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

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*J Immunol* 2004; 173:7647-7653;

doi: 10.4049/jimmunol.173.12.7647

http://www.jimmunol.org/content/173/12/7647
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

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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 CD3ε-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+, T cells grafted with immunoreceptors carrying a single-chain fragment of $K_a < 10^{-8}$ M were activated in a similar fashion against cells with different amounts of ErbB2 on the surface. T cells with a low affinity receptor ($K_a > 10^{-8}$ 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.
Characteristics of the anti-ErbB2 scFvs used in this study and of recombinant immunoreceptors derived thereof.

Table 1.

<table>
<thead>
<tr>
<th>Number</th>
<th>scFv</th>
<th>$K_d$ (M)</th>
<th>$k_{on}$ (M$^{-1}$s$^{-1}$)</th>
<th>$k_{off}$ (s$^{-1}$)</th>
<th>Receptor Derivative</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>C6-B1D2</td>
<td>1.5 x 10^{-11}</td>
<td>6.9 x 10^{5}</td>
<td>0.1 x 10^{-4}</td>
<td>C6-B1D2-scFv-Fc-ζ</td>
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<tr>
<td>2</td>
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<td>1.2 x 10^{-10}</td>
<td>5 x 10^{5}</td>
<td>0.6 x 10^{-4}</td>
<td>C6MH3-B1-scFv-Fc-ζ</td>
</tr>
<tr>
<td>3</td>
<td>C6M3L-9</td>
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<td>7.6 x 10^{5}</td>
<td>7.6 x 10^{-4}</td>
<td>C6M3L-9-scFv-Fc-ζ</td>
</tr>
<tr>
<td>4</td>
<td>C6.5</td>
<td>1.6 x 10^{-8}</td>
<td>4.0 x 10^{5}</td>
<td>6.3 x 10^{-4}</td>
<td>C6.5-scFv-Fc-ζ</td>
</tr>
<tr>
<td>5</td>
<td>C6.5G98A</td>
<td>3.2 x 10^{-7}</td>
<td>4.1 x 10^{5}</td>
<td>1.3 x 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. 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 x 10^{10} to 10 x 10^{11} cell/well) were cocultivated for 48 h in 96-well, round-bottom plates with tumor cells that express different amounts of ErbB2 (each 5 x 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 biotinylated anti-human IFN-γ mAb 4S.B3 (0.5 µg/ml). The reaction product was visualized with 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-sulfophenyl)-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 nonspecific 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 effector cells/OD_{tumor cells without effectors + medium}) x 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 pbuBlet (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., $K_d = 3.2 \times 10^{-7}$ to $1.5 \times 10^{-11}$ 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).

We incubated the same number of T lymphocytes that was grafted with anti-ErbB2 receptors harboring binding domains with different affinities in the presence of serial dilutions of solid bound ErbB2 protein and recorded the secretion of IFN-γ indicating T...
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 $10^{-8} \text{M}$, whereas upon binding of the anti-ErbB2 immunoreceptor with an scFv $K_d$ of $3.2 \times 10^{-7} \text{M}$, 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...
units were regarded as highly positive (ErbB2^+++^), cells with an MFI >10 arbitrary units were regarded as medium positive (ErbB2^++^), and cells with <10 arbitrary MFI units were regarded as weakly positive or negative (ErbB2^-^-^-). Anti-ErbB2 receptor-grafted T cells were cocultivated with each of these cells at different T cell:tumor cell ratios, and specific cytolysis and induction of IFN-γ secretion by grafted T cells were recorded. As summarized in Fig. 5, T cells equipped with immunoreceptors with an scFv of K_d <10^{-8} M lysed SK-OV-3 (ErbB2^+++^), Colo 201 (ErbB2^++^), and MCF-7 (ErbB2^++^) cells, respectively, with similar efficiencies, whereas T cells engrafted with immunoreceptor 5 (C6.5G98A-scFv-Fc-ζ), which harbors an scFv with a K_d >10^{-8} M, were much less efficient. As a control, nonmodified lymphocytes 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, but was only partially dependent on the affinity of the scFv binding domain. Increasing the affinity of the scFv domain, i.e., K_d <10^{-8} M, did not increase the efficiency of cytolysis of target cells with high and medium ErbB2 expressions. In contrast, T cells expressing the low affinity anti-ErbB2 immunoreceptor 5 (scFv K_d = 3.2 \times 10^{-7} M) required a higher E:T cell ratio to result in the same efficiency of cytolysis compared with T cells with high affinity receptors. In contrast, anti-ErbB2 receptor-grafted T cells of any affinity did not lyse Colo320 tumor cells or transformed (HaCat) and primary keratinocytes, respectively, that express only low amounts of ErbB2.

We obtained corresponding results when monitoring IFN-γ 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 immunoreceptor 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 immunoreceptor-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 coincubated 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 summarized in Fig. 7, T cells grafted with immunoreceptors of high and medium affinity lysed ErbB2-positive tumor cells with similar kinetics, whereas T cells equipped with the low affinity immunoreceptor 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 $1.5 \times 10^{-11}$ 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-$\gamma$ 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 cellular activation depends strictly on the affinity of the scFv domain of the immunoreceptor. The level of maximum amounts of secreted IFN-$\gamma$ 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 ($K_d < 10^{-8}$ M), respectively, are activated in a similar fashion, whereas T cells expressing an immunoreceptor with a low affinity binding domain ($K_d, 3.2 \times 10^{-7}$ 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 $K_d < 10^{-8}$ 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 ($K_d > 10^{-8}$ 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 ($K_d, 3.2 \times 10^{-7}$ 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

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**FIGURE 5.** Ag-specific lysis of ErbB2-expressing target cells by receptor-grafted T cells. Peripheral blood T cells were grafted with anti-ErbB2 immunoreceptors with different $K_d$ values of the respective scFv domain and cocultivated for 48 h (0.078 – $10^4$ receptor-grafted T cells/well) with target cells (5 $(10^4$ cells/well) expressing different amounts of ErbB2 on the cell surface. The viability of ErbB2-positive target cells was determined colorimetrically by a tetrazolium salt-based XTT assay, as described in Materials and Methods.
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 × 10^3 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 × 10^3 receptor-grafted T cells/well) with target cells (3 × 10^4 cells/well) that express different amounts of ErbB2 on the cell surface. IFN-γ secreted 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 $\sim 10^{-8}$ 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, MAGE1-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 scFvs 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.

Acknowledgments

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


