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Engineering High-Affinity T Cell Receptor/Cytokine Fusions for Therapeutic Targeting

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1. Introduction

1.1 Background: Inflammatory diseases

Inflammatory diseases or conditions, including autoimmune diseases, allergies, and transplant/graft rejection, constitute a significant health issue. Among these diseases, current treatments include various global immunosuppression strategies such as steroids for autoimmune diseases and asthma, antithymocyte globulin (ATG) for lymphocyte depletion or cyclosporine A for transplant tolerance, and other treatments (reviewed in (Fort & Narayanan 2010)); however, these can leave the patient with increased susceptibility to infection, tumor development, or other immune challenge (reviewed in (Belkaid 2007; Sabat et al. 2010)). Therefore, more targeted, antigen-specific immune tolerance is desirable.

While excessive inflammation is a complex process including many immune effector cells, $CD4^+$ helper T cells are thought to be crucial to disease progression for many conditions (Davidson et al. 1996; Gebe et al. 2008; Reijonen et al. 2002; Sospedra & Martin 2005; Tesmer et al. 2008). Pathogenic $CD4^+$ T cells in these diseases are thought to express T cell receptors (TCRs) that recognize specific peptides bound to class II major histocompatibility complex (MHC) proteins on the surface of antigen-presenting cells (APCs), where the presented peptide is derived not from a pathogen, but from an allergen, transplant antigen, or self protein. Specific peptides derived from allergens or "self" proteins have been identified as linked to the pathological responses in many cases (Haselden et al. 1999; Hemmer et al. 1998; Jahn-Schmid et al. 2002; Muraro et al. 1997; Reijonen et al. 2002; Vergelli et al. 1997), and links between particular class II MHC alleles and disease susceptibility have been identified (Ebers et al. 1996; Oksenberg et al. 1996; Wucherpfennig & Sethi 2011). Hence, one attractive option would be to selectively inhibit only those $CD4^+$ T cells that initiate or sustain pathological inflammation. Some efforts at more targeted induction of tolerance

have been explored, including natalizumab, an antibody against the α4 integrin, which selectively inhibits leukocyte attachment to and extravasation through inflamed vascular endothelium (Rice et al. 2005), but has no epitope or tissue specificity. In another example, a pooled, randomized peptide vaccine called glatiramer acetate (GA) has been approved to induce tolerance in multiple sclerosis (Johnson et al. 1995; Teitelbaum et al. 1988; Teitelbaum et al. 1996), but acts across a variety of epitopes, and may affect the patient's ability to respond normally to novel infectious challenges. Truly selective immunosuppression, therefore, remains an outstanding challenge.

1.2 Immunosuppressive cytokine IL-10

Interleukin-10 (IL-10), first identified as a cytokine synthesis inhibitory factor secreted by Th2 cells (Fiorentino et al. 1989), is a member of the Class II cytokine family largely associated with immune suppression. While IL-10 can be produced broadly by many different T cell subsets, along with B cells, NK cells, dendritic cells, macrophages, and other immune cells (reviewed in (Asadullah et al. 2003; O'Garra et al. 2008; Ouyang et al. 2011; Saraiva & O'Garra 2010)), it is strongly associated with regulatory T cells (Tregs). Tregs both produce IL-10 (and sometimes TGF-β) in the absence of other effector cytokines, and require IL-10 for their function. The ability of IL-10 to support Treg differentiation as well as suppress inflammatory responses in antigen-specific T cells makes it an attractive potential therapeutic.

1.2.1 Biological / immunological effects

IL-10 functions *in vivo* to protect against excessive inflammation and tissue damage. It suppresses the expression of proinflammatory cytokines by adaptive and innate immune cells (reviewed in (Asadullah et al. 2003; Moore et al. 2001)). In the absence of IL-10, exaggerated inflammatory responses to infection occur, often resulting in tissue damage. For example, IL-10-deficient mice develop chronic inflammatory bowel disease after exposure to enteric bacteria (Kuhn et al. 1993; Sellon et al. 1998). Many general immunosuppressants currently used to treat inflammatory diseases are thought to be effective in part by increasing the ratio of IL-10 to other cytokines (O'Garra et al. 2008). Despite its predominant function to suppress inflammatory responses, IL-10 can also enhance certain functions of the immune system, including humoral immunity, NK cell activity, or $CD8^+$ T cell responses (reviewed in (Asadullah et al. 2003; O'Garra et al. 2008)). These various effects depend on the specific conditions and concentrations of IL-10.

Defects in IL-10 levels have been linked to steroid-insensitive asthma (Hawrylowicz et al. 2002), and can increase the pathology of autoimmune diabetes. Clinical trials of systemic administration of IL-10 for treatment of the autoimmune conditions psoriasis, Crohn's disease, and rheumatoid arthritis have been designed to take advantage of the broad immunosuppressive action of this cytokine (Asadullah et al. 2003). While no particular benefit was seen in the treatment of rheumatoid arthritis, some promising results were seen in psoriasis and Crohn's disease; however, the effects were modest, and they depended heavily on finding the appropriate dosing (Asadullah et al. 2003; O'Garra et al. 2008). Studies of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis, suggested that IL-10 is crucial to protect against inflammation and damage in the central nervous system, but localization of the cytokine within the target organ is required

for effectiveness (Bettelli et al. 1998; Kennedy et al. 1992; Samoilova et al. 1998). As a soluble therapeutic, IL-10 seems most effective when combined with targeted delivery methods such as T cells transduced to express IL-10, or genetically engineered *Lactococcus lactis* that produce IL-10, both having shown promise in treatment of murine inflammatory bowel disease (Lindsay & Hodgson 2001; Steidler et al. 2000; Wirtz et al. 1999).

1.2.2 Structure

IL-10, like other Class II cytokine family members, is composed of alpha-helical bundles (six helices designated A-F), similar to common cytokine-receptor γ-chain (γ_c) family cytokines IL-2 and IL-15. However, unlike IL-2, but like interferon gamma, IL-10 exists as an intercalated homodimer, where the E and F helices from one polypeptide chain fold into a bundle with the A-D helices from a second polypeptide chain (Zdanov et al. 1995). As a dimer, its affinity for the high-affinity IL-10 receptor is in the picomolar range (Tan et al. 1995). However, introduction of an extended flexible linker between N116 and K117 allowed folding into a soluble, bioactive, monomeric form with a K_D of 30 nM for IL-10R1 (Josephson et al. 2000).

Some viruses have evolved an ability to take advantage of the suppressive functions of IL-10 to down modulate the immune response by producing viral IL-10 homologues (Fleming et al. 1997; Hsu et al. 1990; Kotenko et al. 2000; Moore et al. 1990; Spencer et al. 2002). Study of these homologues have led to identification of a point mutation, I87A, which, when included in murine or human IL-10, leads to a cytokine with the suppressive, but not stimulatory functions of IL-10 (Ding et al. 2000).

1.3 Specific tissue targeting of immunoactive proteins

Systemic immunomodulation carries potential risks associated with the disruption of the balance of inflammation and tolerance in the immune system (Belkaid 2007; Fort & Narayanan 2010). In contrast, targeted delivery of a cytokine like IL-10 to particular sites of interest could greatly increases its desired immunosuppressive effect (Kennedy et al. 1992; Kuhn et al. 1993; Lindsay & Hodgson 2001). In order to deliver immunomodulatory proteins selectively to the tissues where their actions are desired, strategies involving covalent linkages of soluble targeting elements to immunoactive proteins have been explored with some success. For example, these targeted approaches have been studied for cancer treatments with the hope of directing immunostimulatory cytokines to the tumor microenvironment (Gillies 2009; Kaspar et al. 2007; Kaspar et al. 2006; Schanzer et al. 2006; Sommavilla et al. 2010). By analogy, immunosuppressive treatments for inflammatory disease would be desired locally, rather than systemically. In addition, the tissue-specific, graft-specific, or allergen-specific nature of most inflammatory diseases make them strong candidates for targeted delivery of immunosuppression. Discussion of several specific targeting strategies for the delivery of soluble effector molecules follows.

1.3.1 Antibodies and scFv

The use of monoclonal antibodies that bind specific target epitopes has been a common strategy for many purposes, including diagnostics and treatments. Some antibodies have been used directly for targeting, taking advantage of Fc-mediated effector functions, such as

with rituximab (anti-CD20 for B cell carcinoma) or with infliximab (anti-TNF-alpha for rheumatoid arthritis or Crohn's disease). Although beyond the scope of this article, antibodies have also been linked to bioactive payloads such as toxins, radioisotopes, or chemotherapeutic agents for the treatment of cancer (Ma et al. 2004; Pohlman et al. 2006; Schanzer et al. 2006).

Antibody fragments termed single-chain variable fragments (scFv) that consist of the variable domains of the heavy and light chains of an antibody, connected by a flexible linker into a single polypeptide chain, have been employed to target immunoactive molecules locally to particular tissues. Fusions of scFv specific for tumor-associated epitopes with cytokines including IL-15, IL-12, GM-CSF, TNF-α, and IFN-α have been studied for localized immune stimulation in the case of cancer with some success (Gillies 2009; Kaspar et al. 2007; Kaspar et al. 2006; Schanzer et al. 2006; Sommavilla et al. 2010). In one study, targeting of IL-10 with the scFv L19, specific for an angiogenesis marker, led to significant improvement in arthritis symptoms over irrelevantly-targeted IL-10 (Trachsel et al. 2007). However, ideal cell surface tissue antigens that can be targeted by monoclonal antibodies may not be available for every application, especially if they are unrelated to the progression of the disease being addressed.

1.3.2 Benefits of targeting MHC-restricted epitopes using TCRs

Since many inflammatory diseases are thought to be mediated by T cells that recognize specific, sometimes known, peptide-MHC complexes (Haselden et al. 1999; Hemmer et al. 1998; Jahn-Schmid et al. 2002; Muraro et al. 1997; Reijonen et al. 2002; Vergelli et al. 1997), it is reasonable to target immunosuppressive treatments directly to those complexes. As indicated above, the natural receptor for peptide-MHC is the αβ heterodimeric TCR (reviewed in (Davis et al. 1998)). TCRs have diversity that is similar to antibodies, and they have evolved to recognize peptide-MHC ligands with a high degree of peptide specificity, and thus the ability to distinguish among small variations in these complexes. However, TCRs, unlike antibodies, are not expressed in soluble form, but only as transmembrane proteins, and only as part of a larger TCR complex with additional transmembrane subunits called CD3. The wild-type affinities of TCR:peptide-MHC interactions are typically low—on the order of 1-500 μM (reviewed in (Stone et al. 2009)). While this affinity is sufficient to trigger cellular responses in the T cell, it is likely too low to efficiently target a soluble therapeutic. Nevertheless, there is some precedence for use of a wild-type affinity TCR, expressed as a three-domain soluble fusion with IL-2 or IL-15 (Belmont et al. 2006; Card et al. 2004; Wong et al. 2011). Naturally occurring autoimmune TCRs are thought to be even weaker binders than TCRs against foreign peptide-MHC ligands; in fact, the affinity of one autoimmune-specific TCR was impossible to measure for a soluble monomer by surface plasmon resonance (Li et al. 2005a). However, advances in protein engineering have allowed the generation of soluble TCRs with affinities that are appropriate for targeting.

1.3.3 Affinity considerations / engineering

The goal of using a TCR as a targeting moiety for a soluble immunotherapeutic has become plausible due to advances in engineering and directed evolution (reviewed in (Richman & Kranz 2007)) using techniques such as yeast surface display, phage display, or more recently, T cell display (Chervin et al. 2008). Engineered receptors with 1000-fold or more

increased affinity compared to their original, wild-type TCRs have been isolated, and the soluble forms of these TCRs bind to the targeted peptide-MHC with a high level of specificity (Chervin et al. 2008; Holler et al. 2003; Holler et al. 2000; Li et al. 2005b; Weber et al. 2005). The higher affinities achieved for TCR binding to peptide-MHC (nanomolar and picomolar) are in the range of affinity-matured antibodies, and are thus sufficient to evaluate for their ability to serve as soluble therapeutics.

Obtaining sufficient quantities of purified, soluble, recombinant TCR posed a significant challenge. One solution was to produce in *E. coli* soluble TCRs that contain the full extracellular alpha and beta chains with an additional disulfide bond between the Cα and Cβ domains (Boulter et al. 2003). By analogy with scFv (V_{heavy}-linker-V_{light}) constructs from antibodies, single-chain TCR variable domain, or scTv, constructs (V_{alpha}-linker-V_{beta}) have been generated for several TCRs, and this form would be well suited for covalent fusion to immune effector molecules. Unlike scFvs, scTvs generally require additional engineering to be soluble and stable, a process that has been carried out through yeast display (Kieke et al. 1999; Richman et al. 2009). Also, increased experience with TCR engineering has led to identifying a particular human variable domain called Vα2 that is best suited for engineering, facilitating future efforts at scTv construction (Aggen et al. 2011).

1.4 Model system 3.L2 and high-affinity TCR M15 engineered by yeast surface display

Inflammatory disease is critically linked to specific TCR interactions with class II MHC that present particular peptides on the surface of APCs. Our goal to target the immunosuppressive effects of IL-10 locally to particular class II MHC epitopes may be modeled by targeting the murine class II MHC allele I-E^k presenting a peptide derived from the beta chain of the minor d allele of hemoglobin (Hbβ^d [64-76]). This Hb/I-E^k complex is recognized by a wild-type TCR called 3.L2 with an affinity of 20 μM for the peptide-MHC (Weber et al. 2005). Engineering efforts to improve this interaction are detailed below.

1.4.1 Engineered for stability as a soluble, single-chain TCR variable fragment (scTv)

The variable genes for the 3.L2 TCR were used to generate a scTv construct in a Vβ8.3-linker-Vα18 orientation (Weber et al. 2005). This construct was cloned as a fusion to the yeast mating protein Aga-2 for engineering by yeast surface display. The scTv with wild-type variable sequences was not expressed as a folded protein on the surface of yeast, as has been observed for most scTvs. To identify key mutations that might enable yeast surface display and expression in *E. coli*, random mutations were introduced into the scTv gene by error-prone PCR, and a library of scTv mutants was transformed into yeast. From yeast expressing the library of mutants, a 3.L2 clonotypic antibody was used to select yeast with the most stable 3.L2 scTv mutants. The most stable and highest expressed mutant, called M2, contained six point mutations (Weber et al. 2005).

1.4.2 Engineered for high affinity to Hb/I-E^k complex

Using the M2 gene as a scaffold, directed hypervariable libraries were generated with mutations in the complementarity determining regions 3 (CDR3) of the scTv (Weber et al. 2005). Successive rounds of selection of the yeast libraries for binding to the specific ligand,

Hb/I-E^k, led to isolation of a high-affinity mutant named M15. The M15 mutant and an M1 mutant containing completely wild-type CDR sequences (identical to 3.L2) were expressed in *E. coli* and refolded as soluble scTv proteins. Binding to Hb/I-E^k was measured by surface plasmon resonance for both scTvs, and an 800-fold increase in affinity was found for the high-affinity M15 (K_D=25 nM) as compared to 3.L2 (K_D=20 μM for M1) (Weber et al. 2005). The affinity of M15 for Hb/I-E^k is thus in the range seen for monoclonal antibodies.

1.5 M15-targeted IL-10 fusion molecules

To evaluate the possibility of targeting the immunosuppressive cytokine IL-10 to cells expressing a particular class II MHC-peptide complex, we engineered a series of fusion molecules linking the M15 scTv to various IL-10 proteins; these IL-10 proteins included human, murine, dimeric, monomeric, and I87A mutants. The proteins were produced and purified from *E. coli* or HighFive insect cells, and were found to be stable in solution. The various constructs were evaluated for proper folding of the IL-10 cytokine as detected by ELISA, and the M15 targeting element was shown to bind specifically to cells presenting the proper peptide-MHC. Importantly, the constructs functioned in various IL-10 cytokine-dependent assays, indicating that the approach has the potential to deliver targeted immunosuppressive molecules, and providing a valuable model for further evaluation of this strategy.

2. Methods

2.1 Cloning, production, and purification of scTv M15 and M15:IL-10 fusions

For *in vitro* folding, full-length human IL-10 (hIL-10) was attached to the C-terminus of scTv M15 via either a 18-mer linker (VNAKTTAPSVYPLAPVSG) (Card et al. 2004) or a 15-mer linker $(SSSSG)_3$ (Trachsel et al. 2007). In addition to these wild-type M15:hIL-10 constructs, constructs containing modified hIL-10 sequences were also made. One modification involved inserting six amino acids (GGGSGG) between hIL-10 residues Asn115 and Lys116 (Josephson et al. 2000), in order to prevent formation of IL-10 homodimers that could complicate production of M15:hIL-10 conjugates. The other modification involved a point mutation (I87A) to reduce the affinity of hIL-10 for hIL-10 receptor (hIL-10R) by ~100-fold, while preserving its biological activity (Ding et al. 2000). The rationale for reducing the affinity of hIL-10 for hIL-10R, whose K_D is in the picomolar range (Tan et al. 1995), was to prevent the hIL-10–hIL-10R interaction from potentially dominating the binding of scTv M15 to its peptide–MHC ligand (Hb–I-E^k) on target cells (nanomolar K_D).

Each M15:hIL-10 construct was cloned into the bacterial expression vector pET-26b(+) and produced as inclusion bodies in BL21(DE3) *E. coli* cells. The inclusion bodies were washed with 50 mM Tris-HCl (pH 8.0) containing 5% (v/v) Triton X-100, then dissolved in 6 M guanidine, 50 mM Tris-HCl (pH 8.0), and 10 mM DTT. For *in vitro* folding, the inclusion bodies were diluted into ice-cold folding buffer containing 0.4 M L-arginine-HCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 3 mM reduced glutathione, and 0.9 mM oxidized glutathione to a final protein concentration of ~40 mg/l. After 72 h at 4 °C, the mixtures were concentrated and dialyzed against PBS, prior to purification using sequential Superdex S-200 and Mono Q columns.

Wild-type human and mouse IL-10 (mIL-10) conjugated to scTv M15 were also produced using a baculovirus expression system. The homologous IL-10 genes were fused to scTv M15 in the same way as for *E. coli* expression, using both 18-mer and 15-mer linkers. Accordingly, we cloned the following constructs into the pAcGP67B baculovirus transfer vector: 1) M15:mIL-10 containing the 18-mer linker; 2) M15:SG15:hIL-10 containing the 15-mer linker; and 3) M15:SG15:mIL-10. In addition, the constructs included a C-terminal FLAG tag (DYKDDDDK) for affinity purification.

The pAcGP67B vector uses a baculovirus signal sequence (gp67) to direct secretion of expressed proteins into the culture supernatant. Each construct was co-transfected into Sf9 insect cells with linearized baculovirus DNA to generate a recombinant baculovirus. Secretion of all three constructs was confirmed by Western blotting using the anti-FLAG monoclonal antibody M2. For protein production, 1–3 liters of HighFive insect cells at ~10^6 cells/ml in serum-free medium were incubated with recombinant baculovirus at a multiplicity of infection of ~3 pfu/cell. Culture supernatants were harvested 3–4 days after infection and concentrated ~20-fold. Following dialysis against TBS (pH 7.3), supernatants were loaded on an anti-FLAG M2 agarose column and bound proteins were eluted using 100 µg/ml FLAG peptide. Recombinant M15:mIL-10, M15:SG15:mIL-10, and M15:SG15:hIL-10 were further purified with sequential Superdex S-75 and Mono Q columns.

2.2 IL-10 ELISA

Properly folded IL-10 was detected in preparations of free IL-10 or M15:IL-10 fusions using the Ready-Set-Go sandwich ELISA kit from eBioscience (San Diego, CA). Briefly, a monoclonal antibody against IL-10, murine or human, was diluted in a coating buffer of phosphate-buffered saline (PBS) and coated onto the wells of high-binding, clear, flat-bottomed 96-well plates by incubating overnight at 4˚C. The solution was removed, and wells were then blocked by filling with a solution of 1% bovine serum albumin in phosphate-buffered saline (PBS-BSA), pH 7.4, and incubating for at least 2 hours at room temperature or overnight at 4˚C. After blocking, known concentrations of standard IL-10 or various dilutions of the M15 fusion protein of interest in the provided assay diluent buffer were added to the wells and incubated for 2 hours at room temperature. Wells were washed three times with PBS containing 0.1% Tween-20 detergent (PBST), and then a biotinylated polyclonal detection antibody solution was added to each well and incubated for 2 hours at room temperature. Wells were then washed three times with PBST, and a solution of Avidin conjugated to horseradish peroxidase was added to each well and incubated for 45 minutes at room temperature. After the avidin solution was removed, wells were again washed three times with PBST, and 50 microliters per well of tetramethylbenzidine (TMB) solution was added, which, when acted upon by peroxidase, results in a colored precipitate. After development, the reaction was stopped by adding 50 microliters per well of a 1N sulfuric acid solution. Absorbance in each well at 450nm was measured using an ELx800 Universal Microplate Reader (BioTek Instruments, Inc, Winooski, VT).

2.3 Specific binding to peptide-loaded cells

The I-E^{k+} CH27 immortalized B cell line (Pennell et al. 1985) was loaded incubated with various concentrations of a hemoglobin-derived peptide, Hb^d[64-76] in RPMI 1640 media supplemented with overnight at 37˚C penicillin, streptomycin, hepes, glutamine, beta

mercaptoethanol, and 10% fetal calf serum (complete RPMI media). Cells were then washed in ice-cold PBS-BSA, and incubated with 20 ng/mL of scTv M15:IL-10 fusion constructs or 10 ng/mL biotinylated scTv M15 on ice for 2 hours. Cells were then washed in excess PBS-BSA, and cells stained with M15:IL-10 fusions were incubated for 2 hours on ice with 1:100 diluted biotinylated anti-IL-10 polyclonal antibody from the Ready-Set-Go ELISA kit (eBioscience, San Diego, CA). Cells were again washed with PBS-BSA, and then all the cells incubated for one hour on ice with streptavidin fluorescently labeled with phycoerythrin (1:100, BD Pharmingen, San Diego, CA). Cells were washed twice with PBS-BSA, and then resuspended in PBS-BSA for analysis by analytical flow cytometry using a BD FACSCanto (BD Biosciences, San Jose, CA).

2.4 MC/9 proliferation functional assay

MC/9 mouse liver mast cells (American Type Culture Collection, ATCC, Manassas, VA) were maintained in complete RPMI media supplemented with 5% Rat ConA supernatant (TStim, BD Biosciences, San Jose, CA). In the absence of ConA supernatant, MC/9 cells selectively proliferate in response to functional human or murine IL-10 cytokine. A standard number of MC/9 cells per well were cultured with 5 pg/mL murine IL-4 (insufficient to drive proliferation on its own, but can potentiate responses to IL-10) and various concentrations of IL-10 standard concentrations or scTv M15:IL-10 fusion proteins for 48 hours at 37˚C. After this time, 10 microliters per well of 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT kit, ATCC, Manassas, VA) solution was added, and the cells were cultured an additional 2 hours at 37˚C. Metabolically active cells reduced this to a purple formazan precipitate, which was then dissolved by adding detergent to each well and incubating at least two hours in the dark at room temperature. Absorbance was measured at 570nm using an ELx800 Universal Microplate Reader (BioTek Instruments, Inc, Winooski, VT).

2.5 Inhibition of T cell activation by soluble scTv

CH27 antigen presenting cells ($3x10^4$) were cultured at 37°C for 30 minutes with soluble scTv M15 at the indicated concentrations (0, 10, or 50 μg/ml) and a range of Hb peptide concentrations. 3.L2 T cell hybridoma cells ($1x10^5$) were then added to the antigen presenting cells for 24 hours and activation was determined by measurement of IL-2 in the tissue culture supernatant. Supernatants (100 μl) were added to the IL-2-dependent CTLL-2 line ($5x10^3$ cells), followed by culture for 48 hours. CTLL-2 cells were pulsed with 0.4 μCi $[^3H]$thymidine for the final 18–24 h. IL-2 production was quantified by measurement of $[^3H]$thymidine incorporation.

3. Results

3.1 Cloning, expression, and purification of M15:IL-10 fusions

To conjugate high-affinity TCR M15 to the anti-inflammatory cytokine IL-10, we fused a single-chain version of M15 (scTv M15) to the N-terminus of IL-10. The scTv M15 construct, which was engineered by linking Vβ to Vα via a 17-mer peptide (GSADDAKEDAAKKDGES), was then connected to different versions of hIL-10, including wild-type hIL-10, hIL-10 (I87A) which contains the I87A point mutation, monomeric hIL-10

(mono) which has the GGGSGG insertion, and hIL-10 (I87A, mono) with both the point mutation and insertion. Wild-type IL-10 exists as an intercalated (domain-swapped) homodimer in solution with picomolar affinity for IL-10R (Zdanov et al. 1995). Insertion of GGGSGG into the loop connecting the swapped secondary structural elements of dimeric IL-10 generates a monomeric version of this cytokine with a K_D for IL-10R of 30 nM, without significantly affecting immunosuppressive activity (Josephson et al. 2000).

All M15–IL-10 constructs were tested for *in vitro* folding. Interestingly, only the monomeric versions could be folded successfully, whereas conjugates containing wild-type (i.e. dimeric) IL-10 showed poor folding behavior under a wide range of conditions. As expected, M15–hIL-10 (mono) and M15–hIL-10 (I87A, mono) behaved as monomers in gel filtration (Figure 1A).

The linker used for attaching scTv M15 to IL-10 was either an 18-mer or 15-mer peptide. The 18-mer linker (VNAKTTAPSVYPLAPVSG) (Card et al. 2004) was originally adopted from the hinge region between the V_H and C_H1 domains of an antibody, whereas the 15-mer linker $(SSSSG)_3$ (Trachsel et al. 2007) was designed to maximize flexibility and solubility. Proteins with the two different linkers showed similar properties, except that extensive hydrolysis was detected for conjugates using the 18-mer linker after two weeks storage at 4 °C (Figure 1C).

To express conjugates with wild type IL-10, we adopted the baculovirus expression system. Constructs containing scTv M15 fused to wild type mouse or human IL-10 genes were inserted into the pAcGP67B baculovirus transfer vector, which uses the baculovirus polyhedrin promoter to drive expression. Secretion of M15–IL-10 conjugates was directed by the gp67 signal sequence. Importantly, all three wild-type IL-10 conjugates (M15:mIL-10, 18aa linker, M15:hIL-10, 15aa linker, and M15:mIL-10, 15aa linker) eluted as dimers in gel filtration (Figure 1B), indicating that IL-10 retained its native state when conjugated to scTCR M15. The yield of the conjugates from infected High Five cells was typically 1 mg per liter of culture.

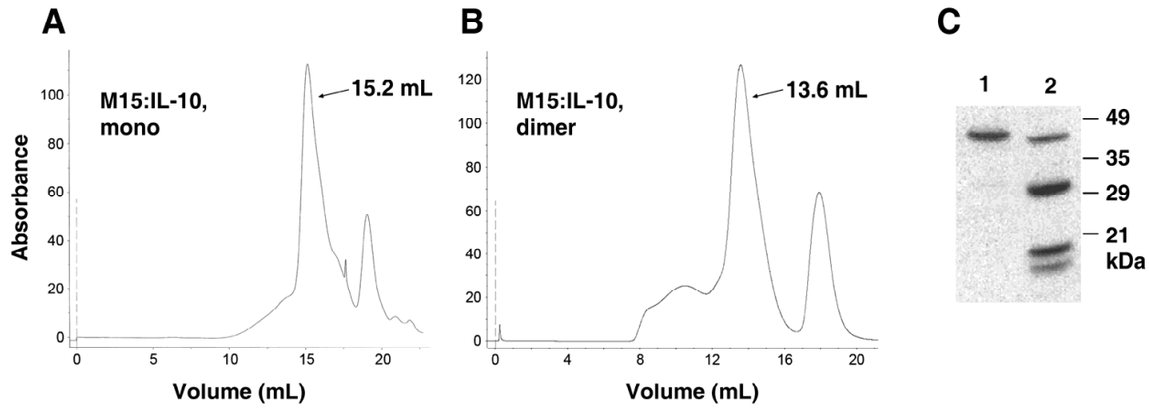

Fig. 1. Purification and characterization of soluble, scTv M15:IL-10 fusion proteins.

Size exclusion chromatography of *(A) in vitro* folded human M15:IL-10, mono, and *(B)* insect cell-expressed human M15:IL-10, dimer. Gel filtration was performed with a Superdex S-200 column in phosphate-buffered saline at a flow rate of 0.5 ml/min. M15:IL-10, mono eluted at

15.2 mL and M15:IL-10, dimer eluted at 13.6 mL. *(C)* SDS-PAGE analysis of M15:IL-10 conjugates with two different linkers. Purified samples of M15:IL-10, I87A, mono, 15aa linker (Lane 1) and M15: IL-10, I87A, mono, 18aa linker (Lane 2), both from *in vitro* folding and containing human IL-10, were stored at 4 °C for two weeks before being analyzed.

3.2 Detection by IL-10 ELISA

To examine initially whether the IL-10 domain of the M15 fusion constructs were folded properly, they were assayed with IL-10-specific antibodies in a sandwich ELISA, as shown in Figure 2. M15:IL-10 fusions expressed in *E. coli* containing human IL-10 with an additional linker to allow for monovalent IL-10 folding (M15:IL-10, mono and M15:IL-10, mono,I87A) were detected equivalently with recombinant, commercially available human IL-10 (Figure 2A). M15:IL-10, I87A, which is designed for IL-10 folding into an intercalated dimer, was detected less efficiently. This corresponds to difficulty folding the dimeric IL-10 construct *in vitro* (See Section 3.1). Additional dimeric constructs expressed in insect cells were more stable, and were detected by ELISA with identical efficiency with commercially available free IL-10 (human M15:IL-10 proteins: Figure 2B; murine M15:IL-10 proteins: Figure 2C). These results show that the M15:IL-10 fusion proteins contain properly folded IL-10.

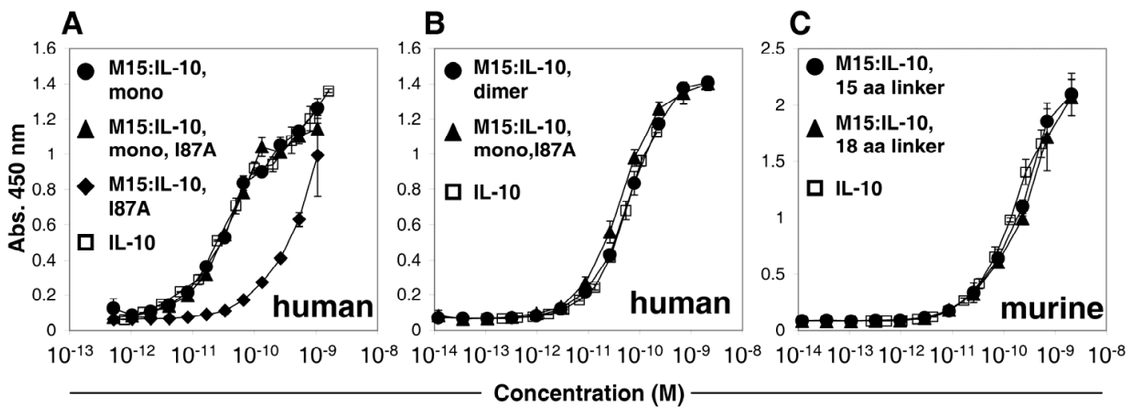

Fig. 2. Properly folded IL-10 was detected in ELISAs of the various soluble M15:IL-10 fusions.

(A-C) Absorbance of the ELISA-linked TMB substrate at 450 nm is shown for various concentrations of IL-10 and M15:IL-10 fusion proteins. *(A)* Human IL-10 was detected by sandwich ELISA for M15:IL-10 fusions produced in *E. coli*: M15:IL-10, mono (closed circles), M15:IL-10, mono, I87A (closed triangles), M15:IL-10, I87A (dimer construct, closed diamonds), and human IL-10 alone (open squares). *(B)* Human IL-10 was detected by sandwich ELISA for M15:IL-10 fusion produced in HighFive insect cells as well as *E. coli*: M15:IL-10, dimer (HighFive protein, closed circles), M15:IL-10, mono, I87A (*E. coli* protein, closed triangles), and human IL-10 alone (open squares). *(C)* Murine IL-10 was detected by sandwich ELISA for dimeric M15:IL-10 fusions produced in HighFive: M15:IL-10, 15 amino acid linker (closed circles), M15:IL-10, 18 amino acid linker (closed triangles), and free murine IL-10 (open squares).

3.2.1 Constructs with human IL-10, produced in *E. coli*

Preliminary tests were carried out with human IL-10 fused to scTv M15 in several configurations, including IL-10 as a monomer with an additional linker inserted between residues 116 and 117 (M15:IL-10, mono), IL-10 as a dimer containing the point mutation I87A (M15:IL-10, I87A), purported to eliminate stimulatory effects of the cytokine while maintaining suppressive effects, and IL-10 as a monomer plus the I87A mutation (M15:IL-10, mono,I87A). Initial protein expression was carried out in *E. coli* as inclusion bodies, followed by solubilization and re-folding *in vitro*. Analysis of these purified proteins by ELISA confirmed that monomeric IL-10 constructs were detected equivalently on a molar basis as free IL-10, with or without the I87A mutation (Figure 2A, closed circles and triangles). The fusion designed to allow IL-10 to form the native intercalated dimer (M15:IL-10, I87A) was detected at much lower levels, corresponding to less stable protein (See Section 2). In order to be able to obtain more stable IL-10 dimer fusions to accurately compare monomer and dimer constructs for biological function, a second, eukaryotic expression system was employed.

3.2.2 Constructs with human IL-10, produced in insect cells

A dimeric M15:IL-10 fusion protein (M15:IL-10, dimer) was designed and expressed as a stable dimer (See Section 2) by HighFive insect cells. This purified protein was compared directly with a similar, monomeric protein from *E. coli* (M15:IL-10, mono,I87A). Both proteins contained a 15-amino acid linker between the scTv and the cytokine. As can be seen in Figure 2B, the dimeric human IL-10 fusion (closed circles) was detected equivalently on a per-molar basis as both the *E. coli* monomer fusion (closed triangles) and free human IL-10 alone (open squares).

3.2.3 Constructs with murine IL-10

Dimeric M15:IL-10 fusions containing murine IL-10 were also expressed in insect cells. Two linkers were analyzed between the scTv and the cytokine: a 15-amino acid linker similar to the human constructs in Figure 2B (M15:IL-10, 15aa linker), and an 18-amino acid linker (M15:IL-10, 18aa linker) similar to the original E. coli constructs in Figure 2A. The 18-amino acid linker had proven to be susceptible to degradation over time. Side-by-side analysis by murine ELISA (Figure 2C) shows that fusion proteins with both linkers are detected equivalently on a per molar basis by ELISA (closed circles and closed triangles), and they are, in turn, detected equivalently with free murine IL-10 (open squares).

3.3 Specific binding/targeting to cells displaying target epitope

Having confirmed proper folding of the IL-10 portion of the fusions by ELISA, specific binding of the scTv portion was tested by detecting binding specifically to APCs presenting Hb/I-E^k. Flow cytometry experiments verified that M15:IL-10 fusion proteins bound specifically, and carried with them covalently-linked IL-10.

3.3.1 Detection of binding through scTv via fused IL-10

CH27 cells (I-E^{k+}) were loaded with peptide by overnight incubation with 1 or 5 μM Hb peptide in culture medium, and were then incubated with soluble, biotinylated scTv M15 or

with various M15:IL-10 fusion constructs on ice. Bound fusion was detected using biotinylated, polyclonal anti-IL-10 followed by fluorescently-labeled streptavidin, while bound scTv was detected by fluorescently-labeled streptavidin only. Staining results are shown in Figure 3.

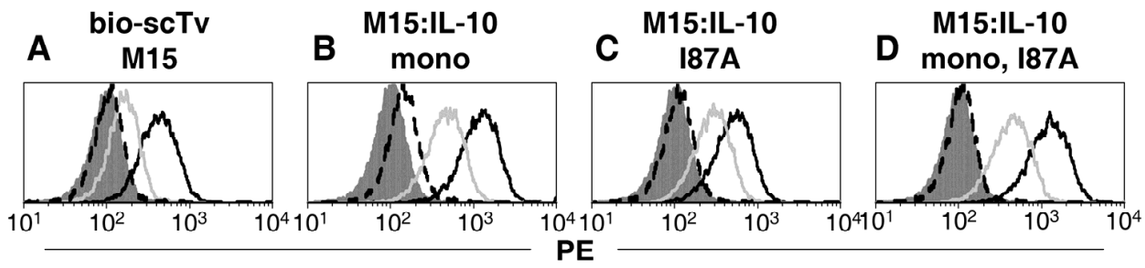

Fig. 3. M15 scTv and M15:IL-10 fusions bind specifically to cells presenting Hb/I-E^k.

(A-D) Flow cytometry histograms are shown in each panel for CH27 cells loaded with 5 μM Hb peptide (solid black trace), 1 μM Hb peptide (solid gray trace), no added peptide (dashed black trace), or secondary detection only (dark gray shaded trace). *(A)* Cells stained with biotinylated scTv M15 without any IL-10, detected by streptavidin phycoerythrin (PE) alone. *(B-C)* Binding of M15:IL-10 fusions refolded from *E. coli* was detected with biotinylated polyclonal anti-IL-10 antibody, followed by streptavidin-PE for *(B)* M15:IL-10 containing monovalent human IL-10, *(C)* M15:IL-10 containing dimeric human IL-10 and the I87A point mutation, and *(D)* M15:IL-10 containing monovalent human IL-10 with the I87A mutation.

Figure 3A shows staining of CH27 cells with free M15 scTv. At 5 μM Hb peptide, the cells stain four-fold brighter than without added peptide (MFI of 46.5 and 11.3, respectively). As can be seen in Figure 3B-D, all of the M15:IL-10 constructs bound specifically to cells which had been loaded with Hb peptide. The magnitude of staining increase seen with the dimeric M15:IL-10, I87A protein was lower (roughly 5-fold) than that seen for the two monomeric constructs containing the additional linker between N116 and K117 (8-fold for M15:IL-10, mono, and 12–fold for M15:IL-10, mono,I87A, respectively). This is consistent with evidence that the dimer construct made in *E. coli* was less stable than the monomer constructs (See Sections 3.1 and 3.2, and Figure 2A).

3.4 Functional assays of M15 scTv and IL-10 fusions

M15 and its fusions can act by direct inhibition of T cell recognition, by preventing the binding of the cellular TCRs on T cells to the peptide-MHC on APCs, or of course could act to immunosuppress resident T cells through the action of the cytokine IL-10. The direct T cell inhibitory potential was examined with soluble M15 scTv (Figure 4), and the biological function of the cytokine portion of the M15-IL-10 fusions was evaluated using a murine mast cell line that selectively proliferates in the presence of IL-10 (Figure 5). This cell line can respond to both human and murine IL-10.

3.4.1 Direct inhibition of T cell activation by soluble M15 scTv

The high-affinity scTv M15 binds to its antigen, a Hb peptide loaded onto I-E^{k+}. For purposes of using this scTv as a model for inhibiting pathogenic T cells that are specific for a

target antigen, a soluble scTv could not only serve as a specific IL-10 targeting agent, but could directly inhibit activation of the T cells by preventing recognition of APCs. To explore this possibility, soluble high-affinity M15 was added in a T cell activation assay (Figure 4). IL-2 secretion from the specific T cells was induced at various concentrations of Hb peptide loaded onto I-E^{k+} APCs and this activity was inhibited by the soluble scTv M15. This suggests that the binding of scTv alone can reduce the magnitude of an immune response against a self- or allegen-associated peptide-MHC complex.

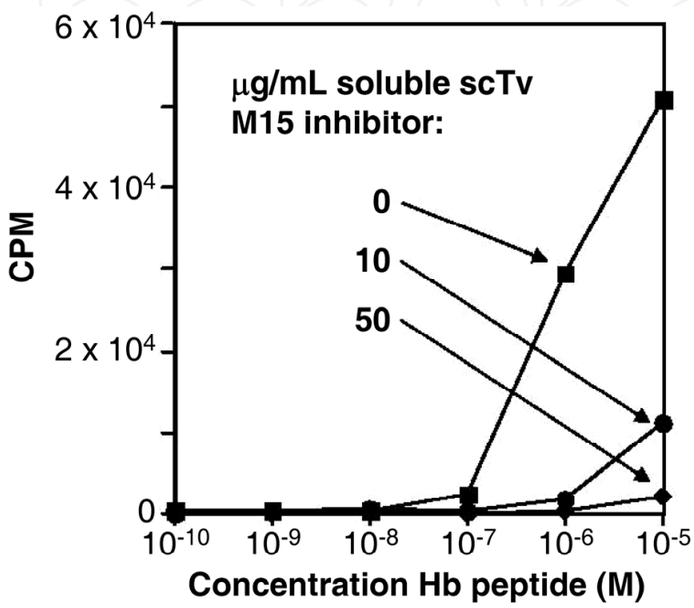

Fig. 4. Activation of 3.L2 T cells is inhibited by binding of scTv M15.

Activation of T cells carrying the 3.L2 TCR (wild-type affinity) by I-E^{k+} APCs incubated with various concentration of stimulatory Hb peptide alone (closed squares), or with 10 μg/mL (closed circles) or 50 μg/mL (closed diamonds) soluble, high-affinity scTv M15. IL-2 secretion by the 3.L2 T cells was detected by proliferation of an IL-2-dependent cell line, measured by incorporation of tritiated thymidine.

3.4.2 Induced proliferation of a cytokine-dependent cell line (MC/9)

To determine whether the M15:IL-10 fusions exhibited functional activity, we determined if they induced proliferation of the IL-10-dependent cell line, MC/9. Although less potent than commercial recombinant IL-10, the M15:IL-10 dimer protein (closed diamonds) was functional in this biological assay, whereas the M15:IL-10, mono-I87A (closed circles) was less potent than the dimer (Figure 5A). The murine M15:IL-10 proteins (closed triangles and closed squares) were equally capable of stimulating proliferation of MC/9 cells (Figure 5B).

(A,B) Proliferation of the cytokine-dependent MC/9 cell line cultured with free IL-10 or different M15:IL-10 constructs was evaluated by MTT reduction by measuring absorbance of solubilized formazan precipitate at 570 nM (See Section 2.4). *(A)* Proliferation induced by human IL-10 (open squares) or the human IL-10 fusion proteins M15:IL-10, dimer (closed diamonds) or M15:IL-10, mono,I87A (closed circles). *(B)* Proliferation induced by murine IL-

10 (open squares) or the murine IL-10 fusions M15:IL-10, 15aa linker (closed triangles) or M15:IL-10, 18aa linker (closed squares).

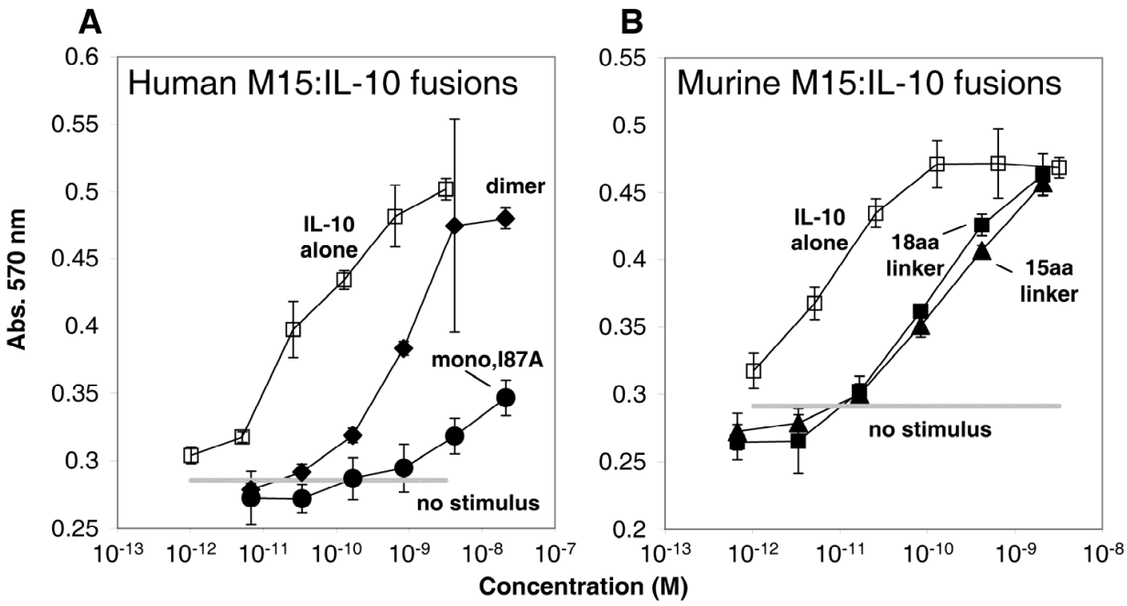

Fig. 5. Functional activity of IL-10 and M15:IL-10 fusion proteins.

4. Conclusions

We have described here the first soluble IL-10 protein fusions targeted by a high-affinity single-chain variable TCR (scTv) protein, M15, directed to a class II MHC-peptide complex (Hb/I-E^k). Many inflammatory diseases are driven by recognition of specific class II MHC-peptide complexes, providing an opportunity to use such scTv molecules to target soluble therapeutics, or as direct inhibitors of pathogenic T cells. To our knowledge, the M15 TCR is the only class II TCR with high affinity for its target peptide-MHC (an 800-fold increased affinity compared to wild type 3.L2). The fusion proteins we generated contained N-terminal scTv linked by a flexible 15- or 18-amino acid peptide to IL-10 at the C-terminus. Proper folding of the cytokine portion of the fusions was verified (Figure 2), as was specific binding of the scTv to the target ligand, Hb/I-E^k (Figure 3).

Functional assays confirmed that the IL-10 fusion proteins could stimulate biological activity in a cytokine-dependent cell line (Figure 5); however, the fusion proteins were less efficient than free murine or human IL-10 at stimulating that activity. It is possible that some steric hindrance from the N-terminal scTv prevents fully efficient signaling that is not ameliorated by extending the linker between the two proteins from 15 to 18 residues (Figure 5B). The ELISA data shown in Figure 2 suggests that most of the epitopes are, indeed, accessible to antibodies. The published structure of IL-10 bound to its high-affinity receptor, IL-10R1 (Josephson et al. 2001) suggests that an N-terminal extension would not interfere with that interaction. Based on modeling and mutagenesis studies, the low-affinity receptor, IL-10R2, has been suggested to dock much more closely to the N-terminus (Yoon et al. 2010). Hence, this lower affinity receptor interaction may be interrupted by the N-terminal scTv in the fusion protein. Switching the domain order may result in more potent function from the

scTv fusions. However, this level of reduced potency may not, in fact, be a disadvantage for these targeted therapeutics, as it may reduce potentially problematic systemic effects of IL-10 (Belkaid 2007; Fort & Narayanan 2010), while allowing the biodistribution to be dominated by the high-affinity scTv targeting element (Weber et al. 2005).

Despite data that monomeric IL-10 created by inserting a 6-amino acid linker between residues 116 and 117 of the protein is still functional (Josephson et al. 2000), the fusions reported here with monomeric IL-10 displayed low activity, even when the I87A mutation, which abrogates stimulatory activities of IL-10 (Ding et al. 2000), was not included (Figure 4A, and data not shown). Perhaps signaling through an IL-10 monomer is particularly sensitive to any steric interference reducing the ability to co-localize IL-10R1 with IL-10R2 resulting from the N-terminal attachment of the scTv domain.

Further characterization of scTv:IL-10 fusions will include examining the effects (including suppressive capacity) of the IL-10 fusions at different doses, ideally for different cell types, and particularly various helper T cell lineages including Th1, Th2, and Th17 cells. Biodistribution and pharmacokinetic studies will also be required to verify appropriate *in vivo* targeting by the scTv, and sufficient serum half-life for effectiveness. Results of these studies may suggest further redesign of either the delivery system for the protein or, possibly, of the fusion constructs themselves.

The use of engineered scTv TCR fragments as targeting elements for soluble molecules is an increasingly attractive and viable option (Aggen et al. 2011; Richman & Kranz 2007). T cells play a central role in many inflammatory diseases, and effective T cell responses can combat serious infections or cancer. Selective modulation of the relevant T cell populations by downregulation or upregulation, respectively, may represent an advance in treatment with reduced side effects compared to systemic treatments. Additionally, in many cases relevant T cell peptide-MHC epitopes related to these diseases are known, and immunodominant or specific TCRs have been isolated. While the wild-type receptors are not suited for incorporation into soluble therapeutics, advances in TCR engineering for affinity and stability as a soluble scTv (Aggen et al. 2011; Kieke et al. 1999; Li et al. 2005b; Richman et al. 2009; Weber et al. 2005) transform this into a more practical strategy.

As has been seen, the location of delivered IL-10 has a dramatic impact on its effect as a therapeutic (Asadullah et al. 2003; Kennedy et al. 1992; Lindsay & Hodgson 2001; O'Garra et al. 2008). Maintenance of an appropriate dose at the site of action is also important, as high doses of IL-10 can be less effective than lower doses at controlling inflammation in patients (Asadullah et al. 2003). Targeting by inclusion of a specific scTv in IL-10 fusions may be effective alone, and may synergize when combined with promising delivery methods used for free IL-10, such as local secretion by virally-transduced cells or by genetically modified bacteria (Lindsay & Hodgson 2001; Steidler et al. 2000; Wirtz et al. 1999). Sustained activity at the desired site, combined with facile, continuous delivery may make targeted IL-10 therapy a valuable addition to current treatments against various excessive inflammatory conditions.

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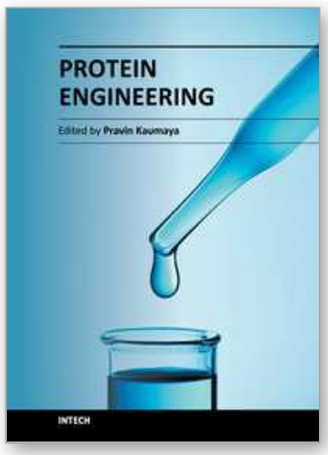

Protein Engineering

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A broad range of topics are covered by providing a solid foundation in protein engineering and supplies readers with knowledge essential to the design and production of proteins. This volume presents in-depth discussions of various methods for protein engineering featuring contributions from leading experts from different counties. A broad series of articles covering significant aspects of methods and applications in the design of novel proteins with different functions are presented. These include the use of non-natural amino acids, bioinformatics, molecular evolution, protein folding and structure-functional insight to develop useful proteins with enhanced properties.

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