EFFECT OF EFALIZUMAB ON NEUTROPHIL AND MONOCYTE FUNCTIONS IN PATIENTS WITH PSORIASIS

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We evaluated the effect of efalizumab on neutrophil and monocyte functions. The *in vitro* pre-incubation with efalizumab concentrations similar to those reached during *in vivo* therapy almost completely saturated CD11a binding sites without affecting the membrane expression of CD11b, CD128a or CD128b. There was a significant reduction in the chemotactic activity of the pre-treated cells toward three different chemo-attractants, whereas their phagocytic capacity and production of oxygen radicals remained unchanged. One month after the administration of efalizumab to five patients with psoriasis (T1) circulating neutrophil counts increased by 34% from pre-therapy (T0) with no change in the number of monocytes. In the same patients the CD11a binding sites on phagocytes were >90% saturated, and there was also a significant down-modulation on neutrophils (44% of T0) and monocytes (63% of T0). In line with *in vitro* results, efalizumab treatment caused a significant deficiency in the chemotactic properties of neutrophils and monocytes, but no changes in phagocytosis, oxidative burst, production of pro-inflammatory cytokines or the membrane expression of CD11b, CD128a and CD128b. Our findings suggest that neutrophils and monocytes may be among the targets of efalizumab activity in patients with psoriasis.

Psoriasis is a chronic, immune-mediated inflammatory disease of the dermis and epidermis that is characterized by an abundance of T cells, increased vascularity and high concentrations of tumour necrosis factor-α (TNF-α) in skin lesions (1). As most of the currently available phototherapies and systemic treatments (i.e. methotrexate and cyclosporin) for moderate-to-severe psoriasis are ineffective in some patients, and have potentially serious side-effects in others that complicate long-term disease management (2), various treatments with improved safety profiles have been proposed.

Better knowledge of the pathogenesis of the disease has suggested some immunological targets such as T-lymphocytes and inflammatory cytokines (3), and a number of biological agents have been approved by the US Food and Drug Administration for moderate-to-severe plaque psoriasis. These include alefacept, a fully human recombinant leukocyte function-associated antigen-3 (LFA-3)/IgG1 fusion protein that inhibits T cell activation and selectively reduces memory T cells (4); infliximab and etanercept, which are TNF-α antagonists (5-6); and efalizumab, a humanized monoclonal antibody (mAb) that binds to the alpha subunit (CD11a) of LFA-1, and inhibits the homing and activation of T

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cells (7).

The efficacy and safety of efalizumab have been demonstrated in many clinical trials (7-9), and the results of the European experience with efalizumab in routine clinical practice have recently been published (10). Its mechanism of action is clearly related to its inhibition of the activation, adhesion and trafficking of T cells (7), but it may also bind to other LFA-1 positive cells such as neutrophils and monocytes (11), which play an important role in maintaining and amplifying T cell-associated inflammation in psoriatic plaques (12-13).

To the best of our knowledge, there are no published data concerning the effect of efalizumab on phagocytic cell function. We therefore evaluated certain phenotypical and functional aspects of neutrophils and monocytes after their *in vitro* pre-incubation with different mAb concentrations, and then repeated the same evaluations using phagocytic cells obtained from psoriatic patients before and after treatment with efalizumab for 1 month. Chemotaxis, phagocytosis, and the production of reactive oxygen intermediates (ROI) and inflammatory cytokines were assessed in peripheral blood neutrophils and monocytes, as was the membrane expression of CD11a, CD11b (the alpha subunit of complement receptor type 3), CD128a/CXCR1 and CD128b/CXCR2, the main interleukin-8 (IL-8) receptors on phagocytes.

MATERIALS AND METHODS

In vitro study

The effect of efalizumab activity on phagocytic cell functions was evaluated *in vitro* using peripheral blood neutrophils and monocytes obtained from five healthy volunteers. The purified cells (see below) were incubated with different drug concentrations (see Figures and Tables) for 30 minutes at 4°C, washed and immediately used in the assays; the final concentrations used *in vitro* were similar to those obtained *in vivo* at steady-state after four weekly doses of efalizumab (1 mg/kg/week) (11).

Ex vivo study

Peripheral blood samples were collected from five selected patients (four men and one woman; mean age 42 years, range 30–45 years) with active psoriasis (Psoriasis Area and Severity Index score >10) (14). All of the patients had failed to respond to, were intolerant of, or had contraindications against at least two systemic therapies, including methotrexate, cyclosporin A, acitretine and psoralen combined with ultraviolet light A, and all gave their written informed consent to participate in the study. The controls were ten healthy age- and gender-matched individuals.

Efalizumab was administered subcutaneously every week. After a first conditioning dose of 0.7 mg/kg, the drug was administered at a dose of 1 mg/kg. Peripheral blood samples were obtained before treatment (T0) and 1 month after the start of treatment (T1).

Peripheral blood neutrophils were obtained by means of density gradient centrifugation (Lymphoprep; Nyegaard, Oslo, Norway) (15). The purified cells consisted of a population of >95% pure, viable neutrophils, as assessed on the basis of morphology and trypan blue exclusion. Peripheral blood monocytes were purified on a Nycoprep 1.068 gradient (Axis-Shield; Oslo, Norway) as described in detail by Boyum (16), which routinely yielded 85–90% monocytes as assessed by Wright staining, non-specific esterase staining and, in some cases, immunofluorescent staining for CD14; viability was >95% by trypan blue exclusion.

Neutrophil and monocyte chemotaxis were evaluated using a modified Boyden chamber assay with blind well chambers and 3 μm (for neutrophils) or 5 μm (for monocytes) micropore filters (Millipore; Bedford, MA, USA) (17). The chemotactic response was determined by evaluating the number of cells per high power field (hpf) that had migrated through the entire thickness of the filter. The chemo-attractants were zymosan-activated serum (ZAS) as a source of C5a (1 mg zymosan/ml serum for 30 minutes at 37°C) at a 1% (vol/vol) final dilution in RPMI 1640 (HyClone Laboratories, Logan, UT, USA); the synthetic peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma) at a final concentration of 10^{-8} M; and IL-8 (Biosource, Camarillo, CA, USA) at a final concentration of 100 ng/ml.

Phagocytosis was evaluated using C3-coated zymosan (C3Zy) as particles for uptake (18). The number of particles ingested per cell (phagocytic index) was established by means of direct light microscopy (x1000 magnification) on at least 200 cells.

Lucigenin (bis-*N*-methylacridinum nitrate; Sigma)-amplified chemiluminescence (CL) was used to evaluate the phagocyte production of ROI (19). A Luminometer 1251 (LKB, Wallac, Turku, Finland) was used to record the background of the light output (in mV) before to the addition of the stimuli: phorbol 12-myristate 13-acetate (PMA; final concentration 5 ng/ml; Sigma) or FMLP (10^{-7} M final concentration) The background counts were subtracted from the values obtained after neutrophil stimulation.

Flow cytometry of purified phagocytes (*in vitro* ex-

Table I. *Effect of efalizumab in vitro on the production of chemiluminescence (CL) and on the phagocytic capacity of neutrophils and monocytes.*

	Efalizumab (μg/ml)			
Test	**0**	**1**	**10**	**100**
PMA-stimulated CL[a]				
Neutrophils	48.7 ± 0.4	42.9 ± 2.1	40.9 ± 2.1	40.4 ± 2.4
Monocytes	12.2 ± 1.6	12.0 ± 2.3	10.5 ± 1.4	11.3 ± 1.5
FMLP-stimulated CL[a]				
Neutrophils	19.2 ± 0.3	18.0 ± 0.3	18.0 ± 0.4	20.2 ± 0.4
Monocytes	2.2 ± 0.3	1.8 ± 0.3	2.1 ± 0.5	2.7 ± 0.5
Phagocytosis[b]				
Neutrophils	1.03 ± 0.03	0.94 ± 0.13	1.00 ± 0.16	1.07 ± 0.05
Monocytes	1.19 ± 0.03	1.20 ± 0.06	1.23 ± 0.03	1.19 ± 0.08

[a]Peak CL values in mV; [b]Phagocytic index
FMLP, N-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol 12-myristate 13-acetate
The cells were preincubated with different concentrations of efalizumab before being tested for chemiluminescence and phagocytosis. Results are means ± standard error of three separate experiments.

periments) (18) or whole blood samples (*ex vivo* studies) (20) was used to determine the membrane expression of CD11b, CD128a and CD128b; specific fluorescent-conjugated mAbs (Becton Dickinson, San Jose, CA, USA) were used in these assays. Neutrophil and monocyte CD11a expression was determined by FACS using two fluorescence-labelled anti-CD11a antibody clones: clone MHM24 (Dako Corporation, Carpinteria, CA, USA) binds to an epitope similar to that used by efalizumab and therefore only to free CD11a (i.e. not bound by efalizumab) (11), whereas clone HI111 (Becton Dickinson) binds to a different CD11a epitope remote from the efalizumab binding site, and therefore binds to all of the CD11a on a cell regardless of whether or not it is also bound by efalizumab (11). The neutrophil and monocyte populations were identified on the basis of their forward- and side-scatter characteristics. A relative measure of antigen expression was obtained using mean fluorescence intensity (MFI), converted from a log to a linear scale, after subtracting the cells' self-fluorescence and the fluorescence of cells incubated with irrelevant isotype control mAbs.

Production of cytokines in vitro

The spontaneous and lipopolysaccharide (LPS)-induced production of TNF-α, IL1-β and IL-8 by leukocytes was evaluated on whole blood samples (21) obtained from the controls and from patients with psoriasis at T0 and T1. The samples (100 μl, diluted 1/10) were incubated at 37°C for 18 hours in humidified atmosphere (5% CO_2) with or without LPS from *Escherichia coli* 055:B5, (Sigma) 10 ng/ml and the cytokine levels were determined in cell-free supernatants using a specific ELISA (Biotrak, Amersham, UK).

Statistical analysis

The data are expressed as mean values ± standard error of the mean (SE), and were statistically analysed using Student's t test for unpaired or paired data as appropriate. A p value of <0.05 was considered statistically significant.

RESULTS

In vitro study

As shown in Fig. 1A, pre-incubation of the neutrophils and monocytes with efalizumab led to dose-dependent saturation of the CD11a binding sites, as

Table II. *Leukocyte and leukocyte subpopulations (cells/μl), chemiluminescence (CL), phagocytosis and cytokine production by controls and patients with psoriasis before treatment (T0) and after treatment with efalizumab for 1 month (T1).*

	Controls	Patients (T0)	Patients (T1)
WBC (range)	6170 (4000–9000)	5740 (4200–6900)	7700 (6200–8900)
Lymphocytes (range)	2000 (1400–2800)	1650 (990–2300)	2650 (2100–3600)
Neutrophils (range)	3380 (2300–4500)	3450 (2300–3900)	4000 (3200–5000)
Monocytes (range)	526 (330–780)	600 (480–820)	610 (470–750)
PMA-stimulated CL[a] (mean ± SE)			
Neutrophils	50.2 ± 6.0	57.7 ± 10.7	50.6 ± 2.64
Monocytes	10.9 ± 0.8	11.9 ± 1.7	10.3 ± 0.9
FMLP-stimulated CL[a] (mean ± SE)			
Neutrophils	18.8 ± 3.6	27.7 ± 3.4	17.5 ± 1.9
Monocytes	2.5 ± 0.4	2.7 ± 0.1	2.7 ± 0.2
Phagocytosis[b] (mean ± SE)			
Neutrophils	1.06 ± 0.09	1.05 ± 0.04	0.99 ± 0.04
Monocytes	1.23 ± 0.21	1.34 ± 0.32	1.29 ± 0.25
LPS-induced cytokines[c] (mean ± SE)			
IL-1β	232.3 ± 105.8	250.3 ± 63.9	423.5 ± 113.8
IL-8	96.6 ± 17.8	96.3 ± 29.3	98.8 ± 13.5
TNF-α	1.93 ± 0.2	2.51 ± 1.05	3.36 ± 0.82

[a]Peak CL values in mV; [b]Phagocytic index; [c]pg/ml/10[5] leukocytes.
FMLP, N-formyl-methionyl-leucyl-phenylalanine; IL, interleukin; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; TNF- α, tumour necrosis factor- α; WBC, white blood cell

demonstrated by the inhibition of mAb MHM24 binding; nearly complete saturation was obtained with an efalizumab concentration of 10 μg/ml, which is similar to that obtained *in vivo* at steady-state after four weekly drug doses (1 mg/kg/week).

There were no changes in the binding of the anti-CD11a mAb HI111 (Fig. 1B) or the anti-CD11b, CD128a and CD128b mAbs (not shown).

Efalizumab had no effect on neutrophil or monocyte chemiluminescence production (spontaneous or induced by PMA or FMLP) or phagocytic capacity (Table I), but it did lead to a dose-dependent inhibition of neutrophil chemotactic activity in response to the three chemo-attractants (only a downward trend in the case of monocytes) (Fig. 2A and B).

Ex vivo study

The number of circulating neutrophils and mono-

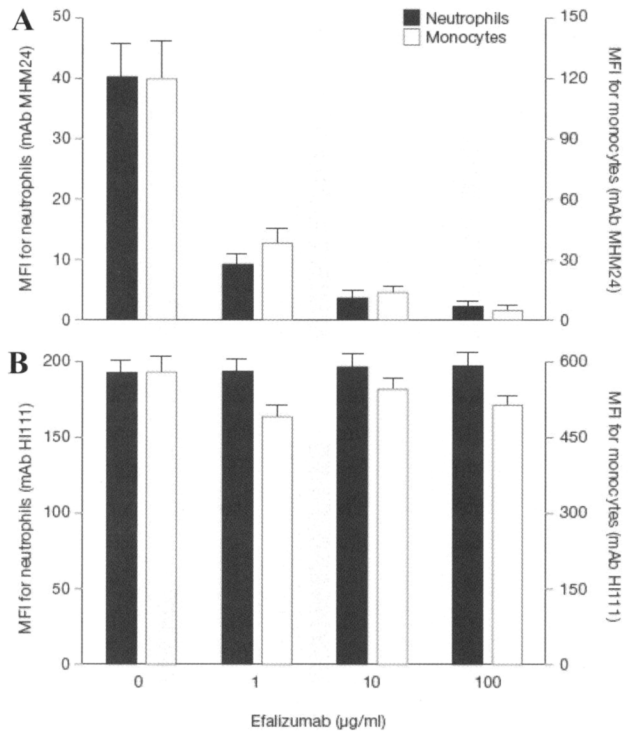

Fig. 1. *Effect of efalizumab in vitro on neutrophil and monocyte CD11a binding sites. The cells were pre-incubated with different concentrations of efalizumab before being stained with the fluorescein 5-isothiocyanate-conjugated anti-CD11a mAb MHM24* ***(A)*** *or the phycoerythrin-conjugated anti-CD11a mAb HI111* ***(B)****. The results are expressed as mean fluorescence intensity (MFI) ± standard error, corrected for non-specific staining from three separate experiments.*

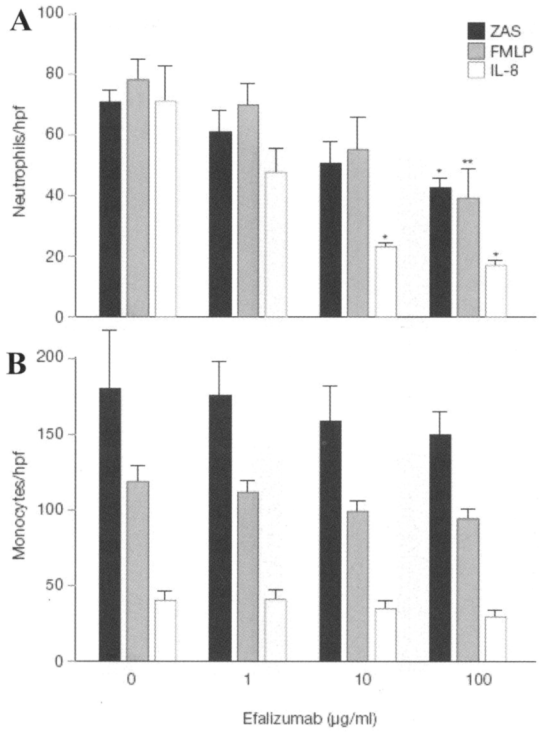

Fig. 2. *Effect of efalizumab in vitro on neutrophil and monocyte chemotaxis. Purified neutrophils* ***(A)*** *and monocytes* ***(B)*** *were pre-incubated with different concentrations of efalizumab before being assayed for migration versus three different chemo-attractants: Zymosan-activated serum (ZAS), N-formyl-methionyl-leucyl-phenylalanine (FMLP) and interleukin-8 (IL-8). The values represent the mean number of migrated cells per high power field (hpf) ± standard error from three separate experiments.*
**p<0.01; **p<0.05 versus medium.*

cytes, their functional activities and the membrane expression of CD11a, CD11b, CD128a and CD128b were evaluated in blood samples taken from five patients with severe psoriasis before treatment (T0) and 1 month after the start of therapy with efalizumab (T1).

There were no differences in total or differential leukocyte counts between the patients and controls at T0 but, as expected, mean circulating white blood cell levels increased by approximately 34% after 1 month of therapy (Table II). Circulating lymphocytes increased by 60% and circulating neutrophils by 34%; there was no change in the number of monocytes (Table II).

Analysis of neutrophil and monocyte membrane antigen expression (CD11a, CD11b, CD128a and CD128b) by FACS did not reveal any differences between patients and controls (Fig. 3A and B for CD11a; not shown for other antigens). After treatment with efalizumab for 4 weeks, the MHM24 mAb showed that the CD11a binding sites on both neutrophils (Fig. 3A) and monocytes (Fig. 3B) were 90% saturated, and the use of the HI111 anti-CD11a mAb, which binds to all CD11a binding sites regardless of efalizumab binding, showed a significant decrease in CD11a expression (neutrophils 44% of T0; monocytes 63% of T0) (Fig. 3A and B); there was no change in the circulating phagocyte membrane expression of CD11b, CD128a or CD128b (not shown).

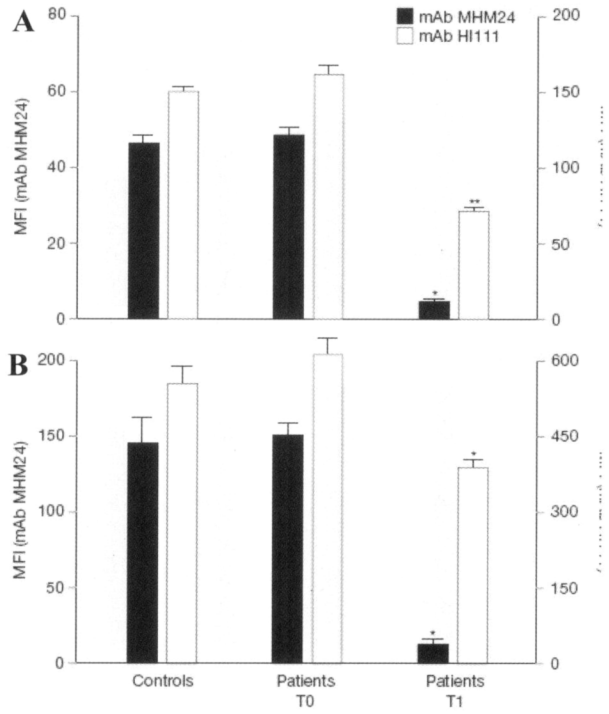

Fig. 3. *Effect of efalizumab therapy on CD11a expression on the membranes of circulating neutrophils* ***(A)*** *and monocytes* ***(B)****. Whole blood samples were obtained from ten controls and five patients with psoriasis before (T0) and after therapy with efalizumab for 1 month (T1), and the neutrophils and monocytes were stained with the fluorescein 5-isothiocyanate-conjugated anti-CD11a mAb MHM24 or the phycoerythrin-conjugated anti-CD11a mAb HI111. The results are expressed as mean fluorescence intensity (MFI) ± standard error, corrected for non-specific staining.*
**p<0.001, T1 versus T0 ; **p<0.02, T1 versus T0.*

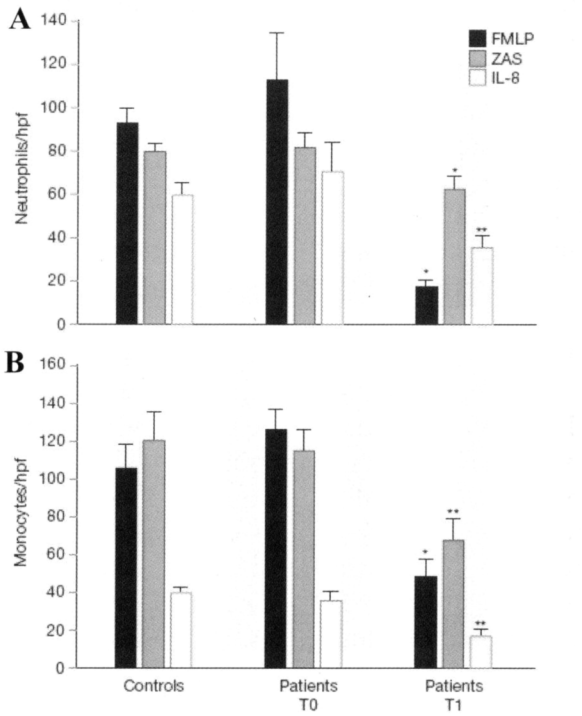

Fig. 4. *Effect of efalizumab on neutrophil and monocyte chemotaxis. Peripheral blood phagocytes were purified from ten controls and five patients with psoriasis before (T0) and after therapy with efalizumab for 1 month (T1). Mean values ± standard error of the number of migrated neutrophils* ***(A)*** *or monocytes* ***(B)*** *per high power field (hpf) using different chemo-attractants: zymosan-activated serum (ZAS), N-formyl-methionyl-leucyl-phenylalanine (FMLP) (10^{-8} M) and interleukin-8 (IL-8) (100 ng/ml).*
**p<0.001, T1 versus T0; **p<0.05, T1 versus T0*

There was no between-group difference in the capacity of neutrophils and monocytes to produce reactive oxygen intermediates, ingest particles (Table II) or migrate toward a chemo-attractant at T0 (Fig. 4). Treatment with efalizumab for 1 month led to no change in chemiluminescence production or phagocytosis (Table 2) but, in line with our *in vitro* results, both neutrophil and monocyte chemotactic activity was significantly inhibited (Fig. 4A and B) in relation to all three chemo-attractants: respectively, 80% and 54% inhibition for FMLP, 41% and 57% for IL-8, and 22% and 43% for ZAS.

Finally, there was no difference in baseline spontaneous (not shown) or LPS-induced production of TNF-α, IL-1β and IL-8 by leukocytes (Table II) between patients with psoriasis (T0 and T1) and controls, even if an upward trend for IL-1β and TNF-α production by the patient's leukocytes was observed at T1.

DISCUSSION

In the present work we found that a concentration of efalizumab similar to that obtained *in vivo* at therapeutic doses (1–10 μg/ml) (11) almost completely saturates CD11a receptors on phagocytes without interfering with the membrane expression of CD11b, CD128a or CD128b. Furthermore, efali-

zumab caused a dose-dependent inhibition of the chemotactic capacity of phagocytes toward three chemo-attractants without modifying phagocytosis or the production of ROI.

In comparison with control cells, there was no significant difference in membrane antigen expression or the functional properties of phagocytes obtained from patients with psoriasis before efalizumab treatment.

In line with our *in vitro* results and previous *ex vivo* studies (11), treatment with efalizumab for 1 month not only saturated CD11a receptors but also significantly down-modulated their membrane expression on both neutrophils (44% of T0) and monocytes (63% of T0). The number of circulating neutrophils increased by 34% between T0 and T1, but there was no change in the number of monocytes. The almost complete saturation and partial down-modulation of the CD11a binding sites did not change phagocytosis or phagocyte production of ROI but, in line with our *in vitro* results, therapy with efalizumab significantly inhibited the chemotactic capacity of both neutrophils and monocytes. This down-modulation of phagocyte chemotaxis has not been previously reported for efalizumab *in vitro* or *ex vivo*. Previous studies have shown that impairing LFA-1/ICAM interactions, using monoclonal antibodies to LFA-1 or LFA-1 knockout mice, suppresses neutrophil and monocyte migration in various models of inflammation (22-23); a similar effect may occur *in vivo* in patients with psoriasis following treatment with efalizumab.

Our *in vitro* and *ex vivo* results indicate that the saturation and/or down-modulation of CD11a by efalizumab inhibits phagocyte migration independently of the inhibition of LFA-1/ICAM-1 interactions. The mechanism underlying this inhibition is unclear, but seemed to be unrelated to chemotactic receptor expression as the defect was found with three different chemo-attractants and there was no change in membrane CD128a/CXCR1 or CD128b/CXCR2 expression, despite a significant defect in chemotaxis toward IL-8. The saturation of CD11a receptors by efalizumab may alter the induction of the migratory phenotype of phagocytes which, among other things, are characterized by LFA-1 redistribution to their leading edge (24).

There is increasing evidence that T cell dysregulation is involved in the development and maintenance of psoriasis (1-3), but the role of neutrophils and monocytes in its pathogenesis has also been described: phagocytes are required for the development of psoriatic plaques and represent an important pathogenic factor potentiating T cell-associated inflammation (12-13, 25-26).

Our data showing that efalizumab saturates CD11a receptors, down-modulates CD11a expression, and significantly inhibits the chemotactic properties of phagocytes may provide an insight into a new and efficient mechanism of action by means of which this biological drug controls plaque psoriasis.

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