

Efalizumab Therapy for Atopic Dermatitis Causes Marked Increases in Circulating Effector Memory CD4 + T Cells That Express Cutaneous Lymphocyte Antigen

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Efalizumab is an mAb directed against CD11a, a molecule involved in T-cell activation and extravasation from blood into tissue. Ten patients with severe atopic dermatitis were treated with efalizumab for 84 days, and peripheral blood mononuclear cells were analyzed for expression of activation and adhesion markers. Efalizumab treatment led to decreases in CD11a mean fluorescence intensity (MFI) on naive, central memory, and effector memory CD4 + and CD8 + T cell subsets. MFI for CD18 was decreased in both CD4 + and CD8 + T cells. Percentages of cells positive for cutaneous lymphocyte antigen (CLA) were increased fourfold in all CD4 + and CD8 + T cell subsets. Increases in the percentages of CD4 + and CD8 + T cells expressing $\beta 7$ and CD49d were also observed. No significant changes were observed in the percentages of CD4 + and CD8 + T cells that produced either IFN- γ or IL-4. In summary, efalizumab treatment resulted in (i) decreases in CD11a and CD18 expression in all circulating T-cell subsets and (ii) increases in the percentages of blood T cells expressing tissue homing markers (CLA, $\beta 7$, CD49d). These data suggest that blockade of T-cell extravasation into tissue is the major pathway by which efalizumab leads to improvement in cutaneous inflammation.

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INTRODUCTION

Lymphocyte function-associated antigen (LFA)-1 is a heterodimer composed of the α -integrin CD11a and the $\beta 2$ -integrin CD18 and is involved in T-cell extravasation from blood into tissue (Butcher and Picker, 1996; Kupper and Fuhlbrigge, 2004). To exit blood vessels, T cells first go through a selectin-mediated rolling phase, and these initially weak interactions are reinforced through binding of LFA-1 on T-cell surfaces with CD54 (intercellular adhesion molecule (ICAM-1)) on endothelial cell surfaces (Butcher and Picker, 1996; Henderson *et al.*, 2001; Kupper and Fuhlbrigge, 2004). Following the rolling phase, T cells anchor via LFA-1 to

endothelial cell ICAMs and migrate into tissue. LFA-1 is involved in the migration of T cells to sites of inflammation as well as the migration of naive T cells into lymph nodes (Berlin-Rufenach *et al.*, 1999; Smith *et al.*, 2007). Additionally, T-cell adhesion to endothelium and naive T-cell transmigration into lymph nodes is impaired in LFA-1 null animals (Berlin-Rufenach *et al.*, 1999; Henderson *et al.*, 2001), demonstrating the importance of LFA-1 in T-cell extravasation. LFA-1 on T-cell surfaces also interacts with CD54 on the surface of antigen-presenting cells to provide co-stimulation and full T-cell activation (Hogg *et al.*, 2002; Joshi *et al.*, 2006).

Efalizumab is a recombinant humanized mAb directed against the CD11a subunit of LFA-1 (Joshi *et al.*, 2006) and is indicated for the treatment of individuals with moderate-to-severe chronic plaque psoriasis. Approximately 30% of patients will have at least 75% improvement of their skin disease following a standard 12-week course of therapy (drug is typically given subcutaneously at a weekly dose of 1 mg kg⁻¹) (Gordon *et al.*, 2003; Lebwohl *et al.*, 2003; Leonardi *et al.*, 2005; Menter *et al.*, 2005). Sustained efficacy in the treatment of psoriasis patients over longer periods of time has also been described (Lebwohl *et al.*, 2003; Leonardi *et al.*, 2005; Menter *et al.*, 2005). As patients improve, T-cell numbers are reduced in healing skin, whereas T-cell numbers are increased in peripheral blood (Krueger *et al.*, 2000;

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Abbreviations: CLA, cutaneous lymphocyte antigen; EASI, eczema area and severity index; ICAM-1, intercellular adhesion molecule; LFA, lymphocyte function-associated antigen; MFI, mean fluorescence intensity; PBMC, peripheral blood mononuclear cells; PE, phycoerythrin

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Gottlieb *et al.*, 2002; Vugmeyster *et al.*, 2004), consistent with efalizumab's role in blocking T-cell extravasation into skin. Although the drug has proven to be relatively safe, with no reported increased incidence of infections in treated patients (Langley *et al.*, 2005), some individuals who discontinue drug undergo a rebound of psoriasis (Carey *et al.*, 2006). Others have postulated a mechanism for disease flare observed following drug discontinuation whereby skin-homing peripheral blood T cells migrate into the skin after efalizumab-induced restraints on extravasation are relieved.

Recently, we evaluated safety and clinical efficacy of efalizumab in a 12-week pilot study in 10 individuals with severe atopic dermatitis (Takiguchi *et al.*, 2007), another common inflammatory skin disease where the number of cutaneous T cells are elevated (Homey *et al.*, 2006; Simpson and Hanifin, 2006; Wittmann and Werfel, 2006). In these patients, reported herein, we examined cell-surface antigen expression of adhesion molecules and homing markers on various T-cell subsets in the blood. In addition, cytokine secretion by activated blood T cells was quantified. Our purpose was to gain further insight into the impact of efalizumab treatment on the composition and activation state of circulating T-cell populations. In addition to finding marked increases in skin-homing T cells in the blood of these patients, we found that treatment with efalizumab caused general increases in circulating effector memory T cells capable of homing to other tissues in the body. These results suggest that efalizumab may be clinically useful for both cutaneous and non-cutaneous T-cell-mediated diseases.

RESULTS

Efalizumab therapy increases the percentage of circulating CD4⁺ effector memory T cells

As described below, T-cell populations were initially gated into either CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells. We defined CD45RA⁺CD27⁺CD62L⁺ cells as naive T cells, CD45RA⁺CD27⁺CD62L⁺ cells as central memory T cells, and CD45RA⁺CD27[−]CD62L[−] as effector memory T cells. The mean ratio of CD4 to CD8 T cells present in the peripheral blood of patients was 3.04, 2.09, 2.21, and 2.32 at days 0, 28, 56, and 84, respectively, reflecting a slight decrease in the percentages of total circulating CD4⁺ T cells and a slight increase in the percentages of total circulating CD8⁺ T cells, although differences were not significant (Figure 1a).

Compared to baseline levels, examination of changes in circulating T-cell subsets revealed significant increases in the percentages of effector memory CD4⁺ T cells at days 56 ($P<0.029$) and 84 ($P<0.007$) following efalizumab therapy (Figure 1b). In contrast, no differences in the percentages of circulating effector memory CD8⁺ T cells were observed. In addition, significant decreases in the percentages of CD4⁺ central memory T cells were observed at days 56 ($P<0.010$) and 84 ($P<0.042$). Minor decreases in CD8⁺ central memory cells were observed, although these differences were not statistically significant. Decreases in the percentages of naive CD4⁺ (but not CD8⁺) T cells at days 28 ($P<0.14$), 56 ($P<0.03$), and 84 ($P<0.13$) were noted, although significance was only achieved at day 56, likely due to high

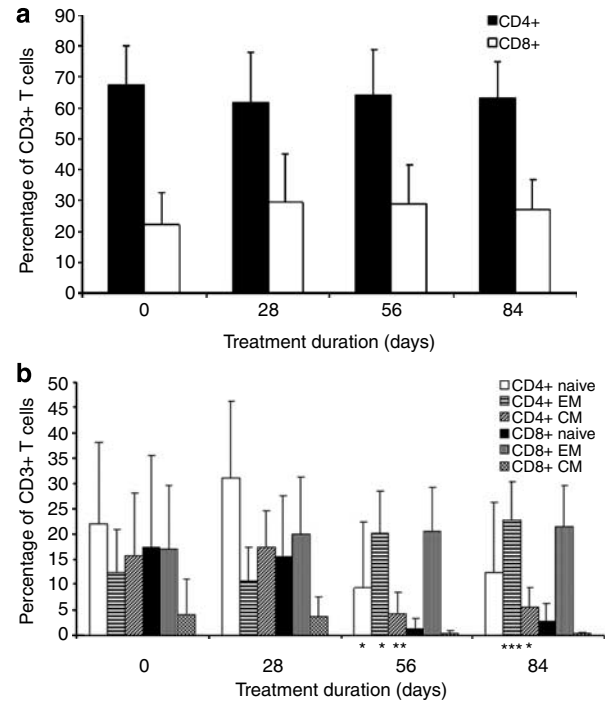


Figure 1. Efalizumab therapy increases the percentage of circulating CD4⁺ effector memory T cells. (a) The percentage of CD4⁺ and CD8⁺ T cells in the blood was determined on the indicated days of the efalizumab treatment, and the CD4⁺/CD8⁺ ratio was slightly decreased during the course of treatment, although differences did not reach statistical significance. (b) The percentages of naive, effector memory, and central memory CD4⁺ and CD8⁺ T cell subsets were determined on the indicated days of efalizumab treatment and revealed an increase in the percentage of effector memory CD4⁺ T cells in the blood; the percentages of CD4⁺ naive and central memory T cells were decreased. No significant changes were observed in the distribution of CD8⁺ T cell subsets, when compared to baseline levels.

interpatient variability. Minor, but not statistically significant, decreases in CD8⁺ naive T cells were observed. These data suggest that efalizumab therapy in humans with atopic dermatitis preferentially redistributes effector memory CD4⁺ T cells, altering the T-cell subset composition of the blood.

Efalizumab therapy decreases CD11a surface expression on circulating T cells and expression levels correlate with clinical improvement of atopic dermatitis

The percentages of CD4⁺ and CD8⁺ T cells expressing CD11a, the target for efalizumab, were significantly reduced at days 28, 56, and 84 (Figure 2a). Mean fluorescence intensity (MFI) of CD11a on both CD4⁺ and CD8⁺ T cells was also significantly reduced compared to baseline levels on all days studied (Figure 2b).

When compared to baseline levels, CD11a expression was significantly decreased on all six T-cell subsets on all days studied (Figure 2c). For naive CD4⁺ T cells, CD11a levels were decreased by 47.7% ($P<0.003$), 49.2% ($P<0.002$), and 47.4% ($P<0.002$) on days 28, 56, and 84, respectively, when compared to baseline levels. For effector memory CD4⁺ T cells, CD11a levels were decreased by 52.3% ($P<0.001$), 60.8% ($P<0.001$), and 59.9% ($P<0.0001$) on days 28, 56,

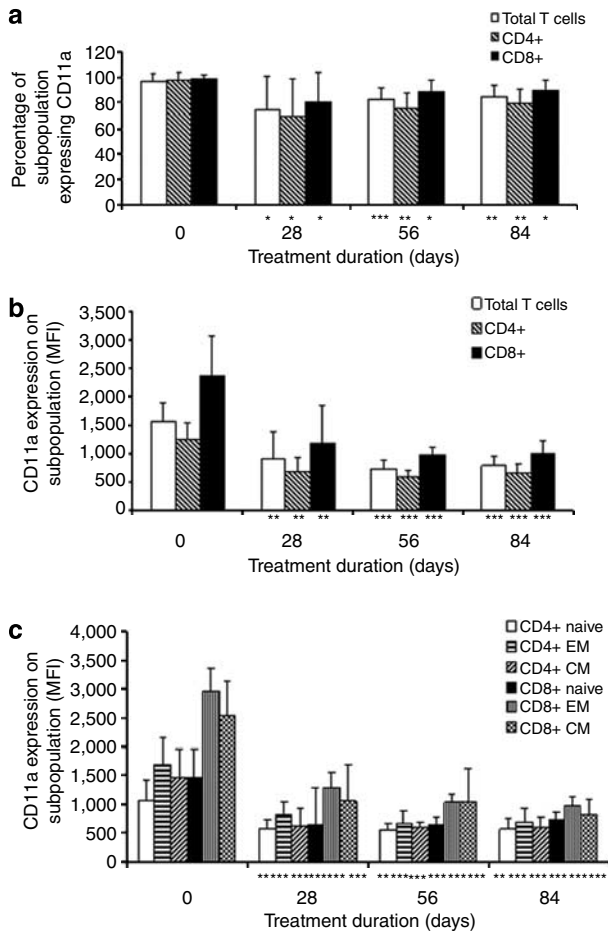


Figure 2. Efalizumab therapy decreases CD11a surface expression on circulating T cells. (a) The percentages of CD11a+ T cells, CD4+ T cells, and CD8+ T cells in the blood during the course of efalizumab treatment were determined on the indicated days and revealed significantly decreased percentages of all T-cell subgroups expressing CD11a, when compared to patient baseline levels. (b and c) Significantly decreased levels of CD11a surface protein expression on circulating T cells, CD4+ T cells, and CD8+ T cells was also observed on the indicated days in response to efalizumab therapy as well as decreased CD11a expression levels on all naive, effector memory, and central memory CD4+ and CD8+ T-cell subset populations.

and 84, respectively. For central memory CD4+ T cells, CD11a levels were decreased by 56.6% ($P<0.0001$), 59.4% ($P<0.001$), and 59.8% ($P<0.0001$) on days 28, 56, and 84, respectively. Similarly, for naive CD8+ T cells, CD11a levels were decreased by 55.3% ($P<0.0001$), 56% ($P<0.0001$), and 51.2% ($P<0.0001$) on days 28, 56, and 84, respectively. For effector memory CD8+ T cells, CD11a levels were decreased by 56.3% ($P<0.0001$), 64.9% ($P<0.0001$), and 66.9% ($P<0.0001$) on days 28, 56, and 84, respectively. Finally, for central memory CD8+ T cells, CD11a expression levels were decreased by 58.4% ($P<0.0001$), 59.5% ($P<0.0001$), and 67.5% ($P<0.0001$) on days 28, 56, and 84, respectively.

Reduction of surface CD11a expression by T cells correlated with clinical improvement in atopic dermatitis as measured by the eczema area and severity index (EASI) score.

Analysis of expression levels of CD11a and EASI scores using Pearson's correlation yielded r values of 0.37 ($P<0.018$) for naive CD4+ cells, 0.41 ($P<0.008$) for effector memory CD4+ T cells, 0.45 ($P<0.004$) for central memory CD4+ T cells, 0.51 ($P<0.0007$) for naive CD8+ T cells, 0.48 ($P<0.0015$) for effector memory CD8+ T cells, and 0.434 ($P<0.005$) for central memory CD8+ T cells. These data indicate that a moderate relationship exists between CD11a surface expression levels and clinical improvement of atopic dermatitis secondary to efalizumab therapy.

Efalizumab therapy decreases CD18 surface expression on circulating T cells

The β -subunit of the LFA-1 heterodimer is CD18 (Butcher and Picker, 1996; Kupper and Fuhlbrigge, 2004). Consistent with a reduction in the number of T cells expressing CD11a, the percentage of total T cells expressing CD18 was decreased by 27.55, 17.16, and 17.23%, although differences were not statistically significant.

The percentage of CD4+ T cells expressing CD18 was significantly reduced by 36.0% ($P<0.043$), 28.3% ($P<0.011$), and 24.8% ($P<0.020$) at days 28, 56, and 84, respectively (Figure 3a). Patients receiving efalizumab exhibited a decrease of 21.0%, 11.5%, and 11.1% in the percentage of CD8+ T cells expressing CD18 at days 28, 56, and 84, respectively, when compared to baseline expression, although differences were not statistically significant. When compared to baseline levels, analysis of CD18 surface protein levels indicated a decrease of 27.6% ($P<0.026$), 34.65% ($P<0.007$), and 33.2% ($P<0.003$) at days 28, 56, and 84, respectively. CD18 expression on CD4+ T cells was decreased by 30.1% ($P<0.013$), 33.1% ($P<0.008$), and 31.33% ($P<0.002$), whereas CD8+ T cells decreased CD18 expression by 41.1% ($P<0.016$), 58.9% ($P<0.005$), and 57.7% ($P<0.006$) on all treatment days studied (Figure 3b).

Levels of CD18 protein were reduced on all CD4+ and CD8+ T-cell subsets examined. Naive CD4+ T cells exhibited 30.7% ($P<0.024$), 29.7% ($P<0.063$), and 26.9% ($P<0.026$) decreases on days 28, 56, and 84, respectively, compared to baseline levels (Figure 3c). On effector memory CD4+ T cells, CD18 levels decreased by 53.4% ($P<0.036$), 27.5% ($P<0.038$), and 40.1% ($P<0.057$) on days 28, 56, and 84, respectively. On central memory CD4+ T cells, CD18 levels decreased by 42.8% ($P<0.0002$), 43.7% ($P<0.0018$), and 43.8% ($P<0.0006$) on days 28, 56, and 84, respectively. CD18 expression by naive CD8+ T cells decreased by 37.2% ($P<0.0005$), 24% ($P<0.051$), and 20.6% ($P<0.124$) on days 28, 56, and 84, respectively, compared to baseline. Effector memory CD8+ T cell expression of CD18 was decreased by 48.1% ($P<0.0006$), 52.2% ($P<0.0008$), and 53.9% ($P<0.0003$) on days 28, 56, and 84, respectively. Finally, central memory CD8+ cells decreased expression by 46.2% ($P<0.0006$), 57.5% ($P<0.0005$), and 56.0% ($P<0.0012$) on days 28, 56, and 84, respectively.

Decreased levels of CD18 correlated with clinical improvement but to a lesser degree than the decreases observed with cell-surface levels of CD11a. CD18 downregulation on central memory CD4+ T cells, naive CD8+ T cells, effector memory

CD8+ T cells, and central memory CD8+ T cells correlated with decreased EASI scores, yielding Pearson correlation coefficient values of 0.41 ($P<0.0094$), 0.35 ($P<0.029$), 0.47 ($P<0.002$), and 0.40 ($P<0.01$), respectively. Decreased CD18 expression by naive and effector memory CD4+ T cells did not significantly correlate with decreased EASI scores.

Efalizumab therapy increases the percentage of circulating T cells that express CLA, a skin-homing marker

Previous studies have shown that cutaneous lymphocyte antigen (CLA) binds E-selectin, which is expressed on dermal endothelial cells, and that this interaction mediates T-cell extravasation into skin (Butcher and Picker, 1996; Kupper and Fuhlbrigge, 2004). Patients receiving efalizumab exhibited significant increases in the percentages of circulating total T cells, CD4+ T cells, and CD8+ T cells expressing

CLA (Figure 4a). Baseline mean values ranged approximately 8–14% in these three T-cell populations, and the mean values increased to 31–48% on the various treatment days, indicating consistent fourfold increases in the percentages of CLA-expressing T cells (Figure 4a). By contrast, there was a trend toward decreased CLA surface-expression levels on most T-cell populations (Figure 4b). Examination of T-cell subsets indicated that the percentage of naive CD4+ T cells in the blood expressing CLA increased over baseline levels by 30.0% ($P<0.02$), 29.2% ($P<0.008$), and 32.3% ($P<0.0001$) on 28, 56, and 84 days of treatment, respectively (Figure 4c). The number of effector memory CD4+ T cells expressing CLA increased by 28.6% ($P<0.011$), 19.7% ($P<0.022$), and

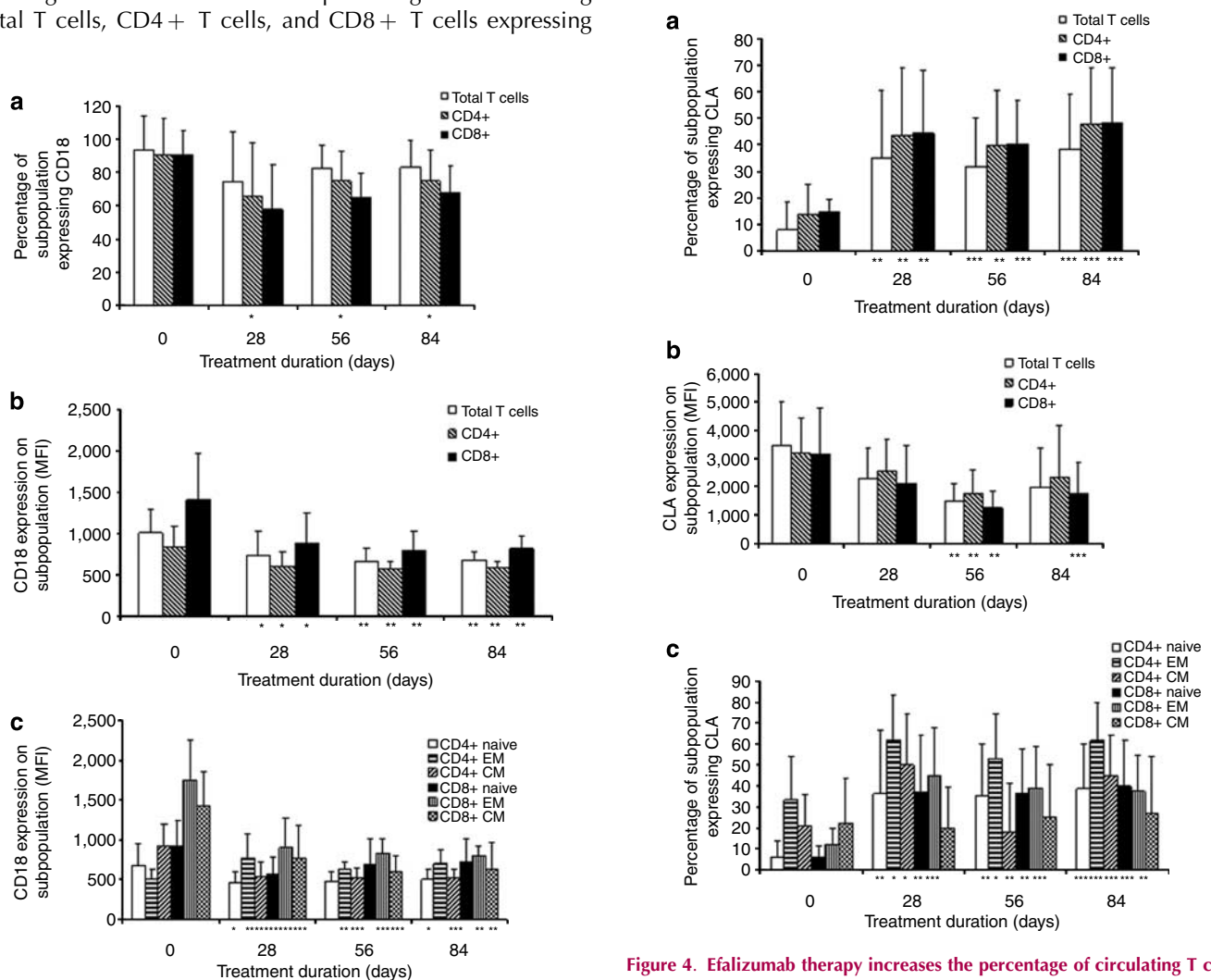


Figure 4. Efalizumab therapy increases the percentage of circulating T cells that express CLA, a skin-homing marker. (a) The percentages of T cells, CD4+ T cells, and CD8+ T cells in the blood expressing CLA during the course of efalizumab treatment were determined on the indicated days and were shown to be significantly elevated when compared to patient baseline levels. (b) By contrast, surface protein expression levels of CLA on total circulating T cells, CD4+ T cells, and CD8+ T cells were decreased on the indicated days of efalizumab treatment. (c) When compared to baseline levels, the percentages of CLA-expressing T cells markedly increased in all T-cell subset populations, except for central memory CD8+ T cells, where increases failed to meet significance.

28.0% ($P<0.0001$), whereas the percentage of central memory CD4+ T cells expressing CLA increased by 23.6% ($P<0.024$), 20.2% ($P<0.007$), and 28.5% ($P<0.0001$) on days 28, 56, and 84, respectively, when compared to baseline levels. The number of naive CD8+ T cells expressing CLA increased by 31.1% ($P<0.008$), 30.4% ($P<0.001$), and 33.8% ($P<0.0001$) on days 28, 56, and 84, respectively. Percentages of effector memory CD8+ T cells expressing CLA increased by 33.1% ($P<0.001$), 27.1% ($P<0.001$), and 25.4% ($P<0.003$) on days 28, 56, and 84, respectively, when compared to baseline levels. Finally, percentages of CLA+ central memory CD8+ T cells increased by 17.3, 11.9, and 14.8% on days 28, 56, and 84 of efalizumab treatment compared to baseline levels, though these differences were not statistically significant. Despite consistent increases in the numbers of CLA-expressing T cells in blood, no significant correlations were detected between increasing blood CLA+ T cells and the clinical EASI score among individual patients.

Efalizumab therapy increases the percentage of circulating T cells that express $\beta 7$ /CD49d, a gut-homing marker

The $\beta 7$ interacts with CD49d, and together this heterodimeric molecule is involved in T-cell movement from peripheral blood into gastrointestinal tissue (Butcher and Picker, 1996; Kupper and Fuhlbrigge, 2004). Percentages of total T cells, CD4+ T cells, and CD8+ T cells expressing $\beta 7$ in the blood increased slightly, although these increases were not

statistically significant (Figure 5a). By contrast, and yet similar to CLA, $\beta 7$ surface expression levels were significantly downregulated on both total T cells and CD4+ T cells. $\beta 7$ surface expression levels of total T cells were decreased by 49.3% ($P<0.020$), 35.7% ($P<0.007$), and 31.7% ($P<0.004$). Expression levels on CD4+ T cells were decreased by 52.2% ($P<0.050$), 40.8% ($P<0.015$), and 32.2% ($P<0.005$) at days 28, 56, and 84, when compared to baseline levels, although differences were not detected in CD8+ T cells (Figure 5b).

Percentages of circulating CD49d+CD4+ T cells markedly increased by 106.5% ($P<0.008$) and 114.9% ($P<0.001$) on days 56 and 84, respectively (Figure 5c). Percentages of total T cells and CD8+ T cells expressing CD49d also increased, although a statistically significant difference was only observed for total CD49d+ T cells on day 84 ($P<0.029$). Consistent with the observed decreases in CLA and $\beta 7$ surface expression levels, decreases in CD49d MFI by 46.7% ($P<0.011$), 61.0% ($P<0.00018$), and 55.2% ($P<0.0006$) were observed on total T cells on days 28, 56, and 84, respectively, when compared to baseline levels (Figure 5d). CD4+ T-cell expression levels of CD49d were significantly reduced by 47.3% ($P<0.013$), 62.6% ($P<0.0001$), and 59.6% ($P<0.0005$) on days 28, 56, and 84, respectively. Finally, CD8+ T cells exhibited a 24.0% ($P<0.236$), 55.4% ($P<0.0034$), and 39.6% ($P<0.065$) reduction in CD49d expression on days 28, 56, and 84, when compared to baseline levels.

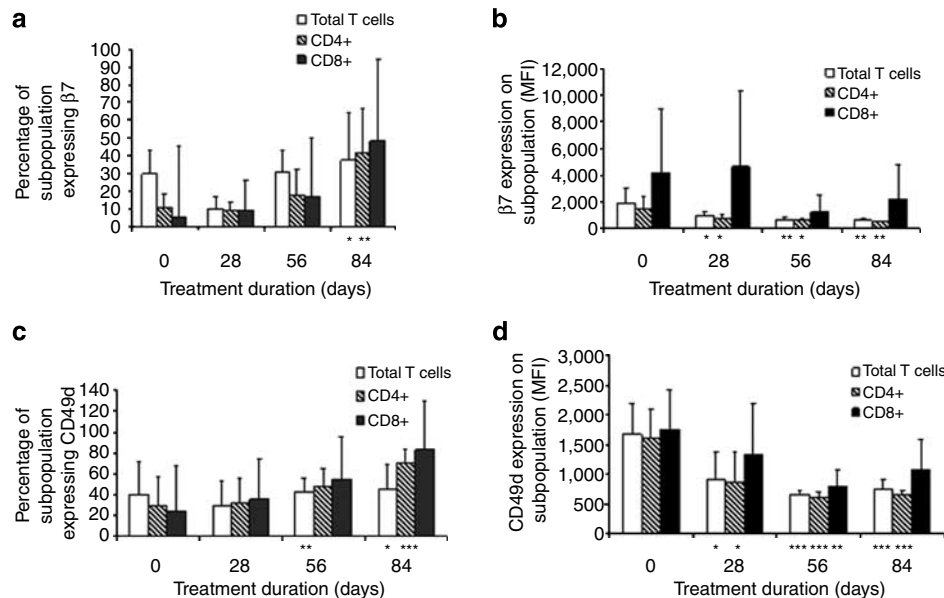


Figure 5. Efalizumab therapy increases the percentage of circulating T cells that express $\beta 7$ /CD49d, a gut-homing marker. (a) The percentages of T cells, CD4+ T cells, and CD8+ T cells in the blood expressing $\beta 7$ during the course of efalizumab treatment were determined on the indicated days and were found to be significantly elevated when compared to patient baseline levels. (b) Surface protein expression levels of $\beta 7$ on total circulating T cells, CD4+ T cells, and CD8+ T cells were determined on the indicated days of efalizumab treatment and were found to be decreased when compared to baseline levels, although decreased $\beta 7$ integrin surface protein expression on CD8+ T cells was not statistically significant. (c) The percentages of T cells, CD4+ T cells, and CD8+ T cells in the blood expressing CD49d during the course of efalizumab treatment were determined on the indicated days and were found to be significantly elevated when compared to patient baseline levels. (d) Surface protein expression levels of CD49d on total circulating T cells, CD4+ T cells, and CD8+ T cells were determined on the indicated days of efalizumab treatment and were found to be decreased when compared to baseline levels, although decreased CD49d surface protein expression on CD8+ T cells was only significant on day 84.

Efalizumab therapy increases CD44 surface expression on circulating T cells

CD44 is an adhesion receptor that is upregulated, when compared to naive T cells, on the surface of memory T cells. In this study, essentially all circulating T cells expressed CD44, and there was no effect on this percentage as a result of treatment (data not shown). By contrast, CD44 surface expression level on total T cells markedly increased by 90.7% ($P < 0.0003$), 81.4% ($P < 0.0001$), and 104.9% ($P < 0.00002$) on days 28, 56, and 84, respectively, of efalizumab therapy (Figure 6). Similarly, CD44 levels on CD4+ T increased by 102.7% ($P < 0.0002$), 89.3% ($P < 0.00002$), and 113.6% ($P < 0.00007$) on days 28, 56, and 84, respectively, post-initiation of treatment. Finally, CD44 MFI on CD8+ T cells increased by 75.5% ($P < 0.0003$), 80.67% ($P < 0.0001$), and 95.7% ($P < 0.00004$) on days 28, 56, and 84, respectively, when compared to baseline.

Minor, but not significant, decreases in the percentages of stimulated T cells that produce IFN- γ during efalizumab therapy

Efalizumab can inhibit full T-cell activation *in vitro* by blocking the co-stimulatory pairing of CD54 on antigen-presenting cells and CD11a on T cells (Joshi *et al.*, 2006). To determine whether efalizumab therapy in humans functioned in this manner, we examined the ability of stimulated 1-day-cultured peripheral blood mononuclear cells (PBMCs), isolated from our efalizumab-treated patients, to produce either IFN- γ or IL-4. Over the course of therapy, we noted slight, but not statistically significant, decreases in the percentages of both CD4+ and CD8+ T cells that produced IFN- γ (Figure 7). There was a slight decrease in the percentage of CD4+ T cells that produced IL-4 and a slight increase in the percentage of CD8+ T cells that produced IL-4, although these observed changes in cytokine production were also not statistically significant.

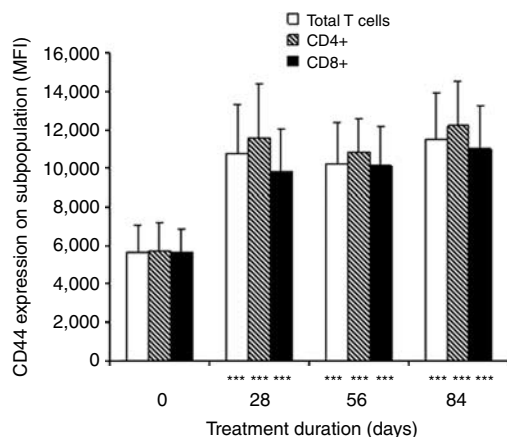


Figure 6. Efalizumab therapy increases CD44 surface expression on circulating T cells. CD44 surface protein expression levels on total circulating T cells, CD4+ T cells, and CD8+ T cells were determined on the indicated days of efalizumab treatment. Expression levels of CD44 were significantly increased on all T-cell populations and on all days when compared to baseline levels.

DISCUSSION

In this report, we found that treatment of severe atopic dermatitis patients with efalizumab, a humanized mAb directed against CD11a, led to striking immunologic changes in circulating T-cell populations. Details on the clinical efficacy seen in our patients have been reported (Takiguchi *et al.*, 2007). Examination of the peripheral T-cell compartment by eight-color flow cytometry during the course of therapy revealed decreases in CD11a, the drug's target molecule, and CD18, the heterodimeric partner of CD11a, on all T-cell subsets (Figures 2 and 3). These data are consistent with internalization of cell-surface CD11a/CD18 heterodimers and are consistent with previous reports that examined efalizumab's mechanism of action in patients with psoriasis (Gottlieb *et al.*, 2002; Vugmeyster *et al.*, 2004; Mortensen *et al.*, 2005). It is also possible that those cells expressing high levels of CD11a bind increased amounts of efalizumab in the blood and are cleared more efficiently from the blood stream, resulting in the selection of a T-cell population that expresses lower levels of CD11a. Downregulation of both CD11a and CD18 correlated with individual clinical responses to therapy.

We also documented dramatic increases in percentages of circulating memory T cells, especially effector memory CD4+ T cells, that expressed tissue homing markers (CLA, $\beta 7$, and CD49d) during efalizumab treatment (Figures 4 and 5). The cellular infiltrate in the skin of atopic dermatitis

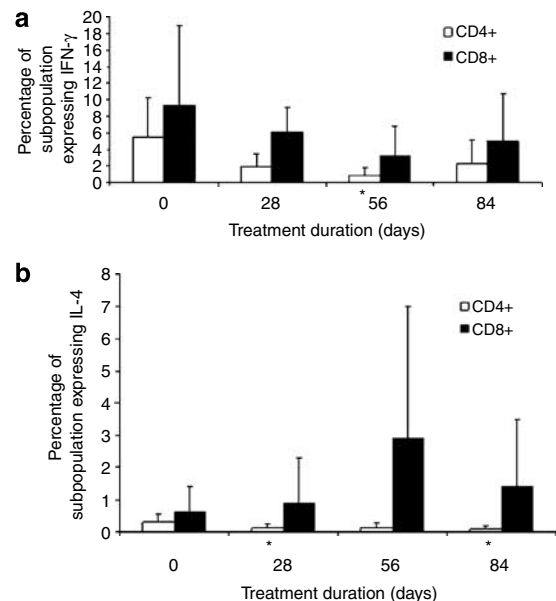


Figure 7. Minor, but not significant, decreases in the percentages of stimulated 1-day-cultured blood-derived T cells that produce IFN- γ during efalizumab therapy. (a) The percentages of cells expressing IFN- γ were determined through intracellular staining of 1-day-cultured T cells on the indicated days and found to be slightly decreased during efalizumab therapy, although differences were not statistically significant. (b) The percentages of cells expressing IL-4 were determined through intracellular staining of 1-day-cultured T cells on the indicated days and found to be slightly increased during efalizumab therapy, although differences were not statistically significant.

patients consists primarily of CD4⁺ memory T cells, and thus it is not unexpected that the number of these T cells would increase in blood in concert with healing of skin lesions in this disease (Wakita *et al.*, 1994; Watanabe *et al.*, 1994). Of these data, perhaps our most striking finding was that percentages of circulating T cells that expressed CLA, a predominant skin-homing marker, increased fourfold during treatment with efalizumab (Figure 4). It has been reported that increased numbers of CLA⁺ T cells are present in atopic dermatitis lesions when compared to non-lesional skin or skin from healthy individuals (de Vries *et al.*, 1997). In addition, there are increased numbers of CLA⁺ T cells in the blood of patients with untreated atopic dermatitis, and a higher percentage of these CLA⁺ T cells express $\beta 7$, CD49d, and CD11a compared to healthy controls (Seneviratne *et al.*, 2007). Although we did not examine lesional skin in our study, a recent report showed that T cells are markedly reduced in previously affected skin following successful treatment of atopic dermatitis with efalizumab (Hassan *et al.*, 2007). Taken together, we believe there are two likely mechanisms involved in the marked accumulation of CLA⁺ T cells in the blood of our patients: (i) T cells could not enter into the skin due to impairment of CD11a function and (ii) CLA⁺ T cells present in inflamed atopic dermatitis skin before therapy returned to the circulation as skin disease improved during therapy. Further study will be required to define the mechanism regulating CLA⁺ T cell accumulation in the blood.

CLA⁺ T cells accumulating during efalizumab therapy may be capable of entering into cutaneous tissue upon cessation of treatment and relief of the inhibition of T-cell trafficking. Indeed, our data regarding increased CLA⁺ T cells in the blood of efalizumab-treated patients likely explain skin disease flares observed in 10–15% of psoriasis patients upon discontinuation of this drug (Leonardi, 2004). This rebound phenomenon was observed in one patient in this study (Takiguchi *et al.*, 2007). Combination drug treatment at the time efalizumab is discontinued (e.g., by adding cyclosporine or methotrexate for a brief period of time) may prevent disease flare and is now often practiced by clinicians. In addition, combination therapy with efalizumab and a drug that induces T-cell apoptosis, such as alefacept (Vaishnaw and TenHoor, 2002; Krueger and Callis, 2003), a tumor necrosis factor- α blocker, or even photopheresis may effectively synergize to kill circulating CLA⁺ T cells that are elevated by efalizumab therapy. If this were the case, efficacy in treating skin disease would be expected to be much greater when two types of treatment with differing mechanisms of action are combined when compared to efficacy achieved with either drug type alone. Of interest, several cases describe successful combination use of efalizumab and tumor necrosis factor- α blockers for patients with severe psoriasis (Lowe *et al.*, 2005).

Increased numbers of $\beta 7$ -positive T cells are also present in atopic dermatitis lesions when compared to non-lesional skin or skin from healthy individuals (de Vries *et al.*, 1997). A significant gut-homing integrin receptor is formed by heterodimerization of $\beta 7$ with CD49d (Hamann *et al.*, 1994). We

observed increased percentages of both $\beta 7$ - and CD49d-expressing T cells in the blood of our patients, whereas the surface protein expression levels of both proteins were decreased. The accumulation of both $\beta 7$ - and CD49d-positive circulating T cells suggests that efalizumab may also act to disrupt T-cell trafficking to mucosal tissue. Of interest, our findings that efalizumab increases percentages of $\beta 7$ and CD49d⁺ gut-homing T cells (Figure 5) and decreases T-cell activation (Figure 7) suggests that this drug may indeed be effective for inflammatory bowel disease. Further study is necessary to determine whether other T-cell-mediated inflammatory conditions may also respond to efalizumab via similar immunologic mechanisms.

$\beta 7$ can also bind with CD103, forming a receptor that interacts with E-cadherin, which is expressed on keratinocytes. The $\beta 7$ /CD103 heterodimer functions to retain T cells within epidermis (Karecla *et al.*, 1996). It has been reported that $\beta 7$ /CD103⁺ T cells accumulate in psoriasis lesions and that $\beta 7$ /CD103 appears to be involved in T-cell recruitment and retention in inflamed skin (Pauls *et al.*, 2001; Rottman *et al.*, 2001). A reduction in T-cell $\beta 7$ /CD103 surface protein expression levels in atopic dermatitis patients may facilitate T-cell migration from the tissue, thereby promoting emigration of resident T cells from the skin and further contributing to the accumulation of $\beta 7$ ⁺ T cells in the blood.

A significant increase in surface expression of CD44, the receptor for hyaluronan, was also observed on circulating T cells during efalizumab therapy. CD44 expressed by endothelial cells binds hyaluronan, which in turn is bound by CD44 expressed by skin-infiltrating T cells (Mohammadzadeh *et al.*, 1998). CD44–hyaluronan interactions allow T cells to anchor to the vascular endothelium, facilitating extravasation (Mohammadzadeh *et al.*, 1998). CD44 surface protein expression is increased on T cells during disease progression in an IL-4 transgenic mouse model for atopic dermatitis (Chen *et al.*, 2005), and CD44 is required for cutaneous T-cell infiltration in a mouse model for atopic dermatitis (Gonda *et al.*, 2005). Thus, we postulate that increased CD44 surface expression of levels on circulating T cells in our patients reflects blockade of effector memory T-cell infiltration into skin, with subsequent accumulation of these cells within the blood.

Efalizumab is theoretically capable of decreasing cutaneous inflammation through several mechanisms: decreased T-cell trafficking from blood into skin through interruption of LFA-1/ICAM-1 interactions, decreased T-cell activation by blocking co-stimulatory signaling between T cells and antigen-presenting cells, decreased naive T-cell trafficking into lymph nodes, and increased clearance of antibody-coated T cells. Our data suggest that inhibition of blood-to-tissue trafficking is the dominant mode of action for this drug in the treatment of patients with atopic dermatitis. Although T cells produced less IFN- γ during efalizumab therapy, indicating T-cell activation levels were dampened, these changes were not statistically significant. Impaired trafficking of naive T cells into lymph nodes likely did not occur in our patients, as populations of memory, not naive, T cells were elevated with treatment. In summary, our findings likely

reflect the fact that efalizumab produces dramatic blockade of memory CD4⁺ T-cell extravasation from blood into skin as well as other tissues, suggesting that efalizumab may be clinically useful for a variety of both cutaneous and non-cutaneous T-cell-mediated diseases.

MATERIALS AND METHODS

Patients and sample collection

The Oregon Health & Science University Institutional Review Board approved all aspects of this study according to the Declaration of Helsinki Principles. After giving informed consent, 10 patients were enrolled in a 12-week open-label study to determine the efficacy and safety of efalizumab in treating moderate-to-severe atopic dermatitis (Takiguchi *et al.*, 2007). All subjects received an initial conditioning dose of 0.7 mg kg⁻¹ of efalizumab at day 0 and doses of 1 mg kg⁻¹ weekly, beginning on day 7 and ending on day 77. Patients were assigned an EASI score. The EASI scores for each patient were determined weekly, and changes in EASI score determined clinical improvement in response to efalizumab treatment. A quantity of 15 ml of blood was collected on days 0, 28, 56, and 84 before the weekly administration of efalizumab, and PBMCs were isolated from blood using Ficoll-hypaque (Amersham, Piscataway, NJ) gradient separation.

Reagents

The following mouse anti-human mAbs and corresponding isotype control Abs were purchased from BD Biosciences (San Jose, CA) unless otherwise noted: unconjugated CD3 (clone: UCHT1), phycoerythrin (PE)-Cy7-conjugated CD4, PE-Cy5.5-conjugated CD8, Pacific Blue-conjugated CD45RA (Caltag, Burlingame, CA), APC-conjugated CD62L, APC-Cy7-conjugated CD27 (Ebioscience, San Diego, CA), FITC-conjugated CD11a (clone HI111 recognizes an epitope distinct from that recognized by efalizumab), CD18, CD44 (Beckman Coulter, Fullerton, CA), CLA, IFN- γ , and PE-conjugated CD11b, CD11c, CD49d, β 7, and IL-4.

Immunostaining and flow cytometry on freshly isolated PBMCs

Freshly isolated PBMCs were suspended in staining buffer (10% fetal bovine serum solution in PBS) and aliquots of 1×10^6 cells were placed in five separate tubes. All mAbs described below were used at a final concentration of 10 μ g ml⁻¹. Four samples were incubated with an anti-CD3 mAb for 30 minutes, washed twice with staining buffer, and incubated with an Alexa-Fluor 430 conjugated goat anti-mouse secondary Ab (Invitrogen, Carlsbad, CA) for 30 minutes at 4°C. Cells were then washed twice with staining buffer and incubated with 10 μ g of whole mouse IgG to block any unbound anti-mouse secondary Ab. Directly conjugated Abs (CD4 PE-Cy7, CD8 PE-Cy5.5, CD45RA Pacific Blue, CD62L APC, CD27 APC-Cy7) were then added to each of the four samples. Next, CD11a FITC and CD11b PE were added to one sample, CD18 FITC and CD11c PE were added to the second sample, CD44 FITC and CD49d PE were added to the third sample, and CLA FITC and β 7 PE were added to the fourth sample. The final tube of cells was incubated with a mixture of fluorophore-conjugated isotype control Abs. Cells were incubated with these combinations of directly conjugated mAbs for 30 minutes, washed twice with staining buffer, and resuspended in staining buffer for flow cytometric analysis using an LSRII and FACS Diva software (BD Biosciences, San Jose, CA). One hundred

thousand total events were collected and analyzed from each sample.

Immunostaining and flow cytometry on cultured PBMCs

An aliquot of freshly isolated PBMCs from each patient on each day was washed twice in sterile PBS. PBMCs were then plated into 12-well culture dishes coated with or without anti-CD3 mAbs containing Roswell's Park Memorial Institute with 10% fetal bovine serum, 100 μ g ml⁻¹ streptomycin, and 100 U ml⁻¹ penicillin (Gibco, Grand Island, NY). Recombinant human interleukin-2 (R&D Systems, Minneapolis, MN) and anti-CD28/CD49d mAbs (BD Biosciences, San Jose, CA) were added to cells incubated with anti-CD3 mAbs to provide co-stimulatory signals. After 20 hours of culture, both unstimulated and stimulated PBMC cultures were treated with a 1,000-fold dilution of Golgi Plug containing brefeldin A (BD Biosciences) and incubated for an additional 4 hours to allow for intracellular cytokine accumulation. PBMCs were then centrifuged, washed twice, and resuspended in staining buffer. Cells were stained for surface antigen expression, as described above, using mouse anti-human CD3, CD4, CD8 CD62L, CD45RA, and CD27 mAbs. PBMCs were then washed twice, fixed and permeabilized using the Cytofix/Cytoperm kit (BD Bioscience), and stained with mouse anti-human IFN- γ and mouse anti-human IL-4. Samples were analyzed by flow cytometry as described above.

Definitions of T-cell subsets and statistical analyses

T-cell populations analyzed by flow cytometry were initially gated into either CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells. We defined CD45RA⁺CD27⁺CD62L⁺ cells as naive T cells, CD45RA⁺CD27⁺CD62L⁺ cells as central memory T cells, and CD45RA⁺CD27⁺CD62L⁺ cells as effector memory T cells on the basis of previous classifications (Butcher and Picker, 1996; Kupper and Fuhlbrigge, 2004). For each antigen (CD11a, CD18, CD44, CD49d, CLA, and β 7) and for each of the six T-cell subsets (naive CD4⁺, naive CD8⁺, central memory CD4⁺, central memory CD8⁺, effector memory CD4⁺, and effector memory CD8⁺) and at each time point (days 0, 28, 56, and 84), the percentage of positive cells and the MFI were determined. Significant changes in antigen expression levels in response to efalizumab treatment were detected using a two-tailed Student's paired *t*-test. The percentage of T cells expressing each antigen and the antigen MFI values at baseline were compared to expression and percentage values obtained at days 28, 56, and 84. Pearson's correlation coefficients were calculated to determine whether a relationship existed between antigen expression levels during the course of efalizumab treatment and patient clinical scores.

CONFLICT OF INTEREST

This was an investigator-initiated study, with all clinical and laboratory study costs being provided by Genentech Corporation through a contract between Oregon Health & Science University and Genentech.

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