRS3-scFv- γ receptor does not (data not shown). No receptor monomers were detected implying that virtually all receptor molecules of both configurations are present as homodimers on the cell surface.

Conjugate formation of receptor grafted T cells

To elucidate whether different receptor configurations, ie with and without an extracellular protein spacer domain, affect binding of receptor grafted cells to cells expressing the corresponding ligand, we monitored conjugate formation between HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor grafted MD45 T cells, respectively, and CD30^{high} (L540) and CD30^{low} (Jurkat) target cells. L540 tumor cells (CD30^{high}) react more than 10 times higher with the anti-CD30 mAb HRS3 than Jurkat cells (CD30^{low}) (data not shown). For comparison reasons, we used receptor grafted MD45 T cells with similar expression levels of the HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor, respectively. Receptor grafted MD45 cells were labeled with the green fluorochrome PKH2; CD30^{high} L540 and CD30^{low} Jurkat cells were labeled with the red fluorochrome PKH26. Conjugates formed by receptor grafted MD45 T cells with CD30⁺ cells (red plus green fluorescence) were analyzed by flow cytometry. The specificity of conjugate formation was assessed by competition with the anti-idiotypic mAb 9G10 and an isotype-matched control mAb, respectively. As shown exemplarily in Figure 3a, coinubation of PKH2-labeled, receptor grafted MD45 T cells with PKH26-labeled CD30^{high} L540 cells resulted in intercellular conjugate formation that is specifically blocked in the presence of the anti-idiotypic mAb 9G10 but not in the presence of a control IgG antibody. We obtained 12.2% and 8.4% specific cellular conjugates, respectively, after co-incubation of L540 cells with HRS3-scFv-Fc- γ and HRS3-scFv- γ receptor grafted cells, respectively, demonstrating that both receptors mediate nearly equally efficient intercellular conjugate formation (Figure 3b). Conjugate formation is dependent on the density of ligand on the surface of targeted cells since specific conjugates of receptor grafted cells were only observed with CD30^{high} L540 but not with CD30^{low} Jurkat cells (Figure 3b).

Activation of receptor grafted cells in the presence of soluble ligand

The anti-CD30 receptor in both configurations, ie HRS3-scFv-Fc- γ and HRS3-scFv- γ , mediates cellular activation upon crosslinking with highly complexed, plastic-immobilized antigen (data not shown). To assess the avidity

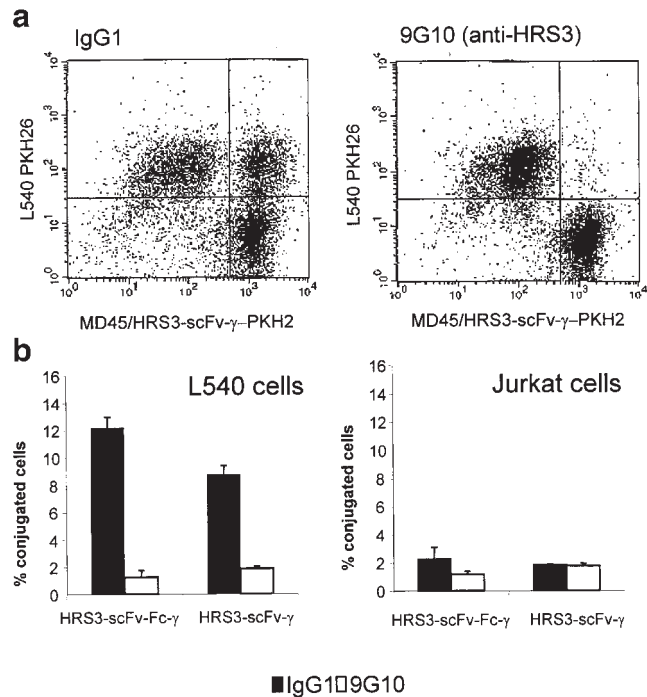


Figure 3 Conjugate formation of receptor grafted MD45 T cells with L540 tumor cells. Receptor grafted MD45 T cells (1×10^5) were labeled with PKH2 and co-incubated with PKH26-labeled CD30⁺ L540 tumor cells (1×10^5) in the presence of an isotype-matched IgG1 mAb or the anti-HRS3 idiotype mAb 9G10 (each 10 $\mu\text{g}/\text{ml}$). Cells were analyzed by flow cytometry and conjugated cells were identified by dual fluorescence. (a) Dot plot analysis of conjugated effector and target cells. HRS3-scFv- γ receptor grafted MD45 T cells form specific conjugates with CD30⁺ L540 target cells. Conjugate formation is prevented in the presence of the anti-HRS3 idiotype mAb 9G10 (right) but not in the presence of an isotype-matched control IgG1 mAb (left). (b) Summary of conjugate-forming assays. HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor grafted MD45 T cells, respectively, were co-incubated with CD30^{high} L540 tumor cells (left) or CD30^{low} Jurkat cells (right) in the presence of an isotype-matched control IgG1 mAb (black bars) or of the anti-HRS3 idiotype mAb 9G10 (white bars). The assay was done in triplicate and the s.e.m. (standard error of means) was determined.

of the receptor in different configurations to ligand, we stimulated receptor grafted MD45 cells by immobilized CD30 antigen and anti-idiotypic mAb 9G10, respectively, in the presence of increasing amounts of soluble CD30 antigen or mAb 9G10. As summarized in Figure 4, up to 10 times higher concentrations of soluble CD30 antigen and 9G10 mAb, respectively, are required to inhibit specific activation of HRS3-scFv- γ compared with HRS3-scFv-Fc- γ receptor grafted cells reflecting again a higher avidity of the HRS3-scFv-Fc- γ receptor for soluble antigen than the HRS3-scFv- γ receptor. On the other hand, the HRS3-scFv-Fc- γ receptor is blocked by lower concentrations of soluble antigen than the HRS3-scFv- γ receptor, thus preventing cellular activation more efficiently. Although both receptors are equipped with identical antigen binding domains that both conserve the idiotype profile of the parental mAb (cf. Figure 2), the protein spacer domain obviously alters the apparent avidity to soluble ligand.

The spacer domain affects cellular activation of receptor grafted T cells by coculture with CD30⁺ tumor cells

Since the extracellular spacer domain of the receptor affects binding to soluble ligand, but not cellular conju-

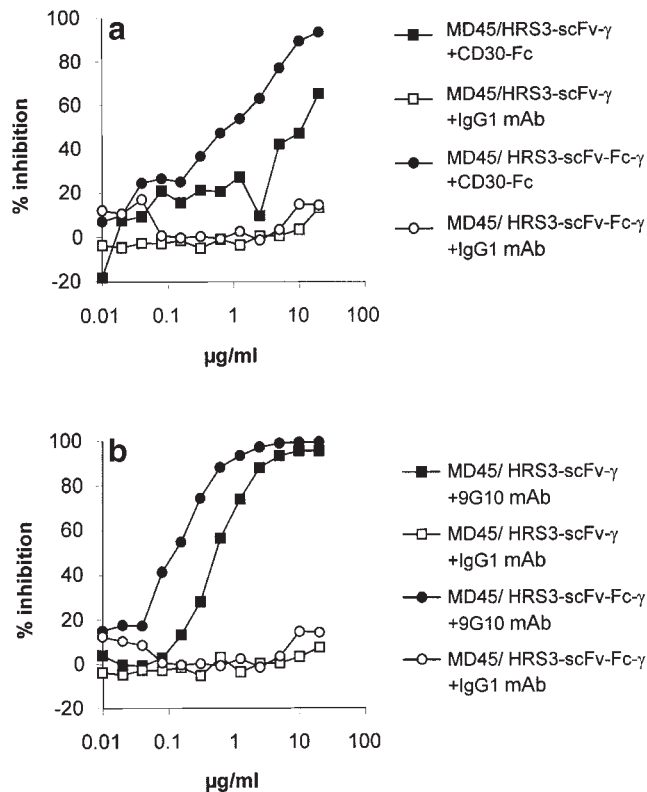


Figure 4 Inhibition of cellular activation by soluble antigen and anti-idiotypic mAb. (a) Recombinant CD30-Fc protein (1 μ g/ml) was coated on to 96-well ELISA plates. HRS3-scFv- γ (squares) and HRS3-scFv-Fc- γ (circles) receptor grafted MD45 T cells (1×10^5 cells per well) were incubated for 48 h in the presence of soluble CD30-Fc protein (closed symbols) or, for control, of an IgG1 mAb (open symbols). (b) The anti-HRS3 idiotype mAb 9G10 (1 μ g/ml) was coated on to 96-well ELISA plates. HRS3-scFv- γ (squares) and HRS3-scFv-Fc- γ (circles) receptor grafted MD45 T cells (1×10^5 cells per well) were incubated for 48 h in the presence of soluble mAb 9G10 (closed symbols) or an isotype-matched IgG1 control mAb (open symbols). IL-2 secreted into culture supernatant was determined by ELISA. Binding inhibition was calculated as follows: inhibition (%) = $(1 - \text{IL-2 concentration in the presence of competitor} / \text{IL-2 concentration without competitor}) \times 100$.

gate formation, we assessed receptor-mediated cellular activation by cell-bound membrane antigen. HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor grafted MD45 cells, respectively, with similar amounts of receptor molecules on the cell surface were cocultured with CD30^{high} L540 cells, CD30^{low} Jurkat cells, and, as control, with CD30^{low} BL60 cells, respectively. Cellular activation was monitored by determining murine IL-2 secreted by receptor grafted MD45 T cells into the culture supernatant. As summarized in Figure 5a, HRS3-scFv- γ receptor grafted MD45 cells were efficiently activated by coculture with L540 cells, but only little by coculture with Jurkat cells. Coculture of HRS3-scFv-Fc- γ receptor grafted MD45 cells with L540 cells resulted in low IL-2 secretion; no IL-2 secretion was detected after coculture with Jurkat cells (Figure 5b). This demonstrates that specific activation of receptor grafted cells by coculture with CD30⁺ cells is five to six times more efficiently mediated by the HRS3-scFv- γ receptor than by the HRS3-scFv-Fc- γ receptor. We conclude that the extracellular spacer domain of the receptor substantially affects cellular activation of grafted cells upon binding to membrane-bound antigen.

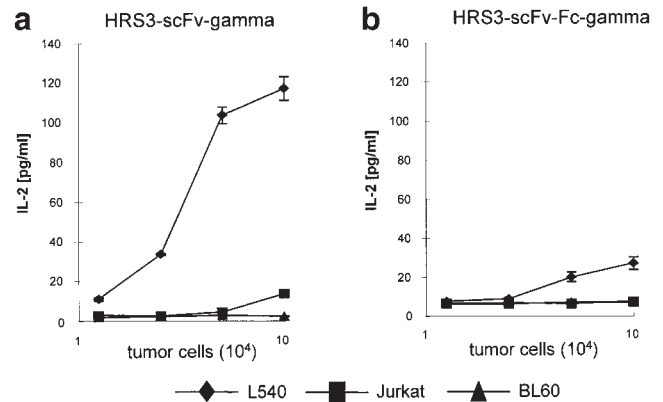


Figure 5 Cellular activation of HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor grafted MD45 cells upon coculture with CD30⁺ L540 tumor cells. HRS3-scFv- γ (a) and HRS3-scFv-Fc- γ (b) receptor grafted MD45 T cells (each 1×10^5 cells per well), respectively, were cocultured for 48 h in round bottom microtiter plates with CD30^{high} L540, CD30^{low} Jurkat, and CD30^{low} BL60 tumor cells (1.25×10^4 – 10×10^4 cells per well). IL-2 in the supernatant was determined by ELISA. The assay was done in triplicate and the s.e.m. was determined.

Cellular activation of receptor grafted peripheral blood T cells

To assess the validity of data obtained from stable transduced cell clones, we tested bulk cultures of peripheral blood T cells grafted with the HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor, respectively. Peripheral blood T cells were transduced by infection with GALV-pseudotyped retroviruses containing the expression cassettes for the HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor, respectively. Two-color fluorescence analysis utilizing the anti-HRS3-specific idiotype mAb 9G10 and the anti-CD3 mAb OKT3 identified T cells expressing the HRS3-scFv- γ and the HRS3-scFv-Fc- γ receptor, respectively (Figure 6). To compare the capability of the receptors to mediate cytokine secretion and T cell proliferation upon specific receptor crosslinking, we cultured HRS3-scFv- γ (14.8%) and HRS3-scFv-Fc- γ (16.1%) receptor grafted peripheral blood T cells, respectively, in microtiter plates that were coated with serial dilutions of CD30 antigen and determined simultaneously IFN- γ secretion and BrdU incorporation. As shown in Figure 7, specific crosslinking of the HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor, respectively, resulted in dose-dependent secretion of IFN- γ into the culture supernatant indicating efficient receptor-mediated activation of peripheral blood T cells. However, HRS3-scFv- γ receptor stimulated secretion of IFN- γ was more efficient and reached a higher maximum level than by the HRS3-scFv-Fc- γ receptor (Figure 7a). Stimulation of antigen-driven proliferation, as determined by BrdU incorporation, was more effective in HRS3-scFv-Fc- γ receptor grafted cells than in HRS3-scFv- γ receptor cells at low antigen concentrations whereas the plateau of maximal proliferation was similar for both receptor grafted cells (Figure 7b). Taken together, these data indicate that the tested cell activation parameters, ie IFN- γ secretion and antigen-driven proliferation, are differentially induced with respect to the receptor configurations.

To confirm these observations with peripheral blood T cells of another donor, we transduced T cells with initial transduction ratios of 20% of T cells expressing the HRS3-scFv- γ and 17% of T cells expressing the HRS3-scFv-Fc- γ

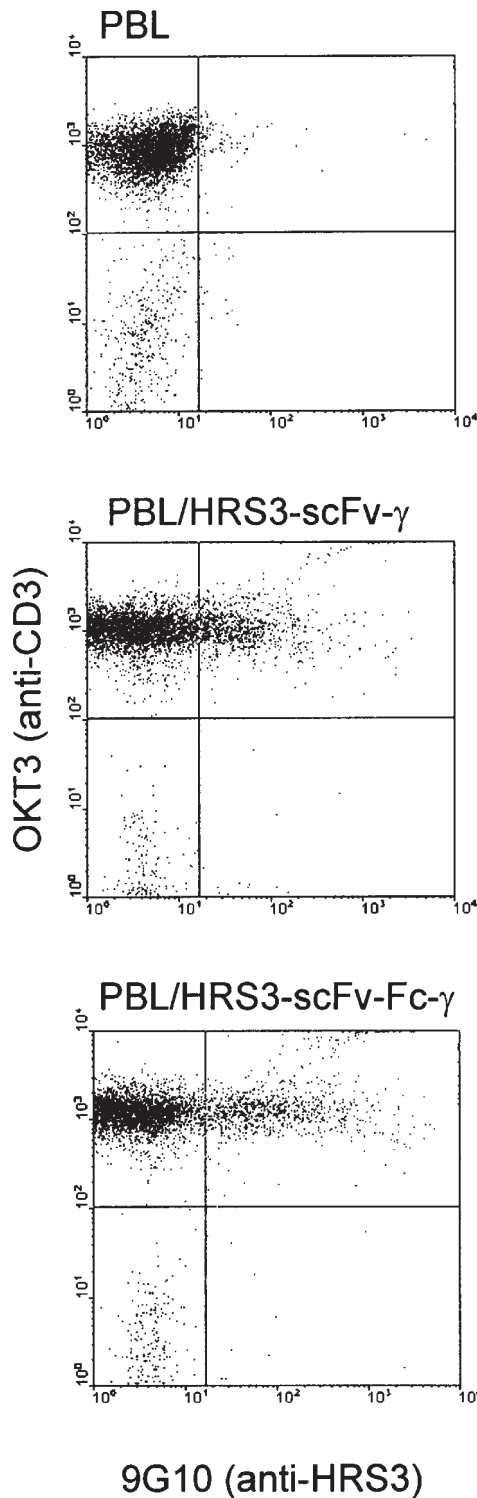


Figure 6 FACS analysis of peripheral blood T cells grafted with chimeric receptors. Peripheral blood T cells were transduced with GALV-pseudotyped retroviruses containing the pSTITCH vector that carries the expression cassettes for the HRS3-scFv- γ receptor and the HRS3-scFv-Fc- γ receptor, respectively. Transduced and non-transduced T cells were analyzed by two-color fluorescence utilizing the anti-CD3 mAb OKT3 (IgG2a) and the anti-HRS3 idiotypic mAb 9G10 (IgG1) (each 5 μ g/ml), respectively. Bound antibodies were detected by FITC-conjugated F(ab')₂-anti-mouse IgG1 and PE-conjugated F(ab')₂-anti-mouse IgG2a antibodies, respectively.

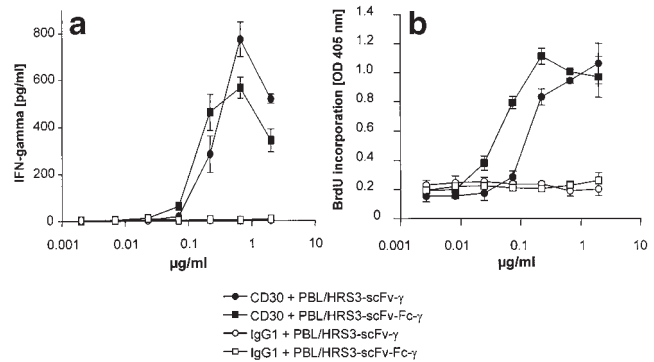


Figure 7 Cellular activation of HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor grafted peripheral blood T cells after binding to immobilized CD30 antigen. HRS3-scFv- γ (circles) and HRS3-scFv-Fc- γ (squares) receptor grafted peripheral blood T cells (each 1×10^5 cells per well) were incubated for 48 h in microtiter plates coated with increasing amounts of the CD30-Fc protein (solid symbols), or a control IgG1 antibody (open symbols), respectively. IFN- γ secretion into the supernatant was determined by ELISA (a). Cell proliferation was determined by monitoring BrdU incorporation (b). The assay was done in triplicate and the s.e.m. was determined.

γ receptor, respectively. Upon subsequent enrichment of receptor expressing T cells by MACS utilizing the anti-idiotypic mAb 9G10, we obtained 65% of T cells that express the HRS3-scFv- γ receptor and 67% of T cells that express the HRS3-scFv-Fc- γ receptor (data not shown). MACS-enriched HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor grafted T cells, respectively, were co-incubated with CD30⁺ L540 and CD30⁻ BL60 target cells, respectively. IFN- γ secreted into the culture supernatant was determined (Figure 8). The kinetics of IFN- γ secretion induced upon antigen-specific receptor crosslinking was found to differ in HRS3-scFv- γ compared with HRS3-scFv-Fc- γ receptor grafted T cells. Signaling by the HRS3-scFv-Fc- γ receptor results in substantially lower amounts of secreted IFN- γ than by the HRS3-scFv- γ receptor. In a second set of experiments, MACS-enriched HRS3-scFv- γ

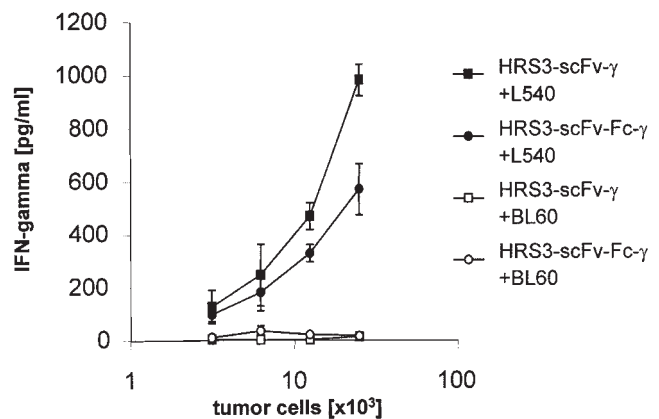


Figure 8 Cellular activation of MACS-enriched receptor grafted peripheral blood T cells upon coculture with CD30⁺ L540 tumor cells. HRS3-scFv- γ (squares) and HRS3-scFv-Fc- γ (circles) receptor grafted T cells (each 1×10^5 cells per well) were cocultured for 48 h in round bottom microtiter plates with CD30⁺ L540 (closed symbols) or CD30⁻ BL60 (open symbols) tumor cells, respectively. The supernatants were assayed for IFN- γ by ELISA. The assay was done in triplicate and the s.e.m. was determined.

and HRS3-scFv-Fc- γ receptor grafted T cells, respectively, were cultured in microtiter plates coated with CD30 antigen in a concentration that induced maximum IFN- γ secretion (cf. Figure 7) and IFN- γ secretion and cellular proliferation was determined simultaneously. Additionally, cells were stimulated in microtiter plates coated with the anti-HRS3 idiotype mAb 9G10 as a surrogate antigen. As summarized in Figure 9, specific crosslinking of the HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor, respectively, resulted in secretion of high amounts of IFN- γ into the culture supernatant indicating efficient cellular activation (Figure 9a). However, HRS3-scFv- γ receptor-mediated IFN- γ secretion was more efficient than activation by the HRS3-scFv-Fc- γ receptor independently of the immobilized ligand utilized for receptor crosslinking. In contrast, receptor-induced T cell proliferation at ligand concentrations that induce maximum IFN- γ secretion was equally efficient for both types of receptors (Figure 9b). These observations obtained by MACS-enriched receptor grafted cells are in accordance with the data obtained by non-enriched T cell preparations (cf. Figure 7).

We compared the cytolytic activity of HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor grafted T cells utilizing target cells that were labeled with BrdU before coculture with receptor grafted cells. Both transduced T cell populations contained about 20% receptor expressing T cells. BrdU-labeled L540 (CD30⁺) and BL60 (CD30⁻) tumor cells, respectively, were cocultured for 48 h with HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor transduced T cells at an effector:target (E:T) cell ratio of 2:1. The content of BrdU-labeled fragmented DNA in the supernatant was determined by ELISA. As demonstrated in Figure 10, both receptors mediate antigen-specific lysis of CD30⁺ target cells (Figure 10a) whereas no lysis of CD30⁻ tumor cells was observed (Figure 10b). HRS3-scFv- γ receptor-mediated cytotoxicity, however, was more efficient than

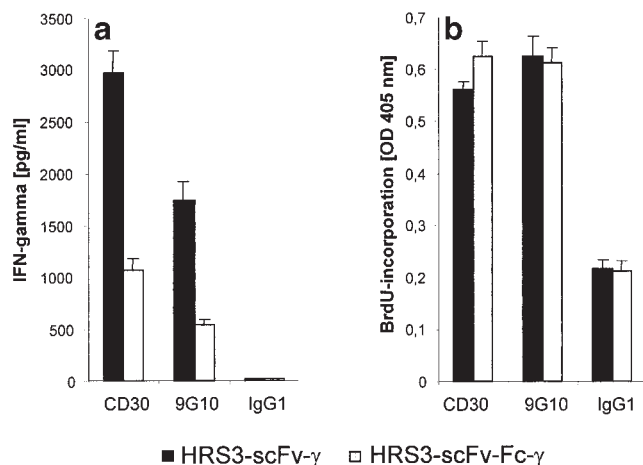


Figure 9 Cellular activation of MACS-enriched HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor grafted peripheral blood T cells after binding to immobilized ligand. HRS3-scFv- γ (black bars) and HRS3-scFv-Fc- γ (white bars) receptor grafted peripheral blood T cells (each 1×10^5 cells per well) were incubated for 48 h in microtiter plates coated with CD30-Fc protein, the anti-idiotype mAb 9G10, or a control IgG1 antibody (each 2 μ g/ml), respectively. IFN- γ secretion was determined by ELISA (a). Cell proliferation was determined by monitoring BrdU incorporation (b). The assay was done in triplicate and the s.e.m. was determined.

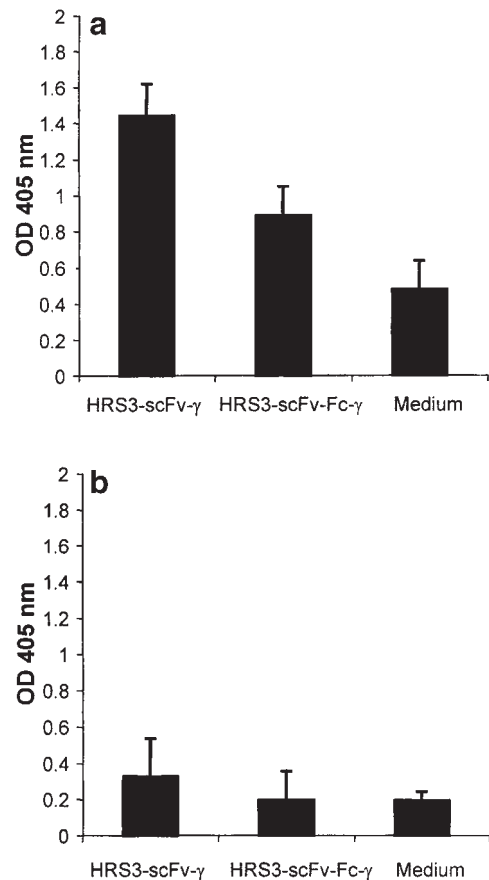


Figure 10 Target cell lysis of HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor grafted peripheral blood T cells. HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor grafted peripheral blood T cells (each 1×10^5 cells per well) were cocultured for 48 h in microtiter plates with BrdU-labeled CD30⁺ L540 cells (a) and CD30⁻ BL60 cells (b) (each 5×10^4 cells per well). The supernatant was harvested and analyzed for BrdU-labeled fragmented DNA by ELISA. The assay was done in triplicate and the s.e.m. determined.

HRS3-scFv-Fc- γ receptor-mediated lysis of CD30⁺ target cells.

Taken together, our data indicate that the recombinant T cell receptor in both configurations induces cellular activation of peripheral blood T cells. Although with different kinetics, signaling by the HRS3-scFv-Fc- γ receptor is equally effective in induction of antigen-driven cell proliferation at the maximum of cellular activation but less effective in induction of IFN- γ secretion than signaling by the HRS3-scFv- γ receptor. The HRS3-scFv-Fc- γ receptor, however, mediates cellular proliferation more efficiently than the HRS3-scFv- γ receptor at antigen concentrations that do not result in the maximum of cellular activation. This may reflect a higher avidity of the HRS3-scFv-Fc- γ receptor and a different kinetic of cell proliferation and IFN- γ secretion. Moreover, the protein spacer domain within the extracellular moiety of the receptor dramatically alters the binding properties to soluble ligand and decreases the efficiency in cellular activation upon binding to antigen, especially in the presence of soluble antigen. Because conjugate formation between receptor grafted and ligand cells is not affected, we conclude that the altered receptor configuration downstream of the antigen binding domain significantly impairs sig-

naling properties without affecting binding to antigen expressing target cells.

Discussion

In this report we compared two recombinant T cell receptors, ie HRS3-scFv- γ and HRS3-scFv-Fc- γ receptor, with different configurations but identical ligand binding and cellular activation domains with respect to the following properties: (1) specific formation of intercellular conjugates; (2) specific binding to immobilized and membrane-bound ligand; and (3) cellular activation initiated by receptor crosslinking. The comparative analysis is based on receptor grafted peripheral blood T cells and on receptor grafted MD45 cell clones that express about the same number of receptor molecules on the cell surface. A number of properties are identical for both receptor configurations: (1) the HRS3-scFv antigen binding domains of both receptors have retained the idiotypic profile of the parental mAb HRS3 as determined by binding to a panel of anti-idiotypic mAbs; (2) both receptors are expressed entirely as homodimers on the surface of grafted T cells; (3) both receptors mediate cellular activation after binding to immobilized CD30 antigen and anti-HRS3 idiotypic antibody 9G10, respectively; and (4) both receptors mediate specific intercellular conjugate formation between receptor grafted cells and CD30⁺ cells. Receptor-mediated cellular activation by immobilized antigen in the presence of soluble antigen, however, revealed a higher avidity of the HRS3-scFv-Fc- γ receptor to soluble ligand compared with the HRS3-scFv- γ receptor. This indicates that insertion of the Fc spacer domain into the extracellular moiety of the receptor alters the binding property of receptor grafted cells to soluble antigen. Most strikingly, both receptor configurations differ in their ability to mediate cellular activation upon co-incubation with CD30⁺ cells resulting in cytokine secretion and target cell lysis, ie the HRS3-scFv- γ receptor mediates cytokine secretion and cytolysis more efficiently than the HRS3-scFv-Fc- γ receptor. Clonal differences of receptor grafted cells are unlikely to account for the different activation patterns upon receptor crosslinking because the same activation properties were recorded for receptor grafted mouse MD45 T cell clones as well as for bulk cultures of receptor grafted peripheral blood T cells. We conclude that the differences in the efficiency to mediate antigen-driven cellular activation are due to intrinsic properties of the receptor configuration itself. Strikingly, CD30 antigen-driven T cell proliferation is mediated equally efficient by both receptor configurations when stimulated by antigen concentrations that induce maximum T cell activation. At lower antigen concentrations, however, the HRS3-scFv-Fc- γ receptor induces T cell proliferation more efficiently than the HRS3-scFv- γ receptor indicating that the kinetics of receptor-mediated cytokine secretion and cell proliferation differ significantly.

Our findings have substantial implications for the understanding of the modular composition of immune receptor molecules. (1) Modification of the basic scFv-signaling chain receptor configuration by insertion of an IgG-derived spacer domain into the extracellular part of the receptor may enhance the stability of receptor expression but substantially impairs its signaling properties upon antigen-specific crosslinking. This modification, however, does not affect intercellular conjugate forma-

tion between receptor grafted cells and antigen-positive target cells. (2) The extracellular constant spacer domain of a chimeric receptor affects its avidity and therefore its signaling properties in the presence of the soluble ligand. This property may also impair its binding to membrane-bound antigen in the presence of soluble tumor antigen and has significant impact on the clinical application of the chimeric receptor strategy in adoptive immunotherapy of malignant diseases. In the presence of high concentrations of soluble tumor-associated antigen, that are frequently found in sera of tumor patients, a receptor molecule with high binding capacity for soluble antigen is more likely to be blocked than a receptor with lower avidity with the consequence that cellular activation by a high avidity receptor is prevented and the therapeutical efficacy is reduced by soluble antigen.

Recombinant immune receptors with the Fc ϵ RI- γ signaling chain are stable and functional without an extracellular spacer domain between the scFv domain and the γ chain. This property stands in contrast to CD3 ζ chain signaling receptors that need an extracellular constant spacer domain for stable expression.⁶ Like γ chain receptors, the spacer domain affects the affinity of ζ chain receptors for ligand and impairs receptor-mediated cytolysis.⁸ Therefore, the high impact of an extracellular constant domain on the signaling function of recombinant receptors calls for a more rational design of the receptor molecule based on the modular components, particularly the signaling domain. Due to stable expression without the necessity of a spacer domain, we suggest that the basic scFv- γ chain receptor configuration is preferred in the application of the chimeric receptor strategy in order to avoid that the extracellular spacer domain itself impairs receptor-mediated signaling and cellular activation.

Compared with the natural T cell receptor complex, recombinant immune receptors exhibit several structurally and functionally based differences. Whereas recombinant receptors with an antibody-derived binding domain are designed to recognize antigens of any structure and chemical composition for which an antibody exists, the natural TCR complex recognizes its specific peptide antigen exclusively in the context of the MHC. Due to marginal differences in the overall structure of peptide-MHC-TCR complexes, the natural TCR allows generalization of the antigen recognition and signaling process. On the other hand, the structural variety of target antigens potentially recognized by the scFv domain of recombinant receptors also implies a high diversity in the recognition process of target antigens. This diversity is moreover potentiated by recombination with different signaling domains for cellular activation. In this situation it has to be taken into account that, besides the structural variety of recognized antigens, the receptor configuration itself, without changing its antigen-binding and signaling domains, also has substantial impact on the recognition and signaling properties. Our analysis provides a rationale in the design of γ chain receptors for MHC-independent T cell targeting in order to use the advantages of the recombinant receptor methodology for immunotherapeutic strategies.

Materials and methods

Cell lines and antibodies

MD45 is a murine T cell hybridoma line,² L540 is a CD30⁺ human Hodgkin's lymphoma-derived cell line,¹³ BL60 is a CD30⁺ Burkitt's lymphoma-derived cell line,¹⁴ Jurkat is a CD30⁺ T cell leukemia line (obtained from the ATCC, Rockville, MD, USA; ATCC TIB 152). All cell lines were cultured in RPMI 1640 supplemented with 10% (v/v) heat inactivated FCS (Gibco, Eggenstein, Germany). The human CD30-Fc fusion protein¹⁵ was purified from supernatants of transfected CHO cells and myeloma cells, respectively, by affinity chromatography on anti-human IgG agarose (Sigma, Deisenhofen, Germany). The anti-CD3 mAb OKT3 was obtained from the ATCC (ATCC CRL 8001). The anti-CD30 mAb HRS3, the anti-HRS3 idiotypic mAbs 9G10, 12D3, 14G9, 14B12 and the anti-idiotypic mAb BW2064/399 with specificity for an anti-CEA mAb have been described elsewhere.^{11,12,16,17}

Chimeric receptors

The generation of the CD30-specific HRS3-scFv- γ receptor has been recently described,^{7,18} the HRS3-scFv-Fc- γ receptor was obtained by replacing the scFv domain within the BW431/26-scFv-Fc- γ receptor⁹ by the HRS3-scFv domain. Briefly, the HRS3-scFv DNA was flanked by *Xba*I (5') and *Bam*HI (3') restriction sites, respectively, by PCR techniques utilizing the following primer oligonucleotides: 5'-GCG GCC CAG TCT AGA ATG GCC CAG-3' (sense); 5'-ACC TGG ATC CGC CCG TTT GAT TTC-3' (antisense) (restriction sites underlined). The BW431/26-scFv- γ DNA was digested with *Xba*I and *Bam*HI and the BW431/26-scFv DNA was replaced by the HRS3-scFv DNA. The expression cassette is under the control of the RSV LTR.² MD45 T cells (1×10^7) were transfected with pRSV-HRS3-scFv-Fc- γ DNA and pRSV-HRS3-scFv- γ DNA, respectively, and cell clones with stable expression of the chimeric receptor were isolated.⁷

Expression of chimeric receptors in peripheral blood T cells

For retroviral transduction of peripheral T cells, the receptor expression cassettes (HRS3-scFv- γ ; HRS3-scFv-Fc- γ) were inserted into the retroviral vector pSTITCH.¹⁹ Briefly, HRS3-scFv- γ ; and HRS3-scFv-Fc- γ were DNA were flanked by *Nco*I (5') and *Bgl*II (3') restriction sites, respectively, by PCR techniques utilizing the following primer oligonucleotides: 5'-TGA TCC ATG GAC TAG TAC GTA ATG GAT TTT CAG GTG CAG ATT TTC-3' (sense); 5'-GGC AGA TCT GAT CAG TCG ACT CTA AAG CTA CTG TGG TGG-3' (antisense) (restriction sites are underlined). PCR amplified DNA was digested and inserted into the *Nco*I and *Bam*HI sites of the vector pSP72 that contains parts of the MMLV splice-acceptor and LTR. The recombinant DNA was inserted into the *Bgl*II and *Xho*I sites of the vector E3qB constituting the MMLV-derived retroviral expression vectors pSTITCH-HRS3-scFv- γ and pSTITCH-HRS3-scFv-Fc- γ , respectively. To generate GALV-pseudotyped retrovirus, the recombinant pSTITCH vector DNA was cotransfected with the retroviral helper plasmid DNA pHIT and pCOLT, encoding the MMLV gag and pol genes and the GALV envelope gene, respectively, into 293T cells.¹⁹ This procedure results in transient production of high titers of infectious retrovirus. Peripheral blood lymphocytes from healthy donors

were isolated by density centrifugation and cultured for 48 h in RPMI 1640 medium, 10% (v/v) FCS in the presence of 400 U/ml IL-2 (Endogen, Woburn, MA, USA) and 100 ng/ml anti-CD3 mAb (OKT3). For retroviral transduction, cells were harvested, resuspended in medium with 400 U/ml IL-2, and cocultured for 48 h with transfected 293T cells. Cells were harvested and receptor expression was monitored by flow cytometry as described below.

Immunofluorescence and flow cytometry

Recombinant receptor expressing cells were identified utilizing a panel of HRS3-mAb specific anti-idiotypic mAbs. T cells (5×10^5 per test) were incubated with different amounts of anti-idiotypic mAbs (0.002–6 μ g/ml) and subsequently with a FITC-conjugated F(ab')₂ anti-mouse antibody (1 μ g/ml) (Immunotech, Hamburg, Germany). CD30 expression of tumor cells was monitored by a FITC-conjugated anti-CD30 mAb BerH2 (5 μ g/ml; Dako, Hamburg, Germany). For two-color immunofluorescence, peripheral T cells were simultaneously incubated with the anti-idiotypic mAb 9G10 (IgG1) and the anti-CD3 mAb OKT3 (IgG2a) (each 5 μ g/ml) and subsequently with a FITC-conjugated F(ab')₂ anti-mouse IgG1 antibody (1 μ g/ml) and a phycoerythrin (PE)-conjugated F(ab')₂ anti-mouse IgG2a antibody (0.5 μ g/ml) (both Southern Biotechnology, Birmingham, AL, USA). Cells were analyzed utilizing a FACScan cytofluorometer equipped with the Cellquest analysis software (Becton Dickinson, Mountain View, CA, USA).

Magnetic activated cell sorting (MACS)

T cells expressing the recombinant receptor were enriched by MACS utilizing the anti-HRS3 idiotypic mAb 9G10. Briefly, transduced T cells were washed with cold PBS, 0.5% (w/v) BSA and incubated with mAb 9G10 (10 μ g/ml) for 15 min on ice, washed twice with cold PBS, 0.5% (w/v) BSA, and subsequently incubated with anti-mouse IgG magnetic beads (Miltenyi Biotech, Bergisch-Gladbach, Germany) for 15 min on ice. Cells were washed twice and separated on magnetic columns utilizing a mini-MACS separator (Miltenyi Biotech).

Conjugate formation assay

The membrane of receptor grafted MD45 cells and of L540 target cells, respectively, was labeled with the green fluorescent dye PKH2 and the red fluorescent dye PKH26 (Sigma), respectively, according to the manufacturer's recommendations. PKH2-labeled MD45 cells (1×10^5) and PKH26-labeled L540 cells (1×10^5) were co-incubated in cold PBS in the presence of the anti-idiotypic mAb 9G10 or an isotype-matched IgG1 control mAb (10 μ g/ml each), sedimented for 15 s at 1000 g, and incubated for 15 min on ice. The cells were carefully resuspended by five times pipetting up and down and analyzed by flow cytometry. Double fluorescent particles represent conjugated MD45-L540 cells.

Specific activation of receptor grafted T cells

To stimulate receptor grafted cells by immobilized antigen, ELISA plates (Polysorb; Nunc, Wiesbaden, Germany) were coated with different amounts of recombinant CD30-Fc protein, anti-HRS3-idiotypic mAb 9G10, or an IgG1 mAb with irrelevant specificity for control,

respectively, and incubated with cells (10^5 cells per well) for 48 h. Cellular activation was monitored by determining the amount of secreted murine IL-2 or human IFN- γ in the culture supernatants. IL-2 was detected by ELISA utilizing a solid phase rat anti-murine IL-2 mAb ($1 \mu\text{g/ml}$) for capture and a biotinylated rat anti-mouse IL-2 mAb ($0.5 \mu\text{g/ml}$) (both Pharmingen, Hamburg, Germany) for detection. IFN- γ was determined by ELISA utilizing a solid phase anti-human IFN- γ antibody ($1 \mu\text{g/ml}$) for capture and a biotinylated anti-human IFN- γ antibody (both Endogen) for detection. Cell proliferation of T cells was determined by incorporation of BrdU (Roche Diagnostics, Mannheim, Germany) and subsequent detection of incorporated BrdU by ELISA utilizing a peroxidase-conjugated Fab-anti-BrdU mAb (Roche Diagnostics). To stimulate receptor expressing cells by membrane-bound CD30 antigen, grafted cells (1×10^5 cells per well) were cocultured for 48 h with different amounts of CD30 $^+$ and CD30 $^-$ target cells (1.25 – 10×10^4 cells per well), respectively, and cytokine release was determined as described above.

Cytotoxicity assay

Cytotoxicity of receptor grafted CTL was determined by ELISA detection of BrdU-labeled DNA fragments that were released upon cytolysis of BrdU-labeled CD30 $^+$ target cells. The assay was performed utilizing a commercially available cellular DNA fragmentation ELISA (Roche Diagnostics) according to the manufacturer's recommendation.

Briefly, L540 tumor cells (4×10^5 cells/ml) were incubated overnight in RPMI 1640 + 10% FCS containing $10 \mu\text{M}$ BrdU. The cells were washed three times and resuspended in medium (2×10^5 cells/ml). BrdU-labeled target cells (2×10^4 cells per well) were cocultured for 12 h with receptor grafted and non-transduced T cells (each 1×10^5 cells/ml), respectively. After coculture the supernatants were removed and the content of BrdU-labeled DNA was determined utilizing BrdU-specific capture and peroxidase-labeled detection antibodies.

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