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13. ABSTRACT *(Maximum 200 Words)*

The main objective of this research project has been to apply the T-body approach for the immunotherapy of breast cancer. To this end, we endowed human lymphocytes with anti-erbB2 specificity by transducing them with a tripartite chimeric receptor made of anti-erbB2 scFv-linked through the homeo-domain, trans membrane and endo-domain of CD28 to the cytoplasmic region of the FcRγ chain. We report on the procedures and protocol developed to construct the optimal configuration of receptor and a protocol for efficient chimeric gene expression in human T cells. The ability of the tripartite chimeric receptor to fully activate primary T cells was demonstrated in vitro and in vivo using transgenic mice made with a model receptor. Transgenic mice that express erbB2-specific receptors from a few scFv have been generated and the ability of it's lymphocytes to reject erbB2 expressing breast tumors has been demonstrated in the SCID mouse model.In another pre clinical model, we have expressed the erbB2-specific tripartite chimeric receptor in murine stem cells and are studying their ability to mature into mature effector cells. Altogether, we expect from the model systems described above to establish the optimal conditions of using T-bodies for breast cancer therapy.

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Abbreviations:

BM – Bone Marrow

CR – Chimeric Receptor

DTH – Delayed Type Hypersensitivity

FcRγ – Receptor for the constant region of IgG

FγG – Fowl gamma globulin

GFP – Green fluorescent protein

GMP- Good Manufacturing Practice

IL-2 – Interleukin 2

IL-2R – IL-2 Receptor

^{125}I-UdR- Radioactive Iodo deoxy uridine

LAK – Lymphokine Activated Killers

mAb – Monoclonal Antibody

MHC – Major histocompatibility complex

NK – Natural killer cells

PBL – Peripheral blood lymphocytes

RN- Retronectin

ScFv – Single chain Fv of an antibody

SCID – Severe combined immune deficiency

T-body – Chimeric receptor expressing T cells

TCR – T cell receptor

Tg - Transgenic

Tg8.7 – A particular transgenic mouse expressing TNP-specific CR

TIL – Tumor Infiltration Lymphocytes

TNP - Trinitrophenol

VH – The variable domain of the antibody heavy chain

VL - The variable domain of the antibody light chain

WT – Wild type

INTRODUCTION

We report here on a research project in which we suggested a novel approach for the immunotherapy of breast carcinoma. The difficulty of treating metastatic breast cancer with conventional therapy, combined with the presence of defined tumor-associated antigens on breast tumors, makes this malignancy an excellent candidate for an immunotherapeutic approach to cancer treatment.

One method developed for the immunotherapy of cancer is to remove lymphocytes from tumors obtained at surgery or biopsy, expand them *ex vivo* in the presence of lymphokines, and reinfuse these cells into the patient. Treatment of cancer by the infusion of such autologous tumor infiltrating lymphocytes (TIL) has produced clinical responses in some patients. Although several explanations can be proposed for the limited response thus far, one possibility is the lack of specificity of the reintroduced lymphocytes. In addition, this technique is limited by the difficulty in obtaining specific TIL for many histologic types of cancer (including breast cancer). In contrast, many monoclonal antibodies have been described that bind tumor-associated antigens shared by tumors of similar histology (e.g. Anti-HER2(anti-erbB2) antibodies). These monoclonal antibodies can be attached to a cytotoxin or to an antibody or growth factor to redirect cytotoxic T cells. However, most clinical attempts using such immunotoxins have not fulfilled expectations. Their therapeutic efficacy is restricted to blood borne tumors, primarily because solid tumors are not sufficiently accessible to antibodies. The recently used antibodies for breast cancer therapy, anti-HER2 antibodies (Herceptin) show benefit to patients yet are not curative and to optimize their anti-cancer effect the addition of inhibitors of cell division (e.g. Taxol) is recommended.

Cancer patients usually mount a poor -if any- immune response against their own tumors due to poor tumor immunogenicity, escape from immune-attack and an immunosuppressed state common to many cancer patients. Several approaches have been attempted to enable the immune eradication of tumor cells. These include various methodologies to augment, non-specifically or specifically, the host immune response and treatment with specific anti-tumor antibodies. Non-specific treatments, such as the use of LAK (lymphokine-activated killer) cells, are not effective in all types of cancer. The requirement for the co-injection of large amounts of IL-2 causes severe side effects which often require the cessation of treatment. The use of antibodies in passive immunotherapy is often of limited efficacy, both because of the difficulty in identifying true tumor-specific antigens, poor tumor penetration, and the short half-life of the antibodies.

Our group has pioneered the "T body" approach, a novel approach for cancer therapy. This approach has several advantages over traditional immunotherapeutic methods. We have joined the two approaches of adoptive immunotherapy and immunotoxin therapy to genetically engineer an improved 'immunocytolysin', which is an antibody recognition unit in the form of single chain variable (scFv) region attached to a cytotoxic T cell signaling molecule. Genes for chimeric T cell receptors have been constructed containing the coding sequence of an antibody-derived scFv directed against a tumor associated antigen, attached to the transmembrane and intracytoplasmic sequences of a T cell signaling molecule. These genes are then transfected into cytotoxic T cells, thereby conferring upon them the ability to specifically recognize and kill tumor cells. The chimeric scFv receptor (scFvR) design we have developed combines antibody recognition and T cell signaling in one continuous protein and has been used to endow murine and human cytotoxic cells with non-MHC-restricted, antibody-derived specificity. This T-body approach combines the advantage of antibody specificity with the homing, tissue penetration, and to the immunotherapy of breast cancer. Accordingly, the research work focused on the preparation and efficient expression of breast-cancer specific chimeric receptors and the establishment of in-vivo pre-

clinical model in which the different aspects of the T-body approach could be evaluated and optimized. In fact, as reported below, we have succeeded to completely eradicate well established breast carcinoma xenograft in the SCID mouse model by intratumoral injection of erb-B2-specific T-bodies.

2) BODY

In the first part of this research project, we constructed a tripartite chimeric receptor (CR) genes made of a continuous construct that encompasses the scFv of an antibody, as the extracellular recognition unit, the homeo-domain, trans-membrane and the intracellular region of CD28 linked to the intracellular part of FcRγ as the cytoplasmic co-signaling and signaling moieties. Such a tripartite CR expressed in murine cells could redirect the T cells with antibody specificity and trigger them for full activation. The construction of the tripartite CR gene, its cloning into a retrovector and efficient functional expression of the tripartite CR in human lymphocytes is reported in the attached paper [1]. In this final report we report on the unique activity of the tripartite CR combining in one chain antibody specificity (in the form of scFv, and the stimulatory and co-stimulatory signals provided by the FcRγ and CD28 respectively). In addition to the model antigen TNP that was used to prove the full spectrum of T cell activation redirected by the tripartite CR, we also developed CR specific to the erbB2 (HER2/Neu), the growth factor receptor over-expressed on more than 30-40% of human breast carcinoma.
In addition to the functional expression of the CR in murine T cell hybridomas and bone marrow, and human PBL- derived T cells, in this project we studied whether the tripartite CR is capable of fully activating, naïve un-primed T cells. This has been achieved by the generation of transgenic mice harboring the TNP-specific CR. We report herein for the first time on our successful efforts to establish transgenic mice harboring the erbB2-specific and functional CR. With regard to the use of the T-bodies for breast cancer therapy, we present herein in detail our improved protocol for the transduction of human lymphocytes from peripheral blood with the erbB2-specific CR and describe our success in demonstrating, in SCID mice, the therapeutic potential of the T-body approach, using human breast xenograft and the genetically modified, erbB2-specific human PBL. A complete cure has been obtained in a large number of mice by a treatment that combines erbB2-specific lymphocytes and the systemic administration of IL-2.

CONSTRUCTION OF THE TRIPARTITE scFv-CD28-γ CHIMERIC RECEPTOR

See the attached paper: Eshhar, Z., Waks, T., Bendavid, A. & Schindler, D.G. Functional expression of chimeric receptor genes in human T cells. *J Immunol Methods* **248**, 67-76. (2001).

TESTING THE TRIPARTITE CR IN CULTURED MURINE CELLS

a. Expression and functional analysis

The ability of the TNP-specific CR to signal was tested first in a T cell hybridoma. A variant of the murine cytotoxic T cell hybridoma MD45, a progeny of the MD hybridoma, derived in our lab. [2], which is lacking TCR expression, MD45.27J sub clone, was transfected with DNA encoding scFv-antiTNP-CD28-γ driven by the RCV promoter and carrying the bacterial neo gene to provide for G-418 resistance in transfectants [3]. A transfectant was selected by drug resistance and shown to produce specific RNA (**Figure 1a**) and surface protein (**Figure 1b**). Membrane compartmentalization has been shown to be important for TCR signaling and a co-stimulation requirement has been shown for the microdomain formation [4-8]. To check whether the CR is localized to the detergent insoluble rafts like the immune synapse, Western immunoblotting analysis was performed (**Figure 2**). The scFv-antiTNP-CD28-γ protein was indeed localized to the detergent insoluble preparation (prepared as described previously [9]. Specific protein, detected only as a high molecular weight complex in the detergent-soluble fraction of cell lysates, was

resolved at the expected size in the detergent-insoluble fraction, suggesting association with detergent-insoluble lipid rafts. In contrast, a chimeric receptor without the CD28 segment was not localized in the insoluble fraction. This is most likely due to the transmembrane motif of the CD28 that is part of the tripartite receptor, which is known to be a raft -associated protein.

The functionality of the chimeric receptor in the hybridoma was checked by examining its response to the TNP antigen. The redirected hybridoma was found to produce IL-2 in response to stimulation with either plastic-bound TNP-fowl γ-globulin (FγG) hapten-carrier protein conjugate or with a panel of TNP-modified target cells (**Figs. 3A and 3B**, respectively). Maximal responses to target cells were obtained by stimulation with the A20 B cell lymphoma, presumably due to residual antigen-presentation properties. Stimulation with TNP-modified P815 or L1210 transfectants expressing the costimulatory molecule B7 or the death receptor Fas, respectively, did not significantly modify responses relative to stimulation with the untransfected parental lines.

The cytolytic function of the hybridoma was demonstrated by determining its effectiveness in killing TNP-modified A20 cells target cells (**Fig. 3C**). Therefore, we concluded that the CD28-based receptor was fully functional in activating the specific helper (IL-2 production) and effector (cytolysis) functions of T cells.

b. Inhibitor studies

Before the introduction of these chimeric receptors into naïve T cells (see generation of transgenic mice, below), it was important to demonstrate the difference between the chimeric scFv-CD28-γ receptor and the scFv-γ receptor that does not contain the CD28 moiety. One possibility of demonstrating it was by the altered drug sensitivity of CD28 compared to TCT/CD3 signaling. CD28 has been known to confer resistance to cyclosporin A and differential sensitivity of CD28 mediated signaling compared to TCR/CD3 mediated signaling was shown [10-12]. In order to determine whether addition of the CD28 signaling module conferred a differential sensitivity to inhibitors, we compared the drug sensitivity of the CD28- γ CR to a CR containing γ alone. Subclones of a hybridoma transfectant expressing scFv-antiTNP-CD28-γand the scFv-antiTNP-γ were stimulated with TNP-FγG in the presence or absence of pharmacological inhibitors and changes in IL-2 production were monitored (**Fig. 4).** Hybridoma transfectant STG-B [3] expressing scFv-antiTNP-γ was used as a control to confirm CD28-mediated effect. Production of IL-2 triggered through the CD28-γ receptor was found to be specifically resistant to inhibition by the calcineurin inhibitor cyclosporin A as reported for signaling via CD28 versus via TCR/CD3 [13].

CD28 contains an amino acid sequence YMNM that is capable of being phosphorylated and binding phosphatidylinositol 3 kinase. This enzyme is very sensitive to wortmannin. This could be the enzyme that regulates T cell survival through Akt/PKB [14]. However, CD28 is not the only receptor in the cell that activates PI3K. In addition, hybridomas often show different drug sensitivities than that of peripheral blood lymphocytes. Nevertheless, our hybridoma did show a differential sensitivity when the CD28 signaling module was part of the CR. In the presence of the PI-3 kinase inhibitor wortmannin, IL-2 production triggered via the scFv-CD28-γ receptor was specifically up-regulated, as reported for signaling via CD28 versus via TCR/CD3 [15].

GF 109203X, a bisindolylmaleimide, is used as a broad specificity inhibitor of the protein kinase C family of enzymes that are ser/thr phosphorylating enzymes [16]. At a concentration of 30 nM, GF109203X was reported to inhibit by 81% the stimulation by anti-TCR/CD3 antibody + PMA, whereas anti-CD28 antibody +PMA stimulated IL-2 synthesis in Jurkat cells was only 22% inhibited, strikingly more resistant [17]. Indeed, in our tripartite CR expressing hybridoma, production of IL-2 triggered through the scFv-CD28-γ receptor was found to be specifically more resistant to inhibition by the PKC inhibitor GF109203X than the scFv-γ receptor, as reported for signaling via CD28 versus via TCR/CD3.

GENERATION OF CHIMERIC RECEPTOR-HARBORING TRANSGENIC MICE

In order to demonstrate that the tripartite receptor can redirect the full activation of naïve unprimed T cells, we have generated transgenic mice expressing it as well as transgenic mice harboring the scFv-gamma without CD28, and tripartite CR in which the cytoplasmic domain was transacted. In all these configurations, the scFv was of the Sp6 antibody that is TNP-specific. Because transgenic mice can also be an excellent source for CR-expressing T cells (T-bodies) and CR-expressing bone-marrow stem cells, which are of prime interest for the adoptive therapy experiments, we have also generated transgenic mice harboring the erbB2-specific tripartite receptor employing the scFv of the N29 mAb. This mouse germline was prepared to create a transgenic mouse for animal models of chimeric T cell receptor therapy of breast cancer (which over express the erbB2 antigen on their surface).

Figure 5 schematically describes the chimeric receptor constructs that we made to create the transgenic mice. Two different systems were used for gene expression. One was a CD3 delta promoter driven expression vector [18] and the other was driven by a CD2 promoter with a locus control region to ensure expression no matter where the gene is inserted in the genome [19]. Locus control regions can overcome heterochromatin induced gene inactivation [20].

a. Generation of transgenic mice containing the chimeric receptor under the CD3-δ promoter

In the first set, transgenic mice were generated putting the CR gene under the CD3-δ promoter that directs the expression of the transgene to only T cells. Table 1 describes the transgenic mouse strain obtained.

Transgenic C57BL/6 mouse (Tg 184) was made with about 50 copies of the transgene scFv-antiTNP-γ driven by the CD3 delta promoter. This construct integrated into the Y chromosome (**Figure 6**) and expressed RNA, particularly well in the thymocytes (**Figure 7**). When outbred with BALB/c or SJL/J mice, expression of mRNA increased as previously reported for a different transgene [21] (**Figure 8**). However, although the splenocytes of this transgenic strain were activated with anti-CD3 antibody, they were not activated by TNP-FγG. Similarly, the anti-erbB2 CR scFvN29-Ig-γ, that could be expressed using the CD3 delta regulatory sequences in MD45/27J cells (**Figure 9**) failed to function in spleen cells of transgenic mice made of it (TgA3). Also in this case, the CR genes integrated into the Y chromosome (**Figure 10**). This gene was expressed as checked by immunoblotting (**Figure 11A**) but very weakly by FACS (**Figure 11B**). Outbred transgenic mice expressed high levels of RNA (**Figure 12A**) and had higher levels of this receptor on their splenocytes (**Figure 12**). However, we were not able show that this receptor is functional. These results by-and-large agree with the data reported by Brocker et al. [22], who succeeded in generating scFv-zeta CR-transgenic mice, but these mice were not functional and needed to be pre-stimulated to demonstrate their activity.

In order to test the effect of the triple chimera containing both the CD28 and Fcε receptor γ chain, a chimeric receptor scFv-antiTNP-CD28-γ driven by the CD3 delta regulatory sequences was integrated into the C57Bl/6 mouse genome in 40-60 copies (Tg5.3 and Tg.5.8, **Figure 13**). mRNA for the scFv-antiTNP-CD28-γ receptor was expressed in the splenocytes of these Tg5.3 Tg.5.8 mice. Immunoprecipitation and immunoblotting analysis also demonstrated expression of transgene protein in splenocytes of these mice (**Figure 14A**). Immunofluorescence analysis of the thymocytes of these mice indicated very low levels of transgene surface expression (**Figure 14B**).

To test the hypothesis that the chimeric receptor can trigger signal transduction pathways capable of activating untreated primary splenocytes, the splenocytes of the transgenic mice were stimulated with immobilized TNP-FγG and then IL-2 production and proliferation were measured at various time points (**Figs. 16, 17**). In a high density stimulation, specific production of IL-2 in response to

stimulation with immobilized TNP-FγG was observed starting at 48 hours post-stimulation, reaching a peak at 72 hours and then declining to background level by 120 hours (**Fig. 16 & 17**, top left). In comparison, IL-2 production induced by stimulation of TCR/CD3 with anti-CD3 mAb gave maximal IL-2 production earlier, at 24 hours, and declined to background levels earlier (**Fig. 18**, left). The maximum levels of IL-2 induced via scFv-antiTNP-CD28-γ were 61% of those induced by TCR/CD3 stimulation.

In low density stimulation, production of IL-2 in response to stimulation with plastic-bound TNP-FγG was induced at 48h post-stimulation and was sustained until the 96h time-point (**Fig. 17**, top right). Comparison of the kinetics of IL-2 production induced by stimulation of TCR/CD3 by positive control stimulation with 2C11 versus that induced by scFv-antiTNP-CD28-γ indicates that stimulation via TCR/CD3 induces maximal IL-2 production earlier, at 24 hours, which then also declines to background levels earlier, by 72 hours (**Fig. 18**, right). By the final time-point at 96 hours, levels of secreted IL-2 induced via scFv-antiTNP-CD28-γ had reached 75% of the maximum levels induced by TCR/CD3 stimulation.

In high density stimulation, proliferation increased slightly at 24 hours post-stimulation in response to stimulation with immobilized TNP-FγG, as calculated by the proliferation index. The proliferation index then decreased at the 48 hour and 72 hour time-points and then drastically increased to the highest levels measured in the assay on the final time-point at 96 hours (**Fig. 17**, bottom left). This sudden elevation in proliferation index late in the assay, when the culture conditions had deteriorated and apoptosis appeared widespread, and therefore is an increase in survival of the TNP-FγG stimulated cells rather than increased growth. This may reflect CD28-specific pro-survival, anti-apoptotic mechanisms mediated by signaling via the CD28 sub-domain of scFv-antiTNP-CD28-γ. In low density stimulation, specific proliferation in response to stimulation with plastic-bound TNP-FγG was observed at 24 hours post-stimulation, increased to maximal levels at 72h and then declined slightly by the final time-point of the assay at 96 h (**Fig. 17**, bottom right).

b. **Generation of transgenic mice containing the chimeric receptor under the CD2 promoter**

It is notable that all transgenic mice made of the CR constructs under the CD3-δ promoter (including the erbB2 specific one, described in the 1st year report), did not express detected amounts of surface protein and this receptor was hardly functional. We have therefore switched to the second system where the transgene expression was driven by a CD2 promoter with a locus control region to ensure expression no matter where the gene is inserted in the genome[20]. **Table 2** describes the transgenic mouse strain obtained. In this system the level of expression was better and functional.

Full activation of primary murine lymphocytes in mice transgenic to TNP-specific chimeric receptor

The transgenic mice (Tg8.3, 8.7 and HTM) were generated by pro-nuclear microinjection of (BALB/c x C57BL/6)F2 embryos with the corresponding CR construct (see **Table 2**). High-level surface expression of the CR in splenic T cells of these mice was demonstrated via immunofluorescent flow cytometric analysis (**Fig 19**). The activity of Tg8.7, expressing the highest levels of the TNP specific tripartite CR, was compared to Tg HTM in which the intracytoplasmic domain of CD28 was deleted, and therefore serves as an appropriate control to evaluate the role of the CD28 moiety in the tripartite CR.

a. Induction of IL-2Rα chain (CD25) by stimulation via anti-TNP CR.

The signal through CD28 molecule can directly induce CD25 expression on T cells, resulting in T cell activation and proliferation.

Stimulation of purified (>90%) splenic T cells from Tg8.7 mice for 72 hr with TNP-modified cells (with or without the expression of B7) showed that this stimulation is independent of B7-CD28 interaction (**Table 3**).

b. Rescue from apoptosis in cells activated via anti-TNP tripartite CR

Co-stimulation through the CD28 receptor plays an important role in enhancing the resistance of antigen receptor-activated T cells to undergo apoptosis.

The proportion of sub-G_1/G_0 cells (apoptotic) in purified (90%) splenic T cells from Tg8.7 mice, stimulated under the same conditions described above for 72 hrs, indicated that stimulation via tripartite CR prevents apoptosis induction (**Table 4**).

These experiments clearly indicate the role played by the CD28 signaling domain. Therefore, in the following experiments only the activity of the T cells of the high expressing tripartite CR transgenic mice (Tg 8.7) was analyzed.

1. Stimulation of IL-2 production

IL-2 as well as other cytokines production is an important component of the T-body's anti-tumor activity. Upon interaction of the T-bodies with the tumor target we expect these cytokines not only to provide growth factors that sustain the T cell reactivity, but also to attract other cells and induce a local inflammatory response, which eventually should cause the elimination of the tumor. We have expected the chimeric receptor to signal the T cell activation in non-MHC restricted manner, and indeed, as demonstrated in the experiments detailed below, the mere binding of the tripartite CR to its plastic immobilized TNP hapten is sufficient to fully activate the T cells.

Figure 20 shows that stimulation of purified (90%) naïve resting splenic T cells of Tg8.7 mice on plastic-bound TNP-FγG for 24 hr induces high levels of specific IL-2 production in response to tripartite CR signaling

2. Stimulation of proliferation by anti-TNP tripartite CR.

Naïve T cells stimulated only through the TCR enter into a state of anergy that is manifested by their inability to proliferate in response to repeated stimulation. Thus, proliferation is the most indicative parameter for CD28 mediated co-stimulation. Proliferation assays of naïve resting splenic T cells of Tg8.7 mice stimulated at $2x10^5$ cells/ml in microtiter plates for 48 hr (**Fig. 21**) indicates that signaling via tripartite CR specifically induces significant degree of proliferation. These levels of proliferation were markedly higher than those induced by stimulation with anti-CD3 antibodies at effective concentration, as determined in control, wild-type mice (WT). These type of experiments clearly establish the co-stimulatory signaling mediated by the tripartite CR.

3. Rescue from apoptosis in cells activated via anti-TNP tripartite CR

Rescue of apoptosis caused by the lack of proper T-cell signaling, or due to antigen-induced cell death, is mediated by the co-stimulatory signaling through CD28 [23-25]. Analysis of apoptosis in bulk splenocytes and in purified (90%) splenic T cells from Tg8.7 mice stimulated at 10^6 cells/ml in 24-well plates for 72 hrs, indicated that stimulation via tripartite CR is effective, as expected from the CD28 co-stimulatory domain (**Fig. 22 & 23**).

4. Up-regulation of the anti-apoptotic transcription factor bcl-X_L by stimulation via anti-TNP tripartite CR

The rescue from antigen-induced apoptosis by CD28 is mediated through the bcl signaling pathway. The bcl-X_L gene has been reported to enhance the intrinsic ability of lymphocytes to resist apoptosis [23]. **Figure 24** shows indeed increased bcl-X_L expression in primary T cells, purified from the Tg8.7 mice and stimulated via anti-TNP tripartite CR.

5. Up-regulation expression of IL-2Rα chain (CD25) by stimulation via anti-TNP tripartite CR
One of the most common markers for T cell activation is the up-regulation of the IL-2Rα chain (CD25) of the IL-2 receptor. Stimulation of purified (90%) splenic T cells from Tg8.7 mice for 60 hr with anti-CD3 mAb generated a low degree of IL-2Rα T cells, whereas a larger number of IL-2Rα T cells were induced by stimulation via anti-TNP tripartite CR (**Fig. 25**).

c. Conclusion of in-vitro experiments
Altogether, these in vitro results demonstrate that stimulation of T cells via the tripartite CR provides both FcRγ - mediated (signal I) and CD28 - mediated (signal II) triggering [26]. It appears that the higher the expression of the CR on the cell surface of the transgenic T cells is, the more potent the cells are.

In-vivo, antigen-specific responses in CR transgenic mice

The TNP hapten, is capable of eliciting delayed type hypersensitivity (DTH) response when applied by skin sensitization. We have therefore compared the ability of the Tg8.7 (generated with TNP-specific CR) to mount TNP-specific DTH without pre-sensitization.
Groups of mice were pre-treated with cyclophosphamide and two days later sensitized with the antigen (skin painting) on their shaved backs. Mice were challenged five days later by painting the right pinnae with the antigen and the left one with the solvent only. One hour later mice were pre-treated with 5-fluorodeoxyuridine and an intravenous pulse of 2 μCi ^{125}I-UdR was given into the tail vain. 24 hr after ^{125}I-UdR administration, the mice were killed and the pinnae cut off at the hairline [27]. The ear reaction reflects a T-cell-dependent DTH response.
The preliminary study depicted in **Figure 26** shows that there was no difference between WT and Tg mice in their ability to elicit DTH response. More importantly - a weak response of the transgenic mice has been observed upon challenging the mice only with the antigen without pre-sensitization. The ratio of radioactivity intake between the ears in the Tg mice is higher than in the WT mice and reflects the extent of inflammatory reaction elicited by the antigen challenge (**Fig. 26**). These responses were associated with an extensive mononuclear and lymphoid cell infiltration (**Fig. 27**).
We thus conclude that the primary T cells harboring the tripartite chimeric receptors are capable of responding in-vivo. These results indicates the feasibility of using the Tg mice and their cells in experimental models aimed to establish the ability of T bodies to reject established breast tumors.

Generation of Transgenic Mice made of anti-erbB2 tripartite CR

For these experiments, the N29-scFv-CD28-gamma chimeric receptor gene was used where N29 and L96 are the anti-erbB2 monoclonal antibodies (mAb) that we have been using throughout this research project.
Transgenic mice were generated by pronuclear microinjection of (BALB/c x C57BL/6)F2 embryos with a construct for expression of (CD2)-N29-CD28-γ. The CD2 tissue-specific promoter [28] allows the gene under its control to be expressed exclusively in T cells and NK cells. The vector we were using proved itself efficient in the Sp6 (anti-TNP) based CR we described above and in the previous report. DNA for microinjection was prepared as follows: linearized scFvR transgenes were separated from the rest of vector sequences by restriction enzyme digestion and gel electrophoresis in low-melting point Agarose. The transgene containing DNA fragments were prepared by melting the Agarose slice in a 10-fold volume of low-salt buffer. Finally, the transgene DNA were purified by affinity chromatography using Elutip-d columns.

Identification of Transgenic Founders. Genomic DNA was isolated from tail tissue of founder mice and was analyzed for integration of transgene by PCR amplification of transgene specific sequence with co-amplification of an endogenous CD3δ specific sequence as an internal control. Out of 70 founder mice obtained, six transgenic founder mice (3 females and 3 males), have tested positive for integration of N29 (anti-ErbB2)-CD28-γ transgene (**Fig. 28**). Reconstruction experiments indicate that between one and twelve copies of transgene are integrated in the germ line of transgenic founder mice.

Surface Expression of the erbB2-Specific Chimeric Receptor in the Transgenic Mice. Surface expression of the anti-erbB2 chimeric receptor in the six mice harboring the tripartite chimeric gene was evaluated by testing their PBL for staining using polyclonal rabbit anti-N29 antibodies. The female mice with the highest copy number also scored positively (**Fig. 29**). Another mouse showed weak staining.

Functional expression of the erbB2-Specific CRr in T cells from the Transgenic Mice
Splenocytes from mice transgenic for anti-ErbB2 tripartite CR were stimulated with plastic-immobilized rabbit anti-N29 idiotypic antibody for 72 hrs. and IL-2 Rα chain induction (Fig. 30) and rescue from apoptosis (**Table 5**) were monitored. The results obtained in these experiments demonstrate the capacity of signals generated via this tripartite CR, to generate productive activation of transgenic T cells.

Construction of a tripartite chimeric receptor made of a new anti-erbB2 mAb

So far, most of our studies used chimeric receptor made of scFv of the N29 anti-erbB2 mAb as their recognition unit [29]. This antibody, like the N29, was developed in the laboratory of Prof. Y. Yarden [30] and was found to be effective in slowing the growth of human tumors in nude mice [31]. It does not react with recombinant erbB2 made in bacteria and is considered to recognize carbohydrate, or carbohydrate-containing epitope in the extracellular part of erbB2 molecule (Y. Yarden personal communication). Human T-bodies, expressing the tripartite CR made of the N29 scFv, injected intra-tumorally into prostate carcinoma xenograft growing in SCID mice, was capable of slowing the growth of this tumor and even curing a significant number of these mice. Nevertheless, in a few mice, tumor growth was resumed after a certain period. When removed and analyzed for surface erbB2 expression, it was interesting to find that the recovered tumor cells were not stained by the N29 mAb, but stained quite well by L96 (**Fig. 31**). Based on such observations, we believe that the erbB2-specific T bodies provide a growth advantage to tumor cells that do not express the N29 epitope. Whether this immunoselection is due to pre-existing variants that do not express the N29 epitope or cells that ceased to express it due to the selective pressure, is being currently studied. Nevertheless, we have concluded from this behavior that for clinical trials it may be beneficial to use another anti-erbB2 mAb in the CR context. Consequently we elected the L96 mAb that strongly stained surface erbB2 on several human adenocarcinomas (see **Figure 32** for comparison of the staining of the human breast adenocarcinoma SKBr3 by N29 and L96). Accordingly, we have cloned the VH and VL from the L96 hybridoma, prepared scFv and used it in the framework of the tripartite CR. The CR gene was inserted into the pBullet retrovector and we prepared and selected a packaging cell producing a high virus titer for the infection of T cells (**Fig. 33**).

Optimization of the transduction procedure and chimeric gene expression in human lymphocytes

In the attached article that is part of this report [1] we also described the procedure we have developed for the preparation and selection of packaging cells producing high titers of the pBullet vector harboring the chimeric receptor and GFP genes. We have further improved and optimized the transduction procedure so as to reproducibly obtain efficient (40-80%) and stable gene transfer into human PBL expression and to follow the fate of the programmed cells in-vivo. Phenotypically, the transfected PBL consisted of 25-30% CD4, 65-70% CD8 and 10-15% CD56 positive cells (data not shown). This shift towards the $CD8^+$ subtype was due to the activation process, using anti-CD3+CD28 antibodies, and culture conditions, since the same distribution of surface markers was seen on activated un-transfected lymphocytes and on GFP negative transfectants. The updated protocol below describes this procedure which may serve as the prototype to be up-graded to GMP conditions:

a. Protocol for transduction of human lymphocytes

Packaging Cell Lines

Cell lines used are the ecotropic GPE86 (ATCC), amphotropic PA317 (ATCC), the PG13 expressing the gibbon ape leukemia virus envelope (GALV env) [32]. The 293T cells were a obtained from R. A. Willemsen (Daniel den Hoed Cancer Center, Rotterdam, The Netherlands). All cells were cultured in DMEM medium (GIBCO-BRL) supplemented with 10%FCS (GIBCO-BRL), L-glutamine solution (2mM), Sodium Pyruvate 1mM, 100 units/ml Penicillin and 100µ/ml Streptomycin (Biological Industries Israel).

1. Preparation of packaging cells

$3x10^5$ GPE86 cells and $2x10^5$ PA317 cells were plated together in a 100mm plate (NUNC). A day later, medium is changed and transfection is performed by adding 20 µg of DNA in CaPO4 (Mammalian Transfection Kit, Stratagene). Following 24 hours the plate is washed with PBS and supplemented with fresh medium. 48-72h later, when the culture is nearly confluent, viral supernatant can be collected.

Pseudotyping was accomplished by infection of PG13 cells. $2x10^5$ PG13 cells are plated in a 100mm plate; the next day the medium is replaced with 5ml of viral supernatants in the presence of 4µg/ml of Polybrene at 37^0C, 7.5% CO_2 for 7 h. 48h later the infection efficiency is evaluated. All vector-containing retroviral supernatants described in this study were harvested after a 24h incubation of near-confluent cells grown in 5ml fresh medium in a humidified incubator at 32^0C, 7.5% CO_2.

2. Enrichment for high vector producing cells by FACS sorting.

To enrich for vector producing packaging cells, after transfection/transduction, the cells were stained under sterile conditions with anti-idiotypic antibodies. Cells were sorted on FACSsort Plus (Becton Dickinson) according to GFP fluorescence and CR expression. Cloning to a single cell/well was accomplished by sorting of positive cells into 96 well plates. Supernatants from the resulting clones were checked for their retrovector as described above and the high titre producing clone was selected as the source for retrovector for the T cell transduction.

Transduction of human lymphocytes

1. Activation of Lymphocytes

Fresh peripheral blood lymphocytes (PBL) of healthy donors were isolated by centrifugation through Ficoll Paque Plus (Pharmacia Biotech), and cultured in RPMI 1640 medium (GIBO-BRL) supplemented with 10%FCS, L-Glutamine solution 2mM, 100 unit/ml Penicillin 100µg/ml Streptomycin and 50µM 2-mercaptoethanol. Cells, (4ml of10^6/ml /well in a 6well plate) were

stimulated for 3 days, on Falcon non-tissue culture treated, 6-well plates, pre-coated with anti-CD3+anti-CD28 antibodies. Immobilization of the antibodies was performed by adding 2.5ml/well of antibody (1.2 μg/ml of each in PBS) to the cells for overnight at 4°C, washed with PBS and blocked with 1% BSA in PBS for 20 min at 37°C.

2. Lymphocyte transduction

1.5ml/well of viral supernatant supplemented with 100 U/ml of IL-2 is added of RN coated 6 well plate and incubated for 30'at 37°c. Activated lymphocytes were harvested from the stimulating plate, washed and resuspended, $2.5x10^6$ cells in 1.5ml of viral supernatant, added to the RN plate. After 4-6h at 37°C 7.5% CO_2, the viral supernatant was gently removed and replaced with 4ml of RPMI-FCS+ 100U/ml IL-2 medium and incubated over night at 37°C 5% CO_2. The same transduction process was repeated on the next day. On the following day lymphocytes were harvested by vigorous pipeting and washing of the wells to obtain all cells. The cells were resuspended in RPMI-FCS medium with 350U/ml of IL-2, and incubated in 37°C 5% CO_2. RN coated plates were prepared by incubating non tissue culture treated 6 well plate (Falcon) with 4ml/well of RN 12μg/ml over night at 4°C, washed with PBS and blocked with 1% BSA in PBS for 20 min at 37°C.

In-vitro effects of erbB2-specific human T bodies on breast cancer cells

Using the above protocol, human lymphocytes were infected with tripartite CR made of the N29 and L96 scFv (anti-erbB2 Abs) and Sp6 (anti-TNP Ab). The infected cells expressed the receptors as verified by GFP expression (Fig. 34) and by rabbit antisera made against the N29 and L96 idiotypes (data not shown).

The functionality of these human 'T-bodies' was analyzed in the following in vitro experiments:

1. *Cytolysis of ErbB-2-expressing breast tumor cell lines by transduced T lymphocytes*

The cytolytic activity of the transduced T lymphocytes was determined using BT-474 human breast cancer cell line as target in a ^{51}Cr release assay (**Fig. 35**). The lymphocytes transduced with the N29 tripartite CR were able to lyse 90% of BT-474 target cells at E:T ratio of 40:1. The transduced T lymphocytes were used one week after the infection. This probably explains the high levels of background (cytolysis of non-infected lymphocytes due to their NK and LAK cell activity).

2. Human lymphocytes expressing the ErbB-2 CR produce a Variety of Cytokines upon their Stimulation with ErbB-2 bearing Target cells.

The capacity of the transduced T lymphocytes to secrete IFN-γ and TNF-α was analyzed following specific interaction with erbB2-expressing target cells (MCF-7, BT-474 and SKBR-3 breast cancer cell lines) (**Fig. 36**). The levels of erbB2 expression in these cells relative to the normal mammary epithelial cell line 184 are: BT-474, 25-fold increase; SKBR-3, 33-fold increase and MCF-7 cells do not overexpress erbB2 (**Fig. 37**). The secretion of these cytokines correlates with the expression of erbB2 on the surface of these target cells (MCF-7 being th lower expressor).

Establishment of human breast tumor experimental model in SCID mice

In addition to the establishment of prostate cancer xenografts from patients' specimen, which is an on-going undertaking in the lab. (done in collaboration with Sheba Medical Center), we have been using the well established BT-474 breast carcinoma cell line as a xenograft in female SCID mice. The BT-474 highly expresses the erbB2 target antigen as judged by immunofluorescent staining with both N29 and L96 mAbs (**see figure 37**). When transplanted sub-coetaneous (s.c.), together with Matrigel, the tissue cultured BT-474 produced tumors in estrogen-dependent manner.

Additional human breast cell lines (SKBR-3 and MCF-7) are being adopted to in-vivo growth in SCID mice. MCF-7 is especially interesting because the level of surface expression of erbB2 is medium-to-low in comparison to the other xenografts and it is of interest to evaluate the in-vivo effect of the T-bodies in relation to the level of erbB2 expression on its target cells.

Effect of the erbB2-specific T-bodies on breast tumor xenografts in vivo

The signaling efficacy of the CR transduced lymphocytes in stimulating T cell effector function was further evaluated in a human tumor rejection assay in vivo. Three consecutive daily intratumoral injections of the transduced lymphocytes (2.0×10^7 each) were administered to female scid mice with well established s.c. BT-474 tumors. In two different experiments the anti-erbB2 CR transduced lymphocytes were able to completely eradicate the BT-474 tumor (**Fig. 38 & 39**). The antigenic-specificity of the anti-erbB2 CR was demonstrated by the complete lack of effect of the anti-TNP CR on the BT-474 tumor.

Histopathological sections of BT-474 tumors two months post three intra-tumoral injections of anti-erbB2 T-bodies also demonstrated the capacity of these T-bodies to infiltrate the tumor and eliminate it (**Fig. 40**). However, the tumors treated with medium or non-specific CR transduced lymphocytes preserved the tumor structure.

Expression of CR genes in murine bone marrow derived stem cells

Another way to obtain mature primary T cells harboring the chimeric receptor genes is by transducing murine bone-marrow stem cells with the chimeric receptor gene ex-vivo, implanting this cells into irradiated mice and allowing the stem cells to differentiate into mature cells in-vivo. In this approach, feasible in cases which require total body irradiation and regeneration of the hematopoietic system by stem cell engraftment (such as in the case of advanced, spread breast cancer), the tripartite receptor is especially attractive component. One can foresee a situation in which part of the reconstituting stem cell graft will include CR expressing cells which upon maturation in the host will home to and reject residual cancer cells.

For transduction of the murine bone-marrow derived stem cells we have used erbB2 and TNP specific chimeric genes in the pBullet retrovector as we have used for lymphocytes, but, rather than using the CMV promoter, we used the PGK one that operates better in murine cells. Packaging cells producing high titre viral supernatants were prepared.

For transduction, mice were treated with 5-Fluorouracil and 48 hrs later, bone marrow (BM) cells (Sca^+, Lin^-) were prepared from the murine long bones and separated on Ficoll. Cells were stimulated in culture in the presence of IL-3, IL-6 and SCF for 48 hrs and then co-cultured on irradiated packaging cells in the presence of the cytokines and polybrene for another 48 hrs. Alternatively, the stimulated BM stem cells were centrifuged with the retrovector supernatants in the presence of protamine at 2000 rpm for 2hrs and then cultured again with the cytokines. The degree of transduction was evaluated by GFP fluorescence and was found to be up to 80% in the co-culture procedure and up to 30% by the centrifugation procedure.

The transduced cells were analyzed for colony formation in methyl cellulose selective medium to assess their hematopoietic potential, cellular differentiation and GFP expression. Most of the transduced cells were injected i.v. into lethally irradiated (900 rad) mice, and engraftment has been evaluated at monthly intervals by looking for surface staining of peripheral blood cells using antibodies against the different hematopoietic lineages.

By the time this report was prepared, we could observe gene transfer into colonies of megakaryocytes, reticulocytes, as well as myeloid and lymphoid lineages. In the mouse sera we could get 20-50% of the peripheral blood cells expressing GFP by 8 weeks following the stem cell transfer. In some cases, the GFP maker was found at 9 months following engraftment. The level of engraftment of T cells and their expression of the chimeric receptor is being studied.

Progress of research in relation to the Statement of Work:

Task 1+2 (Months 1-12): *Cloning of anti-erbB scFv and construction of chimeric receptors.*
All work has been completed.

Task 3 (Months 3-18): *Functional expression of chimeric receptors in lymphocytes and cell lines*
Most of the work has been completed. Following to our success in expressing the CR genes in human lymphocytes, we do not see it necessary to go back to the rat mast cell line (RBL).

Task 4 (Months 1-24): *Establishing in vivo model systems.*
Accomplished: 1. Screening of breast cancer lines for tumor antigens
2. Preparation of transgenic mice
3. Human model in SCID mice
4. Transduction of bone-marrow stem cells and engraftment. In this sub-task we have diverted from our intention to work on human stem cells because they do not mature into effector cells in the SCID mouse model. As described above, we study murine stem cells.
5. Growth of breast cancer xenografts in SCID mice. In this part of the research project we have adapted tissue-culture growing human breast cancer cell lines to growth in SCID mice and also used murine breast cancer cell lines that are transfected with the human erb-B2 gene. These replaced the fresh human xenografts that we started to prepare. Because of the time the establishment of fresh xenografts takes (until the xenograft is being taken in the mouse in a transplantable form and is characterized as a *bona-fide* representative of human breast cancer) the therapeutical experiments were conducted on xenografts developed from the cell lines.

Task 5 (Months 12-36): *Therapeutic model in mice. Analysis and optimization*
Accomplished: 1. A continuous supply of IL-2 is required to maximize the therapeutic potential of T-bodies
2. Long term effect of T-bodies on breast cancer xenografts

Not Accomplished:

We tried to determine the effect of the T-bodies by their systemic administration into tumor bearing mice. We found only marginal effect on sub-cutaneous tumors by the dose ($2x10^7$). Detailed analysis revealed that only a few of the engineered lymphocytes "homed" to the xenografts. Solving this requires further, in depth, study that we fell is beyond the scope of the current project. We plan to apply the DoD for further support on this very issue.

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6) KEY RESEARCH ACCOMPLISHMENTS

- Construction of a tripartite T cell receptor made of scFv-anti-ErbB2-CD28-FcεRγ
- Construction of a tripartite T cell receptor using heregulin as the targeting agent.
- Construction of transgenic mice expressing the gene for a tripartite scFv-anti-TNP-CD28-FcεRγ
- Construction of transgenic mice expressing the gene for a tripartite scFv-anti-ErbB2-CD28-FcεRγ
- Full functional expression of the tripartite chimeric receptor in transgenic mice
- Production of a retrovirus that can efficiently transduce anti-ErbB2 targeting agent tripartite receptors.
- Functional expression in human lymphocytes of erbB2-specific chimeric receptor
- Elimination of established breast cancer xenografts by chimeric receptor expressing human lymphocytes
- Protocol for optimal expression of chimeric receptors in human lymphocytes
- Establishment of pre-clinical model using human breast cancer xenografts in SCID mice (on-going)
- Expression of the tripartite, anti-erbB2 chimeric receptor in murine stem-cells

7) REPORTABLE OUTCOMES

Review article: Eshhar, Z., Waks, T., Bendavid, A., and Schindler, D. G. Functional expression of chimeric receptor genes in human T cells. J. Immunol. Methods, 248: 67-76, 2001

Abstract presented at the 11th International Congress of Immunology, Stockholm, July, 2001: Full activation of naive T cells via a transgenic tripartite chimeric receptor. Eshhar, Z., Bendavid, A., Waks, T., Morvinski, D., and Schindler, D. G.

Abstract presented at the Era of Hope Meeting, Orlando, Sept. 2002. Adoptive Therapy of Breast Cancer Using Effector Lymphocytes Targeted With HER/2 Specific Chimeric Receptors. . Eshhar, Z., Bendavid, A., Waks, T., Morvinski, D.Waks, T. , and Schindler, D. G.

Fulfillment of Ph.D. thesis for Dr . Feigelson

Fulfillment of Ph.D. requirement of Alain Ben-David

Partial fulfillment of Ph.D. requirement of Dr Jehonathan H. Pinthus

Partial fulfillment of Ph.D. requirement of Dinora Morvinski-Friedman

Development of transgenic mouse strains with tripartite chimeric receptors specific to: I. TNP; II. ErbB2 .

Acquired a Grant from the EC 5th Framework program in Quality of Life, The Cell factory key Action

8) CONCLUSIONS:

In this research project we have accomplished the construction of tripartite receptors consisting of a targeting molecule such as a scFv or a ligand, a portion of the co-stimulatory molecule CD28, and the signaling portion of the cytoplasmic domain of the Fcε receptor γ chain. We have optimized the delivery and functional expression of these genes as surface receptors in the splenocytes of transgenic mice, mouse stem cells, and human lymphocytes. We have established a model for human breast cancer xenografts in SCID mice and demonstrated the function and therapeutic potential of human lymphocytes bearing the erbB2-specific chimeric receptor. To avoid escape of such treatment we made chimeric receptors of different specificities. So far our success to cure established breast tumors by the chimeric receptors bearing lymphocytes was by intra-tumoral administration of the engineered lymphocytes with concurrent administration of IL-2. Our research efforts will be directed to establish and demonstrate the potential of of the T-bodiy approach by their systemic administration. This will extend the application of T-bodies, not only to localized disease but also to advanced and disseminated one.

9). TABLES AND FIGURES

Table 1. *Nomenclature and description of CR-transgenic mice generated under the regulation of the CD3δ promoter*

Mouse line	Expressed chimeric receptor	Regulatory sequences	Recognition specificity	Transgene copy number	Genetic background
Tg 5.3	Sp6-CD28-γ	mCD3δ	TNP	60	C57BL/6
Tg 5.8	"	"	"	nd	C57BL/6
Tg 184	Sp6-γ	mCD3δ	TNP	~50	C57BL/6
Tg A3	N29-Ig-γ	mCD3δ	HER-2	75-100	C57BL/6
Tg A4	N29-CD28-γ	mCD3δ	"	100-150	C57BL/6

Table 2. *Nomenclature and description of CR-transgenic mice generated under the regulation of the human CD2 promoter*

Mouse line	Expressed chimeric receptor	Regulatory sequences	Recognition specificity	Transgene copy number	Genetic background
Tg 8.7	Sp6-CD28-γ	hCD2	TNP	nd	C57BL/6 & Balb/c
Tg HTM	Sp6-ΔCD28-γ	hCD2	TNP	nd	Balb/c
Tg N29	N29-CD28-γ	hCD2	HER-2	nd	C57BL/6 & Balb/c

Table 3. *Stimulation of T cells from Transgenic Mice is Independent of B7-CD28 Interaction*
Purified T cells from the CR transgenic mice were co-cultured with the different stimulator cells and analyzed for CD25 (IL-2 R α chain) expression by FACS.

	CD25 Expression (%)			
Mice	WT	Tg HTM	Tg 8.7	Tg 8.7
Stimulating cells				
None	3.0	1.6	1.2	2.0
P815 ($B7^-$)	9.0	8.5	7.0	11.0
P815 ($B7^+$)	10.0	8.5	9.0	14.0
TNP-P815 ($B7^-$)	9.0	10.5	55.0	83.0
TNP-P815 ($B7^+$)	13.0	73.0	66.0	84.0

Table 4. *Rescue from apoptosis of T cells from Transgenic Mice is also Independent of B7-CD28 Interaction*
Purified T cells from the CR transgenic mice were co-cultured with the different stimulator cells as described in the above figure. After 72 hrs, cells were harvested and stained with FITC-Thy1.2 and fixed. PI and RNAase were added for cell cycle analysis. Percent of apoptosis was measured by FACS analysis on Thy 1.2^+ gated cells and calculating the percentage of cells displaying sub-G_1/G_0 DNA content.

	APOPTOSIS (%) - Mice			
Targets	WT	HTM	Tg 8.3	Tg 8.7
P815 ($B7^-$)	48	48	51	56
P815 ($B7^+$)	40	42	50	50
TNP-P815 ($B7^-$)	42	44	40	36
TNP-P815 ($B7^+$)	40	30	40	30

Table 5. *Stimulation via N29-CD28-γ induces rescue from apoptosis in splenocytes of Tg N29 mice (72hrs).* Similar cultures to those described in figure 30 were performed. After 72 hrs, cells were harvested and stained with FITC-Thy1.2 and fixed. PI and RNAase were added for cell cycle analysis. Percent of apoptosis was measured by FACS analysis on Thy 1.2^+ gated cells and calculating the percentage of cells displaying sub-G_1/G_0 DNA content.

Note: GAL 7.1 = rabbit anti-Galectin A polyclonal Ab.

anti-N29 = rabbit anti-N29 idiotype.

Stimulating antibody	Sub G_1/G_0 (%)	G_1 (%)	S (%)	G_2/M (%)
None	76.3	20.4	0.9	1.2
GAL 7.1 10 ug/ml	77.2	20.6	0.4	1.1
anti-N29 10 ug/ml	11.2	51.7	17.5	17.7

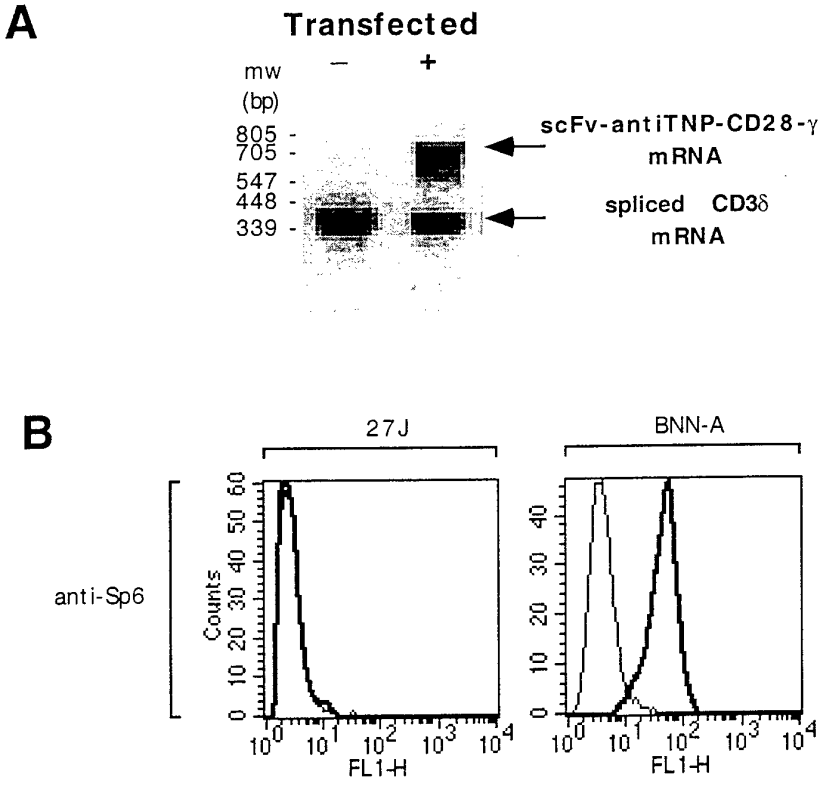


Figure 1. Hybridoma transfectant expresses scFv-antiTNP-CD28-g mRNA and scFv-antiTNP-CD28-γ surface proteins. (A) Complementary DNA was PCR amplified with scFv-antiTNP-CD28-γ specific primers. To control for amplification from spliced RNA and to normalize samples, cDNA was co-amplified with intron-flanking CD3δ primers which generate a 345 bp or a 759 bp amplification product from spliced or unspliced CD3δ mRNA, respectively. Reaction products were separated by agarose gel electrophoresis and visualized by EtBr staining. (B) Immunofluorescence analysis of scFv-antiTNP-CD28-γ surface expression. Primary staining reagents, biotinylated anti-(anti-TNP mAb) Sp6-idiotype mAb GK-20.5 (bold line) or irrelevant staining reagent, biotinylated anti-DNP mAb U-7.6; secondary detection reagent, FITC-streptavidin.

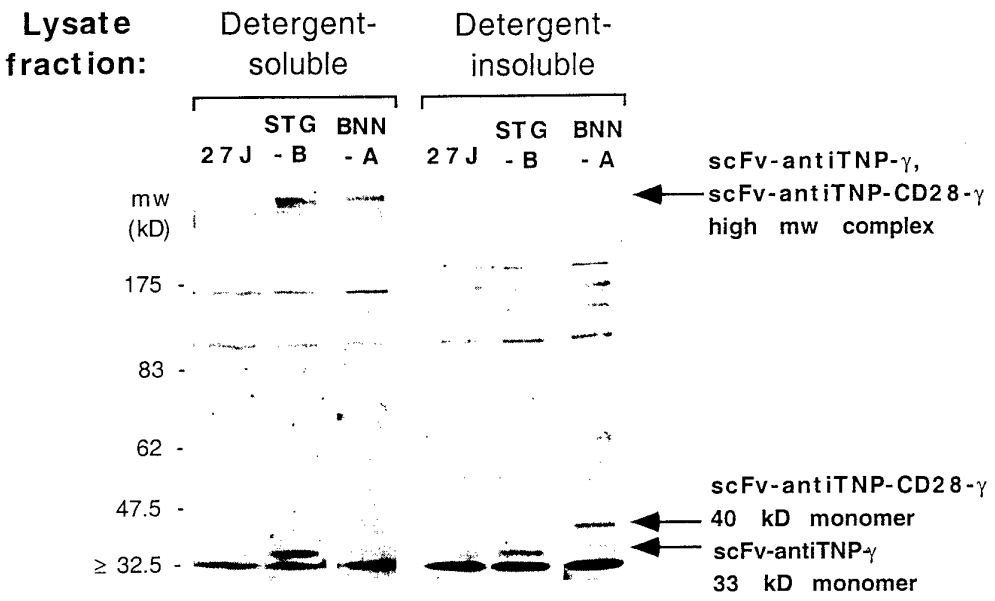


Figure 2. Subcellular localization of scFv-antiTNP-CD28-g protein by Western immunoblotting. Detergent soluble protein fraction represents NP-40 lysate supernatant and detergent insoluble fraction was obtained homogenization of NP-40 insoluble precipitate in RIPA lysis buffer. Lysate proteins were separated by SDS-PAGE under reducing conditions, blotted onto nitrocellulose and stained with rabbit anti-γ serum followed by staining with HRP-protein A and visualized by ECL. The hybridoma transfectant STG-B expressing scFv-antiTNP-γ and the parental untransfected line 27J were used as positive and negative controls, respectively.

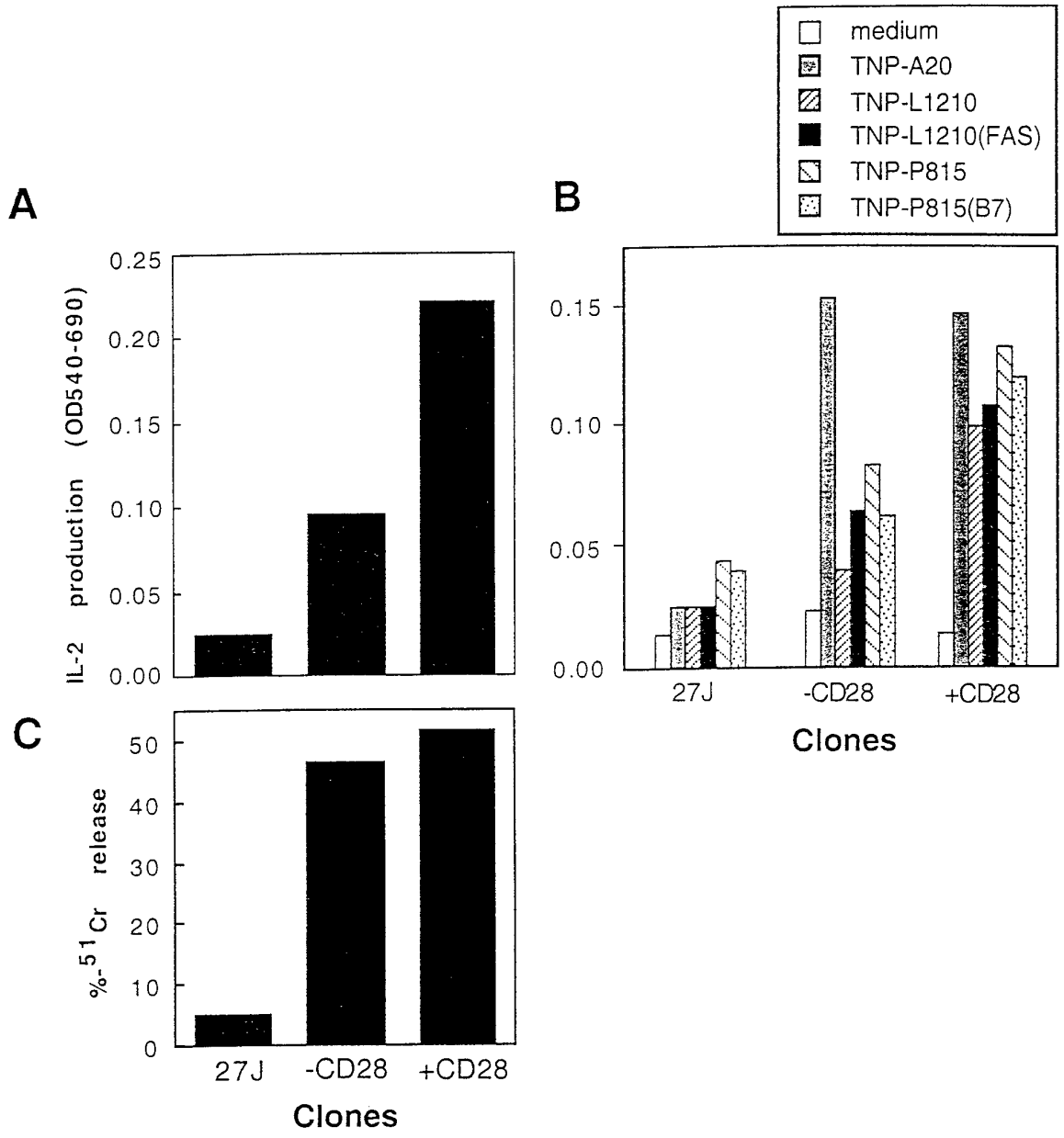


Figure 3. Hybridoma transfectant expressing Sp6-CD28-γ is activated to perform effector functions in response to stimulation with TNP. (A) Stimulation of IL-2 production by plastic-immobilized TNP-FγG. (B) Stimulation of IL-2 production by TNP-modified cells. Target cells; A20, BALB/c B lymphoma-derived cell line; L1210, murine leukemia; L1210(FAS), L1210-derived transfectant expressing fas; P815, murine mastocytoma; P815(B7), P815-derived transfectant expressing B7. (C) Cytolysis of TNP-modified A20 cells. 27J, untransfected parental hybridoma effector negative control; -CD28 is a hybridoma transfectant expressing scFv-anti-TNP-γ.

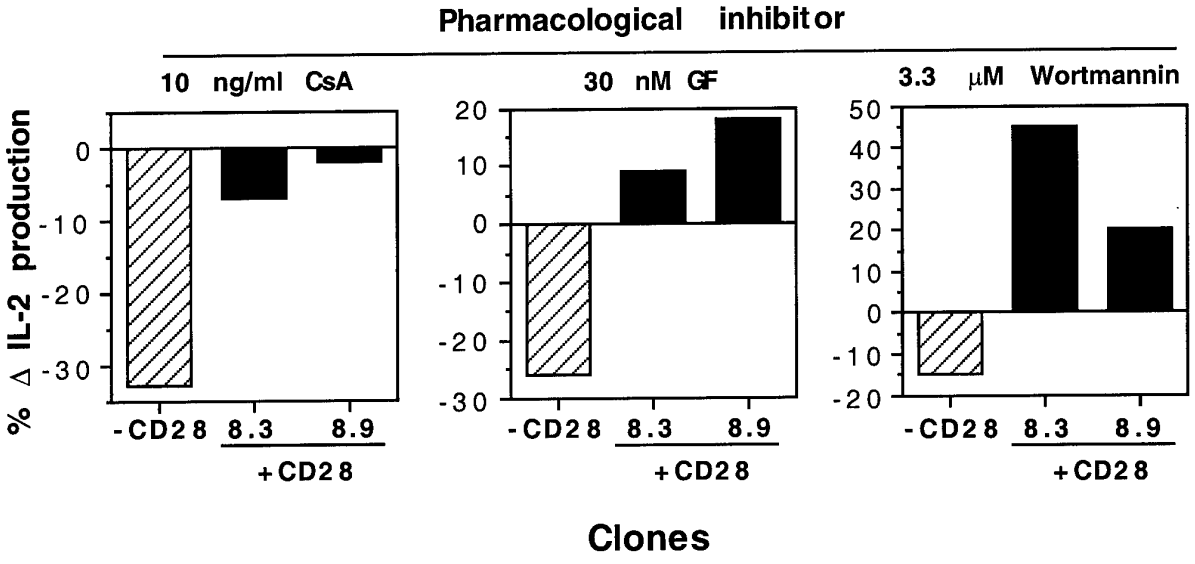
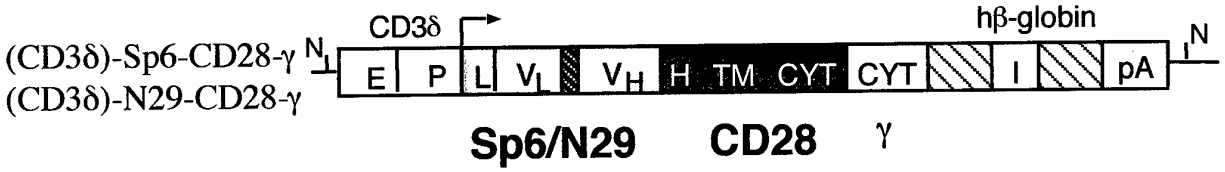
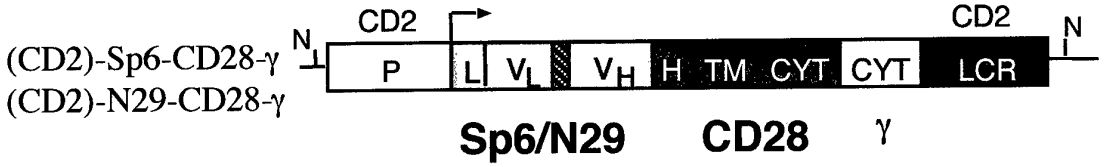


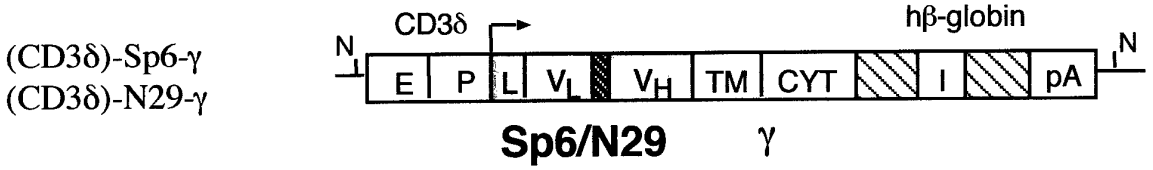
Figure 4. Signaling for IL-2 production by scFv-antiTNP-CD28-γ contains a CD28-specific signaling component. Hybridoma transfectant clones expressing scFv-antiTNP-γ (STG-B) or scFv-antiTNP-CD28-γ (BNN-A subclones 8.3 and 8.9) were stimulated to produce IL-2 by immobilized TNP-FgG in the presence or absence of pharmacological inhibitors. The untransfected parental clone 27J was used as negative effector control and medium and TPA+ionophore were used as negative and positive stimulator controls, respectively. Stimulations were performed with 10^6 cells/well in 24-well plates in a volume of 1 ml. Cells were stimulated for 24h and triplicate supernatant aliquots were harvested. The IL-2 content was determined via CTLL IL-2 bioassay and quantitation of CTLL proliferation by MTT. To compensate for the effect of residual inhibitor on the growth response of CTLL to IL-2, the effect of 27J + inhibitor-conditioned supernatants on the growth response of CTLL to a mid-range concentration of IL-2 was determined. Only GF had such an effect and a multiplication factor (MF) of 1.2 (vs 1.0 for CsA and wortmannin) was used to normalize quantitation of IL-2 production by this inhibitor. The BNN-A clones whose results are shown are those which produced a quantifiable mid-range quantity of IL-2 upon stimulation in the absence of inhibitor, similar to that produced by the reference scFv-antiTNP-γ transfectant clone STG-B.
(%Δ IL-2 production) = 100 x (IL-2 production + inhibitor)x(MF)/(IL-2 production - inhibitor).

Tripartite CR expression constructs





ITAM-CR expression constructs



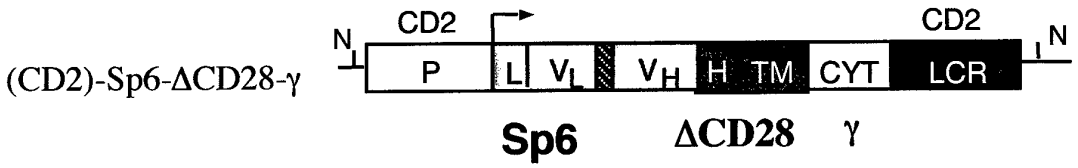


Fig 5. *Chimeric receptor expression constructs.* Constructs of transgene sequences were linearized for pronuclear microinjection at Not1 restriction sites (N).Abbreviations: CYT, cytoplasmic domain; E, enhancer; I, intron (included to improve transgene expression); H, hinge domain; L, Ig leader; LCR, locus control region; LTR, long terminal repeat; P, promoter; pA, polyadenylation sequence; TM, transmembrane domain; V_H and V_L, Ig heavy and light chain variable domains, respectively; ΔCD28 is the CD28 domain compose of part of the extracellular, the transmembrane and without cytoplasmic signaling moiety. Sp6, N29 are scFvs of anti-TNP and anti-ErbB-2 antibodies, respectively.

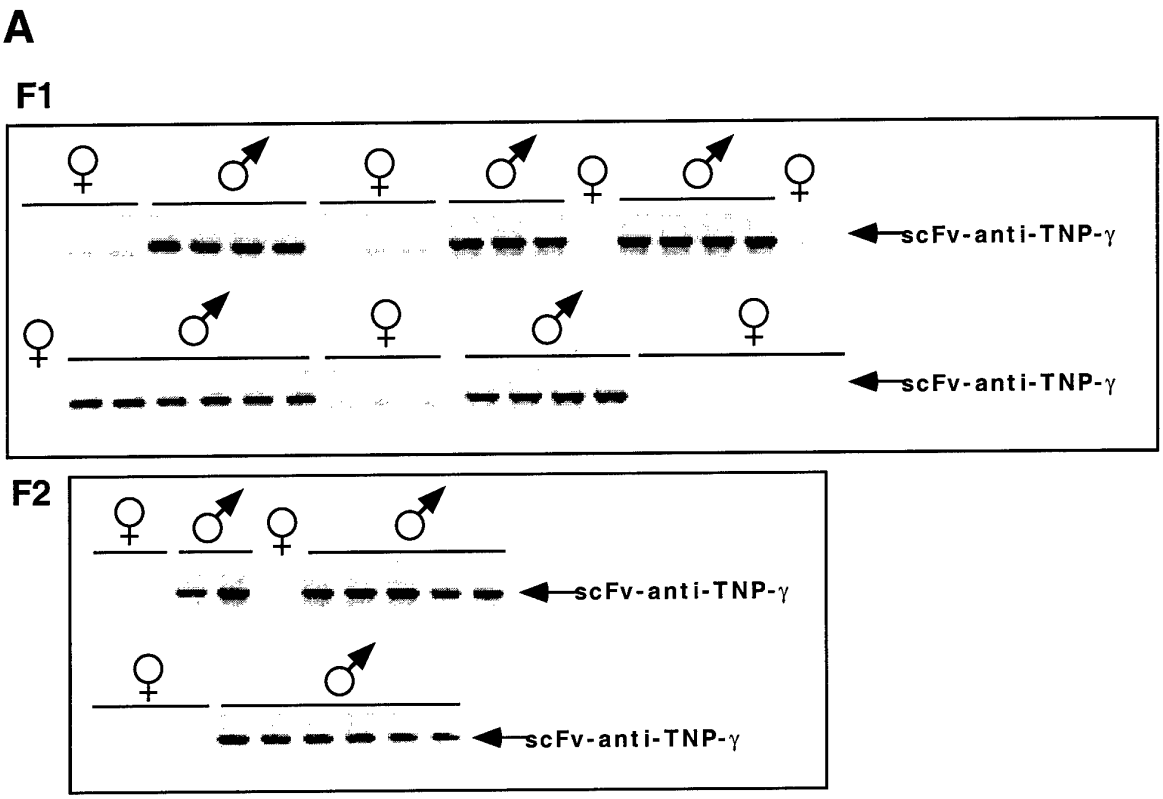
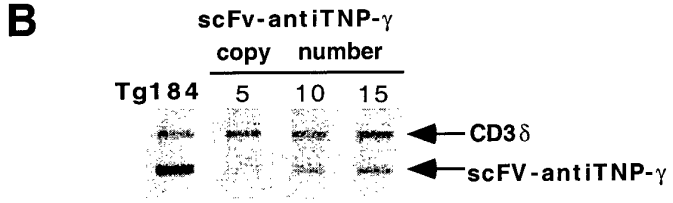


Figure 6. Mouse line Tg184 transgenic for (CD3δ)-scFv-antiTNP-γ bears ~50 copies of transgene integrated in the Y chromosome. (A) Male-specific PCR-amplification of scFv-antiTNP-γ sequences from genomic DNA of F1 and F2 progeny descended from the founder male of line Tg184.



B) Estimation of the number of genomically integrated copies of transgene. Copy number estimation was performed via semi-quantitative PCR amplification of a scFv-antiTNP-γ specific sequence from genomic DNA and from copy number standards composed of C57BL/6 DNA containing dosed quantities of transgene-encoding vector. Amplification products were separated by agarose gel electrophoresis and visualized by EtBr staining.

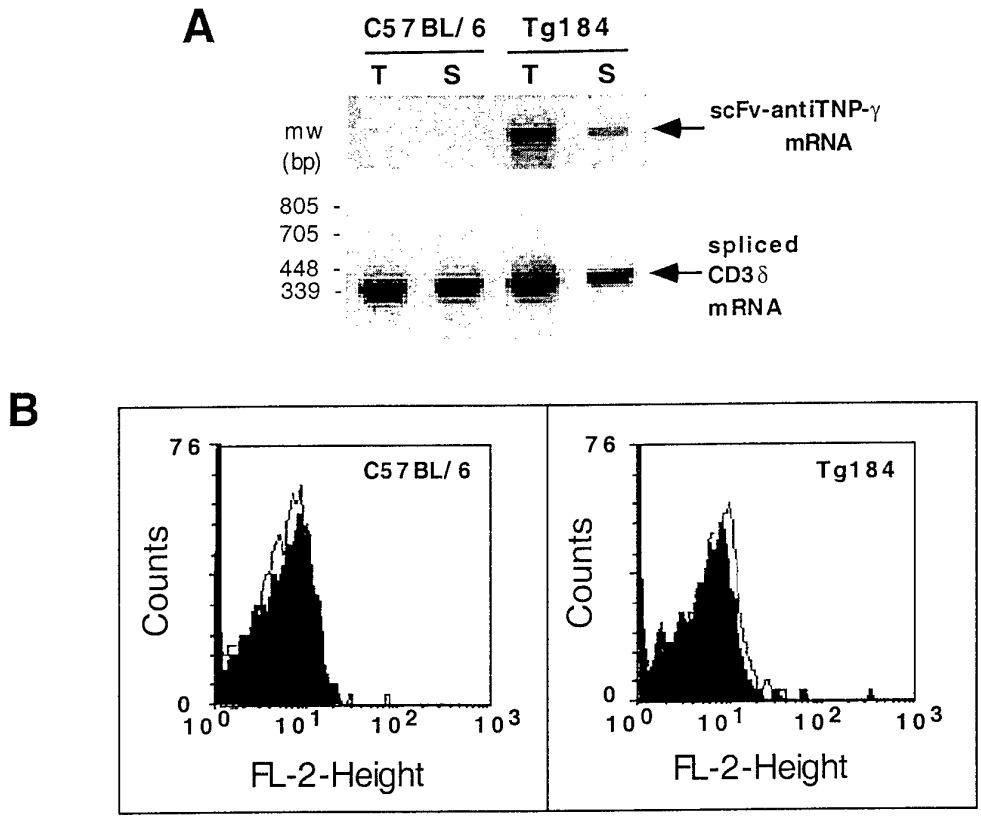


Figure 7. Expression of (CD3δ)-scFv-anti-TNP-γ transgene in splenic T cells of Tg184 mice. (A) Splenic T cells from transgenic mouse line Tg184 express scFV-antiTNP-γ mRNA. Complementary DNA from splenic T cells (S) purified by panning on immobilized Ig and thymocytes (T) was PCR-amplified with Sp6-γ specific primers. To control for amplification from mature spliced mRNA and to normalize samples cDNA was amplified with intron-flanking CD3δ primers which generate a 345 bp or a 759 bp amplification product from spliced or unspliced CD3δ mRNA, respectively. Reaction products were separated by agarose gel electrophoresis and visualized by EtBr staining. (B) Immunofluorescence analysis of scFv-antiTNP-γ surface expression. Primary staining reagent, anti-Sp6-idiotype mAb GK-20.5; irrelevant primary staining reagent, anti-DNP mAb U-7.6 (solid fill); secondary detection reagent; PE-donkey anti-mouse IgG polyclonal Ab.

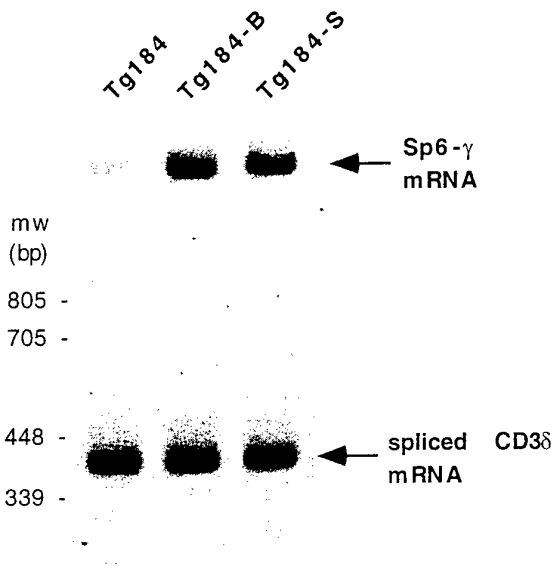


Figure 8. Hybrid Tg184-B and Tg184-S splenocytes express scFv-antiTNP-γ mRNA at higher levels than Tg184. Complementary DNA prepared from whole splenocytes was PCR amplified with scFv-antiTNP-γ specific primers (top). Amplification with intron-flanking CD3δ-specific primers was performed to control for amplification from mature spliced mRNA and to normalize samples (bottom). These primers amplify a 345 bp or a 759 bp sequence from cDNA generated from spliced or unspliced CD3δ sequences, respectively. Reaction products were separated by agarose gel electrophoresis and visualized by EtBr staining.

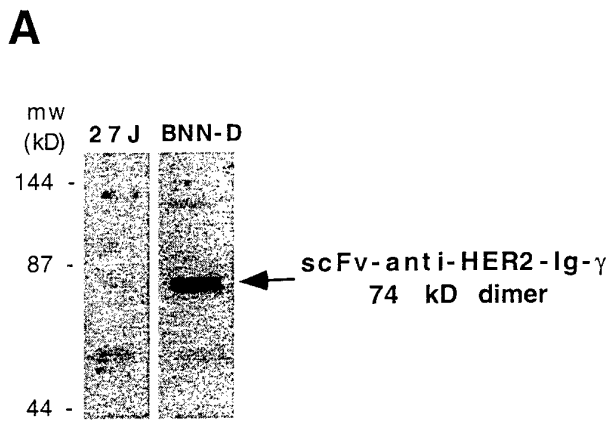
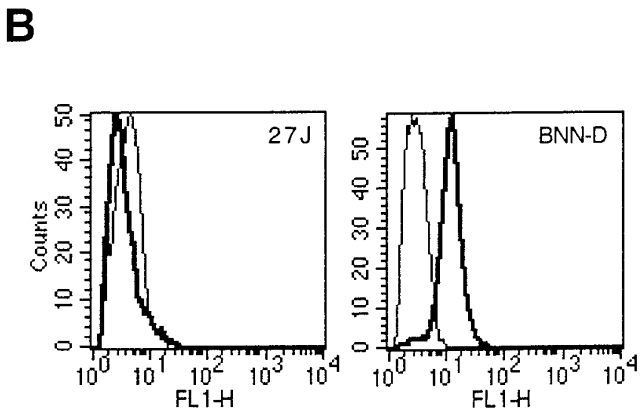


Figure 9. Hybridoma transfectant BNN-D expresses CD3δ-regulated scFV-antiHER2-Ig-γ protein. (A) Immunoblotting analysis of protein expression. Lysate proteins were separated by SDS-PAGE under non-reducing conditions, blotted onto nitrocellulose and stained with rabbit anti-N29 (anti-HER2 mAb) serum followed by staining with HRP-protein A and visualized by ECL. The untransfected parental line 27J was used as negative control.



(B) Immunofluorescence analysis of receptor surface expression. Primary staining reagent, rabbit anti-N29 (anti-HER2 mAb) serum (bold); irrelevant primary staining reagent, normal rabbit serum; secondary detection reagent, FITC-goat anti-rabbit IgG Ab.

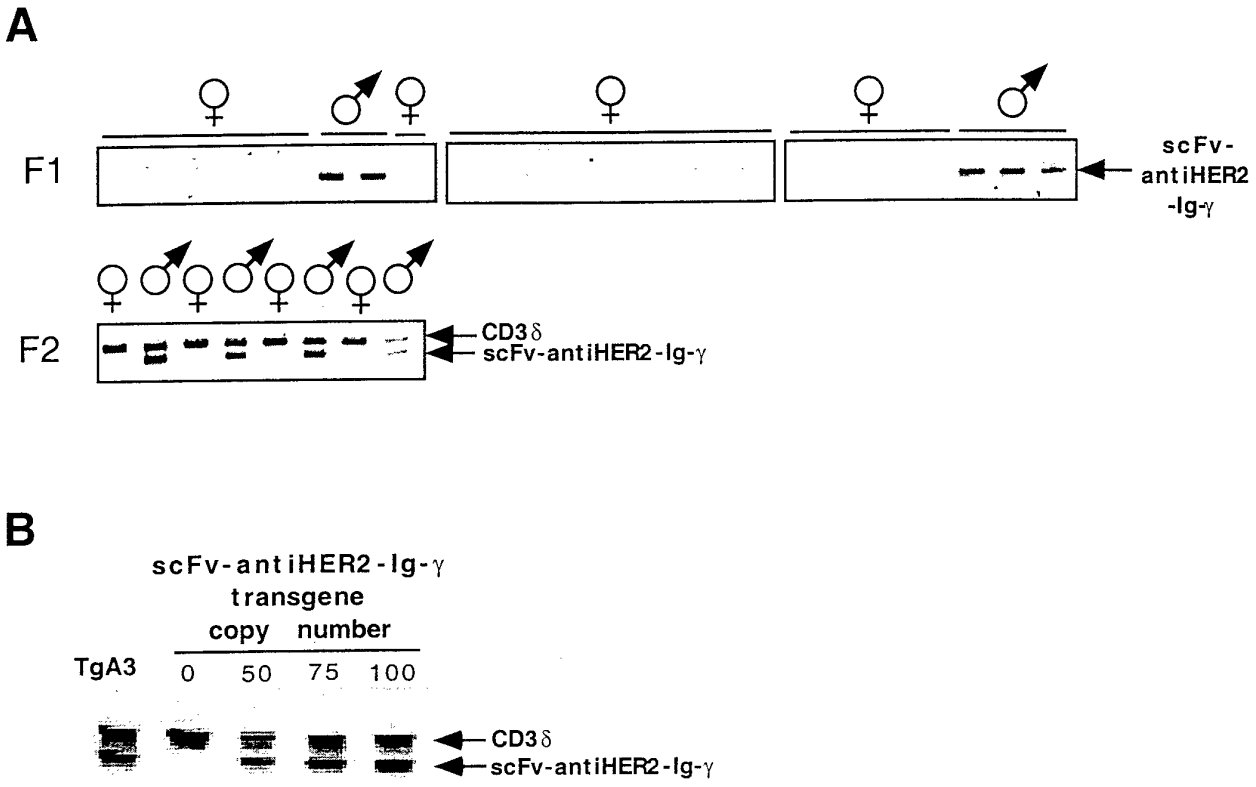


Figure 10. Mouse line TgA3 transgenic for (CD3δ)-scFv-antiHER2-Ig-γ bears 75-100 copies of transgene integrated in the Y chromosome. (A) Male-specific PCR-amplification of scFv-antiHER2-Ig-γ sequences from genomic DNA of F1 and F2 progeny descended from the founder male of line TgA3. (B) Estimation of the number of genomically integrated copies of transgene. Copy number estimation was performed via semi-quantitative PCR amplification of a scFv-antiHER2-Ig-γ specific sequence from genomic DNA and from copy number standards composed of C57BL/6 DNA containing dosed quantities of transgene-encoding vector. Amplification products were separated by agarose gel electrophoresis and visualized by EtBr staining.

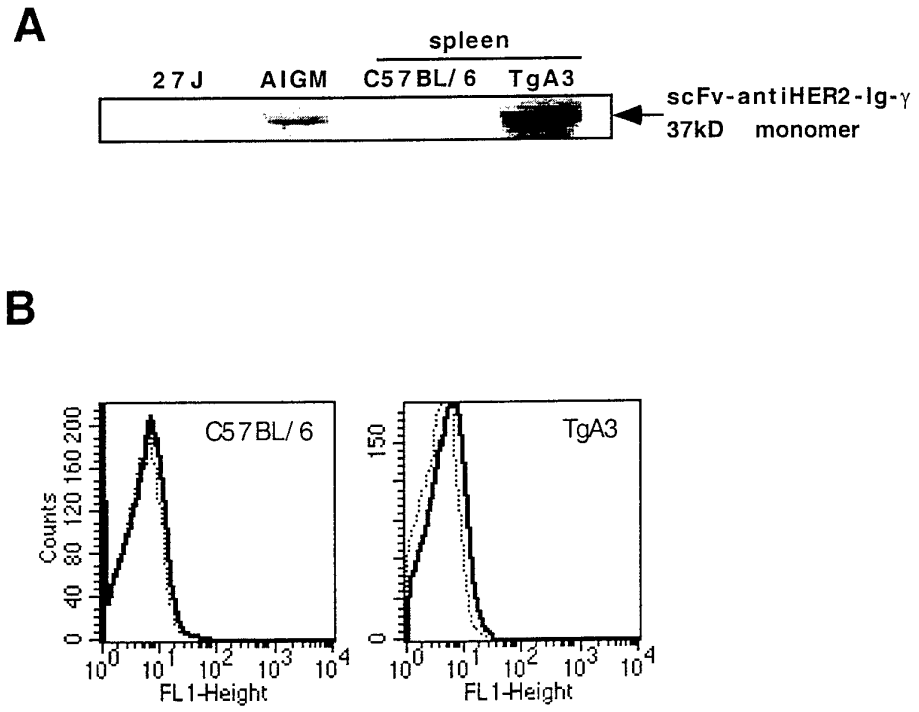


Figure 11. Expression of transgene protein in T cells from mouse line TgA3 transgenic for (CD3δ)-scFv-antiHER2-Ig-γ . (A) Immunoblotting analysis of protein expression in splenocytes. Lysate proteins were separated by SDS-PAGE under reducing conditions, blotted onto nitrocellulose and stained with rabbit anti-N29 (anti-HER2 mAb) serum followed by staining with HRP-protein A and were visualized by ECL. The scFv-antiHER2-Ig-γ expressing hybridoma transfectant AIGM and its untransfected parental line 27J were used for positive control. (B) Immunofluorescence analysis of surface expression in thymocytes of TgA3 mice. Primary staining reagent, rabbit anti-N29 (anti-HER2 mAb) serum; irrelevant primary staining reagent; normal rabbit serum (dotted line); secondary detection reagent, FITC-goat anti-rabbit IgG Ab.

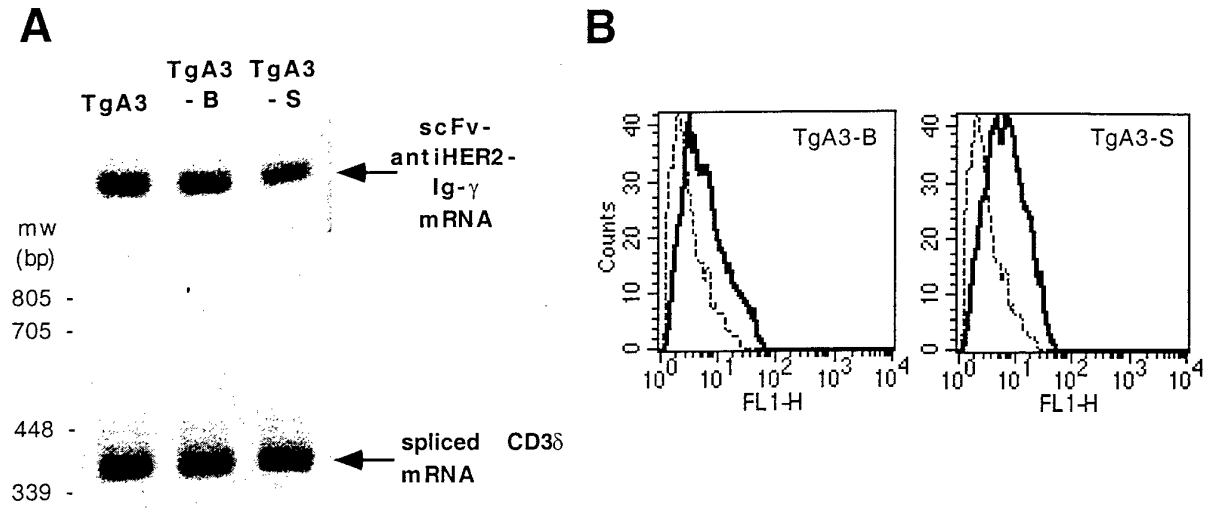


Figure 12. Expression of (CD3δ)-scFv-antiHER2-Ig-γ in splenocytes from hybrid TgA3-B and TgA3-S mice. (A) Splenocytes from TgA3-B and TgA3-S mice express scFv-antiHER2-Ig-γ mRNA. Complementary DNA was PCR-amplified at sub-saturating levels with scFv-antiHER2-Ig-γ specific primers (top). To control for amplification from mature spliced mRNA and to normalize samples, cDNA was amplified with intron-flanking CD3δ-specific primers (bottom). These primers amplify a 345 bp or a 759 bp sequence from cDNA generated from spliced or unspliced CD3δ mRNA, respectively. Reaction products were separated by agarose gel electrophoresis and visualized by EtBr staining. (B) Splenocytes from hybrid TgA3-B and TgA3-S mice express higher levels of scFv-antiHER2-Ig-γ surface protein than TgA3. For immunofluorescence analysis splenocytes were stained with rabbit anti-N29 (anti-HER2 mAb) serum followed by staining with FITC-goat anti-rabbit IgG Ab. Shown here are histogram overlays of the indicated hybrid cells (solid line) over TgA3 cells (dashed line).

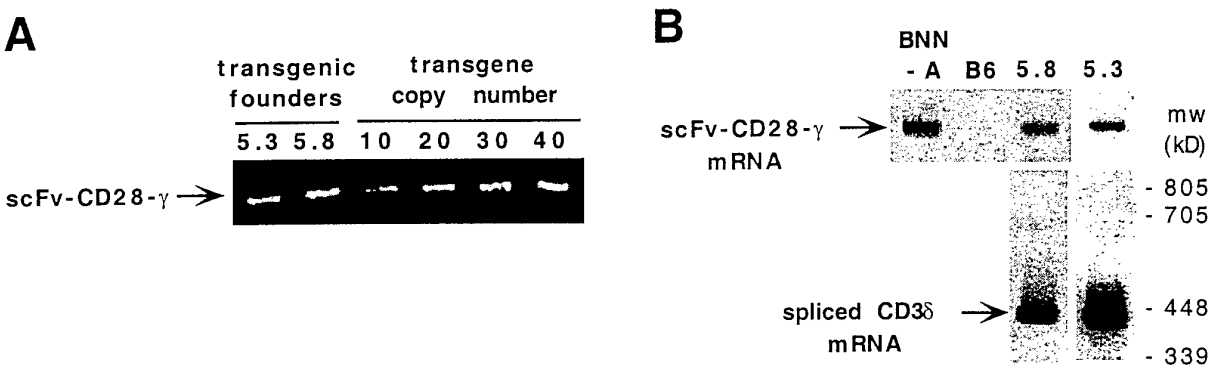


Figure 13. Mouse lines Tg5.3 and Tg5.8 transgenic for (CD3δ)-scFv-antiTNP-CD28-γ have ~40 and ~60 integrated copies of transgene, respectively, and express transgene mRNA. (A) Estimation of genomic transgene copy number. Copy number estimation was performed via semi-quantitative PCR amplification of a scFv-antiTNP-CD28-γ specific sequence from genomic DNA and from copy number standards composed of C57BL/6 genomic DNA containing dosed quantities of transgene-encoding vector. (B) Expression of scFv-antiTNP-CD28-γ mRNA in Tg5.3 and Tg5.8 splenocytes. Complementary DNA was PCR amplified with scFv-antiTNP-CD28-γ specific primers (top). To control for amplification from mature spliced mRNA and to normalize samples, cDNA was amplified with intron-flanking CD3δ primers (bottom). These primers generate a 345 bp or a 759 bp amplification product from mature spliced mRNA or from unspliced sequences, respectively. PCR reaction products were separated by agarose gel electrophoresis and visualized by EtBr staining.

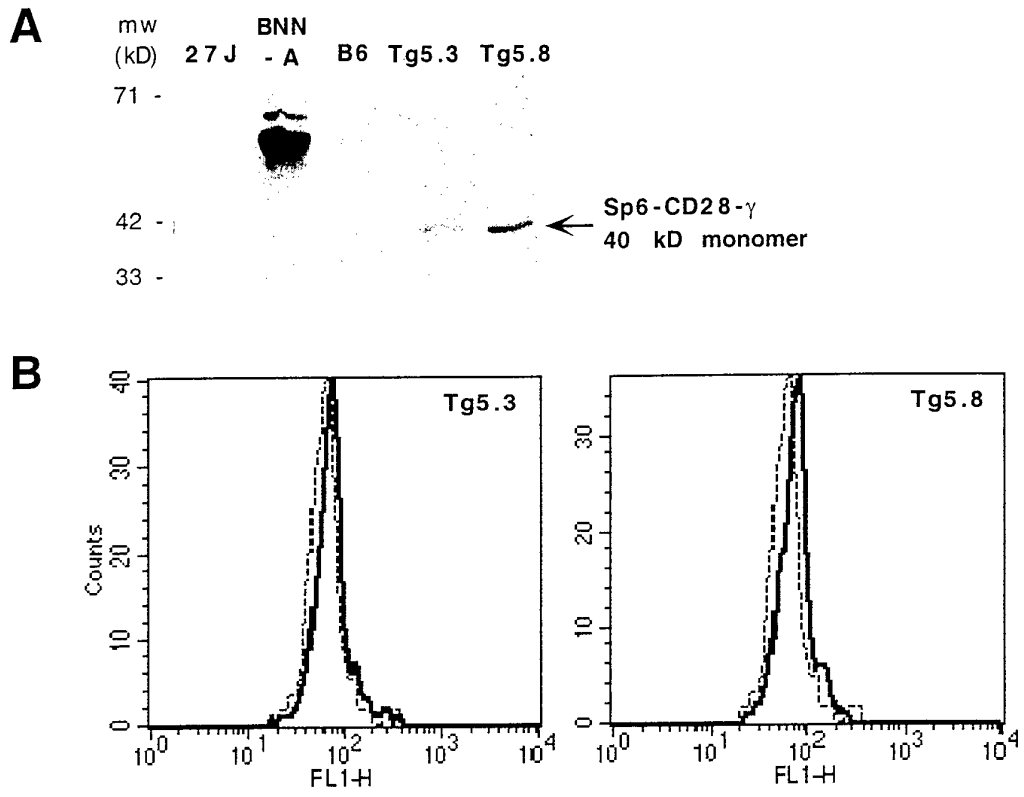


Figure 14. Mouse lines Tg5.3 and Tg5.8 transgenic for (CD3δ)-scFv-antiTNP-CD28-γ express transgene protein. (A) Expression of scFv-antiTNP-CD28-γ protein in Tg5.3 and Tg5.8 splenocytes. Detergent lysates were immunoprecipitated with anti-Sp6 (anti-TNP mAb) idiotype mAb GK20.5 and protein was separated by SDS-PAGE under reducing conditions. Protein was blotted onto nitrocellulose and stained with rabbit anti-γ serum followed by staining with HRP-protein A and visualized by ECL. The scFv-antiTNP-CD28-γ expressing T cell hybridoma transfectant BNN-A and its untransfected parental line 27J were used for positive control. Non-transgenic C57BL/6 (B6) cells were used as negative primary lymphocyte controls. Each lane contains lysate from 50 x 10^6 primary lymphocytes or 6 x 10^6 hybridoma cells. (B) Immunofluorescence analysis of scFv-antiTNP-CD28-γ surface expression in transgenic thymocytes. Cells from the indicated transgenic strain (solid line) and from non-transgenic C57BL/6 mice (dashed line) were stained with biotinylated GK-20.5 and secondarily with FITC-streptavidin.

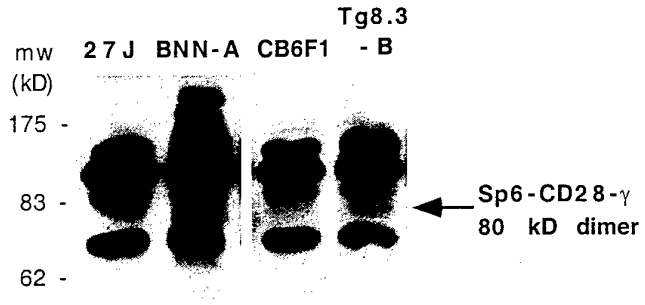


Figure 15. Expression of (CD2)-scFv-antiTNP-CD28-γ protein in T cells of Tg8.3-B mice. Thymocytes of Tg8.3-B mice were analyzed for expression of transgene protein by immunoprecipitation and Western immunoblotting analysis. Detergent lysates were immunoprecipitated with anti-Sp6 (anti-TNP mAb)idiotype mAb GK20.5, separated by SDS-PAGE under partially reducing conditions (required to maintain Sp6 detection epitope) and blotted onto nitrocellulose. Blots were stained with GK20.5 mAb followed by secondary staining with HRP-coupled goat-anti-mouse IgG Ab and specific protein was visualized by ECL. The scFv-antiTNP-CD28-γ expressing T cell hybridoma transfectant BNN-A and its untransfected parental line 27J were used for positive control. Non-transgenic CB6F1 cells were used as negative primary lymphocyte controls. Each lane contains lysate from 50 x 10^6 primary lymphocytes or 5 x 10^6 hybridoma cells.

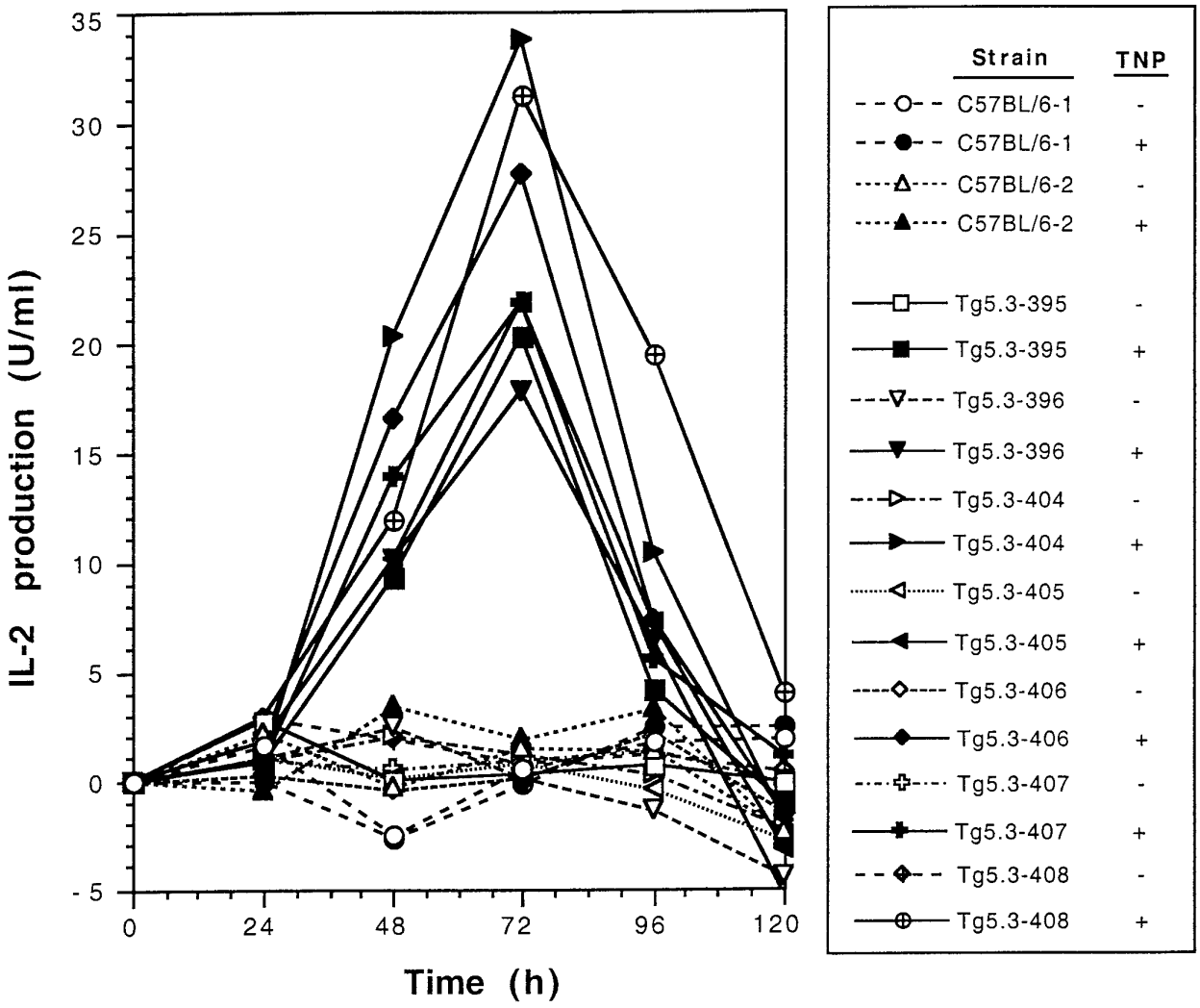


Figure 16. Untreated primary splenocytes from mouse line Tg5.3 transgenic for scFv-antiTNP-CD28-γ produce IL-2 in response to TNP stimulation. Data were derived from parallel stimulations of seven Tg5.3 and two non-transgenic C57BL/6 mice at high cell density.

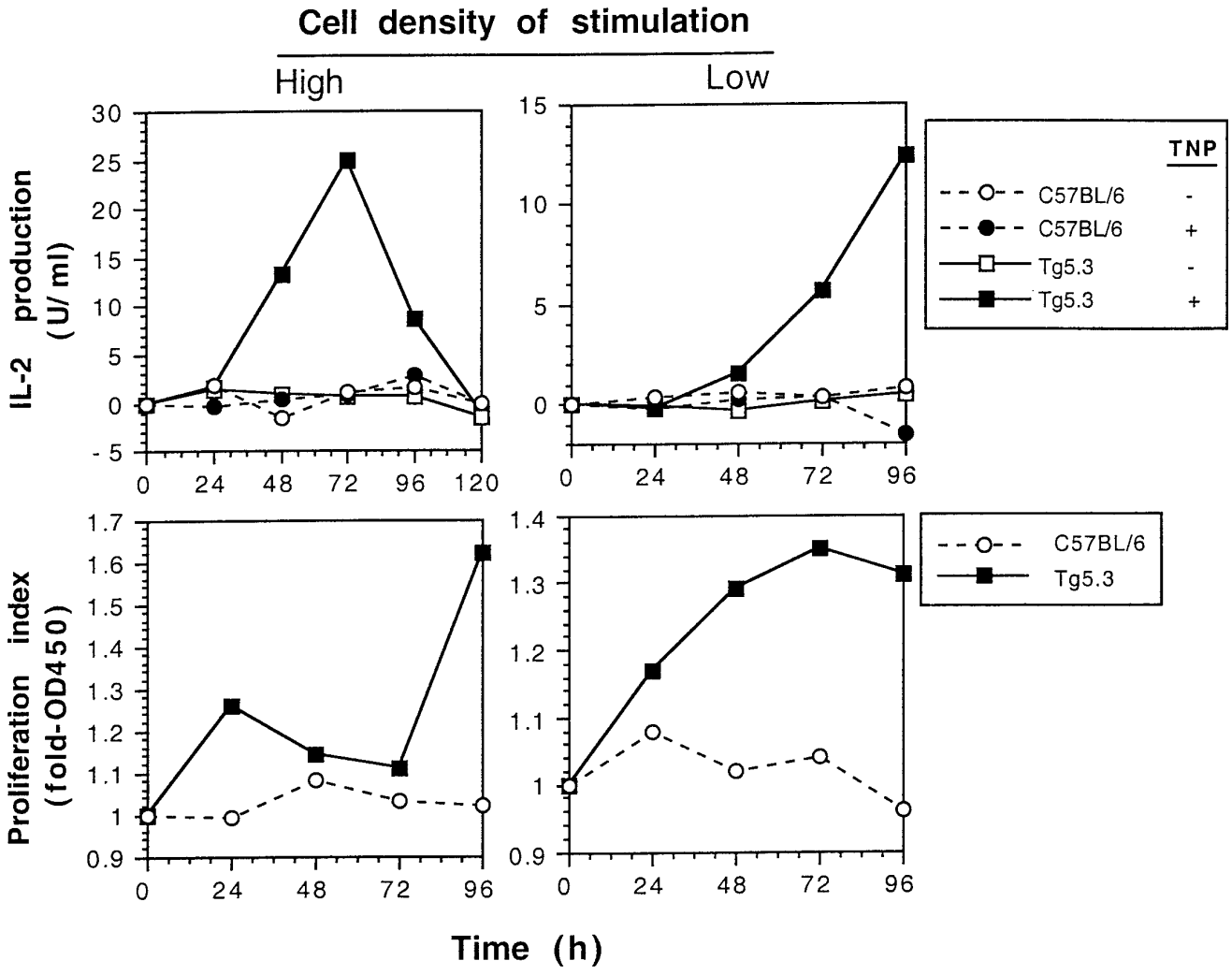


Figure 17. Untreated primary splenocytes from mouse line Tg5.3 transgenic for scFv-antiTNP-CD28-γ proliferate and produce IL-2 in response to TNP stimulation. Data represents average values obtained from parallel stimulations of two non-transgenic C57BL/6 mice and seven Tg5.3 mice.

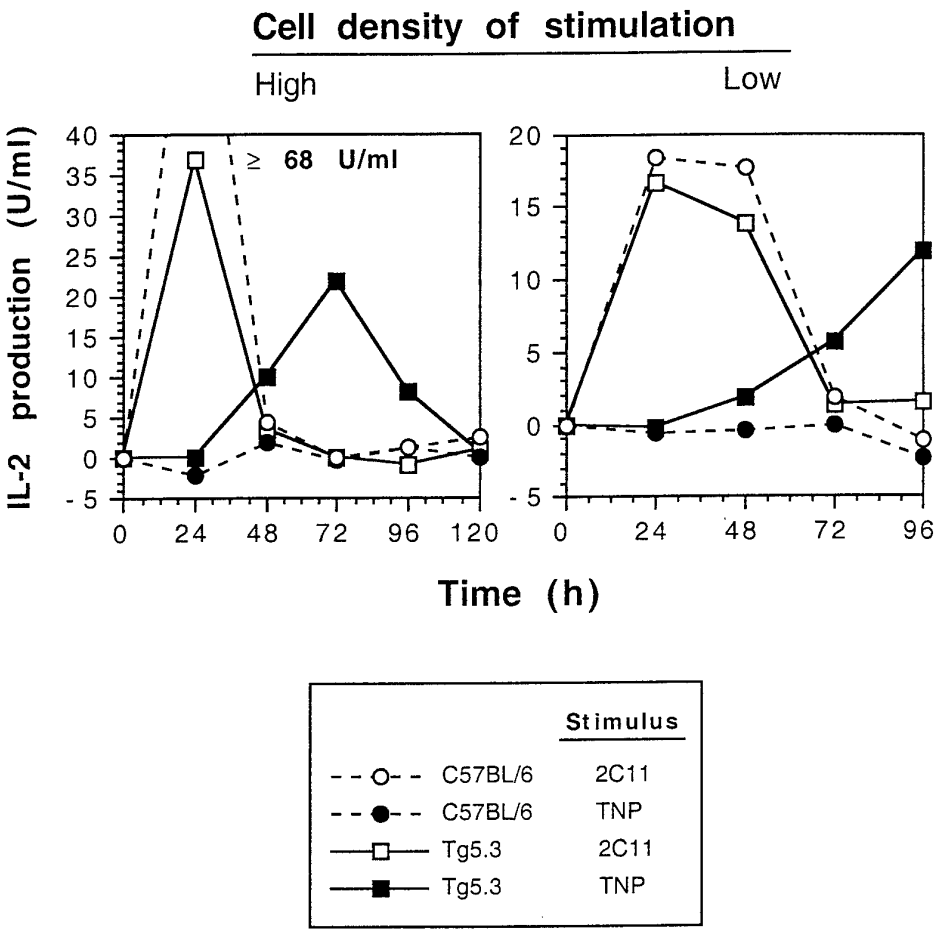


Figure 18. Comparison of IL-2 production by primary Tg5.3 splenocytes transgenic for scFv-antiTNP-CD28-γ in response to stimulation with TNP or 2C11. Data shown represents average values derived from parallel stimulations of two non-transgenic C57BL/6 mice, and seven and five Tg5.3 mice at low and high cell density, respectively.

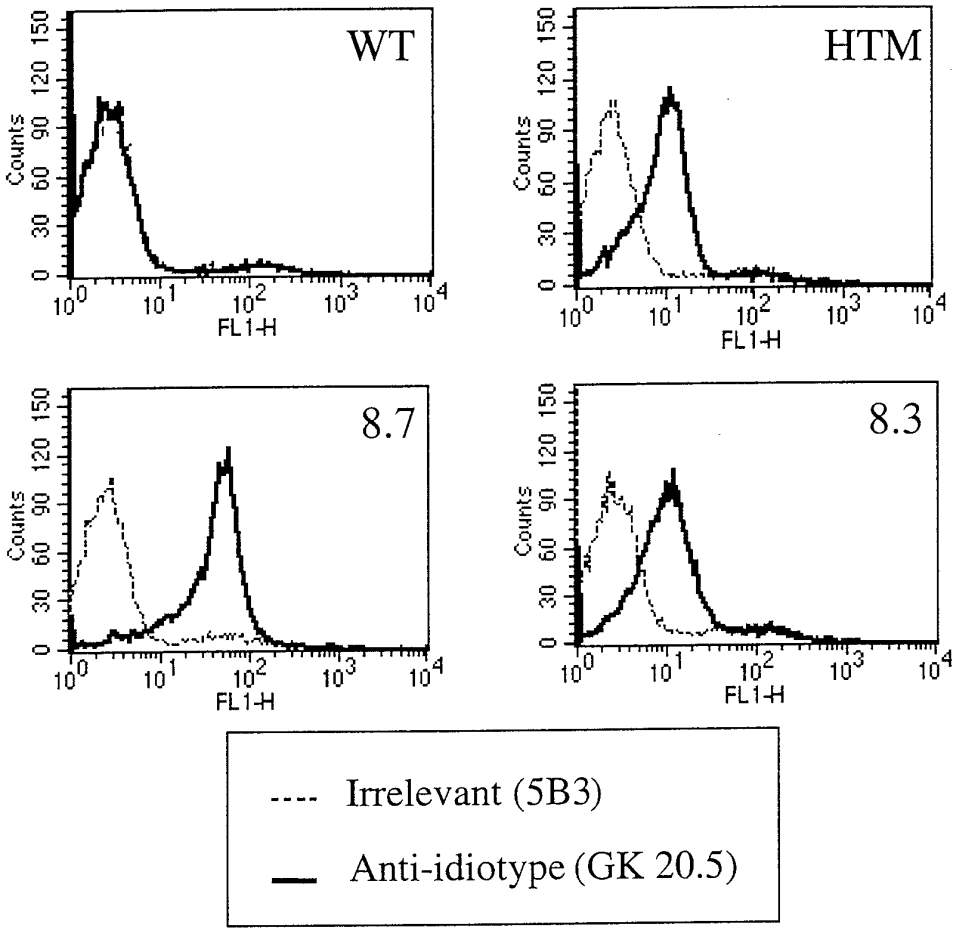


Fig 19. *Surface expression of anti-TNP CR in T cells of transgenic mice from the CD2 series.* Splenic T cells of WT and transgenic mice were analyzed for surface expression of CR by immunofluorescent flow cytometric analysis. Bulk splenocytes from WT or Tg mice were double-stained with PE-conjugated rat anti-mouse Thy1.2 and either biotinylated anti-Sp6-idiotype mAb GK-20.5 (bold lines) or matching biotinylated isotype control anti-DNP mAb 5B3 (dashed lines) followed by secondary staining with FITC-conjugated streptavidin. Histograms were generated by gating on Thy1.2^{+} lymphocytes.

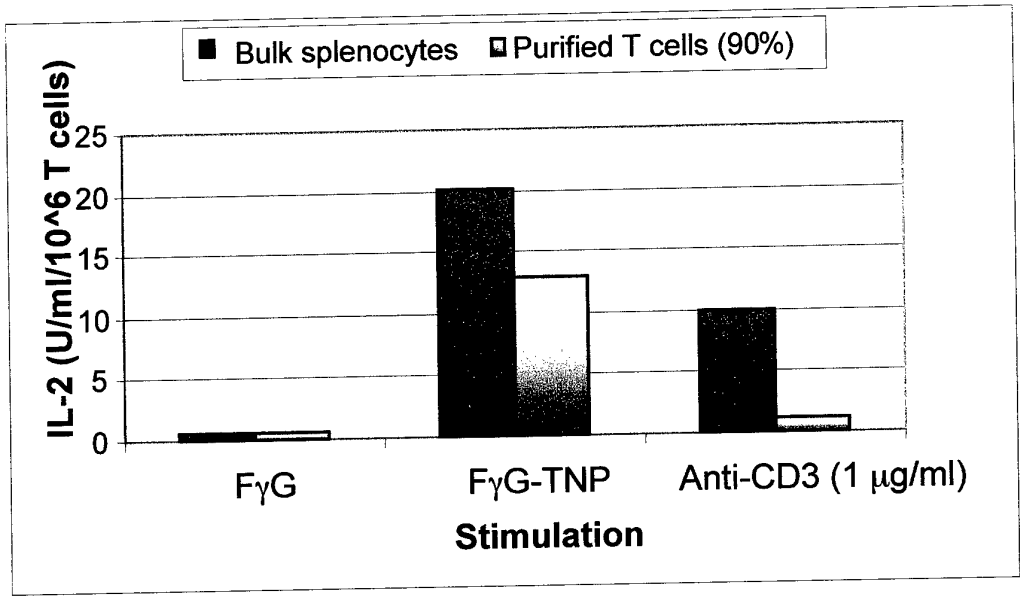


Fig 20. *Stimulation via SP6-CD28-γ induces APC-independent IL-2 production in purified (90%) unprimed naïve splenic T cells of Tg8.7 mice (24 hr).* Bulk and purified splenic T cells were cultured in plates immobilzed with FγG (10 μg/ml), FγG-TNP (10 μg/ml) and anti-CD3 (1 μg/ml). SN were harvested and tested for IL-2 activity.

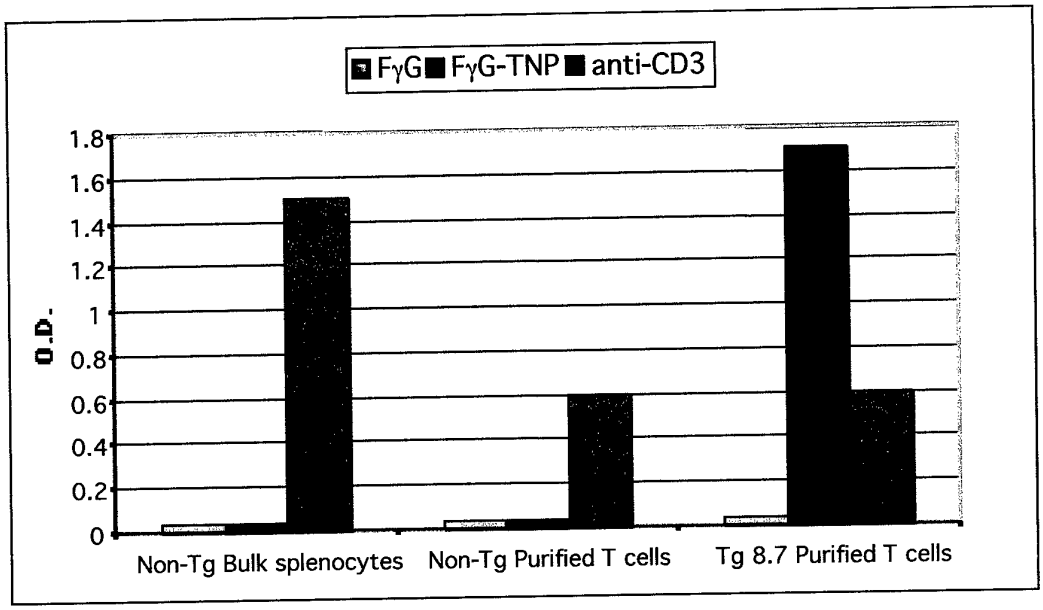


Fig 21. *Unprimed naïve T cells (90%) from Tg8.7 mice proliferate in response to stimulation via Sp6-CD28-γ.* Bulk and purified splenic T cells ($2x10^5$ cells/ml) were cultured in 96-well flat-bottom microplates immobilzed with FγG (10 μg/ml), FγG-TNP (10 μg/ml) and anti-CD3 (2 μg/ml). After 48 hrs, proliferation was assessed via XTT assay.

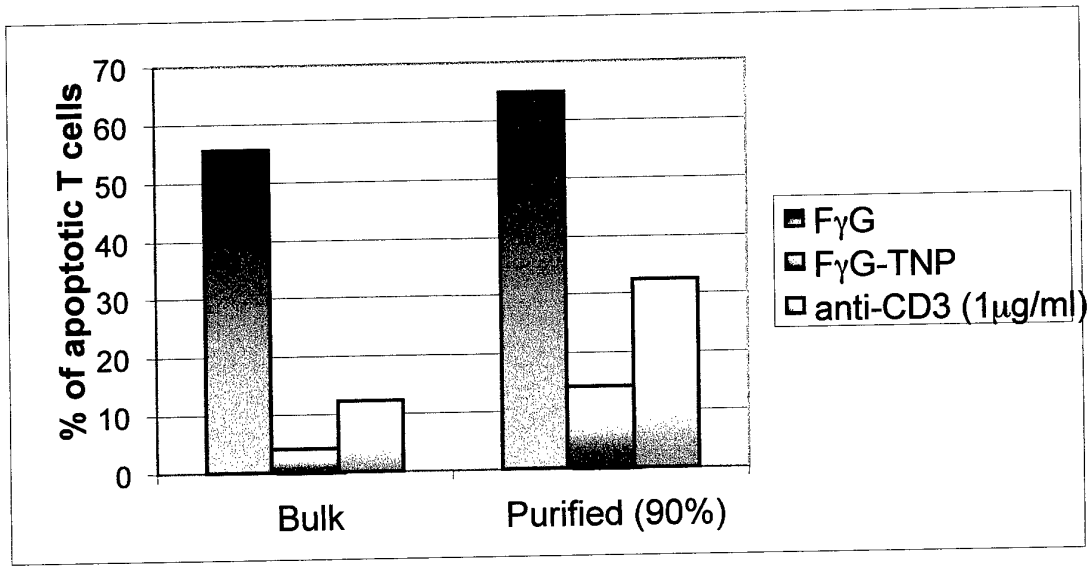


Fig 22. *Stimulation via SP6-CD28-γ induces rescue from apoptosis in purified (90%) unprimed naïve splenic T cells of Tg8.7 mice (72 hr).* Bulk and purified T cells were cultured in 24-well plates that had been coated with the indicated reagents. After 72hrs, cells were stained with FITC-Thy1.2 and fixed. PI and Rnase were added for cell cycle analysis. Percent of apoptosis was measured by FACS analysis on Thy1.2+ gated cells and calculating the percentage of cells displaying sub-G_1/G_0 DNA content.

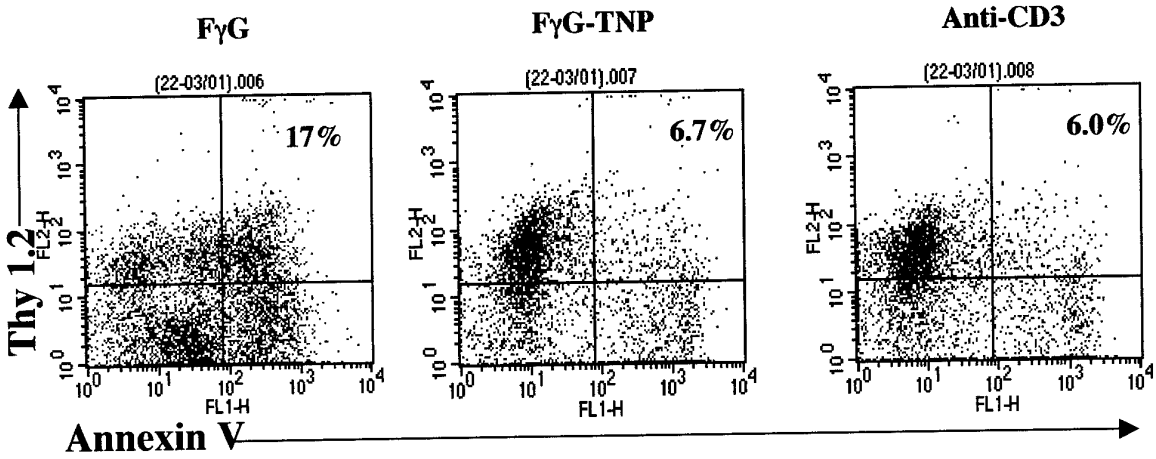


Fig 23. *Rescue from apoptosis (Annexin V staining) in unprimed naïve T cells of Tg8.7 (90%).* Purified T cells were cultured in 24-well plates that had been coated with the indicated reagents. After 72hrs, cells were stained with PE-Thy1.2 and FITC-Annexin V. Necrotic cells were gated out using TOPRO-3 reagent.

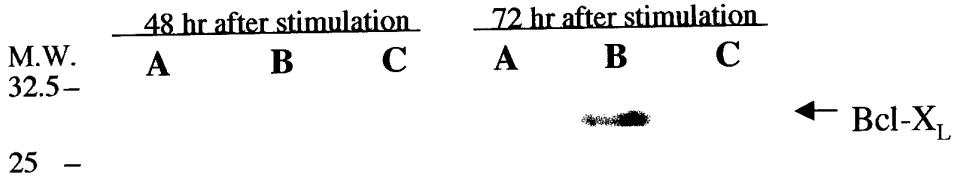


Fig 24. *Induction of bcl-X_L protein after stimulation via anti-TNP-CD28-γ CR.* Purified T cells were cultured on 24-well plates immobilzed with FγG (10 μg/ml)(**A**), FγG-TNP (10 μg/ml)(**B**) and anti-CD3 (2 μg/ml)(**C**). After 48 and 72 hrs., cytoplasmic lysates were prepared, and the lysates from the same numbers of viable cells (5 x 10^5/lane) in each group were subjected to SDS-PAGE and Western blot analysis.

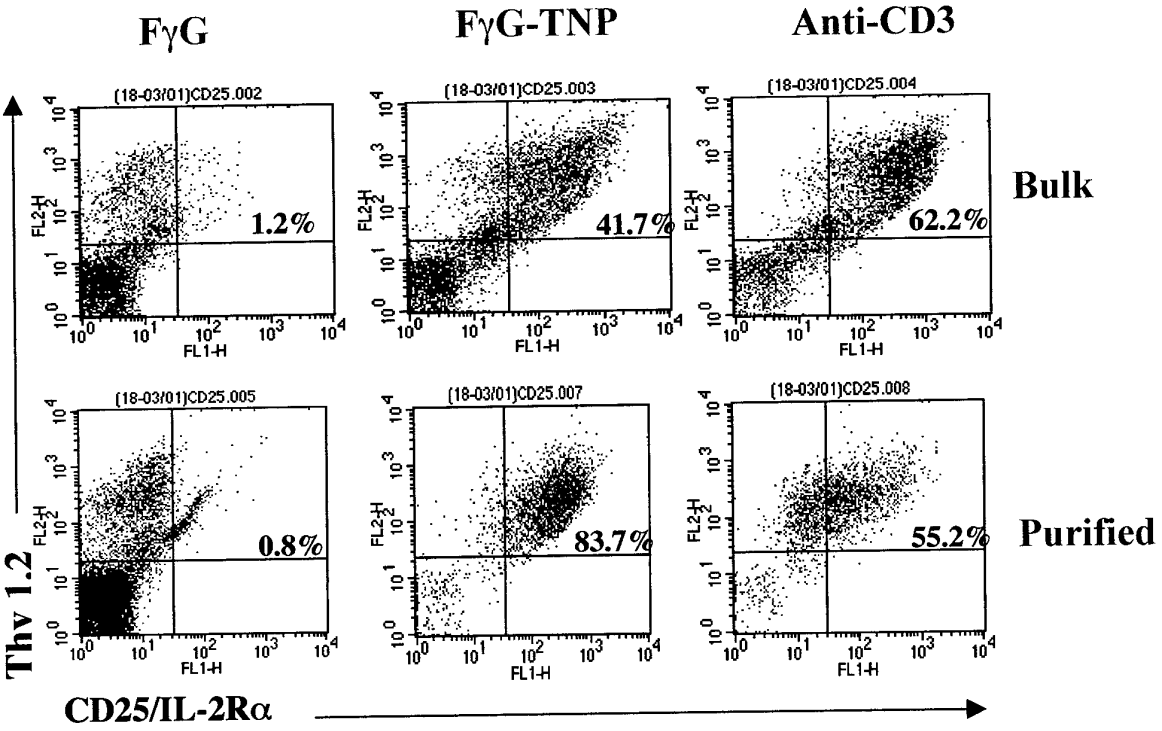


Fig 25. *Up-regulation expression of IL-2R α-chain by stimulation via anti-TNP-CD28-γ in unprimed naïve T cells of Tg 8.7-BH mice (60 hr).* Similar cultures to those in Fig 4 were performed. Sixty hours later, cells were harvested and stained with biotinylated anti-mouse IL-2R α-chain mAb, followed by FITC-conjugated streptavidin and PE-Thy1.2.

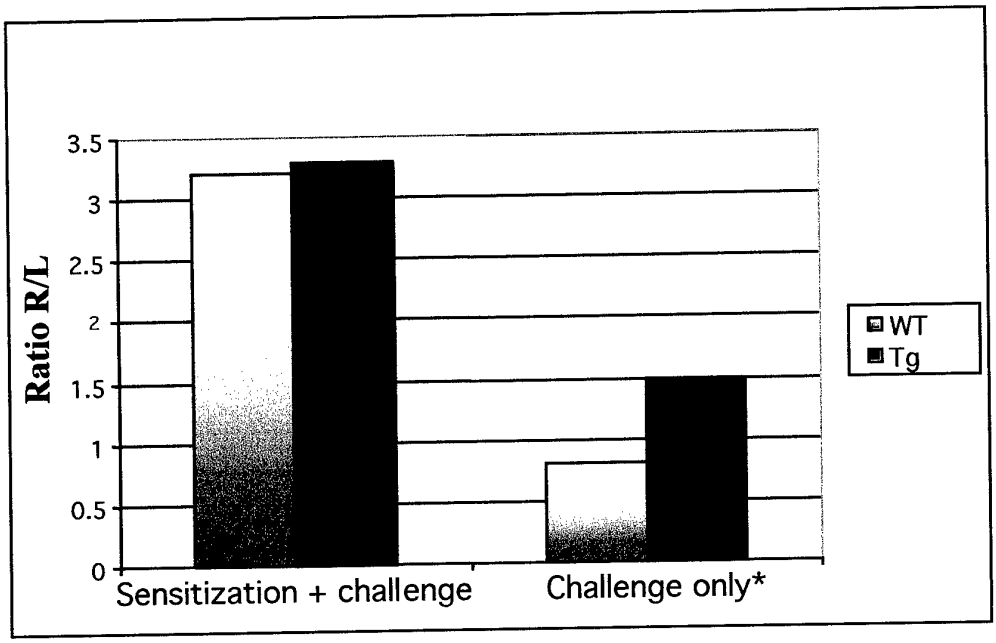


Fig 26. *DTH responses in WT and Sp6-CD28-γ transgenic mice.* All mice were cyclophosphamide pre-treated and then divided in two groups: mice from "sensitization+challenge" group were Ag-sensitized 5 days before challenge. Mice from "challenge only" group were directly challenge with the Ag in the right pinna. Four mice were tested per group. * $p<0.005$.

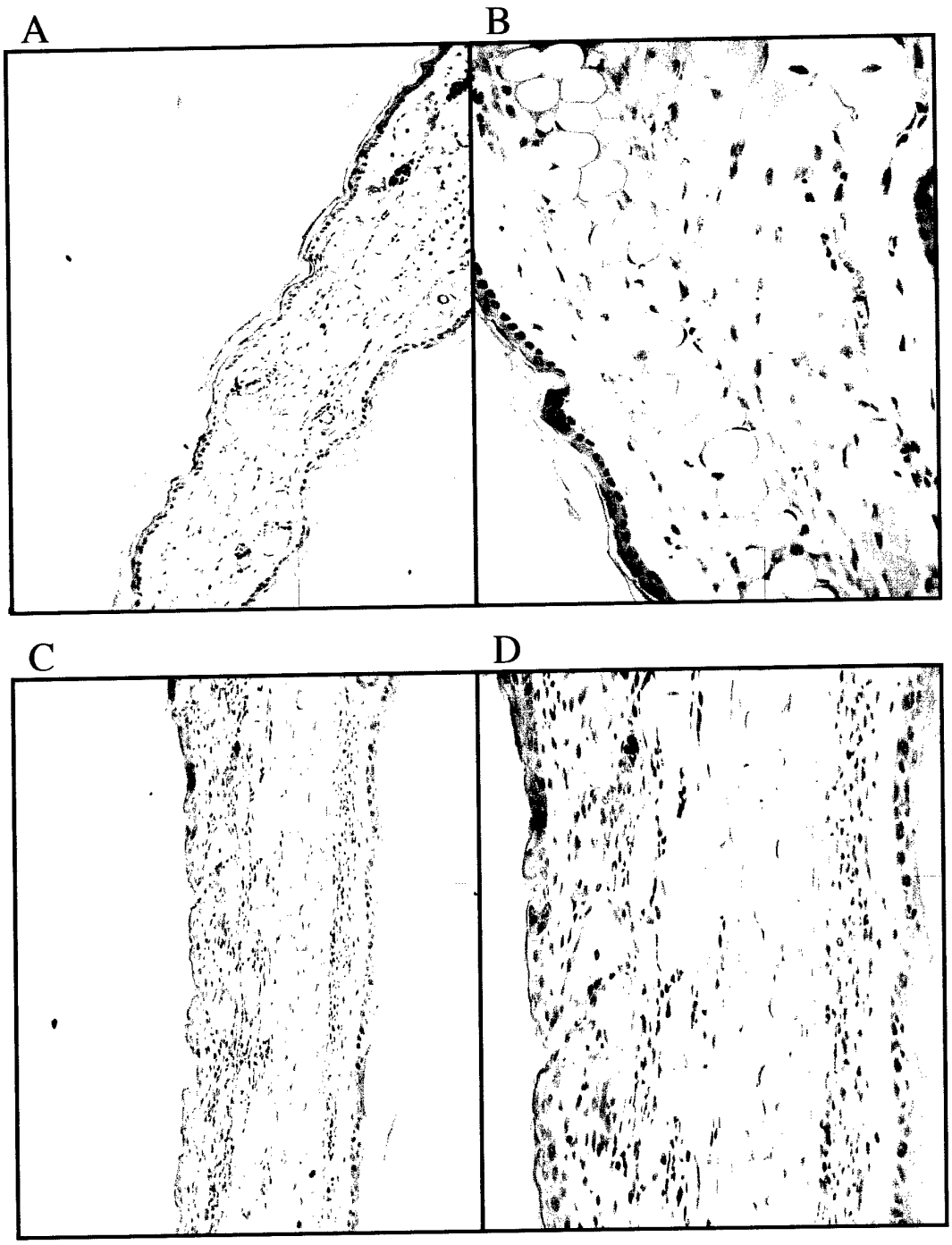


Fig 27.Histology sections of pinnae of Tg 8.7 mouse (representative of "challenge only" group, see Fig 7).Pinnae were fixed in buffered formalin, cut and stained with HE. Note subepidermal and epidermal infiltration of immflamatory cells, mainly neutrophils and lymphoid and plasma cells in the right pinna. **A** and **B** left pinna, **C** and **D** right pinna. A and C: x20. B and D: x40.

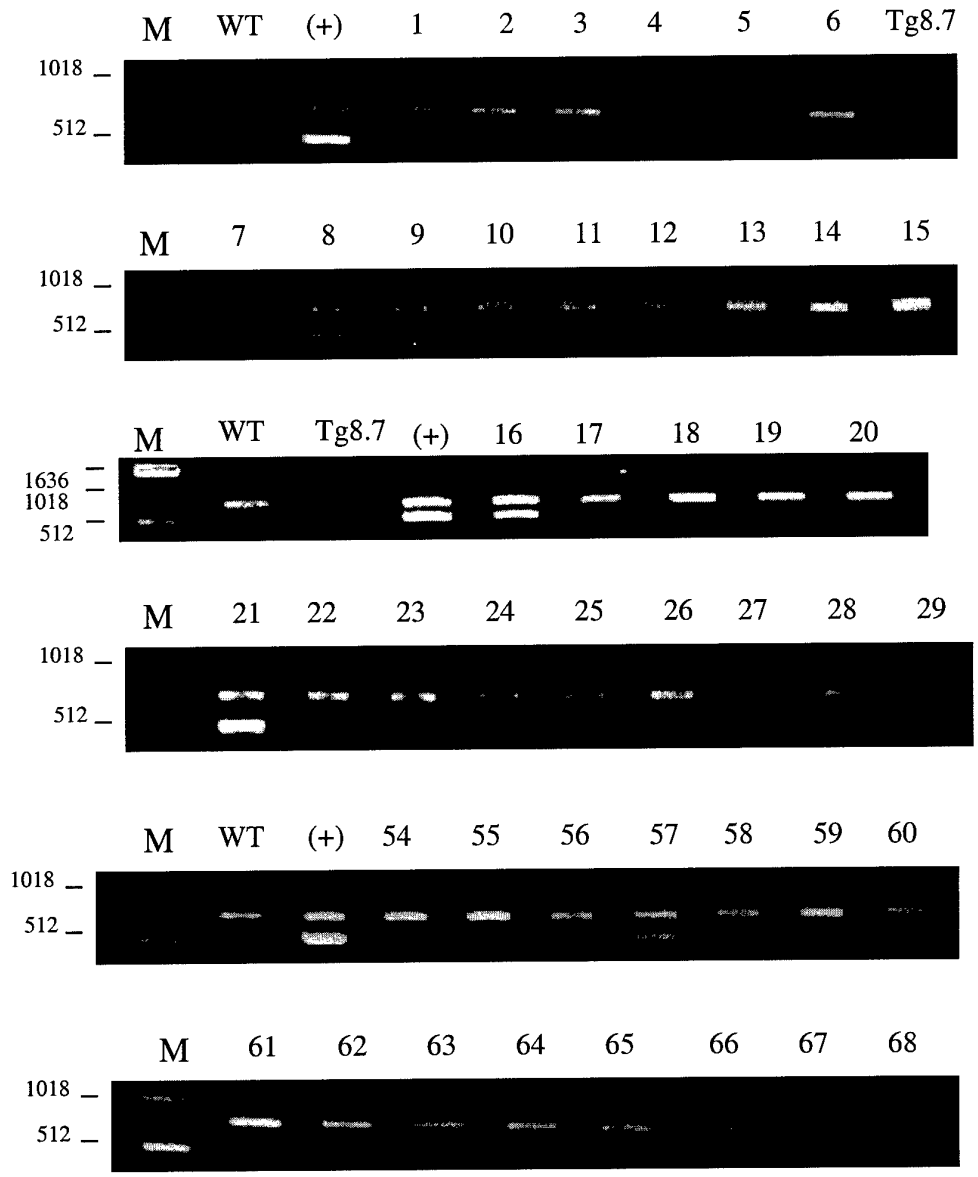


Fig.28. *PCR analysis of transgene integration in TgN29 founder mice*. Representative gels of founders TgN29 1-29 and 54-68, negative controls: CB6F1 genomic DNA (WT) and Tg8.7, positive control (+): CB6F1 genomic DNA doped with closed circular VA-N29-CD28-γ. A 530 bp TgN29 specific sequence was PCR amplified from 0.5 μg of genomic DNA using an upstream primer specific of N29 and a downstream primer located within the γ sequence. PCR products were visualized by agarose gel electrophoresis and EtBr staining.

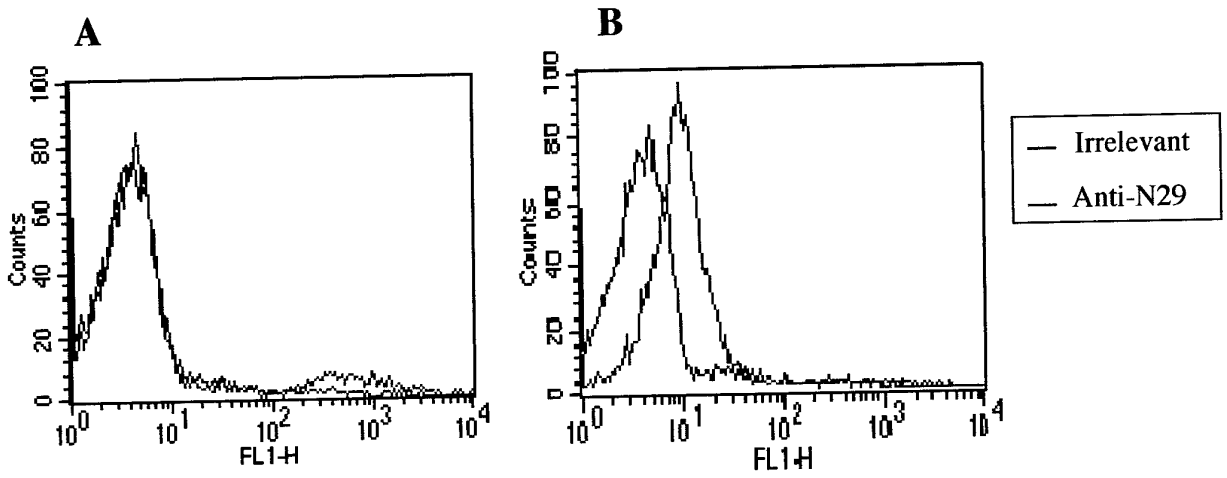


Fig.29. *Surface expression of N29-CD28-γ in T cells of transgenic founder mouse.* Peripheral blood lymphocytes of WT (A) and Tg (B) mice were stained with Thy1.2, anti-N29 idiotype and irrelevant antibody and subsequently analyzed by two-color flow cytometry. Analyzed cells are Thy1.2 gated .

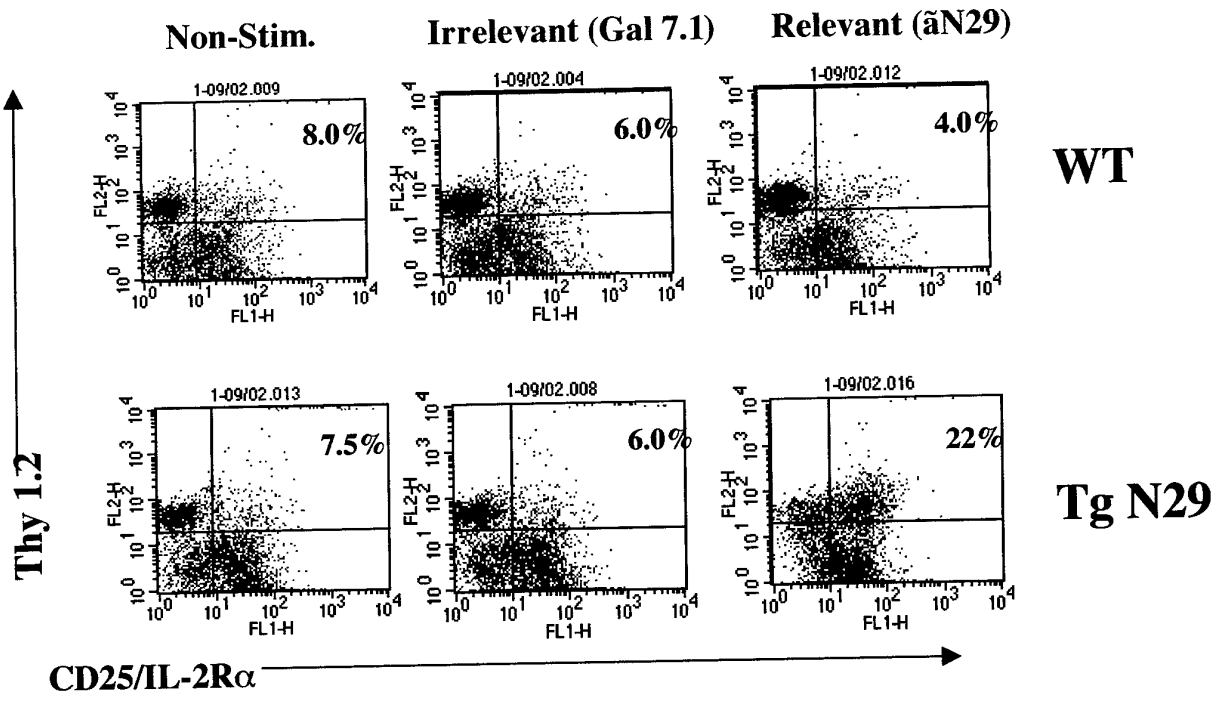


Fig 30. *Up-regulation expression of IL-2 R α-chain by stimulation via anti-ErbB-2-CD28-γ in splenocytes of Tg N29 mice (72hrs).* Bulk splenocytes were cultured in 24-well plates that had been coated with polyclonal Abs Gal 7.1, (rabbit anti-Galectin A; Irrelevant) and anti-N29 (relevant) at a concentration of 10 μg/ml. After 72 hrs, cells were harvested and stained with biotinylated anti-mouse IL-2R α-chain mAb, followed by FITC-conjugated streptavidin and PE-Thy 1.2.

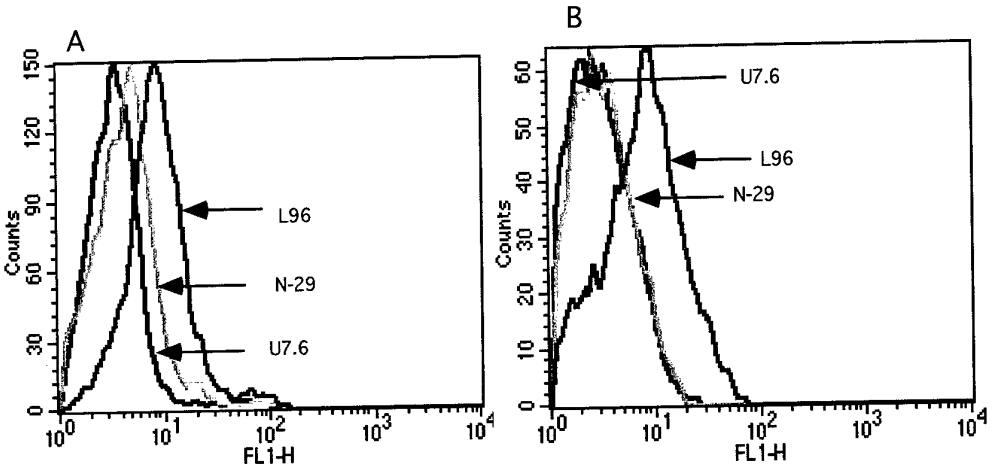


Fig 31. *Expression of the N29 epitopes of HER-2 on CWR22 tumor cells.* A- Tumor from a control animal that was treated with intra-tumoral administration of lymphocytes bearing an irrelevant receptor (anti TNP) with systemic IL-2. B- Re-growth of a tumor from an animal that was treated with intra-tumoral administration of lymphocytes bearing an anti-HER2/N29 receptor with systemic IL-2. Note 20.5% expression of N29 epitopes on the control tumors (B) in contrast to negligible expression of this epitopes on tumors that initially responded to the specific treatment but subsequently regrew. In contrast only a minor reduction in the expression of HER-2 (as indicates by staining with L96 directed against different epitopes) is noted. Staining with the isotype matched U 7.6 antibody directed against irrelevant antigens serves as a background.

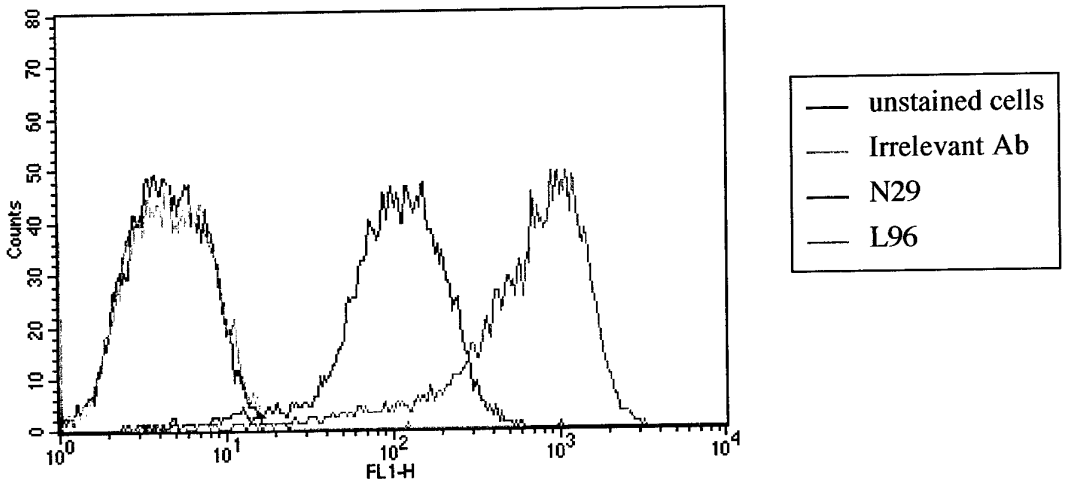


Fig 32. *Surface expression of erbB2 antigen on SKBr3 human breast adenocarcinoma cell line.* Flow cytometry analysis of SKBr3 cells stained with N29, L96 and irrelevant antibody.

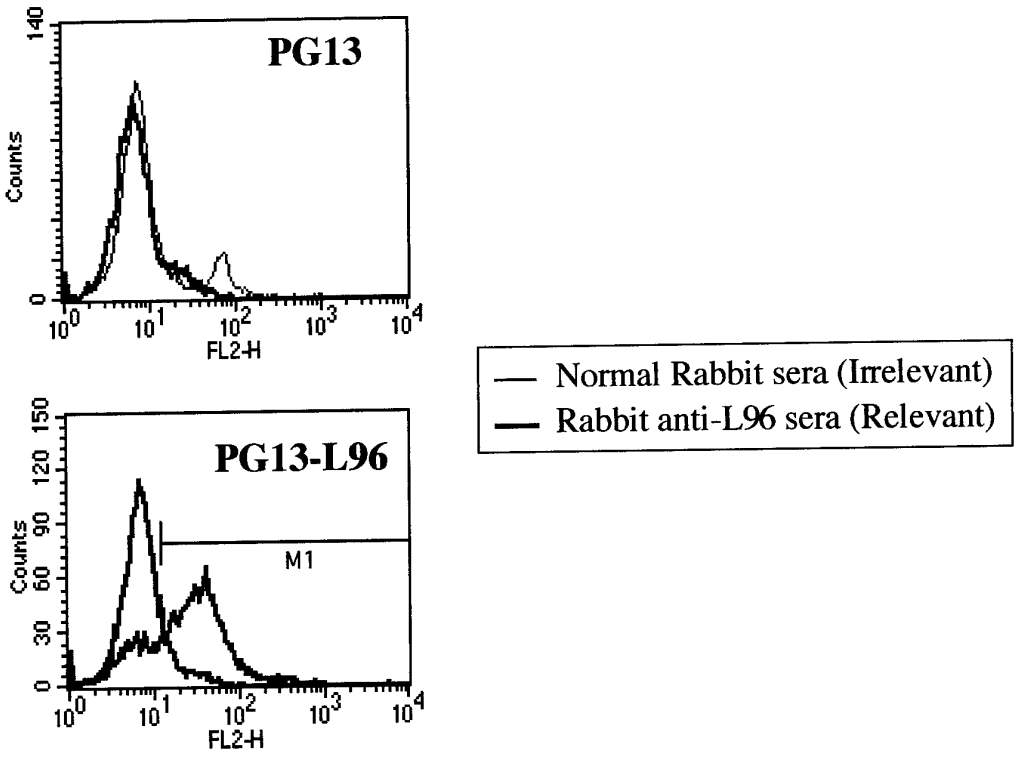


Fig 33. *CR fluorescent pattern of PG13 packaging cell line* . The PG13 packaging cell line and the PG13 cell line transfected with the L96-CD28-γ construct were analyzed for surface expression of the CR by FACS using normal rabbit sera (irrelevant) and anti-L96 anti-idiotype rabbit sera (relevant).

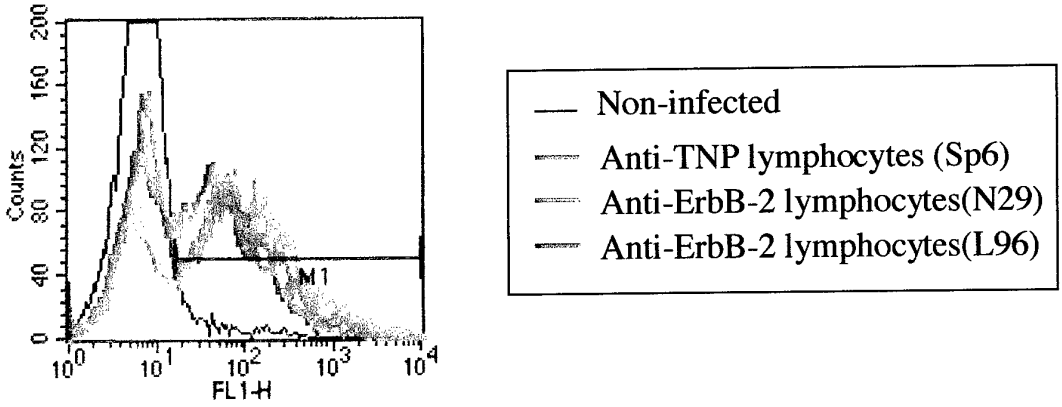


Fig 34. *Expression in human lymphocytes of the tripartite CRs* . High transduction efficacy of the human PBLs is achieved and can be monitored by FACS analysis for GFP expression.

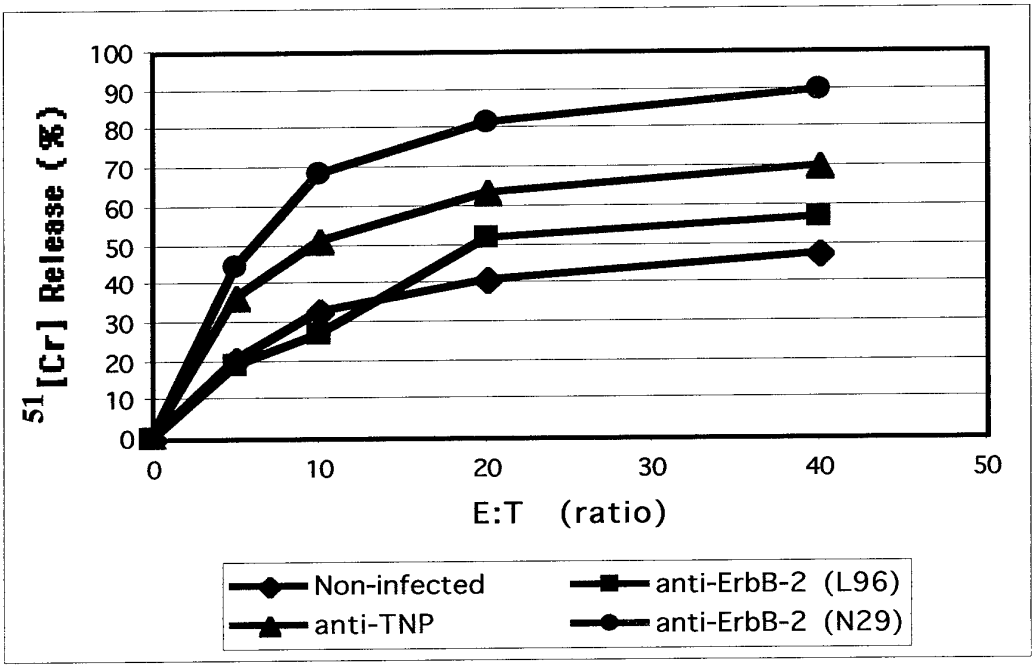


Fig 35. *Cytolysis of ErbB-2-expressing breast tumor cell line by transduced T lymphocytes.* The cytolitic function of the transduced T cells expressing the CR was evaluated in a 13 hrs ^{51}Cr release assay.

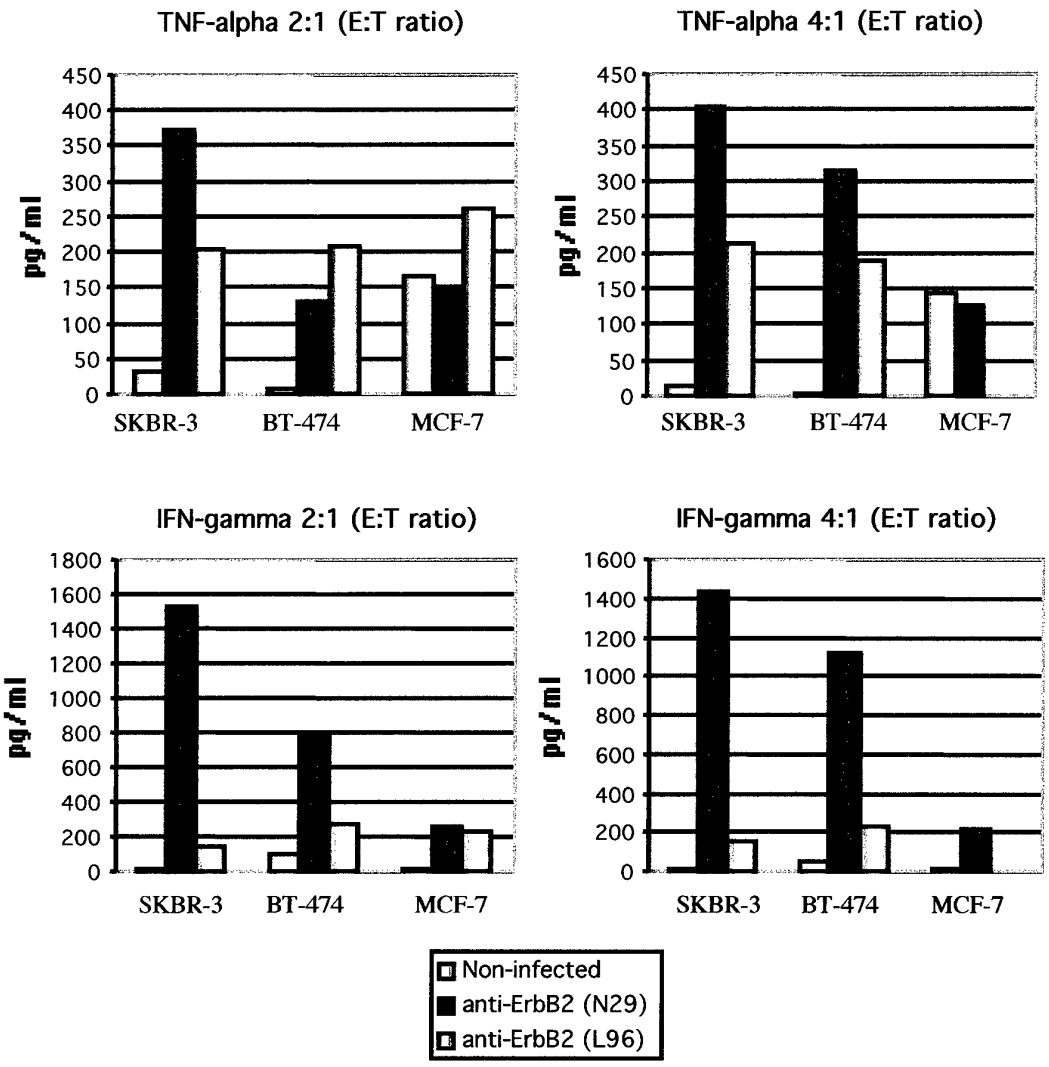


Fig 36. *Human Lymphocytes Expressing the ErbB-2 Chimeric Receptor produce a Variety of Cytokines upon their Stimulation with ErbB-2 bearing Target cells.*

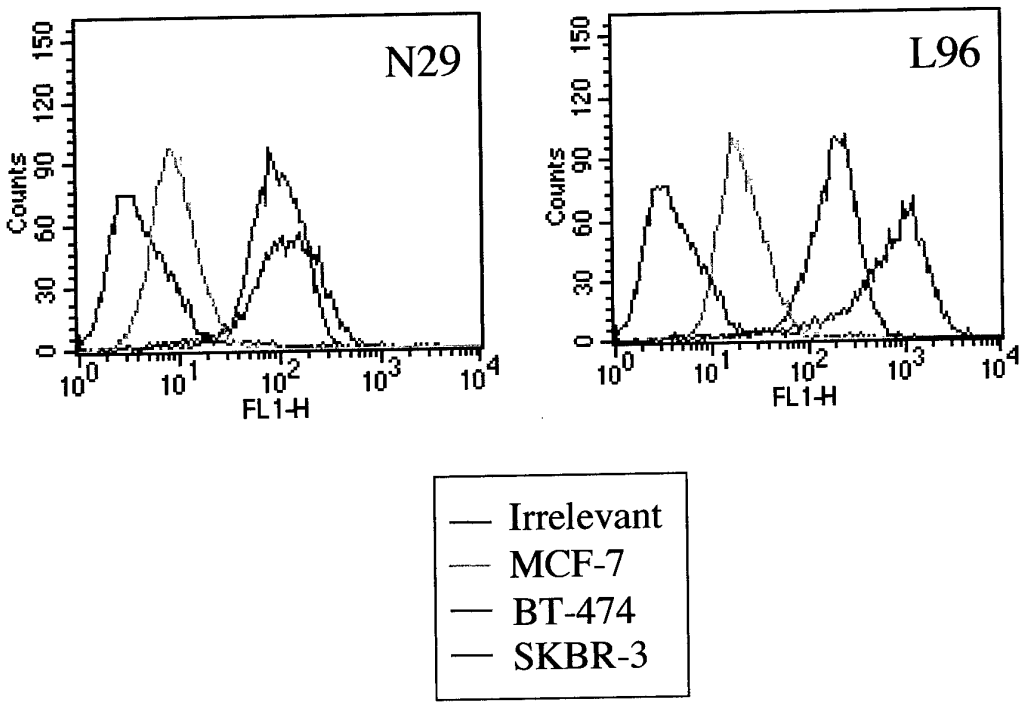


Fig 37. *FACS analysis of the expression of erbB-2 in human breast cancer cell lines*
The indicated breast cancer cell lines were stained with anti-ErbB2 mAbs N29 and L96 and with goat anti-mouse IgG (FITC-labeled) and analyzed by FACS analysis.

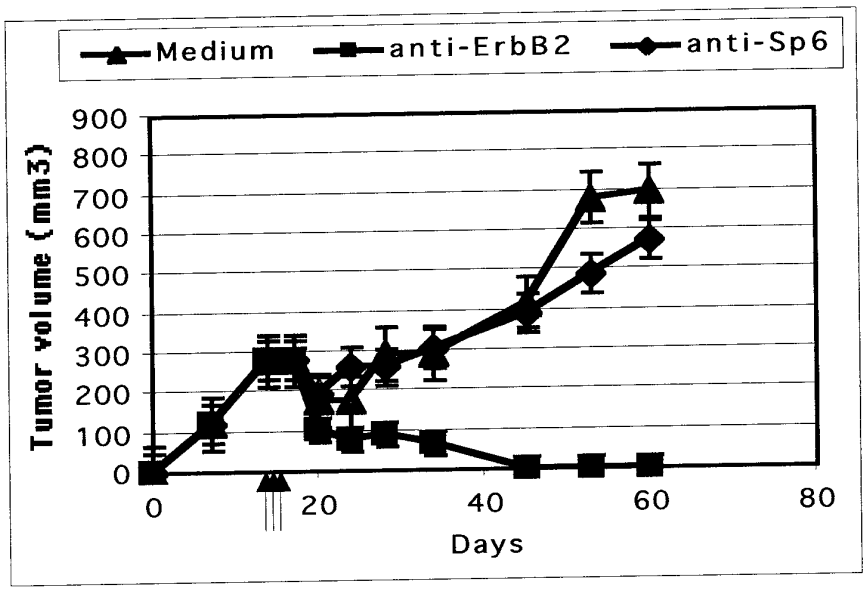
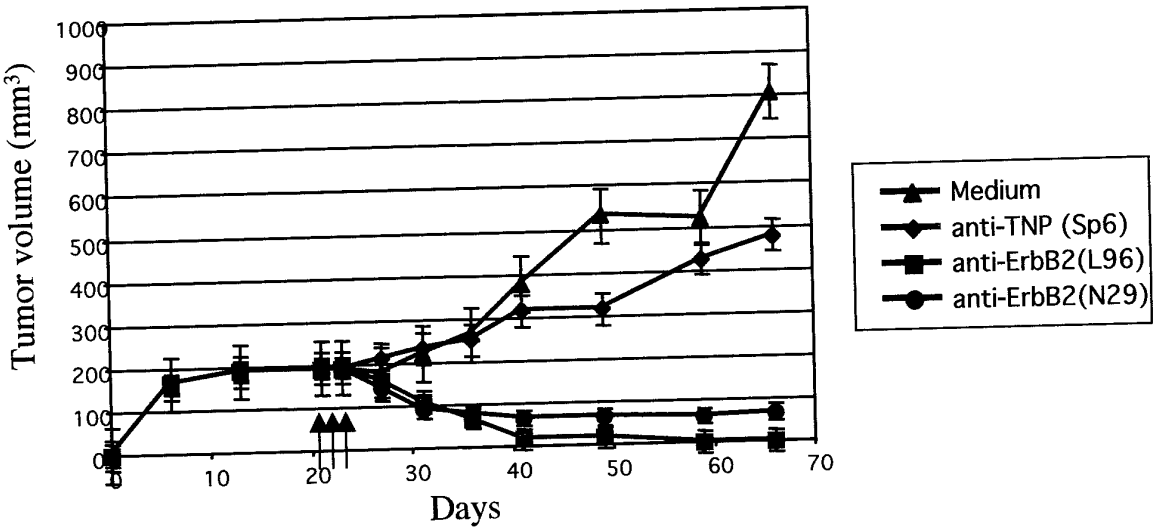


Fig 38. *Treatment of breast carcinoma xenograft (BT-474) with ErbB-2 specific human T-bodies* . Mice bearing well established s.c. BT-474 tumors were randomized to receive 3 consecutive daily intra-tumoral injections (arrows) of $2.0x10^7$ of either anti-ErbB2 CR bearing lymphocytes, anti-TNP CR bearing lymphocytes or HBSS (medium). All mice concurrently received 10^5U/hour IL-2 for 1 week delivered by intra-peritoneal mini-osmotic pump. Results are mean + SE.



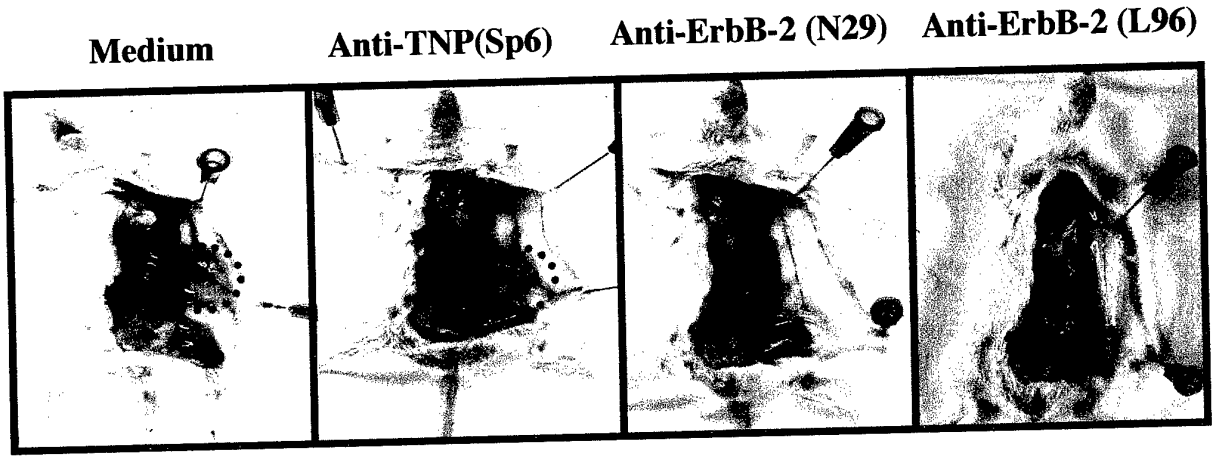


Fig 39. *Elimination of Established Tumors by ErbB2-specific Human Lymphocytes*

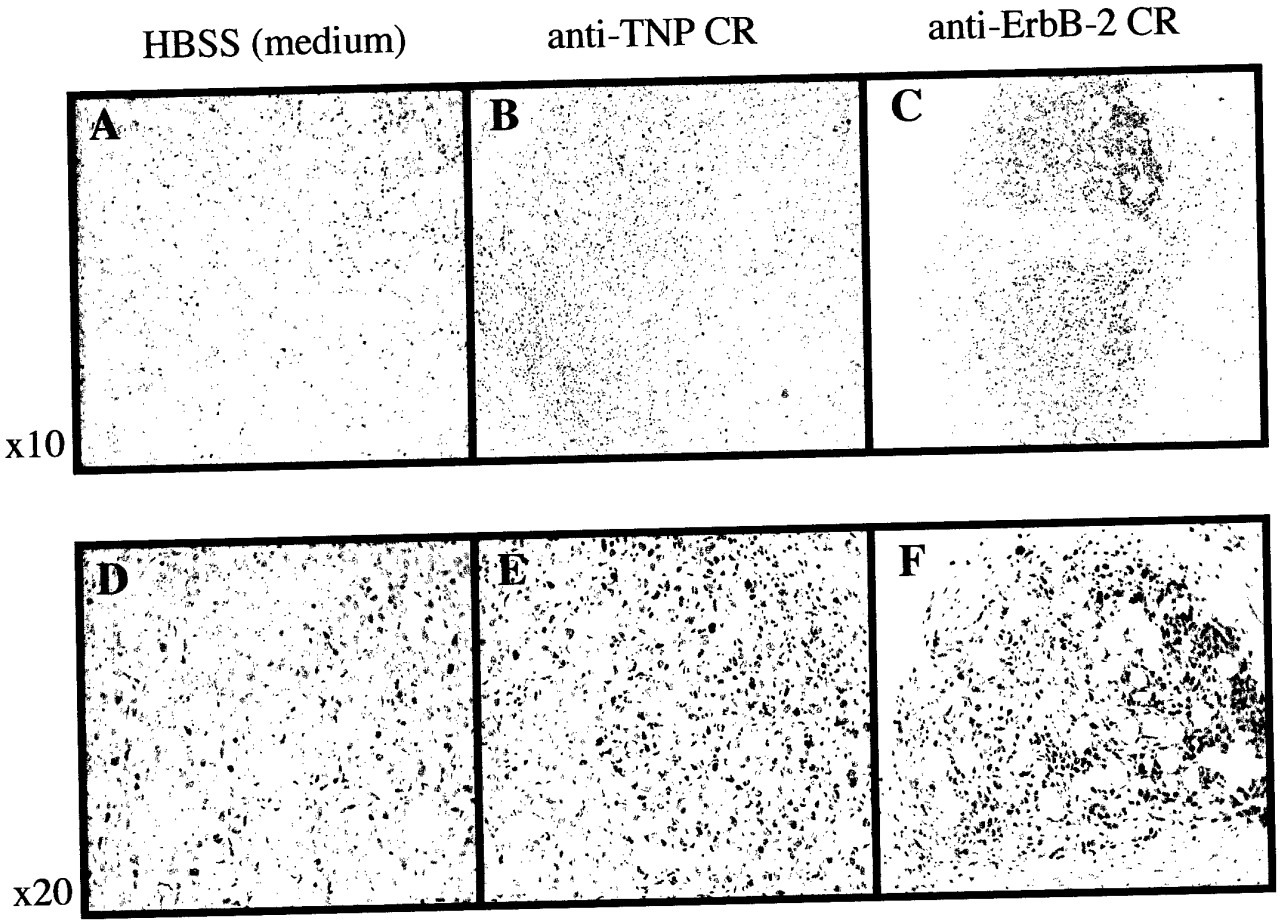


Fig 40. *Histopathological sections of BT-474 tumors two months post three intra-tumoral injections of T-bodies (stained with H&E).* C&F tumor is infiltrated with ErbB-2 specific human lymphocytes. In contrast, tumor injected with medium or anti-TNP CR is preserved with no human lymphocytes infiltration (A&D, B&E)

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Review article

Functional expression of chimeric receptor genes in human T cells

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Abstract

Tumor immunotherapy has been limited to date by the poor antigenicity of most tumors, the immunocompromised state of many cancer patients, and the slow tumor penetration and short half-life of exogenously-introduced anti-tumor antibodies. Our group has developed a model immunotherapy system using a chimeric construct containing an antibody V region fused to a T cell activation molecule (T body) introduced by transfection into cytotoxic T cell lines, or populations of activated primary T or natural killer (NK) cells. In this study we have optimized the conditions needed for efficient transduction of human peripheral lymphocytes (PBL) using retroviral vectors pseudotyped with the gibbon ape leukemia virus (GaLV) envelope. Selection of packaging cells producing high virus titers was performed following transfection with constructs containing the green fluorescent protein (GFP), and FACS sorting. As a model chimeric receptor gene we used a tripartite construct consisting of a single-chain anti-TNP antibody variable region linked to part of the extracellular domain and the membrane spanning regions of the CD28 coreceptor molecule and joined at its 5′ end to a gene fragment encoding the intracellular moiety of the γ activation molecule common to the Fcε and Fcγ receptors. Enriched preparations of retrovectors containing this chimeric receptor and the GFP gene could stably and efficiently transduce human PBL co-activated by anti-CD3 and anti-CD28 antibodies. In routine experiments, the transgene was expressed in 35–70% of the human T cells. Such lymphocytes express the chimeric receptors on their surface and upon stimulation with hapten immobilized on plastic they can produce IL-2. Transfectomas activated in this manner also undergo specific proliferation in the absence of exogenous IL-2. Moreover, the transduced lymphocytes could effectively lyse target cells expressing the TNP hapten on their surface. These studies establish the conditions for the optimal transfection of effector lymphocytes to redirect them against a variety of tumor targets.

Keywords: Chimeric receptor; Retrovectors; T cells; Gene expression; Gene delivery

1. Introduction

To overcome the inability of antibodies to eliminate solid tumors on the one hand as well as the lack of specific of anti-tumor T cells in many malignancies our group has pioneered the T body approach (Gross et al., 1989; Eshhar et al., 1993). In the T-body methodology, we have joined the two approaches of adoptive T cell immunotherapy and

Abbreviations: GFP, green fluorescent protein; scFv, single chain Fv; MuLV, Moloney murine leukemia virus; GaLV, gibbon ape leukemia virus; TNP, trinitrophenol; PBL, peripheral blood lymphocytes; NK, natural killer cells

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antibody therapy to genetically engineer an improved 'immunocytolysin', an antibody V region attached to a cytotoxic T cell signaling molecule and responsible for recognition and activation. Genes for chimeric T cell receptors have been constructed containing the coding sequence of an antibody-derived single-chain Fv directed against a tumor associated antigen, attached to the transmembrane and intracytoplasmic sequences of T cell signaling molecules. These genes are then transfected into cytotoxic T cells, thereby conferring upon them the ability to specifically recognize and kill tumor cells. The scFvR design we have developed combines antibody recognition and T cell signaling in one continuous protein and has been used to endow murine and human effector cells with non-MHC-restricted, antibody-derived specificity. This T-body approach combines the advantage of antibody specificity with the homing, tissue penetration, and target cell destruction mediated by T lymphocytes.

Using this technology, in the past 10 years we have been able to redirect lymphocytes to predefined targets and to endow them with antibody-like specificity. We have designed and developed several receptor configurations, identified the optimal receptor molecules to give a strong effector response, and have chosen a single chain antibody configuration that yields effective antigen binding for virtually all antibodies (Eshhar et al., 1995; Fitzer-Attas et al., 1998). We have transfected effector cells including the mouse MD45 cytotoxic cell line, human NK (Bach and Eshhar, 1995) and rat basophilic cell lines (Bach et al., 1994) and human tumor infiltrating lymphocyte populations.

The use of redirected effector cells has potential in the immunotherapy of virtually any type of cancer in which tumor antigens, not present on normal tissues, are expressed and shared between patients. Using in vivo model systems, lymphocytes transfected with a chimeric receptor of anti-folate binding protein specificity developed in our lab, were shown to eliminate experimental tumors in mice (Hwu et al., 1993, 1995). In addition, we have made a receptor construct containing anti-HER2 antibodies, which is able to mediate cytolysis when transfected into cytolytic mouse hybridoma cells.

For clinical application, patient-derived lymphocytes will be transfected with a chimeric receptor gene encoding an scFv specific to an antigen expressed by the tumor. After expansion in vitro, such genetically engineered cells will be reinfused into the patient where they are expected to undergo activation at the tumor site and reject the tumor, either by direct cytotoxcicity and/or by causing a local inflammatory response. One of the major technical limitations to this scenario is inefficient gene transfer into T cells. For stable gene expression, the only vectors approved for use in humans are adeno-associated and certain retroviral based vectors. While adeno-associated viruses (AAV) fail to infect T cells, some promising results have been obtained using Moloney murine leukemia virus (MuLV)-based vectors for gene delivery into T cells (Mavilio et al., 1994; Bunnell et al., 1995). It was demonstrated that transduction of human T cells can be augmented by pseudotyping the MuLV with the envelope protein (env) of the Gibbon ape leukemia virus (GaLV) (Bunnell et al., 1995; Lam et al., 1966). For integration into the recipient cell genome, retroviral-mediated gene transfer requires dividing cells. A significant enhancement of gene delivery into human T cells has been recently obtained by combining T cell activation using anti-CD3 plus anti-CD28, and transduction in the presence of fibronectin derived peptide (Pollok et al., 1998, 1999; Dardalhon et al., 2000).

Here we increased the efficacy of functional expression of chimeric receptor genes in primary human T lymphocytes by a procedure that combines optimal T cell activation with improved retrovectors. Such a procedure can be readily applied to the immunotherapy of human cancer using ex vivo transduction of patient lymphocytes with chimeric receptors derived from antibodies specific to surface antigens expressed by the tumor in question.

2. Materials and methods

2.1. Retroviral packaging cell lines

The packaging cell lines used include the ecotropic GP+E-86, the amphotropic PA317 (obtained from ATCC), and PG13, which expresses the GaLV env (Miller et al., 1991). 293T cells were a generous gift from R.A. Willemsen (Daniel den Hoed Cancer Center, Rotterdam, The Netherlands). All cells were

cultured in DMEM medium (GIBCO-BRL) supplemented with 10% FCS (GIBO-BRL, Paisley, UK), L-glutamine solution (2 mM), sodium pyruvate 1 mM, 100 Units/ml penicillin and 100 μg/ml streptomycin (Biological Industries, Israel).

2.2. Antibodies and reagents

Anti-human CD3 antibody was purified from the OKT3 hybridoma cell (ATCC) culture supernatant. Anti-CD28.2 was obtained from PharMingen, (San Diego, CA). SP6, an anti-TNP monoclonal antibody (mAb), and 20.5, an anti-Sp6 idiotype mAb, were both provided by G. Kohler (Max-Planck Institute for Immunology) (Rusconi and Kohler, 1985). Fluorescein-labeled anti-mouse Ig antibodies were purchased from Jackson Immuno Research Laboratories Inc (West Grove, PA).

2.3. Transfection/infection

2.3.1. 'Ping-pong' method

First, 3×10^5 GP+E-86 cells and 2×10^5 PA317 cells were co-cultured in a 100-mm plate (NUNC, Roskilde, Denmark). A day later, medium was changed and transfection was performed by adding 20 μg of plasmid DNA in $CaPO_4$ (Mammalian Transfection Kit, Stratagene, La Jolla, CA). After 24 h, the plate was washed with PBS and supplemented with fresh medium. When the culture reached near confluence (24–48 h post transfection), viral supernatant was collected for infection. For infection, 2×10^5 PG13 or 293T cells were plated in a 100-mm plate; the next day, the medium was replaced with 5 ml of viral supernatants in the presence of 4 μg/ml of Polybrene (Sigma) at 37°C, 7.5% CO_2 for 7 h. After 48 h, the infection efficiency was evaluated by monitoring GFP expression (see below). All vector-containing retroviral supernatants described in this study were harvested after a 24-h incubation of near-confluent packaging cells grown in 5 ml fresh medium in a humidified incubator at 32°C, 7.5% CO_2

2.4. Activation of lymphocytes

Peripheral blood lymphocytes (PBL) from healthy donors were isolated by centrifugation through Ficoll Paque Plus (Pharmacia Biotech, Uppsala, Sweden), and cultured in RPMI 1640 medium (GIBO-BRL) supplemented with 10% FCS, L-glutamine solution 2 mM, 100 Units/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-mercaptoethanol.

Cells (10^6/ml in a well from a 24-well plate) were stimulated for 2 days on Falcon non-tissue culture-treated 24-well plates pre-coated with anti-CD3 plus anti-CD28 antibodies. Immobilization of the antibodies was performed by adding 0.5 ml/well of antibody (1 μg/ml PBS of each of the antibodies/well) to the wells overnight at 4°C; the plates were washed with PBS and blocked with 1% BSA in PBS for 20 min at 37°C.

2.5. Lymphocyte transduction

Activated lymphocytes were harvested from the stimulating plate, washed and plated on a RetroNectin™ (FN) (Takara Shuzo Ltd. Otsu, Japan) coated plate at 0.5×10^6/well with 2.8 ml of viral supernatant supplemented with 50 U/ml of IL-2 (recombinant human IL-2 Chiron, Amsterdam, The Netherlands). After 4–6 h at 37°C, 7.5% CO_2, the viral supernatant was gently removed and replaced with RPMI–FCS+50U/mlIL-2 and cells were incubated overnight at 37°C, 5% CO_2. The same transduction process was repeated on the next day. On the day following the second infection, lymphocytes were harvested by vigorous flushing and washing of the wells. The cells were re-suspended in RPMI–FCS medium with 150 U/ml of IL-2, and incubated in 37°C, 5% CO_2. FN-coated plates were prepared by incubating non-tissue culture-treated 24-well plates (Falcon) with 1 ml/well of FN (12 μg/ml) overnight at 4°C, washed with PBS and blocked with 1% BSA in PBS for 20 min at 37°C.

2.6. Flow cytometry

Cells were analyzed using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Green fluorescent protein (GFP) was measured on FL1 (488 nm excitation and 530 nm emission filter). Viability was determined by assessing propidium iodide (PI) (Sigma) staining using FL2 at 488 nm excitation and 585 nm emission. Expression of scFv (Sp6) on the surface of the cells was evaluated by

immunofluorescent staining using anti-Sp6 mAb (20.6) and rhodamine–phycoerythrin (PE) labeled anti-mouse Fab′ antibody, measured on FL2. Cells were sorted on a FACSsort Plus (Becton Dickinson) according to GFP fluorescence. Cloning of packaging cells from single cell/well positive cells sorted directly into 96-well plates.

2.7. Cell proliferation and cytotoxicity assays

The 96-well plates (non tissue culture treated, NUNC) were coated overnight with 2 μg/well of antigen (TNP-FγG) at 4°C, washed with PBS and blocked with 1% BSA on the following day. Alternatively, for stimulation, various targets were irradiated (12 000 R) and TNP-modified as described previously (Gross et al., 1989). Transduced lymphocytes were washed twice and resuspended in culture medium RPMI–FCS with 50 μM 2-mercaptoethanol. Cells were left for 2–4 h in the incubator to eliminate any remaining IL-2. Transduced lymphocytes (1×10^5/well) were plated on antigen (TNP-FγG), coated plates or cocultured with TNP-modified target cells. At different time points, 50 μl of the cell culture was removed to be tested for cell proliferation by the MTT assay (Mosmann, 1983).

Evaluation of the cytotoxicity of transduced lymphocytes was performed by the ^{51}Cr release assay as described before (Gross et al., 1989).

3. Results and discussion

3.1. Vector and chimeric gene constructs

In order to establish the optimal conditions for transduction of primary human T cells, we tried several systems reported to be efficient for gene delivery of chimeric receptor genes. The BULLET system described by Weijtens et al. (1998) was attempted first; we then evaluated the STITCH vector, a modification of the BULLET system which was developed by the same group (Willemsen et al., 2000). This system involves the transient transfection of three plasmids to produce a transducing retrovirus. One plasmid codes for the envelope protein, which could be from an ecotropic virus, an amphotropic virus, or from the gibbon ape leukemia virus (GaLV). Another plasmid supplies the gag and pol proteins. The transfer vector with the LTRs and packaging signal carries an insert with the gene of interest (Fig. 1). In our experiments, this insert was specially designed. The scFv used in our initial experiments was from an antibody with specificity to trinitrophenol (TNP). This facilitates the testing of the genetically modified lymphocytes for their ability to kill a wide variety of cells as any cell can be made a target simply by treatment with picryl chloride to label the cell surface with the TNP group. Another advantage of this scFv is that we have available an anti-idiotypic monoclonal antibody for detecting by FACS expression of this scFv on the cell surface. In the construct used, the co-stimulatory molecule CD28 is genetically fused to this scFv. This provides spacing of the scFv from the cell surface and permits the formation of heterodimers with endogenous CD28 molecules. The CD28 molecule has previously been used in chimeric receptor constructs (Vallina and Hawkins, 1996; Finney et al., 1998). In addition, CD28 signaling has been shown to prevent apoptosis of the lymphocytes. Since CD28 is homologous to CTLA4 and since the structure of CTLA4 has been determined we have employed the latter as a model for the structure of CD28. Alignment of their amino

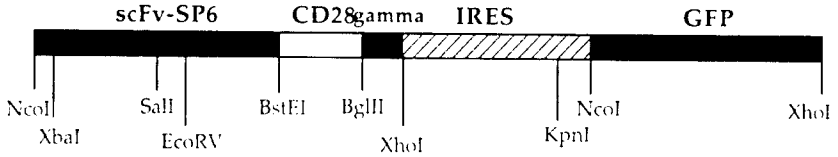

Fig. 1. Structure of the chimeric receptor-GFP gene used in this study. Sp6-scFv is a single chain Fv containing the sequence of the leader, V_L, linker and V_H of the anti-TNP Sp6 antibody. CD28 sequence contains the CD28 hinge region, transmembrane region, and cytoplasmic region. The γ chain sequence contains the cytoplasmic region (starting from the QVR at a *BglII* site). This deletes CRLKI at the beginning of the cytoplasmic region of the Fcε receptor γ and contains the signaling portion of the receptor. IRES is an internal ribosomal entry site (IRES) that permits expression of the GFP from the same transcript as the chimeric receptor.

acid sequences to that of a mouse heavy chain reveals that the sequence in CD28 IHV matches the *Bst*E II site sequence in our single chain VTV (Holm and Sander, 1996). Therefore, the junction was made so that the junction is at a valine which is conserved between the end of the framework 4 beta sheet of the variable region of the antibody (Kabat numbering 111) and a valine which is just after the last beta sheet in CTLA4.

Human CD28 was cloned from PBLs and Jurkat cells using the following primers:

#7966 *Bst*E II primer for homodimer CD28
5′ CCGGTCACCGTGAAAGGGAAACACCTTT-GTCC
#7967 reverse 3′ primer
5′ CGCTCGAGGTGTCAAGATCTATAGGCTG-CGAAGTCGCGTGG

In this way the single chain is attached to the CD28 hinge region, transmembrane region, and cytoplasmic region.

The γ chain cytoplasmic region (starting from the QVR at a *Bgl*II site) was next spliced to the end of the CD28 sequence. This deletes CRLKI at the end of the transmembrane or the beginning of the cytoplasmic region of the Fcε receptor γ chain (Spencer et al., 1993) (Fig. 1). This contains the signaling portion of the receptor and completes the gene for the chimeric T cell receptor.

To enable better tracking of retroviral expression, the gene for green fluorescent protein (GFP) was placed downstream of the chimeric receptor gene separated by an internal ribosomal entry site (IRES) to permit expression of the GFP from the same transcript as the chimeric receptor. Retroviruses with a single transcript containing a desired gene and GFP driven by an IRES have been made previously (Aran et al., 1998; Levenson et al., 1998). In our case, the GFP gene was placed in the retroviral vector pSAM-EN (Morgan et al., 1992) in place of the drug resistance gene, and the chimeric receptor gene was inserted into the cloning site before the IRES. Lack of a second promoter avoids the problem of promoter interference. It also enables linked expression so that GFP fluorescence may be used to assess expression of the scFv. Transduced cells can be observed visually by fluorescence microscopy, thereby permitting the tracking of transduced cells in vivo (Persons et al., 1997; Bagley et al., 1998).

3.2. Establishment of stable vector producing cell lines

Retrovirus-containing supernatants prepared from the 293T cells as described above gave up to 15% transduction of fresh 293T cells as estimated by the proportion of cells exhibiting GFP fluorescence (data not shown). The percentage and level of gene expression in this transient system varied and depended on the transfection efficiency and culture conditions. To obtain a more reproducible system, we have attempted to establish a stable vector-producing cell line by selection for high titer retrovector producing clones. Fig. 2 schematically describes the protocol we have employed for these studies. Briefly, the chimeric receptor gene incorporated in a BULLET or STITCH vector was transfected into the amphotropic PA317 and ecotropic GP+E-86 lines, which were co-cultured in order to increase the viral titer. The supernatant obtained was used to infect a series of packaging cell lines whose supernatants, containing the pseudotyped retrovector, were titered on 293T cells and in parallel tested for their ability to infect activated human PBL (as described in Materials and methods). In several experiments,

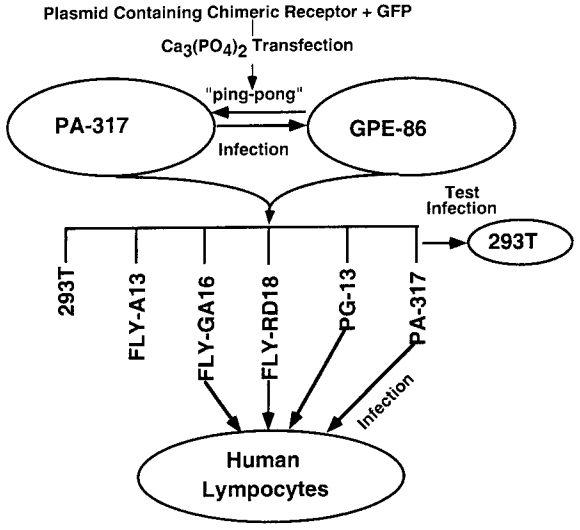

Fig. 2. A diagram of the protocol used for the generation and selection of a high titer packaging cell lines.

the PG13 containing the GaLV envelope, was highly infected, produced the highest titer of virus and resulted in the best gene transfer into the human T cells (data not shown). It was previously shown that the receptor to the GaLV envelope, GLVR-1 is increased following T cell stimulation (Lam et al., 1966). The heterogeneous population of packaging cells obtained was sorted for GFP expression by FACS and the sorted population was further subcloned (Fig. 3). As can be seen in the figure, virtually all the cells expressed GFP following a single cycle of sorting.

3.3. Infection of primary human T cells

For optimal transduction of human peripheral blood lymphocytes with retroviruses, the lymphocytes must be activated. In our protocol, they are grown in RPMI with fetal calf serum without IL-2 with plastic bound anti-CD3 and anti-CD28 antibodies for 48 h. They are then infected with supernatant containing virus from the packaging cell line on plates coated with recombinant fibronectin fragments (Retronectin™). The infection is performed in the presence of IL-2 for 5–7 h, twice over 48 h as described in Materials and methods. Low concentrations of IL-2 were used to prevent the propagation of natural killer cells. Higher concentrations are used for the production of lymphokine-activated killer (LAK) cells (Jadus et al., 1988). RetroNectin™ is a recombinant fibronectin fragment which contains the connecting segment, cell binding domain and heparin binding domain (Hanenberg et al., 1996). Its significantly enhances the infection efficacy, since both the retroviral particles and T cells bind to it (T cells through the VLA integrin, the expression of which is increased following anti-CD3/CD28 stimulation).

Several days following transfection, the cells were phenotyped for surface markers and for expression of the chimeric gene using anti-idiotypic antibody and evaluated for expression of GFP. The percentage of transfected cells, 3–4 days following transduction was in the range of 35–70% and remained stable for about 10–14 days. After this period, the proportion of GFP positive cells began to slowly drop unless they were re-stimulated by anti-CD3/CD28, PHA or allogeneic cells. Phenotypically, the transfected cell population consisted of about 30% CD4, 70% CD8 and 15% CD56 positive cells. The population of NK cells (CD56+) dropped during culture in the low IL-2 concentration.

Since throughout all the selection and sorting procedures we followed GFP expression, and since the chimeric receptor gene and GFP were in two separate cistrons, it was important to check whether the expression of GFP truly indicated chimeric receptor expression. Fig. 4 shows the FACS pattern of transduced T cells that were stained with anti-idiotypic antibody against the Sp6 anti-TNP scFv and also analyzed for GFP expression.. It is clear that most of the GFP expressing cells also expressed the chimeric receptor on their surface.

3.4. Functional expression of the chimeric receptor genes in human T cells

In vivo, effector lymphocytes redirected with chimeric receptors are expected to reach their destination, undergo specific stimulation at the target site to either kill the target or secrete cytokines that in turn will cause inflammation and result in elimination of the tumor. Ideally, the effector lymphocytes may differentiate into memory cells which will stay on-guard and prevent reappearance of the tumor in question. To check the ability of the chimeric-receptor expressing human T cells to perform these functions in vitro, we tested their ability to undergo proliferation and to produce cytokines in response to specific antigen as well as their ability to kill specific target cells.

Fig. 5 depicts the results of an experiment in which T cells expressing the SP6-scFv-CD28-gamma chimeric receptor (anti-TNP specific) were triggered for specific proliferation following stimulation on antigen (TNP-FγG) coated culture-wells and the removal of exogenously supplied IL-2. While lymphocytes did not proliferate following 3 days of culture in the absence of IL-2, in the presence of TNP-FγG (hapten/protein ratio >2) they propagated quite well for at least 1 week. In the presence of antigen excess (TNP_{58}-FγG) cells started to die following 7 days, most likely because of overstimulation or antigen-induced cell death (AICD) that was not prevented by the presence of CD28 in the signaling receptor. Notably, the cell proliferation, and IL-2 production (data not shown), was triggered

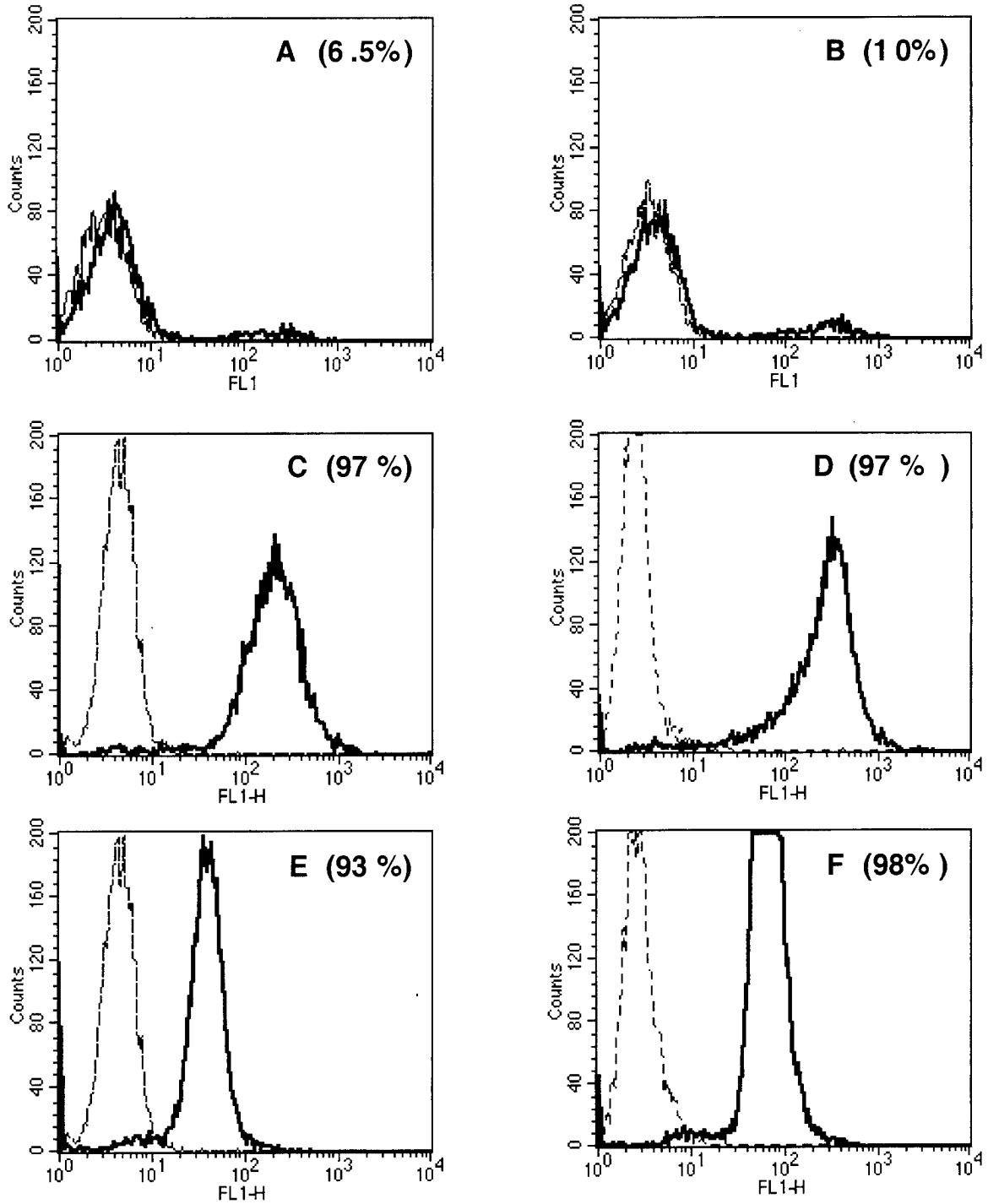

Fig. 3. GFP fluorescent pattern of two packaging cells at different stages of their production. A, C, E represent cell lines derived from the amphotropic PA317 cell; B, D, F represent cell lines derived from the PG13 cell containing the GaLV envelope. A, B-packaging cells 24 h after their transduction by cell-free supernatants obtained from the 'Ping-Pong' step (Fig. 2). C, D represent the GFP expression in bulk-sorted packaging cells. E, F represent clones selected for high vector titer from the bulk-sorted population.

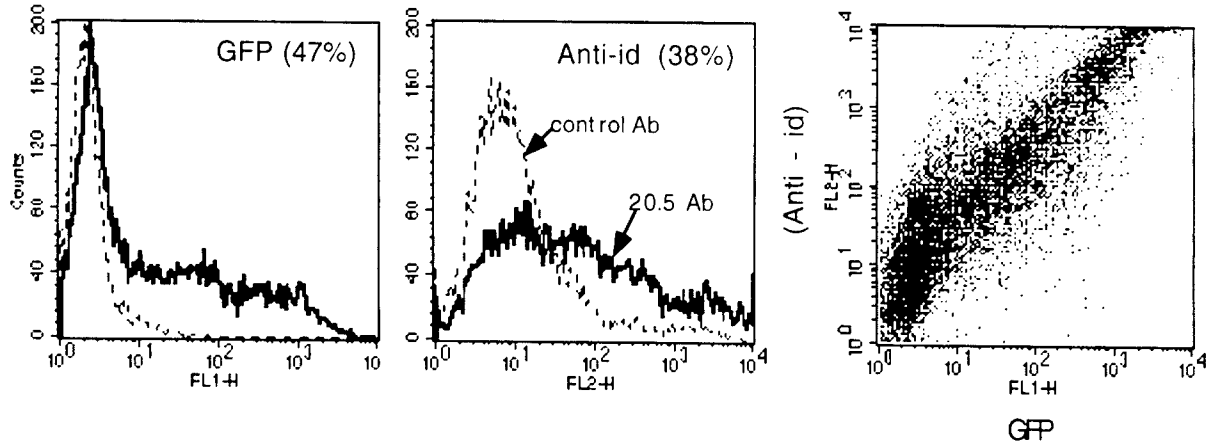

Fig. 4. Co-expression of the chimeric receptors and GFP transduced human T cells. Fluorescent pattern of GFP expression (FL1) and surface expression of the chimeric receptor stained with biotin–anti-idiotypic antibody (20.5) and avidin–phycoerythrin.

by the chimeric receptor in the absence of any other stimulatory and co-stimulatory signal, showing that both signal I and II can be induced by our tripartite receptor. The T cells bearing the chimeric receptors could also specifically and efficiently kill various TNP-modified target cells regardless of their species or tissue origin (Fig. 6). The genetically engineered lymphocytes were able to efficiently cytolyse their targets following 3 weeks culture in the presence of IL-2, even in the absence of preactivation before the cytotoxicity assay. At this stage, the level of non-specific LAK-mediated killing was low.

Taken together, the results described above demonstrate that under the conditions used, PBL-derived T cells can be efficiently transduced to express high levels of chimeric receptors. The chimeric receptor could induce the T cells to specifically proliferate and produce cytokines as a result of specific stimulation by antigen alone without additional stimulation. The genetically engineered cells maintain their abili-

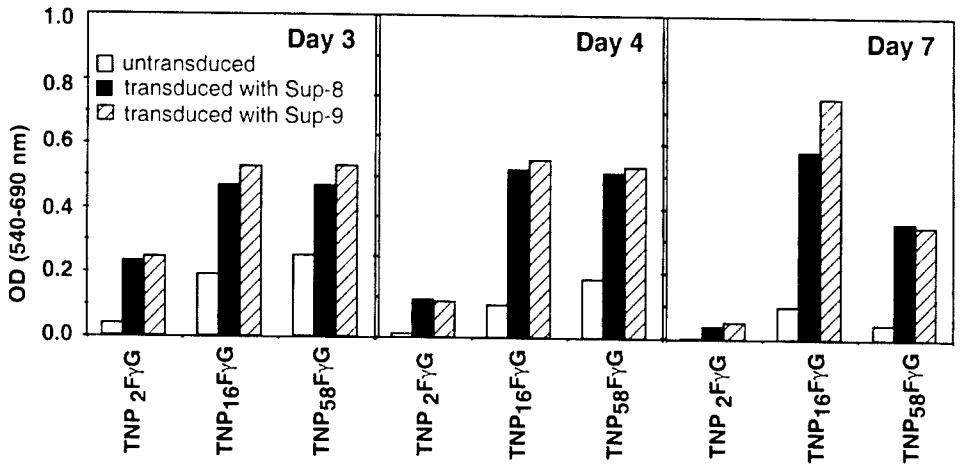

Fig. 5. Proliferation of chimeric receptor expressing lymphocytes stimulated with antigen. T lymphocytes transduced with retrovector preparations made of two packaging clones 8 and 9, as well as non-transduced cells were cultured in the absence of IL-2 on plastic-immobilized antigens containing different hapten groups (TNP) per FγG carrier. Cell proliferation at different days in culture was monitored by the MTT colorimetric assay. Note that the culture medium was changed at day 4.

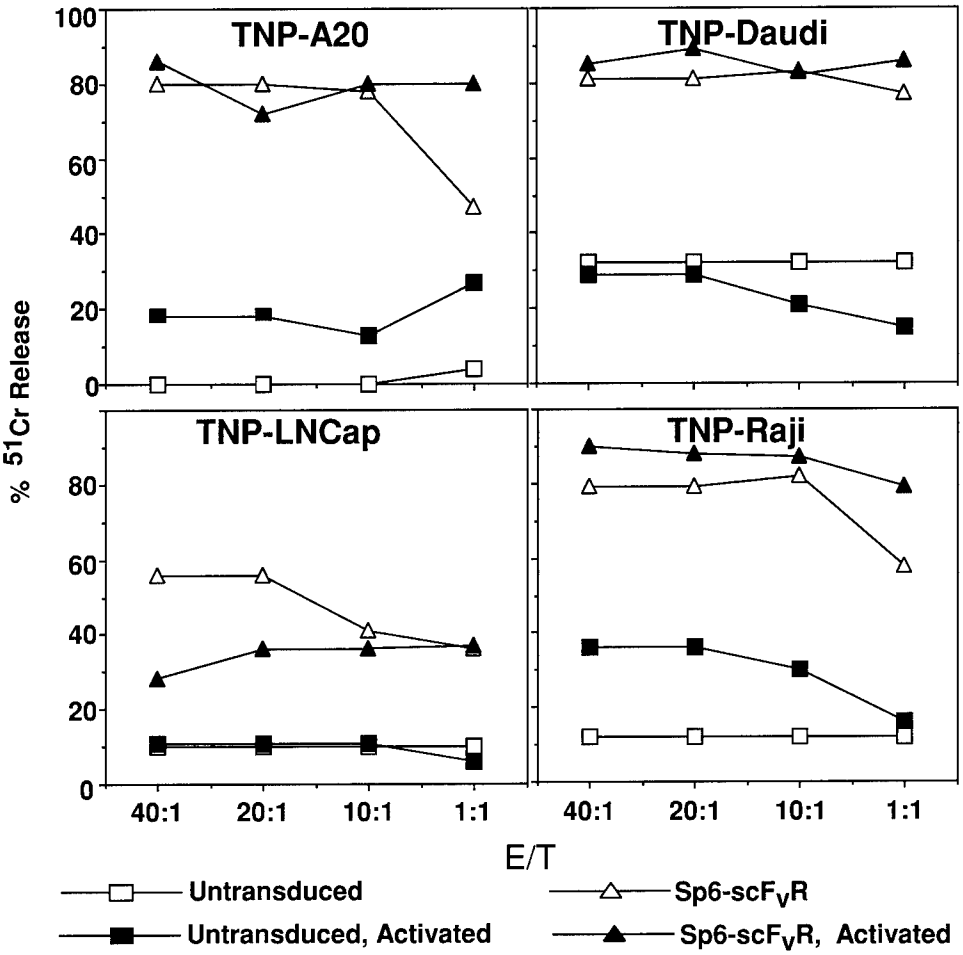

Fig. 6. Specific target cell cytotoxicity mediated by the chimeric receptor expressing lymphocytes. Lymphocytes were harvested 3 weeks after their transduction with chimeric receptor containing vectors. Part of the cells were reactivated on anti-CD3 and anti-CD28 antibodies 24 h before the assay. As target cells served various cell lines modified and unmodified by TNP. Specific ^{51}Cr release from the targets following incubation at different ratios with effector cells (E/T ratio) was determined following 7 h.

ty to kill specific target cells for at least 3 weeks in tissue culture, a period long enough to produce sufficient cells for patient treatment. These results set the grounds for in vivo testing of the T-body approach in animal models for cancer immunotherapy and later in patients.

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