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Chunlei Liu  
*Dartmouth College*

Joel Goldstein  
*Dartmouth College*

Robert F. Graziano  
*Medarex, Inc.*

Jia He  
*Dartmouth College*

Jeremiah K. O'Shea  
*Dartmouth College*

*See next page for additional authors*

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## Authors

Chunlei Liu, Joel Goldstein, Robert F. Graziano, Jia He, Jeremiah K. O'Shea, Yashwant Deo, and Paul M. Guyre

# Fc $\gamma$ RI-Targeted Fusion Proteins Result in Efficient Presentation by Human Monocytes of Antigenic and Antagonist T Cell Epitopes

Chunlei Liu,\* Joel Goldstein,\* Robert F. Graziano,\* Jia He,\* Jeremiah K. O'Shea,\* Yashwant Deo,\* and Paul M. Guyre\*

\*Department of Physiology, Dartmouth Medical School, Lebanon, New Hampshire 03756; and \*Medarex, Inc., Annandale, New Jersey 08801

## Abstract

A major challenge for using native or modified T cell epitopes to induce or suppress immunity relates to poor localization of peptides to antigen presenting cells (APCs) in vivo. In this study, we demonstrate enhanced presentation of antigenic and antagonistic peptides by targeting them to the type I Fc receptor for IgG (Fc $\gamma$ RI, CD64) on human monocytes. A Th epitope of tetanus toxoid, TT830, and the antagonistic peptide for TT830, TT833S, were genetically grafted into the constant region of the heavy chain of the humanized anti-CD64 mAb 22 and expressed as monovalent fusion proteins, Fab22-TT830 and Fab22-TT833S. These CD64-targeted peptides were up to 1,000- and 100-fold more efficient than the parent peptides for T cell stimulation and antagonism, respectively, suggesting that such fusion proteins could effectively increase the delivery of peptides to APCs in vivo. Moreover, the Fc $\gamma$ RI-targeted antagonistic peptide inhibited proliferation of TT830-specific T cells even when APCs were first pulsed with native peptide, a situation comparable with that which would be encountered in vivo when attempting to ameliorate an autoimmune response. These data suggest that targeted presentation of antagonistic peptides could lead to promising Ag-specific therapies for T cell-mediated autoimmune diseases. (*J. Clin. Invest.* 1996. 98:2001–2007.) Key words: autoimmunity • peptide • vaccine • tetanus toxoid • antigen presentation

## Introduction

Increased understanding of antigen recognition by the immune system has provided a theoretical basis for designing immunogenic peptide-based vaccines (1–3). Peptide vaccines can be more effective than traditional vaccines consisting of either killed or attenuated pathogenic organisms for a variety of reasons. By selection from within a given protein Ag of only the epitopes which confer protective immunity, peptide-based vaccines can exclude determinants that could elicit a deleterious immune response and avoid the potentially infectious materi-

als contained in traditional vaccines. Use of peptide-based vaccines to boost immune responses against cryptic tumor antigenic determinants has special significance in the development of immunotherapy for cancer treatment (4, 5), where one goal is to break the immune tolerance of dominant tumor antigens.

However, the development of peptide vaccines has been impeded by the usually poor immunogenicity of peptides due, at least in part, to their inefficient presentation by antigen presenting cells (APCs)<sup>1</sup> in vivo. One way to overcome this problem has been through the development of the so-called antigenized Ab (AgAb) in which antigenic determinants are grafted into the variable region of IgG (6, 7). This method is effective because it increases the half-life of the Ag and facilitates its uptake by APCs via Fc $\gamma$ R. However, some drawbacks still remain. Most notably, uptake of such AgAb by APC is limited due to competition from serum IgG for Fc $\gamma$ R-binding, and by promiscuous capture of Ag by non-APC cells that express Fc $\gamma$ R, such as neutrophils and platelets. Previous research by Gosselin et al. (8) revealed that Ag presentation by human monocytes could be enhanced 100- to 1,000-fold by targeting whole Ag to Fc $\gamma$ RI on monocytes using chemical conjugates of tetanus toxoid (TT) and F(ab')<sub>2</sub> anti-Fc $\gamma$ RI mAb 22. It was postulated that such anti-Fc $\gamma$ RI mAb-based constructs would also be an effective way to increase the immunogenicity of peptides. To test this hypothesis, a Th cell epitope of TT, TT830 (TT830-844) (9), was genetically grafted into the constant region of the humanized anti-Fc $\gamma$ RI mAb 22 to generate a fusion protein, Fab22-TT830. Presentation of peptide TT830 expressed in the fusion protein Fab22-TT830 was found to be  $\sim$ 1,000-fold more efficient than presentation of the peptide alone. These results suggest that specific mAb-based targeting to APCs may have important implications in the development of peptide vaccines.

Peptides containing one or two amino acid changes from native T cell epitopes, termed altered peptide ligands (APL) by Allen and co-workers, have been shown to be agonists, partial agonists, or antagonists for T cell activation (10, 11). Recognition of APL by specific T cells through T cell receptor (TCR) in some cases triggered partial signal transduction and resulted in (a) the inhibition of T cell stimulation by superantigen (12), (b) T cell anergy (13, 14), or (c) modulation of Th1/Th2 differentiation (15–17). Furthermore, immunizing mice with APLs of encephalitogenic peptides of myelin proteolipid protein (PLP) led to the inhibition of PLP peptide-induced experimental autoimmune encephalomyelitis (EAE) in susceptible mice (15, 18, 19), indicating that APL-based treatment may

Address correspondence to Dr. Paul Guyre, Department of Physiology, 740W Borwell Building, 1 Medical Center Drive, Dartmouth-Hitchcock Medical Center, Lebanon, NH 03756. Phone: 603-650-8105; FAX: 603-650-6130; E-mail: paul.guyre@dartmouth.edu

Received for publication 30 May 1996 and accepted in revised form 21 August 1996.

*J. Clin. Invest.*

© The American Society for Clinical Investigation, Inc.

0021-9738/96/11/2001/07 \$2.00

Volume 98, Number 9, November 1996, 2001–2007

1. Abbreviations used in this paper: AgAb, antigenized Ab; APL, altered peptide ligand; EAE, experimental autoimmune encephalomyelitis; Fc $\gamma$ R, IgG Fc receptor; H22, humanized anti-Fc $\gamma$ RI mAb; PLP, proteolipid protein; TCR, T cell receptor; TT, tetanus toxoid.

provide an antigen-specific immunotherapy for T cell-mediated autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (15, 18–20). However, since peptides are poorly presented *in vivo*, nearly 10 times more APL than pathogenic PLP peptide had to be used in association with adjuvants in order to effectively inhibit EAE. To determine whether targeting of APL to FcγRI might improve the presenting efficiency of APL, the influence of targeted APL on the activation of peptide-specific T cells was examined. An antagonist peptide for TT830, TT833S (833K changed to 833S) described by Sette and co-workers (21), was chosen, and the FcγRI-targeted fusion protein Fab22-TT833S was generated. Fab22-TT833S was found to be ~ 100 times more effective than TT833S alone for inhibition of TT830-stimulated T cell proliferation. These studies have revealed, for the first time, a novel approach to enhance the efficiency of TCR antagonists and thus may have potentially important implications in developing targeted-TCR antagonists as Ag-specific therapy for human autoimmune diseases.

## Methods

**Reagents.** AIM V (Gibco Laboratories, Grand Island, NY) was used as culture medium. TT was purchased from Accurate Chemical Co. (Westbury, NY). Sterile and low-endotoxin F(ab')<sub>2</sub> fragment of mouse anti-FcγRI mAb 22 and the bispecific Ab, MDXH210 (consisting of Fab' of humanized Ab 22 chemically linked to Fab' of anti-Her2/neu tumor Ag mAb 520C9), were provided by Medarex, Inc. (Annandale, NJ). The universal Th epitope of TT, TT830-844 (QYISKANSKFIGITEL, termed as TT830 hereafter) (9), and the mutant form of this epitope, TT833S (QYISANSKFIGITEL, lysine at position 833 changed into serine), were synthesized and purified to > 95% by Peptidogenic Co. (Livermore, CA). Another universal Th epitope of TT, TT947-967 (FNNFTVSFWLRVPKVSASHLE, referred to as TT947 hereafter) (> 80% pure) (9), was provided by Dr. William Hickey (Dartmouth Medical School, Hanover, NH) and was used as a control peptide in this study. Commercially available human IgG for intravenous injection (IVIG) was used in blocking experiments.

**Cells.** The monocytic cell line, U937, which expresses FcγRI, was obtained from American Type Culture Collection (Rockville, MD). The method of generating CD4<sup>+</sup>, peptide TT830-specific T cells was modified from a previously described protocol for TT-specific T cell lines (8). Briefly, mononuclear cells were isolated from peripheral blood using Ficoll-Hypaque. 150 × 10<sup>6</sup> mononuclear cells were stimulated in 50 ml of AIM V medium with 10 μM TT830. After 3 d of incubation at 37°C in a 5% CO<sub>2</sub> incubator, nonattached (mostly non-specific cells) were removed by washing the flask once with 10 ml of Hepes-buffered RPMI 1640; specific T cell colonies together with adherent monocytes remained in the flask. 50 ml of AIM-V plus 20 U/ml of human IL-2 (Immunex, Seattle, WA) and 1 ml (2%, final concentration) pooled human serum were added back to the flask. After 10–14 d of total incubation time, T cells were harvested and dead cells were pelleted through Ficoll-Hypaque, yielding a highly enriched population (95–98%) of viable CD4<sup>+</sup>, Ag-specific T cells. The T cells were confirmed to be specific for TT830 peptide as shown in Fig. 3. Large quantities of monocytes were purified from leukaphoresis packs using the cold aggregation method (22) which resulted in 80–90% purity. Both monocytes and T cells were frozen in aliquots for future use and were shown to function normally after being thawed.

**Ag presentation assay.** In proliferation assays, T cells (5 × 10<sup>4</sup>), irradiated monocytes (3,000 rad, 10<sup>5</sup>/well), and various concentrations of peptide TT830 or fusion protein Fab22-TT830 were incubated together in a final volume of 200 μl/well in flat-bottomed 96-well tissue culture plates for 2 d. 10 μl (1 μCi/well) [<sup>3</sup>H]thymidine was then added to each well. After incubating overnight, plates were har-

vested and counted in a liquid scintillation counter. T cell proliferation was expressed as the mean counts per min (CPM) of three replicates ± SD. Background CPM (T cells and monocytes without Ag) was subtracted from all the data points. Experiments with APL were done according to similar protocols reported by Sette and co-workers (21). Briefly, for inhibition assays, irradiated monocytes were treated with various concentrations of TT833S or Fab22-TT833S overnight. 20 nM TT830 and T cells were then added. After a further 2 d of incubation, T cell proliferation was measured as described above. In prepulsing experiments, irradiated monocytes were pulsed with 20 nM TT830 for 4 h. These cells were then washed and added to the 96-well plate, followed by the addition of 10 μM TT833S or 0.1 μM Fab22-TT833S. After overnight incubation, T cells were then added. After a further 2 d of incubation, T cell proliferation was measured as described above. In T cell anergy induction assays, T cells were stimulated with irradiated monocytes and TT833S or Fab22-TT833S for 1 d, recovered after centrifugation over Ficoll-Hypaque, and restimulated with monocytes and various concentrations of TT830 for 2 d. T cell proliferation was then measured by the incorporation of [<sup>3</sup>H]thymidine and the average CPM of three replicates was plotted. In some cases, the percentage of inhibition was calculated by the formula: % inhibition = (CPM<sub>no inhibitor</sub> - CPM<sub>inhibitor</sub>) / CPM<sub>no inhibitor</sub> × 100. All experiments were repeated at least three times.

**Staining and flow cytometry.** Staining procedures were adapted from those previously described (23). Briefly, to individual wells of a 96-well plate at 4°C, 30 μl of myeloid cell line U937 cells was added. This was followed by 30 μl of RPMI + 1 mg/ml BSA containing one of the proteins Fab22-TT830, Fab22-TT833S, or the BsAb MDXH210 at varying concentrations. After 1 h of incubation at 4°C, plates were centrifuged, the supernatants were discarded, and the cells were washed three times with PBS/BSA at 4°C. Cells were then incubated for 1 h with 40 μl/well of FITC-labeled F(ab')<sub>2</sub> goat anti-human IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) followed by three washes with PBS/BSA and resuspended in PBS/BSA containing 1% paraformaldehyde (Eastman Kodak, Rochester, NY). Cells were then examined by FACScan® (Becton Dickinson, Mountain View, CA), and mean fluorescence intensity was measured.

**Cytokine measurement.** Supernatants were collected from the 96-well plates of Ag presentation assays after 2 d of stimulation and frozen until used. The levels of IFN-γ and IL-4 from these samples were measured by specific ELISA. Ab pairs for the IFN-γ and IL-4-specific ELISA were purchased from Pharmingen (San Diego, CA). ELISA assays were performed according to the protocol provided by the manufacturer.

**Generation of H22-TT peptide fusion proteins.** To generate fusion proteins Fab22-TT830 and Fab22-TT833S, synthetic oligonucleotides encoding each peptide were separately engineered into the hinge region in the heavy chain of humanized anti-FcγRI mAb 22 (H22) according to a method we have developed (Graziano, J., R.F. Goldstein, K. Sundarapandian, C. Somasundaram, and Y. Deo, manuscript submitted for publication). The procedures are briefly described as follows.

**Expression and cloning vectors.** mAb 22 has been humanized by grafting its CDR regions into a human IgG1 framework (24). The expression vector for the genomic clone of the heavy chain (pSVgpt) of H22 was modified to allow incorporation of the coding sequence for other molecules, in this case the TT peptides. The BamHI fragment of this vector containing CH1, hinge, and newly engineered XhoI and NotI cloning sites was inserted into the BamHI site of pUC19 to generate the vector pUC19/H22CH1(X + N). This vector was used to clone oligonucleotide sequences encoding TT peptides, as described below.

The oligonucleotide sequences encoding the TT peptides were designed to have an XhoI site on the NH<sub>2</sub> terminus and a NotI site on the COOH terminus of the coding region (Fig. 1 A). These oligonucleotides were synthesized and purified by Genosys Biotechnologies, Inc. (The Woodlands, TX). The synthetic oligonucleotides were then

### A. Synthetic Oligonucleotides coding wildtype(TT830) and mutant (TT833S) TT peptides:

#### TT830: QYIKANSKFIGITEL

5'-TCG AGC CAG TAC ATC AAG GCG AAT TCC AAG TTC ATC GGC ATC ACC GAG CTC TGA-3'

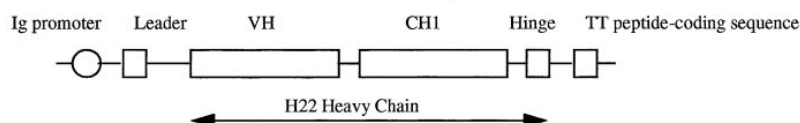
3'-CG GTC ATG TAG TTC CGC TTA AGG TTC AAG TAG CCG TAG TGG CTC GAG ACT CCG-5'

#### TT833S: QYISANSKFIGITEL

5'-TCG AG C CAG TAC ATC AGC GCG AAT TCC AAG TTC ATC GGC ATC ACC GAG CTC TGA-3'

3'-CG GTC ATG TAG TCG CGC TTA AGG TTC AAG TAG CCG TAG TGG CTC GAG ACT CCG-5'

### B. Final H22 chimeric constructs



### C. SDS-PAGE analysis of the affinity-purified fusion proteins:

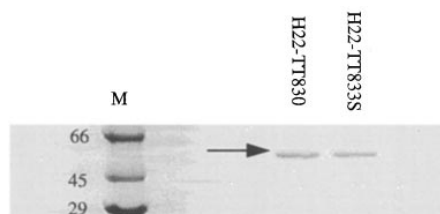


Figure 1. Generation of H22-TT peptide fusion proteins. (A) The sequence of the synthetic oligonucleotides encoding TT peptides. (B) Map of the final genetic constructs. (C) SDS-PAGE analysis of the affinity-purified fusion proteins.

annealed and ligated into the cloning vector pUC19/H22CH1(X + N). Clones which had incorporated the coding sequences for TT peptides were screened by restriction mapping. The BamHI fragment containing CH1, hinge, and TT830 or TT833S was then cut out of pUC19 and inserted into the expression vector which already contained VH. The final expression construct of H22 heavy chain fused with TT peptides is shown in Fig. 1 B.

**Expression.** The murine myeloma NSØ (ECACC 85110503) is a non-Ig synthesizing line and was used for expression of the H22-TT fusion proteins. First, NSØ cells were transfected with the pSVhyg vector containing the H22 light chain coding sequence. The H22 light chain expressing NSØ cells was then transfected with the expression vector construct containing the H22 H-chain Fd sequence fused in frame to the TT coding sequences (Fig. 1 B). An electroporation apparatus (Gene Pulser; Bio-Rad Laboratories, Richmond, VA) was used to carry out the transfection using 200 V and 960  $\mu$ F. 1 or 2 d after transfection, mycophenolic acid (0.8  $\mu$ g/ml; Sigma Chemical Co., St. Louis, MO) and xanthine (2.5  $\mu$ g/ml; Sigma Chemical Co.) were added to the media to select transfectants which had successfully taken up the expression vectors. Individual colonies were isolated based on the binding activity of the culture supernatants to Fc $\gamma$ RI on U937 cells as demonstrated by flow cytometry. The positive colonies were subcloned by limiting dilution.

**Purification.** Clone pW5 expressing the Fab22-TT830 fusion protein and clone pM4 expressing the Fab22-TT833S fusion protein were expanded in roller bottle cultures. The supernatants were clarified and concentrated. Small scale purification was performed by affinity chromatography on an anti-H22 affinity column. SDS-PAGE analysis on a 5–10% acrylamide gradient gel under nonreducing conditions (Fig. 1 C) showed that fusion protein was > 90% pure and had a molecular mass of 50 kD as expected. Protein concentration was determined by absorbance at 280 nm using the extinction coefficient of IgG Fab' = 1.53.

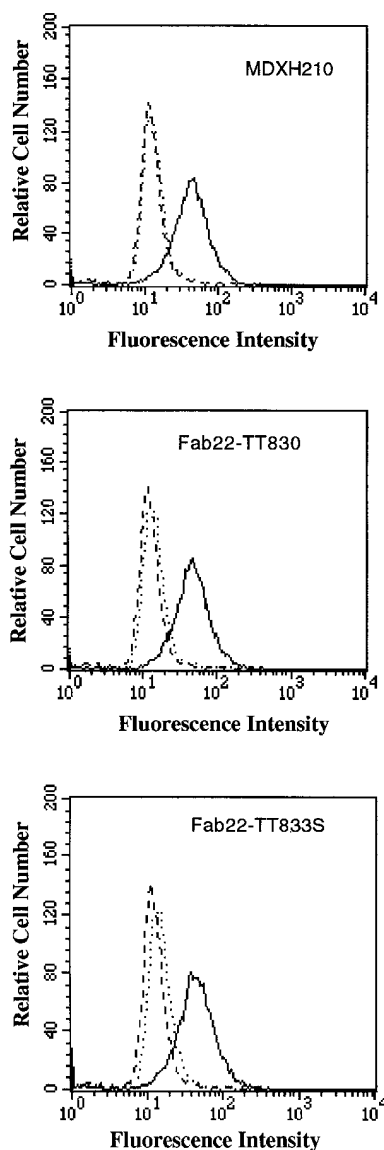
## Results

**H22 fusion proteins bind to U937 cells.** The ability of H22 fusion proteins, Fab22-TT830 and Fab22-TT833S, to bind to Fc $\gamma$ RI was examined first. A previously described bispecific

Ab, MDXH210, which contains the same Fc $\gamma$ RI-binding component (Fab' of humanized mAb 22) (25), was used as a positive control. Binding of fusion proteins and MDXH210 to U937 cells, which constitutively express Fc $\gamma$ RI, was measured by staining with FITC-labeled goat Ab specific for human IgG and flow cytometry. As indicated in Fig. 2, the fusion proteins Fab22-TT830 and Fab22-TT833S bound to U937 cells in a manner similar to MDXH210. The binding of fusion proteins was completely blocked by murine anti-human Fc $\gamma$ RI mAb 22 F(ab')<sub>2</sub>, demonstrating the specificity of fusion proteins for Fc $\gamma$ RI.

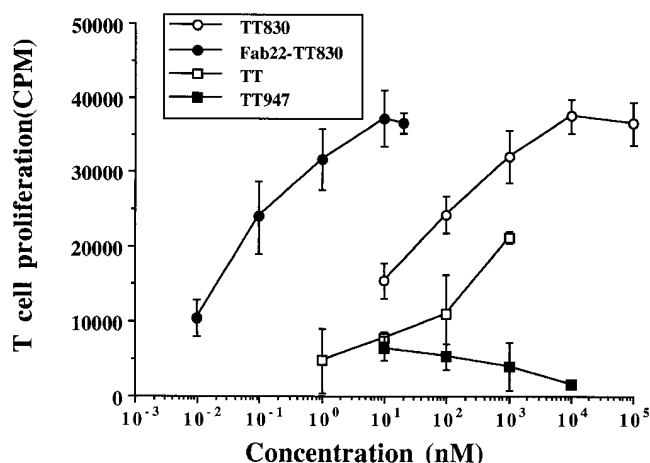
**H22 fusion protein enhances presentation of peptide by 100- to 1,000-fold.** The fusion protein Fab22-TT830 was used in Ag presentation assays to determine whether the Th epitope, TT830, when expressed in the constant region of H22, could be effectively presented by monocytes to autologous T cells. Three T cell lines specific for TT830 were separately generated and used in Ag presentation assays. Fig. 3 shows that  $\sim$  1,000-fold less Fab22-TT830 was required than TT830 peptide to achieve the same level of T cell proliferation. This experiment was repeated for each cell line, and although each exhibited a somewhat different dose-response to TT830, 500- to 1,000-fold enhancement of Ag presentation mediated by the fusion protein Fab22-TT830 was consistently observed. In addition, Fig. 3 also shows that the presentation of Fab22-TT830 was  $\sim$  10,000-fold more efficient than the presentation of the intact TT, suggesting that the enhanced presentation of Fab22-TT830 did not merely result from higher molecular weight or increased stability of Fab22-TT830 as opposed to TT830 peptide. Another antigenic TT epitope, TT947, failed to stimulate the T cells, confirming that the T cells were specific for TT830 peptide. Thus, these results provide clear evidence that Th epitopes expressed in the constant region of H22 can be specifically and efficiently presented.

**Blockade of Fc $\gamma$ RI on monocytes abrogates the enhancement of Ag presentation by the fusion protein.** To directly de-



**Figure 2.** H22-TT peptide fusion proteins maintain the binding ability to Fc $\gamma$ RI on U937 cells. U937 cells were first incubated at 4°C for 1 h with saturating concentration (5  $\mu$ g/ml) of either fusion proteins Fab22-TT830, Fab22-TT833S, or a positive control, bispecific Ab, MDXH210, followed by FITC-labeled goat anti-human IgG Abs as described in Methods. During the first incubation, 100  $\mu$ g/ml murine mAb 22 F(ab')<sub>2</sub> was added to some wells to block the binding of fusion proteins or MDXH210. Samples were then analyzed by FACScan®. Fluorescence histograms for the three samples at 5  $\mu$ g/ml of fusion protein are plotted. The dashed lines represent negative controls in which U937 cells were incubated in medium only followed by secondary Ab. The solid lines denote the staining by fusion proteins or MDXH210 and the dotted lines represent fusion protein binding blocked by murine mAb 22 F(ab')<sub>2</sub>.

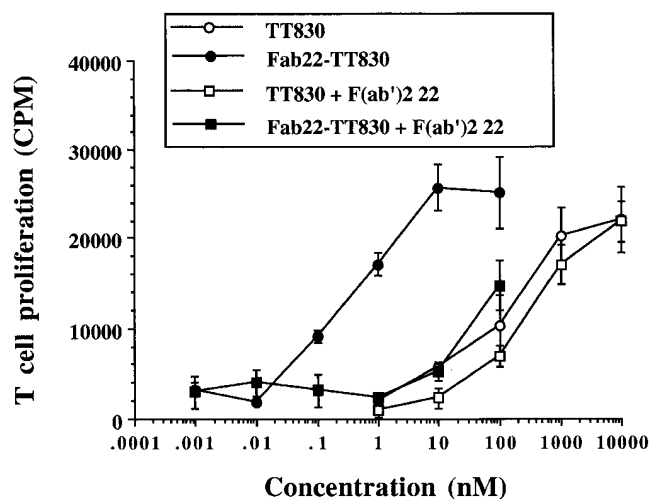
termine whether the enhancement of peptide presentation through the use of the fusion protein is Fc $\gamma$ RI-mediated, binding of Fab22-TT830 to Fc $\gamma$ RI on Ag-presenting monocytes was blocked by treating monocytes with a saturating amount (10  $\mu$ g/ml) of mAb 22 F(ab')<sub>2</sub> for 1 h before the addition of Fab22-TT830 or TT830 peptides at various concentrations. Enhancement of peptide presentation by the fusion protein was abrogated by mAb 22 F(ab')<sub>2</sub>, whereas presentation of TT830 was unaffected (Fig. 4). The fact that the binding of free mAb 22 F(ab')<sub>2</sub> to Fc $\gamma$ RI did not lead to an enhancement of the presentation of free peptides implies that binding of mAb 22 to Fc $\gamma$ RI alone did not alter the functional state of monocytes in a way that enhanced Ag presentation. Therefore, linkage of the peptide to anti-Fc $\gamma$ RI Ab 22 appears to be necessary for the observed enhancing effects on Ag presentation, suggesting that the enhanced presentation is probably a result of efficient Ag capture through Fc $\gamma$ RI. In addition, the enhanced presentation of the TT830 peptide using the fusion protein Fab22-TT830 was not inhibited by IgG (data not shown), in agreement with previous studies showing that mAb



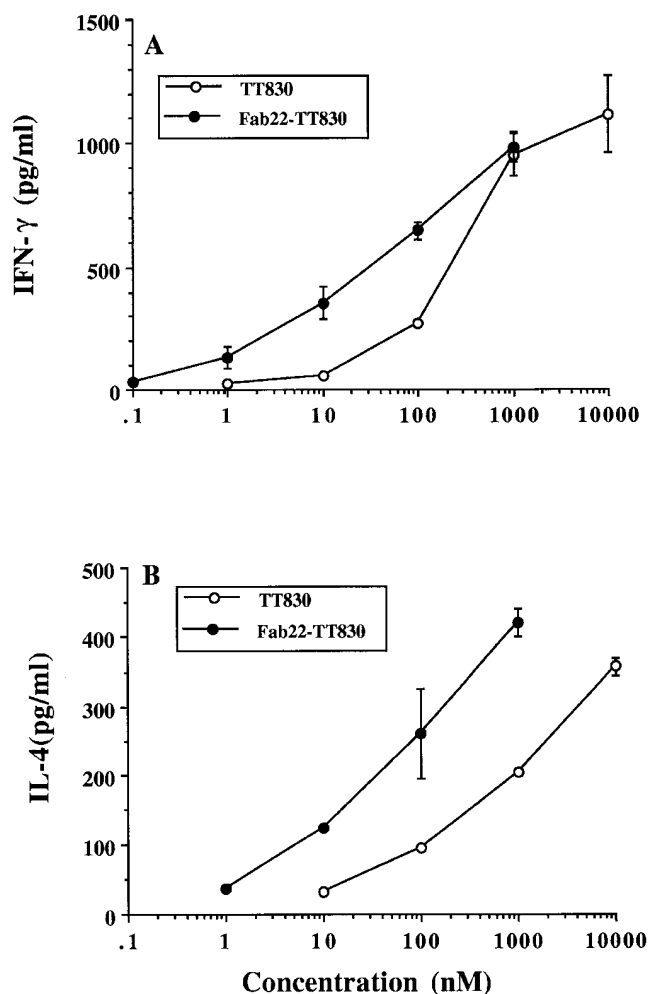
**Figure 3.** The fusion protein Fab22-TT830 enhances the presentation of the Th epitope by  $\sim 1,000$ -fold. The Ag presentation assay was performed as described in Methods. Four different forms of Ag were used in the experiments, antigenic peptide TT830, fusion protein Fab22-TT830, whole TT, and irrelevant Th epitope on TT, TT947. The concentration of Ag is indicated on the x axis.

22 binds to an epitope of Fc $\gamma$ RI outside the ligand binding domain and can thus trigger Fc $\gamma$ R-mediated functions without inhibition by physiological levels of IgG (8, 26).

*IFN- $\gamma$  and IL-4 production is increased after Fab22 fusion protein-enhanced Ag presentation.* Upon activation, T cells not only undergo clonal expansion through proliferation, but also produce cytokines such as IFN- $\gamma$  and IL-4 to exert their effector function on B cell differentiation and monocyte activation (27). Therefore, the production of IFN- $\gamma$  and IL-4 after H22 fusion protein-enhanced Ag presentation was examined. As shown in Fig. 5, A and B, both IFN- $\gamma$  and IL-4 production levels were enhanced by Fab22-TT830, especially at suboptimal Ag concentrations. However, in these experiments, the



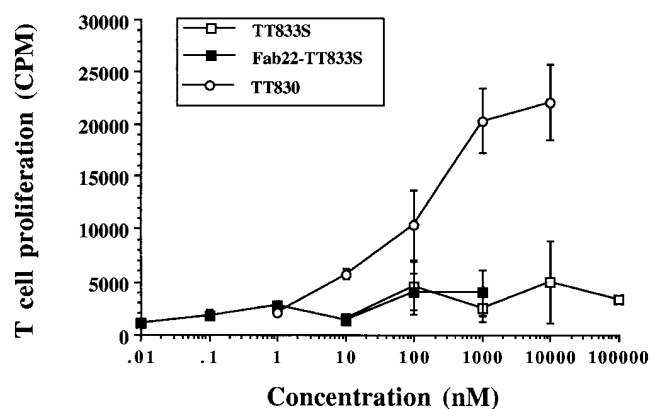
**Figure 4.** The enhanced presentation of fusion protein Fab22-TT830 is Fc $\gamma$ RI dependent. A saturating concentration of mAb 22 F(ab')<sub>2</sub> (10  $\mu$ g/ml) was added to monocytes at the appropriate wells 1 h before the addition of Ag and T cells. The Ag presentation assay was then completed as in Fig. 3. The concentration of Ag is indicated in the x axis.



**Figure 5.** Presentation of TT830 and Fab22-TT830 stimulates IFN- $\gamma$  and IL-4 production. Supernatants from Ag presentation assays (described in Fig. 3) were collected after 2 d of stimulation. The levels of IFN- $\gamma$  and IL-4 were assayed by ELISA and are plotted in A and B, respectively.

enhancement for cytokine production ( $\sim 20$ -fold) was less than that for T cell proliferation ( $\sim 600$ -fold).

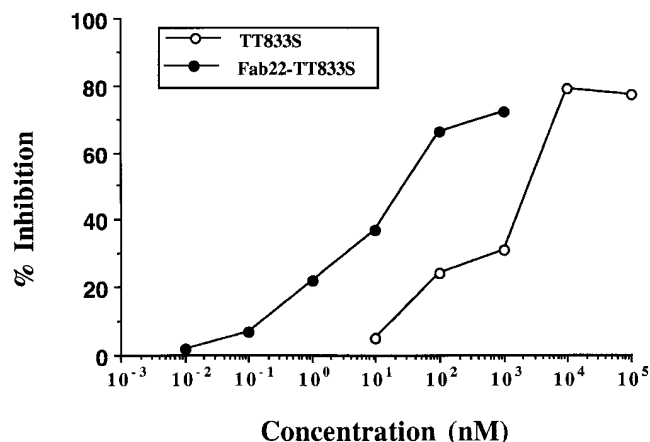
*Fab22-TT833S is at least 100 times more effective than TT833S in inhibiting T cell activation.* We next performed experiments to determine whether the activity of an APL could be enhanced by Fc $\gamma$ RI-targeting. To do this, we synthesized TT833S peptide, an antagonist peptide for T cell epitope TT830. As shown in Fig. 6, even at doses as high as 100  $\mu$ M for TT833S and 1  $\mu$ M for Fab22-TT833S, no significant proliferation of TT830-specific T cells was observed. Both peptide TT833S and Fab22-TT833S also failed to stimulate the production of IFN- $\gamma$  or IL-4 (data not shown). This indicates that changing lysine to serine at position 833 of the TT830 peptide eliminated T cell reactivity of this T cell epitope. Additionally, when peptides TT830 and TT833S were simultaneously presented to TT830-specific T cells, T cell proliferation in response to TT830 was inhibited by TT833S in a dose-dependent fashion, showing that TT833S can function as an antagonist for TT830-specific T cells (Fig. 7). Furthermore, Fab22-TT833S was  $\sim 100$  times more effective than TT833S in inhibiting



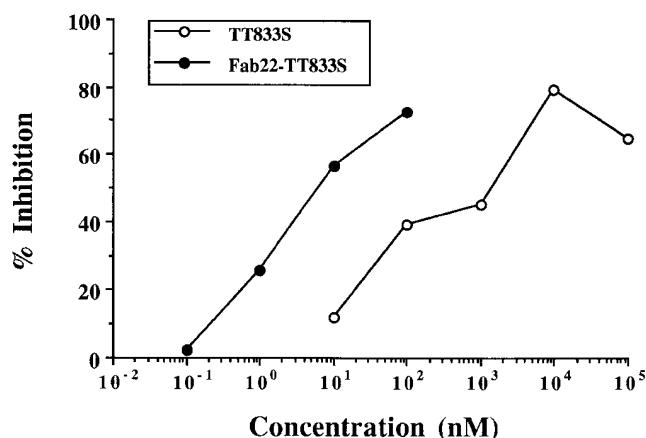
**Figure 6.** Presentation of APL with use of TT833S or Fab22-TT833S fusion protein fails to stimulate T cell proliferation. The concentrations of Ag are indicated on the x axis while T cell proliferation (CPM) is plotted in the y axis. Experiments were done as in Fig. 3.

TT830-stimulated T cell proliferation (Fig. 7). This suggests that the APL, TT833S, when expressed in the constant region of mAb H22, can be correctly and effectively presented by APC. The increased antagonistic efficacy of fusion protein Fab22-TT833S on T cell proliferation probably reflects more efficient Ag capture mediated by Fc $\gamma$ RI as compared with free peptides.

*Inhibition appears to be a result of competition for TCR rather than for MHC class II binding.* The antagonist effects of APL TT833S and fusion protein Fab22-TT833S might be through competition at the level of MHC-binding or TCR-binding, or both. To gain insight into the mechanisms involved, prepulsing experiments, first described by Sette and co-workers (21), were performed. This experimental setting allows agonist (TT830) to bind to MHC class II in the absence of competition from the inhibitor (TT833S), and thus only TCR antagonists but not pure MHC blockers would be effective in



**Figure 7.** Fab22-TT833S is  $\sim 100$  times more effective than TT833S in inhibiting T cell activation. Monocytes were incubated with various concentrations of TT833S or Fab22-TT833S overnight. 20 nM TT830, which stimulates 50% of the maximal T cell response, and T cells were then added. After a further 2 d of incubation, T cell proliferation was measured. The percentage of inhibition was calculated as described in Methods.



**Figure 8.** APL-mediated inhibition appears to be a result of competition for TCR rather than for MHC class II binding. Monocytes were pulsed with 20 nM TT830, which stimulates 50% of the maximal T cell response, 4 h before the addition of various concentrations of TT833S or 0.1  $\mu$ M Fab22-TT833S. After overnight incubation, T cells were then added. After a further 2 d of incubation, T cell proliferation was measured.

inhibiting agonist-stimulated T cell proliferation (21). Specifically, Ag-presenting monocytes were pulsed for 4 h with a concentration (20 nM) of TT830, which stimulated 50% of the maximal response of this particular T cell line. This permitted TT830 to bind with MHC class II in the absence of competition from TT833S. The APL, TT833S or Fab22-TT833S, was then incubated with the prepulsed monocytes for an additional 16 h. Responding T cells were added and their proliferation was measured as usual. Even under such conditions where MHC blockade plays a minimal role, T cell proliferation was still inhibited. T cell antagonism by the fusion protein Fab22-TT833S was at least 100-fold more efficient than by the nontargeted antagonist, TT833S (Fig. 8). Thus, the inhibition appears to be a result of competition for TCR rather than for MHC class II binding.

## Discussion

The observation that immunogenicity is increased by up to 1,000-fold by targeting antigenic peptides to Fc $\gamma$ RI using an anti-Fc $\gamma$ RI mAb 22-based fusion protein reveals a potentially useful vehicle for peptide-based vaccines for infectious diseases and cancer. This peptide-targeting method has several distinct advantages over the AgAbs previously described by other investigators (6, 7). Since the binding site for H22 fusion proteins is outside the ligand-binding domain of Fc $\gamma$ RI, there is no competition for their binding to APC from abundant serum IgG (8, 26, and data not shown). This contrasts with the binding of AgAb to Fc $\gamma$ RI, which would be adversely affected by competing serum IgG, preventing APCs from efficiently concentrating the Ags. Furthermore, H22 fusion proteins bind only to Fc $\gamma$ RI which in healthy subjects is restricted to monocytes, macrophages, and dendritic cells (28), all of which are "professional" APCs. AgAb, on the other hand, could be sequestered by a variety of Fc $\gamma$ RII or Fc $\gamma$ RIII-bearing non-APCs such as neutrophils and platelets. Engineering peptides into the constant domains of human mAb that are specific for particular APC surface molecules may thus represent a gen-

eral approach to increase the antigenic potency for peptide-based vaccines. The resultant constructs would bind very selectively to professional APCs *in vivo* where Ag dilution and the short half-life of peptides would be even more significant than in the cell culture system studied thus far. In addition, targeting Ag to Fc $\gamma$ R, or to different molecules on APCs, might have the potential to qualitatively alter the type of response elicited.

Studies reported in this paper have demonstrated that Th epitopes expressed in the constant region of anti-Fc $\gamma$ RI mAb H22 can be effectively processed and presented by human monocytes, leading to enhanced T cell activation and cytokine production (Figs. 3–5). The effective presentation of the fusion protein Fab22-TT830 appears to result from more efficient Ag capture through specific binding to Fc $\gamma$ RI, as the murine mAb 22 abrogated the enhancement in Fab22-TT830 presentation but had no effect on T cell stimulation by the nontargeted peptide TT830 (Fig. 4). Furthermore, the antagonistic peptide, TT833S, similarly expressed in the constant region of H22, could also be effectively presented by monocytes. As a result, the fusion protein Fab22-TT833S was  $\sim$  100 times more effective than free TT833S in antagonizing T cell activation stimulated by the wild-type peptide TT830 (Figs. 7 and 8).

Our studies also revealed the potentially interesting phenomenon that the Fc $\gamma$ RI-targeted fusion protein Fab22-TT830 enhanced T cell proliferation by up to 1,000-fold as compared with the peptide TT830 (Figs. 3 and 4), whereas enhancement of cytokine (IFN- $\gamma$  and IL-4) production by Fab22-TT830 was only 20-fold (Fig. 5). This difference in the stimulation of T cell proliferation and cytokine production is in agreement with previous studies of TCR partial agonists showing that cellular proliferation and cytokine production are separable events of T cell activation and probably require different degrees of interaction between accessory molecules (29, 30). Another possible explanation is that the Fc $\gamma$ RI-targeted fusion protein Fab22-TT830 alters the production by monocytes of IL-10 and IL-12, both of which are known to significantly influence the production of IFN- $\gamma$  and IL-4 by T cells. We are currently developing similar fusion proteins consisting of Fab22 and specific epitopes of the model antigen ovalbumin. These fusion proteins will enable us to evaluate differential stimulation of T cell proliferation and cytokine production using a well-defined mouse model system (31).

APLs, peptides with one or more point mutations from a Th epitope, have been reported to act as agonists, partial agonists, or antagonists (10, 11). Agonist peptides retained the full activity of wild-type peptides to stimulate T cell activation. As mentioned above, partial agonists stimulated some T cell functions such as IL-4 production by T cells, but not others such as T cell proliferation (29). In some cases, partial agonists were capable of inducing T cell anergy (13, 14). Certain APLs do not trigger any detectable signaling events upon interaction with TCR, but can function as TCR antagonists to inhibit T cell proliferation in response to wild-type peptide antigen and are thus called TCR antagonists (21, 32). In this study, the altered peptide TT833S did not stimulate proliferation or the production of IL-4 and IFN- $\gamma$  by T cells specific for the native peptide TT830 (Fig. 6 and data not shown). TT833S also failed to induce T cell anergy. However, TT833S did inhibit the proliferation of TT830-specific T cells responding to TT830, indicating that TT833S belongs in the category of antagonists first described by Sette and co-workers (21).



The results reported here have potentially important implications for the use of TCR antagonists as Ag-specific therapy for autoimmune diseases. The therapeutic potential of APL in autoimmune diseases has been demonstrated by in vivo treatment using altered encephalitogenic peptides of PLP, which led to the inhibition of PLP-induced EAE in susceptible mice (18, 19). Similar APL-based treatment could provide an antigen-specific immunotherapy for T cell-mediated autoimmune diseases such as rheumatoid arthritis and multiple sclerosis. However, the efficacy for this kind of APL-based treatment of autoimmune diseases is limited by the fact that peptides are poorly presented in vivo, and thus coadministration of adjuvant has been necessary for the in vivo effects in mice. We have demonstrated (Fig. 8) that FcγRI-targeted APL can efficiently antagonize activation of native peptide-reactive T cells even when the APC has been prepulsed with native peptide sufficient for T cell activation. In addition, we have previously shown that the parent molecule of the fusion proteins described in this report, humanized mAb 22, specifically and efficiently binds to human FcγRI in vivo and is an unusually potent immunogen (31). Thus, targeting of APL to FcγRI using mAb 22-based fusion proteins may efficiently deliver APLs to APCs in an adjuvant-free manner in vivo and may effectively inhibit the activation of autoreactive T cells. Overall, the approaches described in this report may lead to more potent vaccines and to promising Ag-specific treatments for autoimmune diseases.

## Acknowledgments

We thank Kathy Wardwell for her help in preparation of this manuscript.

This work was supported by National Institutes of Health grants AI37212 and AI10953. Flow cytometry was performed in the Herbert C. Engler Cell Analysis Laboratory, which was established with a grant from the Fannie E. Rippel Foundation and is supported in part by the core grant of the Norris Cotton Cancer Center (CA-23108).

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