FR255734, a Humanized, Fc-Silent, Anti-CD28 Antibody, Improves Psoriasis in the SCID Mouse-Psoriasis Xenograft Model

Siba P. Raychaudhuri[1,2,3], Smriti Kundu-Raychaudhuri[1], Kouichi Tamura[4], Taro Masunaga[4], Kaori Kubo[4], Kaori Hanaoka[4], Wen-Yue Jiang[1], Leonore A. Herzenberg[1] and Leonard A. Herzenberg[1]

In psoriasis, CD28/B7 costimulatory molecules are well characterized. Here, using the severe combined immunodeficient (SCID) mouse-psoriasis xenograft model, we report therapeutic efficacy of a humanized anti-CD28 monoclonal antibody (FR255734; Astellas Pharmaceuticals Inc., Tokyo, Japan). Transplanted psoriasis plaques on the SCID mouse were treated weekly for 4 weeks with intraperitoneal injections of FR255734 at 10, 3, and 1-mg kg^{-1} doses. Groups treated with doses of 10 and 3 mg kg^{-1} had significant thinning of the epidermis and reduced HLA-DR-positive lymphocytic infiltrates. The length of the rete pegs changed from 415.2 ± 59.6 to $231.4 \pm 40.4 \mu m$ ($P < 0.005$) in the 10-mg kg^{-1} group, and from 323.4 ± 69.6 to $237.5 \pm 73.6 \mu m$ in the 3-mg kg^{-1} group ($P = 0.002$). Positive controls treated with CTLA4-Ig and cyclosporine had significant histological improvement, whereas plaques treated with saline and isotype controls (human and mouse IgG2) remained unchanged. In vitro studies have shown that FR255734 effectively blocked T-cell proliferation and proinflammatory cytokine production. These observations warrant studies to evaluate the efficacy of FR255734 in human autoimmune diseases.

INTRODUCTION

Optimal activation of T cells requires interactions of T-cell receptor with major histocompatibility complex-peptide antigens, and the engagement of a costimulatory receptor with its respective ligand. A number of costimulatory molecules have been shown to influence T-cell activation. The most well-characterized T-cell costimulatory ligands are CD28 and cytotoxic T lymphocyte-associated antigen-4 (CTLA4) (CD152), which engage CD80 and CD86 receptors on antigen-presenting cells (APCs) (Mueller *et al.*, 1989; Linsley *et al.*, 1991). Among these, a principal signal is delivered by engagement of CD28 on T cells with CD80 (B7-1) and CD86 (B7-2) on APCs. This process enhances T-cell activation by stabilization of cytokine mRNA and upregulation of antiapoptotic genes. In contrast, CTLA4-Ig binds to B7-1 and B7-2 molecules on APCs and blocks the CD28-mediated costimulatory signal for T-cell activation. Thus, the B7 family of molecules on APCs regulates T-cell activation by delivering antigen-independent stimulatory signals through CD28 and inhibitory signals through CD152. This unique mechanism of T-cell activation has provided several target molecules for therapeutic manipulation of immune responses.

The effectiveness of costimulatory signal blockade as a therapeutic device was shown over a decade by demonstrating that CTLA4-Ig inhibited graft rejection and induced long-term tolerance in mice (Lenschow *et al.*, 1992). Encouraging results in animal models have led to successful clinical trials with CTLA4-Ig in psoriasis and rheumatoid arthritis (Abrams *et al.*, 1999; Kremer *et al.*, 2003; Genovese *et al.*, 2005). In this study we took an alternative approach to develop immunomodulatory drug by manipulating CD28CD80/86 interactions using a monoclonal anti-CD28 antibody (FR255734) prepared by Fujisawa Pharmaceutical Co., Ltd. (now Astellas Pharmaceuticals Inc., Tokyo, Japan). FR255734 is a humanized IgG2κ anti-human CD28 antibody that has the complementary determining regions of the mouse anti-human monoclonal antibody TN228 and the Fc domain of human IgG2M3 in which two amino acid mutations (V234A, G237A) have been introduced into the human γ2 chain to eliminate binding of the antibody to FcγR. The original TN228 cell line was generated by immunizing BALB/c mice

[1]Department of Genetics, Stanford University School of Medicine, Stanford, California, USA; [2]Division of Rheumatology, Stanford University School of Medicine, Stanford, California, USA; [3]Division of Rheumatology, Allergy and Clinical Immunology, University of California at Davis and VA Medical Center, Sacramento, California, USA and [4]Department of Immunology, Pharmacology Research Lab, Astellas Pharmaceuticals Inc., Tokyo, Japan

Correspondence: Dr Siba P. Raychaudhuri, University of California at Davis, 1911 Geneva Place, Davis, California 95618, USA.
E-mail: sraychaudhuri@ucdavis.edu

with human CD28-transfected mouse fibroblast L cells, and fusing immune splenocytes with P3 U1 myeloma cells. The purified molecule consists of two heavy chains and two light chains, which are 447 amino-acid residues (C2177H3358N575O669S19; MW 48898.64) and 218 amino-acid residues (C1043H1628N279O342S7; MW 23772.21) in length, respectively. FR255734 binds to a human CD28−mouse IgG Fc fusion protein ($K_d = 3.72 \times 10^{-8}$) and inhibits proliferation of human T cells stimulated with anti-CD3 and P815/human CD80+ cells in a concentration-dependent manner. FR255734 does not cross-react with mouse CD28 (Shiao *et al.*, 2007). Here, we are providing results of both *in vivo* and *in vitro* studies documenting the therapeutic efficacy of FR255734.

RESULTS

In vitro studies—Regulatory role of FR255734 on activated T cells

Effect of FR255734 on T-cell proliferation. Activated human T lymphocytes purified from human peripheral blood mononuclear cells (PBMCs) were used for this assay. Stimulation by anti-human CD3 mAb + human-CD80/P815 strongly induced proliferation of T cells, but anti-CD3mAb alone or human-CD80/P815 alone did not (data not shown), indicating that proliferation depends on CD28 costimulatory signaling. Humanized, Fc-silent anti-CD28 antibody inhibited T-cell proliferation in a dose-dependent manner (Figure 1a). Maximum suppression was obtained by the 300-ng ml^{-1} dose and at that dose it reached the plateau effect. Isotype controls (mouse IgG2 and human IgG2) did not show inhibition of T-cell proliferation. The Figure 1a showed that isotype control, mouse IgG2, did not inhibit T-cell proliferation. Human IgG2 had the similar non-inhibitory effect (data not shown).

Cytokine assay by ELISA to investigate the inhibitory effect of FR255734 on T-cell function. Supernatants of PBMCs stimulated with anti-human CD3/CD28 antibodies and tetanus toxoid, a recall antigen were used for this assay. As expected CD3CD28 antibody stimulation induced IFN-γ and IL-2 secretion (Figure 1b). FR255734 antibody inhibited IFN-γ secretion induced by CD3–CD28 stimulation ($P<0.005$, *t*-test) (Figure 1b), whereas isotype controls (mouse and human IgG2) did not inhibit IFN-γ production. These results are summary of experiments using PBMCs from six individuals. Each experiment was performed in triplicate from each individual. Effect of this antibody was similar to the inhibitory effect of CTLA4-Ig ($P<0.005$, *t*-test). When only CD3 antibody was used for stimulation, IFN-γ production by PBMCs was ⩽30 pg ml^{-1}, which was similar to that by unstimulated PBMCs (Figure 1b), indicating that CD28 was necessary for proper stimulatory effects. FR255734 has similar effects on IFN-γ production by tetanus toxoid, a recall antigen (732 ng ml^{-1}) stimulated PBMC ($P<0.005$, *t*-test) (Figure 1c). Experiments were performed using PBMCs from six individuals who had been vaccinated with tetanus toxoid for other purposes. Each experiment was performed in triplicate. FR255734 also had significant inhibitory effects on IL-2 production by CD3CD28 and tetanus toxoid-stimulated PBMCs ($P<0.005$, *t*-test) (Figure 1d and e). Similar inhibitory effects were observed with CTLA4-Ig. Isotype controls (mouse and human IgG2) did not show inhibitory effects on IL-2 and IFN-γ production by CD3CD28 or tetanus-stimulated PBMCs ($P=0.9$, *t*-test) (Figure 1).

In vivo studies

Evaluation of therapeutic efficacy of FR255734 using the SCID mouse-psoriasis xenograft model. Shave biopsies (2.5 × 2.5 cm) were obtained from active plaques located on the thigh or arm of psoriatic patients. Each piece of biopsy was divided into four equal parts of approximately 1 cm^2 size. Eighty severe combined immunodeficient (SCID) mice were transplanted using biopsies obtained from 20 patients. This study includes 60 successful grafts collected from these 20 psoriasis patients. The protocol for selecting FR255734, saline, isotype controls, or the positive control treatment of the successful grafts obtained from a single patient was based on the principle that FR255734 should be evaluated against the controls in the grafts of the same patients as many times as it is possible. If two grafts from the same patients were successful, one was treated with FR255734 and the other one with either positive or negative controls; if three grafts were successful, one graft was treated with a positive control in addition to FR255734, and the other one with saline/isotype control. In case of all four successful grafts, treatment of fourth graft was by a different dose or the same dose of FR255734. In case of only one successful graft, any of the agents from the study groups or the control groups was used. However, like any other mouse studies, some of the transplanted mice died in the middle of the study and additional grafts had to be recruited according to the need of a particular group. First, we evaluated the efficacy of FR255734 at the dose of 10 mg kg^{-1} body weight (group A). This dose was selected on the basis of our *in vitro* studies. Twenty-four successful grafts collected from eight patients were used for the controls ($n=12$) and for group A (FR255734 10 mg kg^{-1}, $n=12$). To obtain proper controls for group A (FR255734, 10 mg kg^{-1}), six grafts each from the same patients were treated with saline/isotype control and 10 mg kg^{-1} FR255734, four grafts each with CTLA4-Ig and 10 mg kg^{-1} FR255734, and two grafts each with cyclosporine and 10 mg kg^{-1} FR255734. Once we observed therapeutic efficacy of FR255734 at the dose of 10 mg kg^{-1}, we looked for therapeutic efficacy of FR255734 at the dose of 3 mg kg^{-1} (group B) and 1 mg kg^{-1} (group C) body weight. In group B (FR255734, 3 mg kg^{-1}, $n=8$), grafts treated from the same patients were 3 and 2, respectively, for saline/isotype control and CTLA4-Ig. In group C (FR255734, 1 mg kg^{-1}, $n=8$), grafts treated from the same patients were 3, 2, and 2, respectively for saline, CTLA4-Ig, and cyclosporine. For each graft, biopsies were taken before and after treatment and histological changes were compared.

Table 2 shows various treatment groups, doses, and schedules. Transplanted plaques treated with FR255734 at 10 and 3 mg kg^{-1} had significant clinical and histological improvement compared with controls. Histological
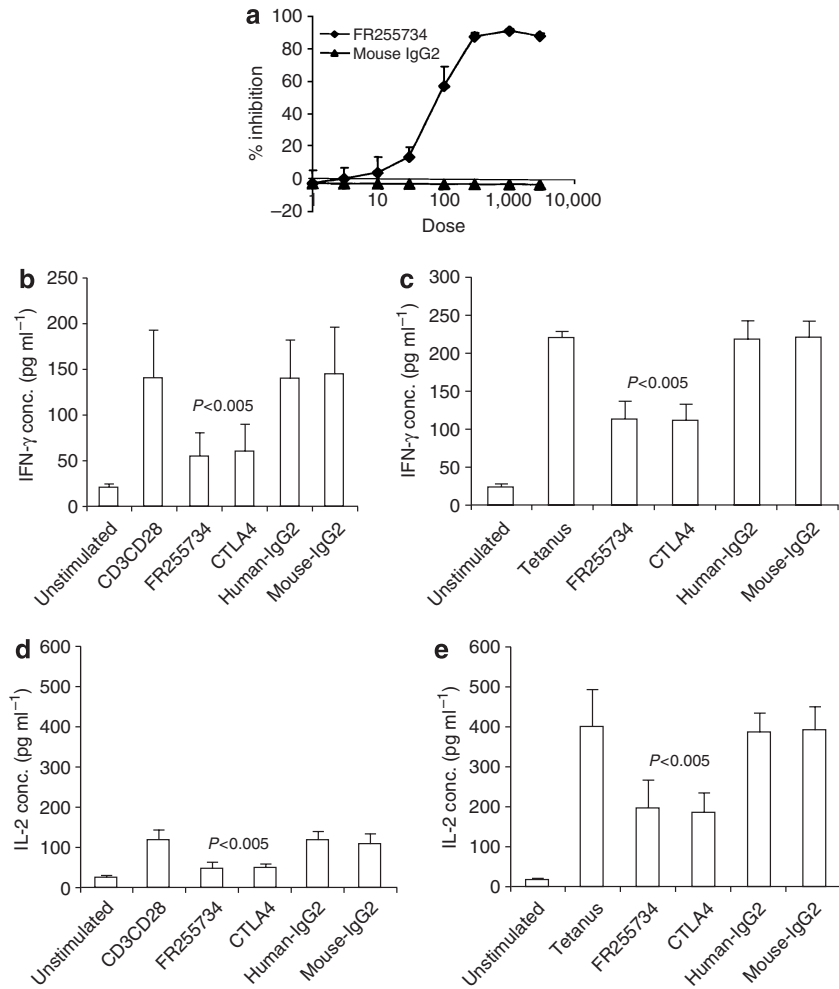
Figure 1. **FR255734 inhibits T-cell proliferation and cytokine production.** (**a**) Humanized, Fc-silent anti-CD28 antibody inhibited T-cell proliferation in a dose-dependent manner. T cells were cultured with antihuman CD3 and CD28 antibody and human CD80/P815. Several doses of FR255734 (3–10,000 ng ml^{-1}) were added to the culture to determine the inhibitory effect. Maximum inhibitory effect was seen at 300 ng ml^{-1} dose where this effect reached a plateau. Results were expressed as mean ± SD of triplicate wells of six individuals. Mouse IgG2 isotype control did not inhibit T-cell proliferation. Each experiment was performed in triplicate. (**b**) FR255734 at 300 ng ml^{-1} significantly inhibited IFN-γ production by anti-human CD3CD28 antibody stimulation. CTLA4-Ig, a positive control, showed similar inhibitory effect. Unstimulated PBMCs produced very small amount of IFN-γ ≤30 pg ml^{-1} compared with CD3CD28 stimulated PBMCs. Human IgG2 and mouse IgG2 isotype controls did not inhibit IFN-γ production. (**c**) FR255734 at 300 ng ml^{-1} dose inhibited IFN-γ production significantly after tetanus toxoid stimulation. CTLA4-Ig had shown similar inhibition. Unstimulated PBMCs produced very small amount was ≤30 pg ml^{-1} of IFN-γ compared with tetanus toxoid stimulated PBMCs. Each experiment was performed in triplicate. Human IgG2 and mouse IgG2 isotype controls did not inhibit IFN-γ production. (**d**) FR255734 antibody at 300 ng ml^{-1} dose inhibited IL-2 production significantly after CD3CD28 antibody stimulation. CTLA4-Ig showed similar effect. Unstimulated PBMCs produced very small amount, ≤20 pg ml^{-1}, of IL-2 compared with CD3CD28 antibody-stimulated PBMCs. Human IgG2 and mouse IgG2 isotype controls did not inhibit IL-2 production. (**e**) FR255734 antibody at 300 ng ml^{-1} inhibited IL-2 production significantly after tetanus toxoid stimulation. CTLA4-Ig also significantly inhibited IL-2 production. Unstimulated PBMCs produced very small amount, ≤20 pg ml^{-1}, of IL-2 compared with tetanus toxoid-stimulated PBMCs. Results are expressed as mean ± SD of results from six individual experiments. Each experiment was performed in triplicate. Human IgG2 and mouse IgG2 isotype controls did not inhibit IL-2 production.

improvement in the treated plaques was evidenced by reduction of hyperkeratosis, acanthosis, and lymphomononuclear cellular infiltrates (Figure 2). In plaques treated with 10 and 3 mg kg^{-1}, there was significant thinning of the epidermis (Table 1). The length of the rete pegs changed from 415.2 ± 59.6 to 231.4 ± 40.4 μm ($P<0.005$) with the 10-mg kg^{-1} dose, and 323.4 ± 69.6–237.5 ± 73.6 μm with the 3-mg kg^{-1} dose ($P=0.002$, paired t-test; Table 1). Plaques treated with 1 mg kg^{-1} FR255734 did not show significant improvement.

Rete peg lengths in the pretreated plaques and 4 weeks following treatment with mouse IgG2 ($n=5$) were 276 ± 101 and 286 ± 62 μm, respectively. Similarly, rete peg lengths in the pretreated plaques and 4 weeks following treatment with human IgG2 ($n=5$) were 300 ± 43 and 284 ± 30 μm, respectively. Transplanted psoriasis grafts treated with these istoype controls did not have any significant histopathological or immunological improvement ($P>0.2$, paired t-test; Table 1; Figure 3). In the saline control group, the before- and after-therapy rete peg lengths were 345.6 ± 105.1 and

322.9 ± 108.6 µm ($P > 0.1$, paired t-test). The control plaques treated with isotype controls and saline for the same duration did not show any significant improvement. These observations imply that histological improvement was specifically due to therapeutic efficacy of FR255734. Also, FR255734 not therapeutically effective at the dose of 1 mg kg^{-1} further suggests that therapeutic efficacy of FR255734 was not because of the class effect of human and mouse IgG2. The transplanted psoriasis plaques are reported to lose the characteristic features of psoriasis with time. To address this variation, we kept untreated plaques and plaques treated with saline as controls. We observed that immunological and histological features of psoriasis were maintained in the transplanted plaques for 4–6 months. Other investigators have similar observations as well. In this study, we completed all experimental works in each mouse within 8 weeks of transplantation.

We observed that inflammatory infiltrates (hematoxylin and eosin staining), HLA-DR-positive lymphocytic infiltrates, and dermal CD3 + lymphocytic infiltrates were significantly

reduced in plaques treated with FR255734 at the effective doses of 10 and 3 mg kg^{-1} body weight. Figure 2 demonstrates marked reduction of CD3 + lymphocytic infiltrates in graft treated with FR255734 at the dose of 10 mg kg^{-1} per week. In pre- and post-treated tissues, CD3 + lymphocytic infiltrates were calculated by using a reticule as described earlier (Raychaudhuri *et al.*, 1998, 1999, 2001, 2004). In group A plaques (FR255734, 10 mg kg^{-1}), the number of the CD3 + lymphocytes per square millimeter of dermis reduced from 166 ± 51 to 22 ± 8 following 28 days of treatment ($P < 0.01$, t-test). The number of the CD3 + lymphocytes in pretreated plaques and in plaques 4 weeks following treatment with the isotype control, mouse IgG2 ($n = 5$), was 172 ± 30 and 152 ± 36, respectively, per square millimeter of dermis. In pretreated plaques and in plaques 4 weeks following treatment with the isotype control, human IgG2 ($n = 5$), CD3 + lymphocytes per square millimeter of the dermis were 168 ± 43 and 155 ± 30, respectively. In the saline control group, the number of CD3 + lymphocytes per square millimeter of dermis before and after treatment was 158 ± 48 and 140 ± 42. Changes in the number of CD3 + lymphocytes per square millimeter of dermis in pre- and post-treated transplanted psoriasis graft following treatment
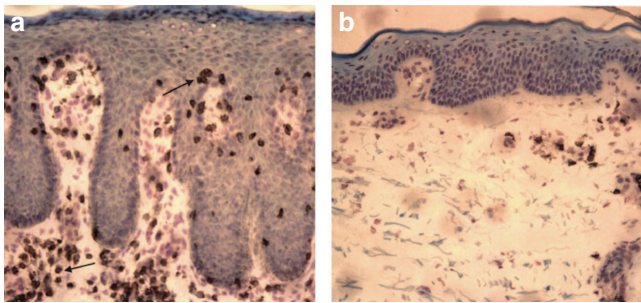
Figure 2. **Histopathological and immunohistochemical studies in pre- and post-treated biopsies of transplanted psoriasis plaques.** Transplanted psoriasis graft on a SCID mouse was treated with FR255734 at 10 ng ml^{-1} per week for 4 weeks. Pretreatment (**a**) and post-treated (**b**) tissues were stained for expression of CD3 and counterstained with hematoxylin. Histopathological studies of the post-treated (**b**) graft demonstrated significant thinning of the epidermis and marked reduction of CD3-positive infiltrates. Dark brown positive cells marked by arrows indicate CD3 + lymphocytes. Original magnification × 200.
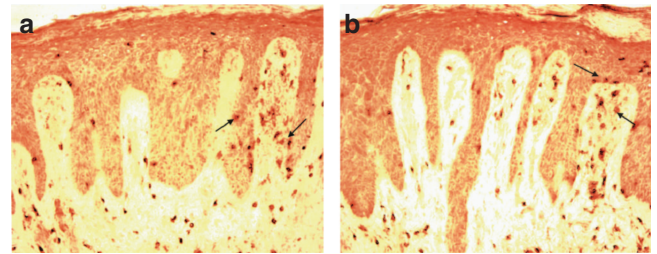
Figure 3. **Immunohistochemical studies for CD3 + lymphocytes in pre (a) and post (b) treated biopsies from a psoriasis graft treated with human IgG2.** Compared with Figure 2 there is no histopathological improvement in the post-treated (**b**) section. In both pre- (**a**) and post-treated (**b**) biopsies, the same degree of epidermal thickening typical for psoriasis along with marked CD3 + lymphocyte infiltrates is seen within dermis and epidermis. Black arrows indicate brown-colored CD3 + lymphocytes.

Table 1. Pre- and post-treatment rete peg length (thickness of epidermis) in different study groups

| Group (no. of mice) | Treatment | Dose | Rete peg length (avg.(µm) ± SD) | | P-value |
			Pre-treat	Post-treat	
A (12)	FR255734	10 mg kg^{-1}	415.2 ± 59.6	231.4 ± 40.4	$P < 0.005$
B (8)	FR255734	3 mg kg^{-1}	323.4 ± 69.6	237.5 ± 73.6	$P = 0.002$
C (8)	FR255734	1 mg kg^{-1}	283 ± 45.8	267.4 ± 71.5	$P = 0.24$
D (5)	Human IgG2	10 mg kg^{-1}	300 ± 43	284 ± 30	$P = 0.25$
E (5)	Mouse IgG2	10 mg kg^{-1}	276 ± 101	286 ± 62	$P = 0.24$
F (12)	N.Saline	10 ml kg^{-1}	345.6 ± 105.1	322.9 ± 108.6	$P = 0.1$
G (6)	CTLA4-Ig	10 mg kg^{-1}	360.6 ± 100.4	209.5 ± 60.4	$P = 0.001$
H (4)	Cyclosporine	2 mg kg^{-1}	360.5 ± 104.8	171.2 ± 55.1	$P < 0.01$

N.Saline, normal saline; CTLA4-Ig, cytotoxic T lymphocyte-associated antigen-4-Ig.

with normal saline and isotype controls (human and mouse IgG2) were not of any statistical significance. In Figure 3, immunohistochemical staining for CD3+ lymphocyte demonstrates that the degree of CD3+ lymphocyte infiltrates remained unchanged in pre- and post-treated biopsies following treatment with human IgG2. Similarly, marked reduction of tumor necrosis factor-α (TNF-α) expression in dermal infiltrates, and reduction of ICAM-1 in the lesional blood vessels were seen in psoriatic grafts treated with FR255734 at doses of 10 and 3 mg kg^{-1} body weight. Figures 4 and 5 demonstrate marked reduction of TNF-α expression in dermal infiltrates, and reduction of ICAM-1 in the lesional blood vessels in grafts treated with FR255734 at the dose of 10 mg kg^{-1} per week. TNF-α in psoriasis is mainly expressed in dermal dendrocytes, monocytes, and mast cells, and can be expressed in the keratinocytes and activated T lymphocyte
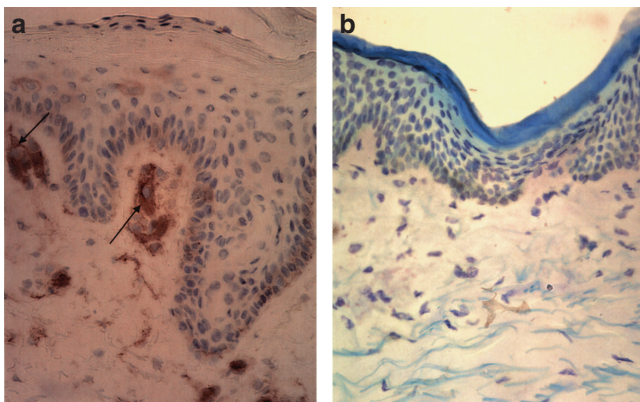
infiltrates (Nickoloff et al., 1991; Ackermann and Harvima, 1998). We only counted discrete dermal TNF-α-positive cells as shown Figure 4. The large size of the TNF-α-positive cells in this figure suggests that these cells are likely mast cells or monocytes. In group A plaques (FR255734, 10 mg kg^{-1}), the number of the TNF-α-positive cells per square millimeter of dermis reduced from 60 ± 22 to 8 ± 8 following 28 days of treatment ($P < 0.01$, t-test). In the saline control group, the numbers of the TNF-α-positive cells per square millimeter of dermis before and after treatment were between 68 ± 18 and 58 ± 16 ($P = 0.1$, t-test). Because of non-availability of tissues, staining for TNF-α could only be performed in two grafts treated with human IgG2. These pre- and post human IgG2-treated biopsies did not show significant difference in the numbers of TNF-α-positive cells. Because of non-availability of tissues, staining for ICAM-1 was not performed in grafts treated with human and mouse IgG2. In saline controls, marked expression of ICAM-1 was noticed in both pre- and post-treated biopsies.

To substantiate this observation, we further investigated whether similar improvement of psoriasis could be attained by blocking interaction of CD28/B7 with CTLA4-Ig. Results in Table 2 demonstrate that, as expected CTLA4-Ig is also therapeutically effective for psoriasis in this SCID mouse-psoriasis xenograft model. Similarly cyclosporine, a known immunomodulatory treatment for psoriasis, was found to be therapeutically effective in this model as well (Table 1). These two treatment groups were positive control groups. Mice remained healthy and active throughout the study period. No weight loss, lack of activity, or infection occurred during the study period and 3 months after the experiments were concluded. Thus, there were no apparent toxic detected despite the inhibitory effects on T-cell stimulation.

Figure 4. **TNF-α-positive dermal infiltrates in pre- and post-treated biopsies following treatment with FR255734, 10 mg kg^{-1} per week for 4 weeks.** Pretreatment (**a**) and post-treated (**b**) tissues were stained for TNF-α with a rabbit anti-human polyclonal antibody and counterstained with hematoxylin. Post-treated (**b**) graft demonstrated significant thinning of the epidermis and marked reduction of TNF-α-positive dermal infiltrates. Brown positive cells marked by arrows indicate intracellular expression of TNF-α. Original magnification × 400.

DISCUSSION
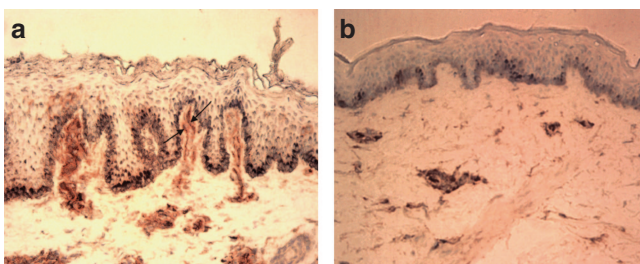
Psoriasis is a multifactorial disease of unknown etiology that affects approximately 2% of the global population (Farber and Peterson, 1961; Raychaudhuri and Farber, 2001). Psoriatic lesions are characterized by a clinical triad consisting of skin induration, scaling, and erythema. Histological findings include inflammation, abnormal keratinocyte

Figure 5. **ICAM-1 expression in pre- and post-treated biopsies following treatment with FR255734, 10 mg kg^{-1} per week for 4 weeks.** Pretreatment (**a**) and post-treated (**b**) tissues were stained for ICAM-1 with a rabbit anti-human polyclonal antibody and counterstained with hematoxylin. Post-treated (**b**) graft demonstrated significant thinning of the epidermis and marked reduction of ICAM-1 in the blood vessels. Brown positive cells marked by black arrows indicate expression of ICAM-1 on the luminal endothelial cells on both sides of a blood vessel extending from deep dermis to the papillary dermis. Original magnification × 200.

Table 2. Study treatment outline

Group	No.	Treatment	Dose	Schedule	Volume
A	12	FR255734	10 mg kg^{-1}	Once a week	10 ml kg^{-1}
B	8	FR255734	3 mg kg^{-1}	Once a week	10 ml kg^{-1}
C	8	FR255734	1 mg kg^{-1}	Once a week	10 ml kg^{-1}
D	5	Human IgG2	10 mg kg^{-1}	Once a week	10 ml kg^{-1}
E	5	Mouse IgG2	10 mg kg^{-1}	Once a week	10 ml kg^{-1}
F	12	Saline	Saline	Once a week	10 ml kg^{-1}
G	6	CTLA4-Ig	10 mg kg^{-1}	Once a week	10 ml kg^{-1}
H	4	Cyclosporine	2 mg kg^{-1}	Thrice a week	10 ml kg^{-1}

CTLA4-Ig, cytotoxic T lymphocyte-associated antigen-4-Ig.

proliferation/terminal differentiation, and dermal angiogenesis. Inflammatory infiltrates, particularly pronounced at the dermal–epidermal junction, consist largely of activated T cells and APCs and precede development of epidermal hyperproliferation. Upregulation of B7-1 (CD80) and B7-2 (CD86) by dendritic cells in psoriatic lesions suggests a critical role for the CD28/B7 costimulatory system in the pathogenesis of psoriasis, and provides logistics for development of therapies targeting these key immunoregulatory molecules (De Rie *et al.*, 1996; Gottlieb *et al.*, 2004). The aim of this study is to develop novel therapeutic approach for T-cell autoimmune conditions by blocking the CD28/B7 costimulatory interactions, a critical molecular event for functional activation of the T cells.

Here, in a series of *in vitro* studies, we have demonstrated that FR255734 effectively inhibits CD28/B7 costimulation and T-cell activation. This antibody inhibits T-cell proliferation (Figure 1a), proinflammatory cytokines, IL-2 and IFN-γ production (Figure 1b–e). For *in vivo* studies, we have used the SCID mouse-psoriasis xenograft model (Nickoloff *et al.*, 1995). Clinical, histopathological, and immunological features of human psoriasis plaque remain intact for 4–6 months in this model (Raychaudhuri *et al.*, 2001). T-cell infiltrates in the transplanted psoriasis grafts on SCID mice express activation markers (HLA-DR and CD69); and dendritic cells and activated T cells express CD80 and CD86, the ligands of the costimulatory molecules. Upregulation and maintenance of CD80/CD86 molecules in transplanted grafts suggest that the CD28/B7 costimulatory system is functional and plays a critical role in the pathogenesis of psoriasis. Several studies, including that by our group, have successfully used this model to evaluate the therapeutic efficacy of established and novel therapies specifically targeted against key biological events associated with pathogenesis of psoriasis (Boehncke *et al.*, 1999; Schon *et al.*, 2002; Raychaudhuri *et al.*, 2004). Thus, this model is unique, as it allows to evaluate the therapeutic efficacy of a pharmacological agent by treating the transplanted human psoriatic plaques.

In this study, FR255734, an antagonist of the CD28 molecule, demonstrated significant therapeutic efficacy for psoriasis. As shown in the Table 1 and Figures 2, 4, and 5, following 4 weeks of therapy with FR255734, we noticed significant thinning of the epidermis along with reduced infiltrates of CD3+ lymphocytes and downregulation of inflammatory markers such as TNF-α and ICAM-1. It is believed that in psoriasis/psoriatic arthritis, similar to other autoimmune diseases, memory T cells are selectively recruited through tight control of homing molecules at the site of the disease. Subsequent recruitment, these specific memory T cells interact with a hypothetical antigen, which activates these cells and initiates signal transduction cascades for upregulation of various inflammatory cytokines (TNF-α and IFN-γ). Our results clearly demonstrate significant reduction of CD3+ T cells in the treated plaques compared with controls. These observations suggest that the antagonism to the CD28/B7 costimulatory system by FR255734 is likely responsible for improvement of psoriasis. Upregulation of

TNF-α and ICAM-1 in psoriasis plays a critical role in the pathogenesis of psoriasis. Role of TNF-α and ICAM-1 in psoriasis is further substantiated by clinical evidence that treatment based on antagonizing TNF-α and ICAM-1 is highly effective in psoriasis (Gottlieb *et al.*, 2000; Chaudhari *et al.*, 2001). Thus, downregulation of these two critical inflammatory mediators following treatment with FR255734 suggests that FR255734, by regulating T-cell activation signals, eventually inhibits inflammatory cascades associated with the pathogenesis of psoriasis.

In conclusion, we have demonstrated by *in vitro* studies that FR255734 effectively inhibits cell activation by blocking CD28/B7 costimulatory interactions. This encouraged us to evaluate the clinical efficacy of FR255734 in T-cell-mediated disease. To evaluate the therapeutic efficacy of FR255734 as a costimulatory antagonist, we have used the SCID mouse model of psoriasis. We noticed significant improvement in the thickness of the epidermis and reduction in infiltrates in the FR255734-treated group ($P<0.005$ at $10\,mg\,kg^{-1}$, and $P=0.002$ at $3\,mg\,kg^{-1}$). In the normal saline-treated group and isotype controls (negative controls), epidermal thickness and the amount of infiltrates remained unchanged (Table 1; Figure 3). Transplanted plaques treated with cyclosporine and CTLA4-Ig (positive controls) also demonstrated significant histopathological improvement. The results of our study substantiate a novel approach for treatment of T-cell-mediated diseases by specifically manipulating the interaction of CD28 and B7 costimulatory molecules of activated T cells. Also, observations of this study provide the basis for clinical trials with FR255734 in diseases known for an active role of T cells such as psoriasis, rheumatoid arthritis, multiple sclerosis, and others.

MATERIALS AND METHODS
In vitro studies—regulatory role of FR255734 on activated T cells
Effect of FR255734 on T-cell proliferation. Peripheral blood mononuclear cells were isolated from blood samples, obtained with informed consent from healthy volunteers, by Ficoll–Hypaque density centrifugation. These experiments were performed at both Astellas Pharmaceuticals Inc. and Stanford University, USA. Blood was obtained after getting informed consent signed. These studies adhered to the Declaration of Helsinki Principles. Human T cells were purified from PBMCs using nylon fiber column (Wako, Osaka, Japan). Purity was >95%, checked by flow cytometry using anti-CD3 antibody conjugated with pacific blue fluorochrome. A total of 1×10^5 T cells were cultured with $1\,ng\,ml^{-1}$ of anti-CD3 antibody (clone, OKT3; Janssen Pharmaceutical, Tokyo, Japan) and 1×10^5 irradiated cells of human CD80 expressing mouse mastocytoma cell line (hCD80/P815) for 3 days. FR255734, mouse, and the human isotype control, IgG2 (both mouse and human IgG2 were gifts to the Principal Investigator of this project from Dr Keting Chu, Department of Infectious Diseases, Stanford University School of Medicine), were added to the culture concomitant with stimulation. To evaluate proliferation of T cells, 6 hours before termination of culture, wells were pulsed with $0.27\,\mu Ci$ of ^{3}H-labeled thymidine (Moravek Biochemicals, Brea, CA) and incorporation was measured using a β-counter.

Cytokine assay by ELISA to investigate the inhibitory effect of FR255734 on T-cell function. Peripheral blood mononuclear cells were stimulated with anti-human CD3CD28 antibodies (BD Pharmingen, San Diego, CA) and tetanus toxoid, a recall antigen (Wyeth-Ayerst Pharmaceuticals, Madison, NJ). Wells of a 24-well plate were coated with human anti-CD3 and CD28 antibodies (5 µg ml^{-1} each) by overnight incubation at 4 °C, then wells were washed with complete medium (RPMI-1640 with 10% human AB serum, penicillin streptomycin, and glutamine). One million PBMCs in 1 ml of complete medium were cultured in these wells for 72 hours. Each set of experiments consisted of three wells without CD3CD28 antibodies; three wells with CD3CD28 antibodies; three CD3CD28 coated wells with FR255734 antibody, 300 ng ml^{-1}; three CD3CD28 coated wells with mouse IgG2, 300 ng ml^{-1}; three CD3CD28 antibody coated wells with human IgG2, 300 ng ml^{-1} and three CD3CD28 coated wells with CTLA4-Ig antibody, 0.2 ng ml^{-1} (positive control); and three wells coated with CD3 antibody alone. After 72-hour incubation, supernatants were collected and stored for IFN-γ and IL-2 assay by ELISA (Endogen-Pierce, Woburn, MA). Similar experiments were performed using tetanus toxoid (732 ng ml^{-1} per well) to stimulate T cells, and in combination with FR255734, isotype controls, and CTLA4-Ig to determine the effect of FR255734 on recall antigen responses. These experiments were performed using PBMCs from six volunteers. This part of *in vitro* studies was carried out at Stanford University.

In vivo studies

Establishment of psoriasis SCID xenograft model. Human psoriasis plaques were transplanted on to the SCID mice. This study was approved by the Stanford University Institutional Review Board. These studies adhered to the Declaration of Helsinki Principles. Patients were recruited and evaluated at the Dermatology Clinic at Stanford University School of Medicine. The patients had generalized plaque psoriasis involving 5–10% of the total skin. They did not receive any systemic treatment for psoriasis or phototherapy in the past 6 months and did not receive any topical preparations other than emollients in past 6 weeks. Shave biopsies (2.5 × 2.5 cm) were obtained from active plaques located on the thigh or arm of 20 psoriatic patients. Each piece of biopsy was divided into four equal parts of approximately 1 cm^2 size. Thus, 80 SCID mice were transplanted using shave the biopsies obtained from 20 patients.

CB17 SCID mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed at the Stanford University Research Animal Facility (Palo Alto, CA) in a pathogen-free environment. The Animal Experimental Protocol Review Committee of Stanford University approved the protocol for animal experiments. Under general anesthesia, a graft bed of approximately 1 cm^2 was created on the shaved area of the back of a 7- to 8-week-old mouse by removing a full-thickness skin sample, keeping the vessel plexus intact on the fascia covering the underlying back muscles. The partial thickness human skin obtained by shave biopsy was then orthotopically transferred onto the graft bed. Nexaband, a liquid veterinary bandage (Veterinary Products Laboratories, Phoenix, AZ) was used to attach the human skin to the mouse skin and an antibiotic ointment (bacitracin) was applied. The psoriatic grafts were accepted in approximately 3–4 weeks.

Methodologies for evaluation of therapeutic efficacy of FR255734. Punch biopsies (2 mm) were obtained on day 0 (before treatment) and day 28 (after treatment) of the study period. Biopsies were snap frozen and cryosections were made for histopathological and immunohistochemical studies: (i) hematoxylin and eosin staining was performed on sections obtained from the biopsies to determine rete peg length, an indicator of epidermal thickness; (ii) immunocytochemical staining was performed using rabbit polyclonal anti-human CD3, HLA-DR, TNF-α, and ICAM-1 antibodies obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Immunohistochemical staining methodologies were as per our standardized earlier publications (Raychaudhuri *et al.*, 2001, 2004). Therapeutic efficacy was determined by comparing pre- and post treatment data: (i) rete peg lengths to determine the effect on epidermal thickness and (ii) the level of lymphomononuclear cell infiltrates to determine the effect on inflammatory cellular infiltrates.

Treatment regimen. FR255734, isotype controls (mouse and human IgG2), saline, and CTLA4-Ig were administered intraperitoneally once a week for 4 weeks (Table 2). Cyclosporine was administered 3 times per week for 4 weeks.

REFERENCES

Abrams JR, Lebwohl MG, Guzzo CA, Jegasothy BV, Goldfarb MT, Goffe BS *et al.* (1999) CTLA4Ig-mediated blockade of T-cell costimulation in patients with psoriasis vulgaris. *J Clin Invest* 103:1243–52

Ackermann L, Harvima IT (1998) Mast cells of psoriatic and atopic dermatitis skin are positive for TNF-alpha and their degranulation is associated with expression of ICAM-1 in the epidermis. *Arch Dermatol Res* 290:353–9

Boehncke WH, Kock M, Hardt-Weinelt K, Wolter M, Kaufmann R (1999) The SCID-hu xenogeneic transplantation model allows screening of anti-psoriatic drugs. *Arch Dermatol Res* 291:104–6

Chaudhari U, Romano P, Mulcahy LD, Dooley LT, Baker DG, Gottlieb AB (2001) Efficacy and safety of infliximab monotherapy for plaque-type psoriasis: a randomised trial. *Lancet* 357:1842–7

De Rie MA, Cairo I, Van Lier RA, BOS JD (1996) Expression of the T-cell activation antigens CD27 and CD28 in normal and psoriatic skin. *Clin Exp Dermatol* 21:104–11

Farber EM, Peterson JB (1961) Variations in the natural history of psoriasis. *Calif Med* 95:6–11

Genovese MC, Becker JC, Schiff M, Luggen M, Sherrer Y, Kremer J *et al.* (2005) Abatacept for rheumatoid arthritis refractory to tumor necrosis factor alpha inhibition. *N Engl J Med* 353:1114–23

Gottlieb A, Krueger JG, Bright R, Ling M, Lebwohl M, Kang S *et al.* (2000) Effects of administration of a single dose of a humanized monoclonal antibody to CD11a on the immunobiology and clinical activity of psoriasis. *J Am Acad Dermatol* 42:428–35

Gottlieb AB, Kang S, Linden KG, Lebwohl M, Menter A, Abdulghani AA *et al.* (2004) Evaluation of safety and clinical activity of multiple doses of the anti-CD80 monoclonal antibody, galiximab, in patients with moderate to severe plaque psoriasis. *Clin Immunol* 111:28–37

Kremer JM, Westhovens R, Leon M, Di Giorgio E, Alten R, Steinfeld S *et al.* (2003) Treatment of rheumatoid arthritis by selective inhibition of T-cell activation with fusion protein CTLA4Ig. *N Engl J Med* 349:1907–15

Lenschow DJ, Zeng Y, Thistlethwaite JR, Montag A, Brady W, Gibson MG *et al.* (1992) Long-term survival of xenogeneic pancreatic islet grafts induced by CTLA4Ig. *Science* 257:789–92

Linsley PS, Brady W, Grosmaire L, Aruffo A, Damle NK, Ledbetter JA (1991) Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin 2 mRNA accumulation. *J Exp Med* 173:721–30

Mueller DL, Jenkins MK, Schwartz RH (1989) Clonal expansion *versus* functional inactivation: a costimulatory signaling pathway determines the outcome of T cell antigen receptor occupancy. *Annu Rev Immunol* 7:445–80

Nickoloff BJ, Karabin GD, Barker JN, Griffiths CE, Sarma V, Mitra RS *et al.* (1991) Cellular localization of interleukin-8 and its inducer, tumor necrosis factor-alpha in psoriasis. *Am J Pathol* 138:129–40

Nickoloff BJ, Kunkle SL, Burdick M, Strieter R (1995) Severe combined immunodeficiency mouse and human psoriatic chimeras—validation of a new model. *Am J Pathol* 146:580–8

Raychaudhuri SP, Dutt S, Raychaudhuri SK, Sanyal M, Farber EM (2001) Severe combined immunodeficiency mouse-human skin chimeras: a unique animal model for the study of psoriasis and cutaneous inflammation. *Br J Dermatol* 144:931–9

Raychaudhuri SP, Farber EM (2001) The prevalence of psoriasis in the world. *J Eur Acad Dermatol Venereol* 15:16–7

Raychaudhuri SP, Jiang WY, Farber EM (1998) Psoriatic keratinocytes express high levels of nerve growth factor. *Acta Derm Venereol* 78:84–6

Raychaudhuri SP, Jiang WY, Farber EM, Schall T, Ruff M, Pert C (1999) Identification of high levels of RANTES in the psoriatic keratinocytes. *Acta Derm Venereol* 79:9–11

Raychaudhuri SP, Sanyal M, Weltman H, Raychaudhuri SK (2004) K252a, a high affinity NGF receptor blocker improves psoriasis: an *in vivo* study using the SCID mouse–human skin model. *J Invest Dermatol* 122:812–9

Schon MP, Krahn T, Schon M, Rodriguez ML, Antonicek H, Schultz JE *et al.* (2002) Efomycine M, a new specific inhibitor of selectin, impairs leukocyte adhesion and alleviates cutaneous inflammation. *Nat Med* 8:366–72

Shiao SL, McNiff JM, Masunaga T, Tamura K, Kubo K, Pober JS (2007) Immunomodulatory properties of FK734, a humanized anti-CD28 monoclonal antibody with agonistic and antagonistic activities. *Transplantation* 83:304–13