Blockade of CD11a by Efalizumab in Psoriasis Patients Induces a Unique State of T-Cell Hyporesponsiveness

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Efalizumab (anti-CD11a) interferes with LFA-1/ICAM-1 binding and inhibits several key steps in psoriasis pathogenesis. This study characterizes the effects of efalizumab on T-cell activation responses and expression of surface markers on human circulating psoriatic T cells during a therapeutic trial. Our data suggest that efalizumab may induce a unique type of T-cell hyporesponsiveness, directly induced by LFA-1 binding, which is distinct from conventional anergy described in animal models. Direct activation of T cells through different activating receptors (CD2, CD3, CD3/28) is reduced, despite T cells being fully viable. This hyporesponsiveness was spontaneously reversible after withdrawal of the drug, and by IL-2 in vitro. In contrast to the state of anergy, Ca\(^{2+}\) release is intact during efalizumab binding. Furthermore, lymphocyte function-associated antigen-1 (LFA-1) blockade resulted in an unexpected downregulation of a broad range of surface molecules, including the T-cell receptor complex, co-stimulatory molecules, and integrins unrelated to LFA-1, both in the peripheral circulation and in diseased skin tissue. These observations provide evidence for the mechanism of action of efalizumab. The nature of this T-cell hyporesponsiveness suggests that T-cell responses may be reduced during efalizumab therapy, but are reversible after ceasing efalizumab treatment.


INTRODUCTION

Lymphocyte function-associated antigen-1 (LFA-1) is a heterodimeric glycoprotein belonging to the \(\beta_2\)-integrin family (Carlos and Harlan, 1994; Werther et al., 1996), also known as \(\alpha\L-\beta_2\) or CD11a/CD18, which binds intracellular adhesion molecules 1–3 (ICAM-1, ICAM-2, and ICAM-3). LFA-1 plays a pivotal role in several important homeostatic processes, including T lymphocyte recirculation, trafficking to sites of inflammation, antigen presentation by dendritic cells, and other cells such as activated keratinocytes (Stewart et al., 1996; Hogg et al., 2003; Kinashi, 2005), and T-cell co-stimulation (Van Seventer et al., 1990, 1991, 1992; Kanner et al., 1993).

LFA-1 is a molecular target for therapeutic modulation of immune responses in disease states, as tissue inflammation in experimental models has been blocked by administration of LFA-1 antibodies (Ferrara et al., 1990; Isobe et al., 1992; Scheynius et al., 1993; Tanaka et al., 1993; Gordon et al., 1995; Moriyama et al., 1996; Willenborg et al., 1996). However, little is known about the therapeutic mechanisms of targeting integrins in human subjects. This issue is important, since the humanized anti-CD11a antibody (efalizumab) is currently an approved agent for treatment of psoriasis vulgaris (Gottlieb et al., 2002; Lebwohl et al., 2003). As psoriasis affects about 25 million people in North America and Europe, new psoriasis therapies are likely to be used in large numbers of patients.

Anergy may be broadly defined as a functional state where a T cell fails to react to its cognate antigen, and it is often considered to be a mechanism to maintain peripheral tolerance (Appleman and Boussiotis, 2003; Schwartz, 2003; Macian et al., 2004). Additional features of anergic T cells include a restoration of functional inactivity by exogenous IL-2, and a lack of \(\text{Ca}^{2+}\) mobilization, which normally occurs during T-cell activation.

This study was designed to characterize the effects of efalizumab on T-cell activation responses and expression...
of T-cell surface molecules during a therapeutic trial. Here we demonstrate two unexpected effects on T cells obtained from psoriasis patients. First, direct stimulation of T cells with mitogenic antibodies was significantly decreased during active treatment (anti-CD2, anti-CD3, and combined anti-CD3/CD28), but remained normal with phorbol-12-myristate-3-acetate (PMA) and ionomycin. This is in contrast to the increase in proliferative responses seen with a polyclonal stimulus in murine T cells treated with LFA-1 antibodies (Bohmig et al., 1994). Thus, only in the human experiments were T cells hyporesponsive to direct stimulation via surface molecules. Second, down-regulation of numerous activation-controlling surface molecules on T cells was produced by efalizumab treatment, including those associated with the T-cell receptor (TCR) complex, co-stimulatory pathways, and integrins unrelated to LFA-1. Since both of these effects were not anticipated from the study of LFA-1 antibodies in model systems, they reinforce the need to study biologic effects of new immune antagonists in human populations receiving these agents.

RESULTS

**In vivo, T-cell activation through anti-CD3-, anti-CD2-, and anti-CD3/CD28 is inhibited by efalizumab (anti-CD11a)**

We determined whether *in vivo* exposure of lymphocytes to efalizumab reduced responsiveness through surface molecule ligation. Anti-CD3 stimulation of peripheral blood lymphocytes from 12 untreated psoriatic patients showed dose-dependent activation, as measured by increased expression of CD69 on CD2-expressing lymphocytes (both mean fluorescence intensity (MFI) and percent positive cells increased) (Figure 1a). After 14 days of efalizumab treatment, CD3-mediated activation was greatly reduced at all doses in all subjects. This non-responsiveness of lymphocytes to activation by anti-CD3 was maintained throughout the treatment period with efalizumab (day 84). After washout of efalizumab (day 168), CD3-mediated T-cell activation returned to normal (Figure 1a), in line with re-expression of CD11a on T cells (Figure 1b).

In a second group of 15 patients, we tested additional activating stimuli, including soluble anti-CD3 antibody, a mitogenic pair of anti-CD2 antibodies (T11.1 and T11.2), and bead-bound anti-CD3 and anti-CD28 antibodies (Figure 1c and d). Consistent with results in the first group of patients, T-cell activation (CD69 upregulation) to soluble anti-CD3 antibodies was inhibited in all 15 patients (*P*<0.001; Figure 1d). T-cell activation induced by CD2 ligation was also consistently inhibited during *in vivo* treatment with efalizumab (*P*= 0.001). However, T-cell activation with a stronger stimulus (bead-bound anti-CD3/anti-CD28 antibodies) induced greater expression of CD69 compared with soluble CD3 or CD2 antibodies (Figure 1c and d). Although eight efalizumab-treated patients had reduced T-cell activation responses to this combined stimulus (example shown in Figure 1c), there was only about a 20% average reduction in responses to CD3/CD28 activation (*P*= 0.37). The hyporesponsiveness of T cells under efalizumab therapy was seen in all patients, and this was unrelated to the therapeutic efficacy.

We also examined T-cell activation induced by PMA and ionomycin to determine whether T-cell hyporesponsiveness in efalizumab-treated patients was due to deletion of cells with activation potential (Figure 1e and f). We considered that this might occur even in the setting of efalizumab-induced relative leukocytosis, (Vugmeyster et al., 2004). Upon PMA/ionomycin activation, virtually all T cells showed increased expression of CD69, and neither the frequency of cells showing activation (data not shown), nor the levels of CD69 induced on individual cells, were reduced during efalizumab treatment. This indicated that the activation potential of the T cells was intact.

We next determined whether a functional response of T cells was impaired by *in vivo* efalizumab treatment. Given that IFN-γ-producing T cells are excessively expanded and activated in psoriasis patients (Szegedi et al., 2003), we examined T cells for their ability to synthesize this cytokine in response to activation by soluble anti-CD3 or anti-CD2 antibodies, by bead-bound anti-CD3/anti-CD28 antibodies, and by PMA/ionomycin activation before and during treatment with efalizumab (Figure 2). With soluble anti-CD3 antibodies, T cells from psoriasis patients treated with efalizumab for 2 weeks showed consistently reduced ability to synthesize IFN-γ (*P*<0.02) (Figure 2a–c). As with induction of CD69, nearly complete inhibition of IFN-γ synthesis in response to CD3 ligation was observed, whereas a lesser inhibition of response to CD3/CD28 co-ligation was measured (data not shown). The average frequency of IFN-γ-producing cells was not altered by efalizumab treatment when PMA/ionomycin was used as the activation stimulus (*P* = 0.2), confirming the presence of responsive T cells, cell viability, and the ability of the cells to produce this cytokine (Figure 2b and c).

The differential T-cell activation response to soluble anti-CD3 antibodies versus PMA/ionomycin suggested that early events in signal transduction through the TCR receptor complex might be blocked by efalizumab exposure *in vivo*. In contrast, T-cell anergy induced by other methods is associated with reduction in the signaling cascade that leads to intracellular calcium release (Haque et al., 1998). To test whether efalizumab induced a similar alteration in T-cell responsiveness, intracellular calcium release was monitored by Indo-1, a protein which fluoresces at different wavelengths depending on its binding to Ca++². As illustrated for two patients with psoriasis in Figure 3b, T cells showed excellent Ca++² flux responses to stimulation with anti-CD3 antibody plus surface cross-linking of this antibody with anti-mouse IgG. Those results were equivalent to the Ca++² flux responses from normal volunteers (Figure 3a). T cells taken from patients at 2 weeks of treatment with efalizumab had no reduction in the intracellular calcium release response, including both the kinetics of release and the overall magnitude of the response. Similar results to those illustrated in Figure 3 were observed in eight sequential patients studied that were stimulated with anti-CD3 (Figure S1) or anti-CD3 and anti-CD2 (Figure S2).
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Hyporesponsiveness of T Cells by Efalizumab Therapy

In vitro, T-cell activation through anti-CD3 and anti-CD3/CD28 is inhibited by efalizumab (anti-CD11a), and this hyporesponsiveness is instantly reversed by IL-2.  

Anti-CD3 stimulation of T-lymphocytes in whole blood from healthy volunteers was measured by induced expression of CD69 on CD2-expressing lymphocytes. In vitro incubation with efalizumab resulted in dose-dependent inhibition of the activation response, with the IC$_{50}$ of $0.1-0.3$ µg/ml$^{-1}$ (Figure 4a). Furthermore, the maximal inhibition was measured near the levels of efalizumab that would occur in vivo in psoriasis patients ($\sim 10$ µg/ml$^{-1}$) (Figure 4a). The murine monoclonal MHM24 (parent of efalizumab) and Fab fragments of efalizumab also strongly inhibited the T-cell proliferation response to CD3 ligation (Figure 4b). Similarly, T-cell proliferation triggered by CD3 and CD28 co-ligation was impaired in the presence of efalizumab and this inhibition was fully reversed by IL-2 (Figure 4c).

Peripheral blood T cells show down-modulation of cell-surface expression of CD3, CD2, TCR, CD4, CD8, VLA-4, and CD28 in efalizumab (anti-CD11a)-treated patients  

As two types of intracellular activation systems (calcium response and PMA/ionomycin response) were functioning correctly in T cells from efalizumab-treated patients, we next analyzed the expression of cell-surface molecules that might control T-cell activation responses before and during efalizumab treatment. We examined the levels of an array of different surface molecules on both the peripheral T cells and on T cells obtained from psoriasis skin lesions by short-term incubation.
Previous studies have demonstrated that efalizumab binds to CD11a and saturates binding sites and also has the ability to downregulate both subunits of LFA-1 (Vugmeyster et al., 2004). First, we studied changes in surface antigens in peripheral blood of efalizumab-treated patients (Figure 5b–g, left side). Saturation of CD11a on circulating T cells was determined using a competing custom-conjugated FITC-efalizumab antibody (Figure 5b), whereas downregulation of CD11a was assessed with CD11a (clone 25.3), which binds to a non-competitive epitope (Figure 5c). There was almost complete saturation of CD11a with efalizumab during therapy (P<0.00001) (Figure 5b). As expected, both subunits of LFA-1 were significantly downregulated in all subjects. The expression of CD11a was reduced by 85–73% (P<0.0001), whereas the expression of CD18 was reduced by 76–63% (P<0.0001).

Other surface receptors unrelated to LFA-1 were also quantified (Figure 5). The levels of CD3 were not affected by efalizumab (P>0.2) (Figure 5d and e). The majority of patients showed downregulation of CD28 on circulating T cells with a mean reduction of 22% (P=0.02). Both the CD28hi and CD28lo populations were reduced (Figure 5d). We were particularly interested in studying the modulation of VLA-4 (CD29 and CD49d) during efalizumab therapy, as it is an important alternative integrin for lymphocyte trafficking in the presence of LFA-1 blockade. MFI values for both components of VLA-4 on circulating CD3+CD8+ cells decreased significantly at day 14 (Figure 5e and f, Figure 6). There was a 52–76% reduction in CD29 (P<0.0007) and a 75–68% reduction in CD49d (P=0.001) (Figure 5c and f). The CD29hi population was largely eliminated, whereas the CD29lo population was less affected (Figure 5f and g). Similarly, the CD49dhi population was significantly decreased by approximately 80% (P=0.006) (Figure 6).
CD2 levels were significantly downregulated (although still quantifiable) with efalizumab treatment by 32–50% (P < 0.03) (Figure 5a and g). The expression of the CD8 co-receptor on CD3⁺CD8⁺ T lymphocytes was significantly decreased as early as day 14, with 58–65% reduction (P < 0.003). CD8 MFI values showed a clear trend of recovery to pretreatment levels after therapy termination (Figure 6c). Finally, there was also a significant down-modulation of CD4 and TCR to about 64–82% of baseline values (data not shown).

Psoriatic lesional T cells show down-modulation of cell-surface expression of LFA-1, CD28⁺, CD29⁺, CD8⁺ and CD3⁻ in efalizumab (anti-CD11a⁻)-treated patients

T cells obtained from psoriatic skin lesions before and during efalizumab therapy were examined for the expression of cell-surface markers involved in T-cell trafficking and activation for comparison to circulating T cells (Figure 5a and b-g, right side). Not all the changes observed in circulating T-cell surface antigens described above were present in psoriatic lesional T cells.

CD11a and CD18 median levels were 55 and 44% of baseline, respectively (P < 0.001), which was a pattern similar to circulating T cells, but greater in magnitude. There was also an extremely consistent down-modulation of CD28 expression to approximately 68% of the baseline level (P = 0.001), which was also a greater reduction relative to that on circulating T cells. CD29 levels were moderately but significantly reduced to 83% of baseline (P = 0.01). CD8 expression was significantly reduced to about 78% of baseline (P = 0.03). However, unlike peripheral blood lymphocytes, CD3 expression on T cells in psoriatic lesions was significantly decreased in 8 out of 14 cases. Overall the mean reduction in the MFI for the CD3 marker was 22% (P = 0.0004). There were no significant changes in the expression of TCR, CD2, CD4, and CD49d. Hence, although efalizumab saturates T-cell CD11a and downregulates LFA-1 in both circulating and skin compartments, there are interesting differences in the effects on other surface molecules. For example, the mean reduction in CD28 is higher in skin infiltrating T cells, whereas changes in CD2 and TCR are apparent only in circulating cells.

DISCUSSION

In this study, we describe a unique T-cell hyporesponsiveness induced by anti-CD11 binding through the humanized monoclonal therapeutic antibody efalizumab. We postulate that efalizumab directly induces an alteration of the T-cell activation threshold for CD3 or CD2. This altered activation threshold induced by LFA-1 antagonism in humans is distinct from anergy described in mouse models (Appleman and Boussiotis, 2003), since (a) the suppression is antigen-independent, as demonstrated by CD2 and CD3 effects; (b) the hyporesponsiveness is spontaneously reversible upon washout of the drug; and (c) Ca²⁺ flux is preserved after CD3 ligation. However, similar to true anergy, hyporesponsiveness...
in efalizumab-treated patients was instantly reversible with IL-2.

We excluded other explanations for this reduced T-cell activation, such as depletion of reactive T-cell populations or reduced T-cell activation potential. Efalizumab-treated psoriasis patients have increased circulating T cells, so it is unlikely that there would be depletion of reactive T cells (Vugmeyster et al., 2004), and furthermore, the cells were...
viable (annexin V/propidium iodide staining; data not shown) and fully activatable by PMA/ionomycin. Peripheral CLA+ T cells were not investigated, although it might be interesting to consider this T cell subset in a future study.

Efalizumab is efficacious in treating psoriasis and it is assumed that key mechanisms of action include reduced migration of T cells into the skin, as well as reduced T-cell activation through interference with immune synapse formation between T cells and antigen-presenting cells, as suggested by animal models (Ferrara et al., 1990; Isobe et al., 1992; Scheynius et al., 1993; Tanaka et al., 1993; Gordon et al., 1995; Moriyama et al., 1996; Willenborg et al., 1996). In superantigen T-cell activation experiments from psoriasis patients, T-cell activation was also reduced (unpublished data), possibly reflecting interference with immune synapse formation. Here, we demonstrate reduced T-cell responsiveness independent of immune synapse formation, suggesting a direct effect on T cells.

The mechanism of this reduced responsiveness is important to understand. If conventional anergy and possibly tolerance can be induced by “signal 1 without signal 2” (Schwartz, 1990; Huppa et al., 2003; Jenkinson et al., 2005; Lin et al., 2005), it is possible that efalizumab-induced effects are caused by “signal 2 without signal 1”. One model of T-cell activation via the immune synapse has LFA-1 serving to increase initial adhesion, with main signal transduction occurring via the TCR complex (Huppa et al., 2003). However, it is now clear that LFA-1 also is capable of directly transducing distinct activation signals. Hence, ligation of LFA-1 by efalizumab might deliver signals (signal 2) that make a T-cell hyporesponsive to later T-cell activation signals delivered via the TCR/CD3 complex (signal 1).

Broad re-organization of activation-controlling surface molecules on T cells is also produced by efalizumab. In vivo efalizumab administration leads to down-modulation not only of CD11a/CD18 as expected (Gottlieb et al., 2002; Vugmeyster et al., 2004), but also of multiple surface molecules, which may be involved in T-cell activation, including CD3, TCR, CD4, CD8, CD28, and the integrin VLA-4. This occurs in T cells both in the peripheral circulation as well as in the tissue, although the effect is more restricted in the tissue. Possible mechanisms of down-regulation of such an extensive array of surface molecules include capping of surface molecules by efalizumab (Mortensen et al., 2005), alteration of cytoskeletal components that are involved in immune synapse formation such as talin, or incorporation of surface molecules into lipid rafts (Hogg et al., 2003; Kinashi, 2005). T-cell activation often leads to incorporation of integrins into lipid rafts and other membrane proteins may complex with the integrins for colocalization within the rafts (Meiri, 2005). There is now evidence that efalizumab clearance is, at least partially, mediated by internalization via CD11a, which supports such a hypothesis (Coffey et al., 2005).

These data provide insight into the mechanism of action of efalizumab contributing to its effect on T-cell-mediated autoimmune diseases such as psoriasis. Moreover, there are interesting conclusions to be made with regards to the response to self- and foreign neo- and recall antigens. However, the mechanism of downregulation of these surface molecules is difficult to assess because we are unable to reproduce these findings in vitro by incubation of peripheral blood with efalizumab (unpublished data). If efalizumab induced specific antigen-dependent anergy, it could be
speculated that long-lasting tolerance even beyond efalizumab presence could be induced. Our data do not support this hypothesis, but rather suggest a transient decrease in T-cell activation, which is reversible by IL-2 exposure or by antibody withdrawal.

Indeed, in a recent human trial (Genentech ACD2244g) investigating the immune response to model antigens during efalizumab in psoriasis patients, T-cell-dependent antibody responses to the specific model neoantigen bacteriophage φX174 were greatly reduced during treatment, but responses to repeated administration of the same antigen normalized after withdrawal without tolerance induction, contradicting previous data from murine studies (Fischer et al., 1986; Benjamin et al., 1988; Krueger et al., 2005) (JG Krueger et al. Effect of therapeutic integrin (CD11a) blockade with efalizumab on immune responses to model antigens in humans. Manuscript submitted for J Immunol, 2007). Taken together, these data emphasize the extent to which T-cell responses are blocked in efalizumab-treated patients and are in good agreement with the results of this study.

We observed that T-cell responses to CD3/CD28 co-ligation were less suppressed than cell activation via CD3 or CD2 alone, suggesting that secondary immune responses in efalizumab-treated patients could be less impacted by treatment. Consistent with this observation, the human immunization trial demonstrated only a moderate reduction, but not abrogation, of responses to skin recall antigens measured by delayed-type hypersensitivity testing.

The unique set of cellular effects seen with efalizumab is clearly distinct from the genetic defects of β2-integrins in humans (leukocyte adhesion deficiency) or mice (Hogg and Bates, 2000; Shaw et al., 2001; Bunting et al., 2002). Overall we have observed unexpected cellular immune effects of LFA-1 antagonism, which are different from those reported in any model system, and could not have been predicted from animal studies. Given the emerging role of the IL-17 pathway in psoriasis (Lowes et al., 2007), future studies to evaluate the therapeutic effects of efalizumab on this pathway will be considered.

**MATERIALS AND METHODS**

**Study design**

Fourteen patients (11 males, 3 females, ages 28-59 years, median 48 years) and 17 patients (4 males, 13 females, ages 29-71, median 47 years) with moderate to severe psoriasis were enrolled in two consecutive studies, study 1 and study 2, respectively, which were approved by the Rockefeller University Hospital Institutional Review Board. All patients were consented and the study was conducted according to the Declaration of Helsinki Principles. The patients were treated with weekly, subcutaneous efalizumab at 1 mg kg⁻¹ per week for 12 weeks, and followed for an additional 12 weeks. A range of clinical responses to efalizumab therapy was observed, from complete clearing of psoriasis to minimal improvement. One and two patients withdrew from the first and second studies, respectively, and did not have final biopsies. Five healthy volunteers (2 males, 3 women, ages 20-68, median 46 years) were also enrolled for assessing the dose-dependent activation response to efalizumab treatment in vitro. Response was determined both histologically and clinically, as previously described (Wittkowski, 2004; Lowes et al., 2005). In study 1, 11 patients were categorized as responders and two as non-responders. In study 2, 11 patients were categorized as responders and four as non-responders.

**Skin biopsies**

Skin biopsies were obtained at baseline and 2 weeks after the first dose and processed as previously described (Ferenczi et al., 2002). Briefly, skin samples were cut into small pieces and placed in 5 ml EDTA (Fisher Scientific, Pittsburgh, PA) acid at 4 °C (pH 7) for 4 hours with continuous gentle agitation. The resultant cell suspension was filtered through a nylon mesh filter, centrifuged, washed with phosphate-buffered saline, and resuspended at 10⁵ cells ml⁻¹ for staining and analysis by FACS.

**Peripheral blood samples**

Peripheral blood draws were taken at baseline and at either 2, 12, and 24 weeks (study 1) or at 24 hours, 2, 6, and 12 weeks (study 2). Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized samples by using standard Ficoll-Hypaque (Pharmacia Biotech, Uppsala, Sweden) density-gradient sedimentation. For study 1, whole blood was used for activation assay, whereas PBMCs were used for T-cell phenotype by FACS, with the exception of CD11a expression for which both whole blood and PBMCs were analyzed. For study 2, PBMCs were used for activation assays, FACS, and Ca²⁺ flux experiments, whereas whole blood was used to determine cytokine production and T-cell phenotype by FACS.

**Whole blood and PBMC activation assays**

Heparinized whole blood was incubated with either 1 μg ml⁻¹ of mouse anti-CD3 antibody (clone UCHT1; PharMingen, San Diego, CA) overnight at 37 °C in the presence of varying dilutions (0.001-100 μg ml⁻¹) of efalizumab (in vitro assay; Figure 1a); 1.6-1,000 ng ml⁻¹ of mouse anti-CD3 antibody (clone UCHT1) overnight at 37 °C (ex vivo assay; Figure 2a); or 25 ng ml⁻¹ PMA, 2 μg ml⁻¹ ionomycin, and 10 μg ml⁻¹ brefeldin A for 4 hours at 37 °C (ex vivo assay; Figure 2e and f). Upon red blood cell lysis (168 μM NH₄Cl, 10 mM KHCO₃, 0.089 mM Na₄EDTA), cells were stained with anti-CD2 FITC and anti-CD69 phycoerythrin (PE)-cy5 (Becton Dickinson, San Jose, CA (BDi)). Geometric mean fluorescence intensity (GMFI) or MFI of CD69 on CD2⁺ lymphocytes was determined by FACS analysis for the in vitro assay (Figure 1a) or ex vivo assay (study 1; Figure 4a, e, f), respectively. For the in vitro assay, percent inhibition was calculated. The IC₅₀ was calculated using a four-parameter curve fit in KaleidaGraph Software.

For study 2 (Figure 1c and d), PBMCs were activated for 4 hours at 37 °C, using 1 μg ml⁻¹ of the OKT3 antibody (anti-CD3) (OrthoBiotech, Bridgewater, NJ), 0.5 μg ml⁻¹ T11.1 + 0.5 μg ml⁻¹ T11.2 (anti-CD2) (Immunotech, Cedex, France), or anti-CD3/CD28 Dynabeads (Dynal Biotech, Invitrogen Corp., Carlsbad, CA) (three beads per T cell) in the presence of 10 μg ml⁻¹ brefeldin A (Sigma Aldrich, St Louis, MO). After removing Dynabeads on Dynal magnetic particle concentrator, PBMCs were washed with FACSWash (2% fetal bovine serum in phosphate-buffered saline, 0.1% sodium azide (Sigma Aldrich)), and permeabilized using FACSperm (BD). MFI of CD69 on CD3 lymphocytes (with anti-CD3 clone SK7 that does not directly compete with OKT3 for T-cell binding) was determined by FACS analysis. Two out of 14 and two of 17 patients in study 1 and study 2, respectively, were excluded from analysis.

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in calculations of the mean, as they had baseline (day 0) T-cell activation below the acceptance criteria (less than 15% CD69 T cells) or had missing samples.

Peripheral blood lymphocyte proliferation assay using healthy volunteers

Cell proliferation was determined using \(^{3}\)H-labeled thymidine incorporation as previously described (van Kooyk et al., 1996; Khanna and Hosenpud, 1999). Briefly, 96-well plates were coated for 1 hour at 37°C with suboptimal concentration of anti-CD3 (T3b, 0.07 \(\mu\)g ml\(^{-1}\)) or anti-CD2 (0.07 \(\mu\)g ml\(^{-1}\)) in RPMI 1640 (Gibco/BRL, Rockville, MD), 1% BSA (100 \(\mu\)g ml\(^{-1}\), 30 minutes at 37°C), and efalizumab (100 ng well\(^{-1}\), 1 hour at 37°C), as described previously (van Kooyk et al., 1996). Resting peripheral blood lymphocytes were added (100,000 cells well\(^{-1}\)) and cultured for 3 days, as described previously (van Kooyk et al., 1996). On day 3, cells were pulsed for 16 hours with \(^{3}\)H-labeled thymidine (1.52 TBq mmol\(^{-1}\), 0.5 \(\mu\)Ci well\(^{-1}\); Amersham Corp., Arlington Heights, IL), and uptake was quantified to measure cell proliferation in the presence of either efalizumab, M1-I2 (efalizumab parent monoclonal murine non-humanized antibody), or the Fab fragment of efalizumab (all at 0.07 \(\mu\)M, from Genentech Inc., South San Francisco, CA).

Whole-blood cytokine analysis

Heparinized whole blood was activated for 4 hours using either anti-CD3 (1 \(\mu\)g ml\(^{-1}\) OKT3), anti-CD2 (0.5 \(\mu\)g ml\(^{-1}\) T11.1 + 0.5 \(\mu\)g ml\(^{-1}\) T11.2), anti-CD3/CD28 Dynabeads, or PMA/ionomycin (25 ng ml\(^{-1}\) PMA, 2 \(\mu\)g ml\(^{-1}\) ionomycin), and anti-CD2/LFA-3 (1 \(\mu\)g ml\(^{-1}\)) and cultured for 3 days, as described previously (van Kooyk et al., 1996). On day 3, cells were thawed with 2 mM EDTA for 10 minutes at room temperature (RT). Red blood cells were then lysed with FACSLyse solution (BD) and centrifuged and washed to obtain PBMCs, which were frozen in media containing RPMI medium 1640 (Gibco/BRL, Rockville, MD), with 1% HEPS buffer (Sigma Aldrich), 0.1% gentamycin (Gibco/BRL), and 5% normal human serum (C-Six Diagnostic, Germantown, WI) with 10% dimethyl sulfoxide. Unactivated controls were processed in parallel. Before cytokine staining, PBMCs were thawed, washed, and permeabilized with FACSPerm. The fraction of IFNy-producing T cells (SK7+ lymphocytes) was determined by flow cytometry. Percent change in IFNy-producing ability was calculated.

FACS analysis

FACS was used to analyze heparinized whole blood, PBMCs, or single cell suspensions from skin biopsies. When whole blood was used, cells were stained with antibodies for 10 minutes at RT, lyzed with FACSLyse for 10 minutes at RT, and washed with FACS Wash. PBMCs were stained for 15 minutes at RT. The following antibodies were used: CD18 (PE), CD3 (PerCP), CD2 (FITC), TCR (FITC), CD49d (PE), CD29 (FITC), CD4 (FITC), CD8 (Per) CD25 (PE), CD28 (PE), CD69 (FITC), IFN-\(\gamma\) (FITC), and IL-2 (FITC) (BD). Staining for CD11a was performed with two different anti-CD11a mAbs: efalizumab–FITC (Genentech, custom design) and clone 25.3–FITC (Immuno- tech). Appropriate IgG isotype controls were added (BD). Cells were washed with FACS Wash and resuspended in 1.3% formaldehyde (Fisher Scientific) in FACS Wash. Samples were analyzed with four-color staining using a FACSCalibur Flow Cytometer and CELLQuest software after calibration with Calibrite beads and FACSComp software (BD).

Ca\(^{2+}\) flux

PBMCs (10\(^{7}\) per milliliter) were loaded with 2 \(\mu\)M Indo-1 (Molecular Probes, Invitrogen Corp., Carlsbad, CA) for 30 minutes at 37°C in RPMI. The cells were washed with warm RPMI, resuspended in RPMI at RT, and acquired at baseline using a BD LSR flow cytometer. OKT3 (anti-CD3) (OrthoBiotech) activator was added and after an additional 20 seconds, a cross-linking mouse IgG\(_{2a}\) was added. The Ca\(^{2+}\) flux was measured as the ratio between bound and unbound Indo-1 versus time.

Statistical analysis

Student’s t-test and two-sided sign test were performed, with the assumption of equal probability of an increase or decrease from day 0 value. Significance was accepted as \(P<0.05\).

CONFLICT OF INTEREST

YV and SB were, and WD, BH, and KH are, employed by Genentech Inc. JGK has served as a consultant for Genentech. All other authors have no conflicting financial interests.

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SUPPLEMENTARY MATERIAL

Figure S1. Anti-CD3-mediated intracellular Ca\(^{2+}\) flux response is normal in T cells from efalizumab-treated patients.

Figure S2. Anti-CD3 and anti-CD2-mediated intracellular Ca\(^{2+}\) flux response is normal in T cells from efalizumab-treated patients.

REFERENCES


