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T Cell Activation by Antibody-Like Immunoreceptors: Increase in Affinity of the Single-Chain Fragment Domain above Threshold Does Not Increase T Cell Activation against Antigen-Positive Target Cells but Decreases Selectivity

Markus Chmielewski,* Andreas Hombach,* Claudia Heuser,* Gregory P. Adams,† and Hinrich Abken²

Chimeric TCRs with an Ab-derived binding domain confer predefined specificity and MHC-independent target binding to T cells for use in adoptive immunotherapy. We investigated the impact of receptor binding affinity on the activation of grafted T cells. A series of anti-ErbB2 single-chain fragment binding domains with a $K_d$ ranging from $3.2 \times 10^{-7}$ to $1.5 \times 10^{-11}$ M was linked to CD3ζ-derived immunoreceptors and expressed in human PBL. Solid phase bound ErbB2 protein triggered activation of receptor-grafted T cells in a dose-dependent manner. The activation threshold inversely correlated with the affinity of the receptor binding domain. The maximum level of cellular activation, however, was the same and independent of the binding affinity. Upon binding to ErbB2⁺ cells, T cells grafted with immunoreceptors carrying a single-chain fragment of $K_d < 10^{-8}$ M were activated in a similar fashion against cells with different amounts of ErbB2 on the surface. T cells with a low affinity receptor ($K_d > 10^{-8}$ M), however, were activated exclusively by cells with high amounts of ErbB2. In conclusion, recombinant immunoreceptors of higher affinity do not necessarily induce a more potent activation of T cells than low affinity immunoreceptors, but the higher affinity immunoreceptors exhibit less discrimination between target cells with high or low Ag expression levels. The Journal of Immunology, 2004, 173: 7647–7653.

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2 Address correspondence and reprint requests to Dr. Hinrich Abken, Klinik I für Innere Medizin, Tumorgenetik, Kliniken der Universität zu Köln, and Center for Molecular Medicine Cologne, Köln, Germany; and *Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA 19111

Materials and Methods

Cell lines and reagents

293T cells are human embryonic kidney cells that express the SV40 large T Ag (12). SK-OV-3 (ATCC HTB77; American Type Culture Collection, Manassas, VA), MCF-7 (ATCC HTB22) Colo201 (ATCC CCL 224), and Colo320 (ATCC CCL 220.1) were derived from various adenocarcinomas expressing different amounts of ErbB2. HaCat is a transformed human keratinocyte cell line (13) and was a gift from Dr. N. Fusenig (German Cancer Research Center, Heidelberg, Germany). Primary human keratinocytes were prepared and maintained as described previously (14). OKT3 (ATCC CRL 8001) is a hybridoma cell line that produces the anti-CD3 mAb OKT3. 293T cells were cultured in DMEM supplemented with 10% FCS.
(v/v) FCS, all other cell lines were cultured in RPMI 1640 medium and 10% (v/v) FCS (all from Invitrogen Life Technologies, Paisley, U.K.). OKT3 mAb was affinity-purified from hybridoma supernatants using goat anti-mouse IgG2A Abs (Southern Biotechnology Associates, Birmingham, AL) that were immobilized on N-hydroxy-succinimide-ester-activated Sepharose as recommended by the manufacturer (Amersham Biosciences, Freiburg, Germany). Human IgG2 Abs and the PE- and FITC-conjugated anti-CD3 mAb UCHT1 were purchased from DakoCytomation (Hamburg, Germany). The goat anti-human IgG Ab and its FITC- and PE-conjugated Fab(′), derivatives were purchased from Southern Biotechnology Associates. The anti-ErbB2 mAb Ab5 was purchased from Oncogene (Cambridge, MA), and the recombinant ErbB2-IgG Fc fusion protein was purchased from R&D Systems (Minneapolis, MN). The anti-human IFN-γ mAb NIB42 and the biotinylated anti-human IFN-γ mAb 4S.B3 were purchased from BD Biosciences (San Diego, CA). Generation of the anti-ErbB2 scFvs was described in detail previously (11). The binding parameters of the anti-ErbB2 scFv are summarized in Table I.

### Generation of recombinant immunoreceptors

To generate the retroviral expression cassettes for ErbB2-specific recombinant immunoreceptors, the DNA coding for C6.5 scFv and its derivatives (gift from Dr. J. Marks, University of California, San Francisco, CA; Table I) were amplified by PCR and flanked by Ncol (5′) and BamHI (3′) restriction sites, respectively, using the following set of primer oligonucleotides: 5′-CGTACCATGGATTTTGAGGTGCAGATTTTCAGCTTCCTTGCTAATCGTGCTCCTGCTAACTATGCTAATGCTAGGGCCTGATGCCCACACGGTTG-3′ (sense) and 5′-TTCTGGATCCGACCTAGGACCTGAGCTCTCCCTTGCTAATCGTGCTCCTGCTAACTATGCTAATGCTAGGGCCTGATGCCCACACGGTTG-3′ (antisense; restriction sites are underlined). The BW431/26-scFv DNA of the anti-CEA receptor BW431/26-scFv-Fc receptors in pBull (15, 16) was cleaved out by Ncol and BamHI and replaced by the digested anti-ErbB2 scFv PCR products.

### Expression of recombinant immunoreceptors

To express recombinant receptors in T cells from the peripheral blood, the expression cassettes were inserted into the retroviral vector pBull (15, 17) as recently described (18). Retroviral transduction of T cells with recombinant receptors was described in detail previously (15, 17, 19), and receptor expression was monitored by flow cytometric analysis. Recombinant receptors were also expressed in 293T cells after transfection of the receptor expression cassettes were inserted into the retroviral vector pBullet (15, 17) as recently described (18). Retroviral transduction of T cells with recombinant receptors was described in detail previously (15, 17, 19), and receptor expression was monitored by flow cytometric analysis. Recombinant receptors were also expressed in 293T cells after transfection of the vector DNA by calcium phosphate coprecipitation (20 μg of DNA/2 × 10^8 cells). Cells were harvested after 48 h and subjected to analysis.

### Immunofluorescence analysis

ErbB2 expression was determined by flow cytometry using the anti-ErbB2 mAb Ab5 and an isotype-matched control mAb (BD Biosciences; each 10 μg/ml). Bound Abs were detected by an FITC-conjugated Fab(′)-anti-mouse IgG Ab (5 μg/ml; Southern Biotechnology Associates), and mean fluorescence was determined. Recombinant receptor-grafted T cells were identified by two-color immunofluorescence using a PE- or FITC-conjugated Fab(′), anti-human IgG Ab (1 μg/ml) and an FITC- or PE-conjugated anti-CD3 mAb (UCHT1; 1/20). Immunofluorescence was analyzed using a FACScan cytofluorometer equipped with CellQuest research software (BD Biosciences, Mountain View, CA). To identify T cells with recombinant receptor expression, we set markers with 99% of nontransduced T cells beyond.

### Receptor-mediated activation of grafted T cells

T cells were harvested after 48 h and subjected to analysis. After 48 h, supernatants were removed and analyzed by ELISA for IFN-γ as described below. In a second set of experiments, anti-ErbB2 receptor-grafted T cells (1.25 × 10^5 to 10 × 10^5 cells/well) were cocultivated for 48 h in 96-well, round-bottom plates with tumor cells that express different amounts of ErbB2 (each 5 × 10^5 cells/well). The culture supernatants were harvested and analyzed for IFN-γ by ELISA. Briefly, IFN-γ was bound to the solid phase anti-human IFN-γ mAb NIB42 (1 μg/ml) and detected by the biotinylated anti-human IFN-γ mAb 4S.B3 (0.5 μg/ml). The reaction product was visualized by a peroxidase–streptavidin conjugate (1/10,000) and ABTS. The specific cytotoxicity of receptor-grafted T cells against target cells was monitored by a 2,3-bis-(2-methoxy-4-nitro-5-sulphonyl)-5-[(phenyl-amino)carbonyl]-2H-tetrazolium hydroxide (XTT)-based colorimetric assay according to Jost et al. (20). Briefly, receptor-grafted and nontransduced T cells were cocultivated with ErbB2+ or ErbB2− tumor cells as described above. After 48 h, XTT reagent (1 mg/ml; Cell Proliferation Kit II; Roche, Mannheim, Germany) was added to the cells and incubated for 30–90 min at 37°C. Reduction of XTT to formazan by viable tumor cells was monitored colorimetrically at an absorbance wavelength of 450 nm and a reference wavelength of 650 nm. Maximal reduction of XTT was determined as the mean of six wells containing tumor cells only, and the background as the mean of six wells containing RPMI 1640 medium and 10% (v/v) FCS. The specific formation of formazan due to the presence of effector cells was determined from triplicate wells containing effector cells in the same number as in the corresponding experimental wells. The number of viable tumor cells was calculated as follows: % viability = (OD(exp. wells − corresponding number of effectors)/OD(tumor cells without effectors − medium)) × 100.

### Results

To monitor the impact of the affinity of the scFv domain on receptor-mediated T cell activation, we generated expression cassettes for the anti-ErbB2 immunoreceptors and inserted them into the retroviral vector pBull (15, 17) as described in Materials and Methods. These anti-ErbB2 scFv-Fc-CD3ζ immunoreceptors harbor the same intracellular signaling, transmembrane, and extracellular spacer domains, but an anti-ErbB2 scFv of different affinity, i.e., k_d = 3.2 × 10^{-7} to 1.5 × 10^{-11} M. The anti-ErbB2 scFv domains of various affinities were derived from the anti-ErbB2 scFv C6.5 by site-directed mutagenesis (21) (Table I). We grafted peripheral blood T cells from healthy donors with the recombinant immunoreceptors by retroviral gene transfer. FACs analyses revealed high expression of all anti-ErbB2 immunoreceptors with nearly the same density on the cell surface (Fig. 1). C6.5-scFv-Fc-ζ immunoreceptor-grafted T cells lysed ErbB2-expressing SK-OV-3 and MCF-7 target cells with high efficiency, whereas anti-CEA BW431/26-scFv-Fc-ζ immunoreceptor-grafted T cells lysed solely MCF-7 tumor cells expressing both Ags. As the control, nonmodified lymphocytes did not lyse the tumor cells, demonstrating the specificity of recombinant immunoreceptor-mediated T cell activation (Fig. 2, A and B). We obtained similar results monitoring IFN-γ secretion of anti-ErbB2 and anti-CEA receptor-grafted T cells upon cocultivation with tumor cells (Fig. 2, C and D).

### Table I. Characteristics of the anti-ErbB2 scFvs used in this study and of recombinant immunoreceptors derived thereof

<table>
<thead>
<tr>
<th>Number</th>
<th>scFv</th>
<th>K_d (M)</th>
<th>k_d (s^{-1})</th>
<th>k_u (M^{-1} s^{-1})</th>
<th>Receptor Derivative</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C6-B1D2</td>
<td>1.5 × 10^{-11}</td>
<td>6.9 × 10^8</td>
<td>0.1 × 10^{-4}</td>
<td>C6-B1D2-scFv-Fc-ζ</td>
</tr>
<tr>
<td>2</td>
<td>C6MH3-B1</td>
<td>1.2 × 10^{-10}</td>
<td>5 × 10^6</td>
<td>0.6 × 10^{-4}</td>
<td>C6MH3-B1-scFv-Fc-ζ</td>
</tr>
<tr>
<td>3</td>
<td>C6ML3-9</td>
<td>1.0 × 10^{-9}</td>
<td>7.6 × 10^5</td>
<td>7.6 × 10^{-4}</td>
<td>C6ML3-9-scFv-Fc-ζ</td>
</tr>
<tr>
<td>4</td>
<td>C6.5</td>
<td>1.6 × 10^{-8}</td>
<td>4.0 × 10^5</td>
<td>6.3 × 10^{-4}</td>
<td>C6.5-scFv-Fc-ζ</td>
</tr>
<tr>
<td>5</td>
<td>C6.5G98A</td>
<td>3.2 × 10^{-7}</td>
<td>4.1 × 10^6</td>
<td>1.3 × 10^{-4}</td>
<td>C6.5G98A-scFv-Fc-ζ</td>
</tr>
</tbody>
</table>

Numbers reflect the order of the binding affinity of anti-ErbB2 scFvs.
cell activation. As demonstrated in Fig. 3A, anti-ErbB2 receptor-grafted T cells were activated by solid phase bound Ag in a dose-dependent fashion. The amount of ErbB2 required to induce grafted T cells to half-maximal IFN-γ secretion depended on the affinity of the scFv domain of the receptor (Fig. 3A). Maximum amounts of secreted IFN-γ were nearly the same for T cells grafted with immunoreceptors with an scFv \( K_d \) of \( 10^{-8} \) M, whereas upon binding of the anti-ErbB2 immunoreceptor with an scFv \( K_d \) of \( 3.2 \times 10^{-7} \) M, induction of IFN-γ secretion was extremely low, even after binding to the highest concentration of ErbB2 in the assay (Fig. 3A). As the control, receptor-grafted T cells were activated by cross-linking the extracellular IgG1 CH2CH3 spacer domain with a solid bound anti-human IgG Fc Ab that results in secretion of the same amounts of IFN-γ for all receptors (Fig. 3B), indicating identical activation properties of the signaling domains despite different scFv binding domains of the immunoreceptors.

We asked whether the receptor affinity has an impact on activation of receptor-grafted T cells upon binding to tumor cells that express ErbB2 in different amounts on the cell surface. For this purpose we estimated the ErbB2 expression of several cell lines and primary keratinocytes, respectively, by flow cytometry (Fig. 4). Cells with a mean fluorescence intensity (MFI) >100 arbitrary

**FIGURE 1.** Two-color immunofluorescence of anti-ErbB2 receptor-grafted peripheral blood T cells. Non-transduced peripheral blood T cells and T cells grafted with anti-ErbB2 immunoreceptors with different affinities of the binding domain (no. 1–5) were simultaneously incubated with a PE-conjugated anti-CD3 mAb and an FITC-conjugated anti-human IgG1 Ab and analyzed by flow cytometry.

**FIGURE 2.** Ag-specific activation of receptor-grafted T cells. Peripheral blood T cells were grafted with the anti-ErbB2 immunoreceptor C6.5-scFv-Fc-ζ and the anti-CEA immunoreceptor BW431/26-scFv-Fc-ζ, respectively, and cocultivated for 48 h (0.078–10 × 10^4 receptor-grafted T cells/well) with target cells (5 × 10^5 cells/well) expressing only ErbB2 (SK-OV-3) or both ErbB2 and CEA (MCF-7) on the cell surface. A and B, The viability of target cells was determined colorimetrically by a tetrazolium salt-based XTT assay, as described in Materials and Methods. C and D, IFN-γ secreted by receptor-grafted T cells into the supernatant was determined by ELISA.
cells with high and medium ErbB2 expressions. In contrast, T cells
did not lyse tumor cells. The efficiency of target cell lysis
was dependent on the amount of ErbB2 on the surface of the target
cells (data not shown), which was demonstrated to suppress IFN-
secretion of receptor-grafted T cells (Fig. 6). Coincubation of
ErbB2++ (SK-OV-3) tumor cells with T cells expressing
anti-ErbB2 immunoreceptors with an scFv of $K_d < 10^{-8}$ M
resulted in secretion of similar amounts of IFN-γ that was
dependent on the ErbB2 expression level on the tumor cells
rather than on the affinity of the recombinant immunoreceptor.
In contrast, T cells equipped with low affinity anti-ErbB2 immu-
noreceptor 5 (C6.5G98A-scFv-Fc-ζ) were only induced to IFN-γ
secretion by incubation with SK-OV-3 cells that express high
amounts of ErbB2. Colo320 cells or transformed (HaCat) or
primary keratinocytes that express only low amounts of ErbB2 on
the cell surface activated neither high nor low affinity immunore-
ceptor-grafted T cells to IFN-γ secretion. Notably, in medium,
high ErbB2-expressing Colo201 cells did not induce substantial
IFN-γ secretion in anti-ErbB2 receptor-grafted T cells, whereas
high affinity receptor-grafted T cells lysed these cells efficiently
(cf., Fig. 5). This may be due to IL-10 secretion of Colo201 tumor
cells (data not shown), which was demonstrated to suppress IFN-γ
secretion of activated T cells (22).

Because the affinity of the scFv binding domain with a $K_d$
$< 10^{-8}$ M has no obvious impact on receptor-mediated cellular
activation by coincubation with ErbB2++ cells, we asked
whether the affinity of the binding domain may affect the kinetics
of target cell lysis and induction of IFN-γ secretion. We coincu-
bated T cells equipped with anti-ErbB2 receptor with various
ErbB2-expressing tumor cells and monitored tumor cell lysis and
induction of IFN-γ secretion at different time points. As summa-
rized in Fig. 7, T cells grafted with immunoreceptors of high
and medium affinity lysed ErbB2-positive tumor cells with similar
kinetics, whereas T cells grafted with the low affinity immunore-
ceptor 5 (C6.5G98A-scFv-Fc-ζ) lysed only ErbB2++ SK-OV-3
cells, but substantially slower than T cells equipped with one of the
high affinity receptors (Fig. 7. A, C, and E). Analysis of IFN-γ
secretion over time revealed similar results; T cells equipped with
immunoreceptors of high affinity (scFv $K_d < 10^{-8}$ M) secrete
IFN-γ in a similar fashion, but T cells with the low affinity receptor
did not secrete detectable amounts of IFN-γ in the presence of
SK-OV-3 cells in this experiment (Fig. 7, B, D, and F).
Discussion

We have generated five nearly identical recombinant immunoreceptors that bind to the same epitope of ErbB2, but with different affinities. The $K_d$ of the scFvs used to bind ErbB2 range from $3.2 \times 10^{-7}$ to $1.5 \times 10^{-11} \text{ M}$ (Table I). All immunoreceptors are equally capable of mediating cellular activation to the same extent, as demonstrated by cross-linking the extracellular spacer domain with an Ab. In contrast, the amount of solid phase bound ErbB2 required for half-maximal induction of IFN-γ secretion by grafted T cells depends on the affinity of the scFv domain of the receptor (Fig. 3). Thus, within this range, the threshold of immobilized Ag required for half-maximal induction of IFN-γ secretion by grafted T cells depends on the affinity of the scFv domain of the receptor. The level of maximum amounts of secreted IFN-γ by activated grafted T cells, however, is independent of the binding affinity of the immunoreceptor.

In the situation of cellular activation upon binding to ErbB2-positive target cells, the correlation between the affinity of the binding domain and cellular activation is not as obvious. T cells grafted with medium and high affinity anti-ErbB2 immunoreceptors ($K_d < 10^{-8} \text{ M}$), respectively, are activated in a similar fashion, whereas T cells expressing an immunoreceptor with a low affinity binding domain ($K_d, 3.2 \times 10^{-7} \text{ M}$) are activated with substantially lower efficiency. Accordingly, for T cell activation via medium and high affinity immunoreceptors, the expression level of the target Ag on the cell surface, rather than the affinity of the binding domain, determines the efficiency of T cell activation with respect to target cell lysis and cytokine secretion. As a consequence, an increase in the affinity of the binding domain above the level of $K_d < 10^{-8} \text{ M}$ does not additionally improve the T cell activation properties of the recombinant receptor. In contrast, an immunoreceptor with a low affinity anti-ErbB2 binding domain ($K_d > 10^{-8} \text{ M}$) efficiently activates grafted T cells only against cells with high densities of ErbB2 on the cell surface.

Our data obtained from cell activation experiments using solid phase bound receptor ligand support the concept that the affinity of this type of recombinant TCR is directly correlated to the number of Ag-bound receptor molecules that are required for cellular activation. This conclusion favors the concept of T cell activation by an increasing number of receptor ligands (9), rather than the concept of serial triggering of few receptor molecules (10) to generate efficient T cell activation. As a cautionary note, the immunoreceptors used in this study have Ab-derived binding domains for MHC-independent recognition, which bind to Ag with higher affinity than TCRs whose affinity for MHC-bound peptide ligands is generally several orders of magnitude lower. Even the lowest binding affinity analyzed ($K_d, 3.2 \times 10^{-7} \text{ M}$) is much higher than the average affinity of the TCR for binding MHC-peptide complexes (23). Because the concept of serial TCR triggering requires low, rather than high, affinity binding, it is unlikely that serial receptor triggering significantly contributes to T cell activation via the
FIGURE 6. Ag-specific IFN-γ secretion by receptor-grafted T cells upon cocultivation with ErbB2-expressing target cells. Peripheral blood T cells were grafted with anti-ErbB2 receptors with different $K_d$ values of the scFv binding domain and cocultivated for 48 h (0.078–10 × 10^4 receptor-grafted T cells/well) with target cells (5 × 10^4 cells/well) expressing different amounts of ErbB2 on the cell surface. IFN-γ secreted by receptor-grafted T cells into the supernatant was determined by ELISA.

FIGURE 7. Time course of Ag-specific activation of receptor-grafted T cells upon cocultivation with ErbB2-expressing target cells. Peripheral blood T cells were grafted with anti-ErbB2 receptors with different $K_d$ values of the scFv binding domain and cocultivated (5 × 10^4 receptor-grafted T cells/well) with target cells (3 × 10^5 cells/well) that express different amounts of ErbB2 on the cell surface. IFN-γ secreted by receptor-grafted T cells into the culture medium and target cell lysis were monitored at different time points. A, C, and E, Viability of ErbB2-positive target cells was determined colorimetrically by a tetrazolium salt-based XTT assay. B, D, and F, IFN-γ secreted by receptor-grafted T cells into the culture supernatant was determined by ELISA.
MHC-independent, Ab-derived immunoreceptors used in this study. Moreover, our results indicate that from the practical point of view the efficiency of immunoreceptor-mediated target cell lysis depends on the affinity of the binding domain. Below a $K_d$ of $10^{-8}$ M of the scFv domain, the immunoreceptors activate grafted T cells with similar efficiency against Ag-positive target cells with different levels of ErbB2. Chames et al. (24) recently reported that recombinant immunoreceptors with specificity for HLA-A1-bound, MAGEl-A1-derived peptide mediate target cell lysis in an affinity-dependent fashion. In this study two Ab-derived binding domains with $K_d$ of $2.5 \times 10^{-7}$ and $1.4 \times 10^{-8}$ M, respectively, were inserted into the recombinant receptor molecule. This is in accordance with our data showing that for scFv with $K_d$ values between $10^{-7}$ and $10^{-8}$ M, the affinity correlates with the efficiency of target cell lysis. Systematical analysis, however, revealed that raising the scFv affinity above this value did not result in improved cellular activation and target cell lysis, not even toward tumor cells with low Ag expression (Figs. 4 and 5). These data are in striking contrast with cellular activation of grafted T cells via solid phase bound ligands (Fig. 3), implying that Ag binding and receptor clustering may occur in a different fashion.

In contrast, a low affinity immunoreceptor is capable, at least in part, of discriminating between target cells with high and low ErbB2 expression levels. This property will be helpful for the targeting of cells with Ags, such as ErbB2, that are overexpressed in tumor tissues, but are also found in low densities on normal cells. Although keratinocytes with low ErbB2 expression were not lysed by both low and high affinity anti-ErbB2 immunoreceptors in our in vitro system, there may be the risk for autoagression using a recombinant receptor with high affinity for clinical application. In summary, raising the affinity of the scFv domain with MHC-independent ErbB2 binding properties could be ambiguous on adoptive immunotherapy: 1) immunoreceptors with high affinity ($K_d < 10^{-8}$ M) are more efficient in cellular activation and target cell lysis than low affinity receptors, but increases in affinity beyond $10^{-8}$ M do not result in additional increases in receptor-mediated cellular activation; and 2) in T cell activation, immunoreceptors with high affinity scFv domains discriminate less between high and low Ag-expressing target cells than do immunoreceptors with low affinity scFv domains.

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We thank Dr. James D. Marks (University of California, San Francisco, CA) for providing us with the anti-ErbB2 scFv C6.5 and derivatives thereof.

References


