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RESEARCH ARTICLE

Glutathione peroxidase activity in the blood cells of psoriatic patients correlates with their responsiveness to Efalizumab

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Abstract

Biological treatment of psoriasis, a chronic inflammatory immune-mediated pathology of huge social impact, has become a recent revolutionizing breakthrough in the management of the disease. Apart from anti-TNF-alpha biologics, recombinant proteins-inhibitors of the T lymphocytes-antigen presenting cells interaction, Efalizumab among them, have been successfully used in the therapy of psoriasis. Serious concern regarding safety and efficacy of biologics remains because they induce numerous adverse effects and a significant number of patients are non-responders. Up-to-now, there are no biochemical or/and immunological markers of the clinical efficacy of these drugs. This study searches for immunological and redox markers of the clinical response in the group of psoriatic patients treated with Efalizumab. Clinical response to Efalizumab was assessed by Psoriasis Area and Severity Index and correlated with suppression of T-cell functions, plasma cytokines, membrane-associated polyunsaturated fatty acids (PUFAs), antioxidant enzymes and markers of oxidative stress. A 12-week Efalizumab therapy did not affect abnormal plasma levels of pro-inflammatory cytokines and lower-than-normal content of PUFAs esterified in phospholipids of red cell membranes. It did, however, suppress T-cell-mediated functions and decrease nitrites/nitrates and malonyl dialdehyde levels independently on the clinical outcome. On contrast, activities of glutathione peroxidase (GPx) and glutathione S-transferase in granulocytes were remarkably increased and catalase decreased exclusively in non-responders vs complete or partial responders. High baseline GPx in erythrocytes decreased in responders. It is concluded that clinical response to Efalizumab correlates with GPx activity in the blood cells, suggesting that high hydroperoxide levels are involved in psoriasis persistence.

Keywords: *Catalase, cytokines, Efalizumab, glutathione peroxidase, glutathione-S-transferase, MDA, psoriasis, PUFAs*

Abbreviations: *AA, arachidonic acid; BrdU, 5-bromo-2'-deoxyuridine; CAT, catalase; CDNB, 1-chloro-2,4-dinitrobenzene; CL, chemiluminescence; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte macrophage colony stimulating factor; GPx, glutathione peroxidase; GST, glutathione-S-transferase; IFN-γ, interferon gamma; IL, interleukin; IP-10, interferon gamma-produced protein of 10 kDa; HNE, 4-hydroxy-2-nonenal; LFA-1, lymphocyte function-associated antigen 1; MCP-1, monocyte chemotactic protein 1; MDA, malonyl dialdehyde; MIP, macrophage inflammatory protein; PASI, psoriasis area and severity index; PBMC, peripheral blood mononuclear cells; PDGF, platelet-derived growth factor; PMA, phorbol 12-myristate 13-acetate; PMN, polymorphonuclear leukocytes; PUFAs, polyunsaturated fatty acids; ROS, reactive oxygen species; RNS, reactive nitrogen species; ROS, reactive oxygen species; RPMI, Roswell Park Memorial Institute (cell culture medium); RT-PCR, real time reverse transcript polymerase chain reaction; SDS, sodium dodecyl sulphate; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TNF-α, tumour necrosis factor alpha; VEGF, vascular endothelium growth factor.*

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Introduction

Psoriasis is a chronic relapsing skin disease with a strong social impact, involving 1–3% of the population worldwide [1]. Psoriasis can manifest in many ways, depending on disease activity, location and severity. The clinical sub-type by far more represented is psoriasis vulgaris (PV), with well demarcated, erythematous, scaly lesions commonly found on the elbows, knees, scalp, lower back and umbilicus. These lesions are characterized by hyper-proliferation of keratinocytes associated with an inflammatory infiltrate consisting mostly of interferon (IFN)-γ-producing (Th1) and IL-17/IL22-producing (Th17) helper T-cells [2]. A substantial proportion of patients with psoriasis develop a form of inflammation at the joints and associated connective tissue known as psoriatic arthritis. Recently, psoriasis has also been defined as a metabolic syndrome disease, due to characteristically impaired lipid metabolism, chronic generalized inflammation and increased risk of cardio-vascular pathologies associated with atherosclerosis, type II diabetes and obesity [3,4].

Along with the distinct features of immune-mediated pathology, active psoriasis displays many systemic and lesion-restricted signs of severe redox imbalance [5]. Thus, increased levels of malonyl dialdehyde (MDA) in the plasma and red blood cells were interpreted as the fingerprint of the exhaustion of natural enzymatic and non-enzymatic antioxidant defence and consequently the prevalence of deleterious peroxidative processes in the cell membranes and plasma lipids. Notably, erythrocytes from psoriatic patients presented a statistically significant decrease in erythrocyte superoxide dismutase (SOD) and glutathione peroxidase (GPx) [6], accompanied by a significant decrease of erythrocyte number and erythrocyte membrane damage [7]. Decreased GPx activity was also reported in the neutrophils of patients with chronic psoriasis and the inverse correlation between Psoriasis Area and Severity Index (PASI) and neutrophil GPx was found [8]. Other works demonstrated decreased total antioxidant capacity of the plasma, along with higher-than-normal activity of extracellular SOD and elevated MDA levels, although there was no correlation between these parameters and the disease severity [9]. Taken together, these results support the view that both skin lesion limited and generalized redox imbalance is a characteristic feature of psoriasis [10,11].

The use of biological drugs represents one of the greatest advances made in the therapeutic medicine in the last 10 years due to their great selectivity of action that, in most cases, provides a fast and significant therapeutic efficacy [12]. Compared to systemic treatments with non-specific, heavily toxic immunosuppressive drugs such as methotrexate, cyclosporine A or corticosteroids, biological drugs act instead against single molecular targets of the immune response. Nonetheless, due to their intrinsic immunosuppressive activity, also biological drugs present relevant safety concerns [12]. Among currently available biological drugs for treating psoriasis, four are against pro-inflammatory cytokines implicated in pathogenesis of the disease, in particular against either membrane bound (Infliximab and Adalimumab) or soluble tumour necrosis factor (TNF)-α (Etanercept) and the cytokines IL-12 and IL-23 (Ustekinumab). Finally, there is a *T*-cell modulator (Alefacept), which opposes the binding of the co-stimulatory receptor CD2 on T-cell surface, to its ligand LFA-3 on antigen-presenting cells [13]. Efalizumab was the second T-cell modulator available for the therapy of psoriasis until 1 year ago, when it was withdrawn from the market, since its risk/benefit ratio was too high [14].

Efalizumab is a humanized IgG_1 monoclonal antibody against the alpha sub-unit (CD11a molecule) of the integrin lymphocyte function-associated antigen 1 (LFA-1), a $\alpha_L\beta_2$-type membrane receptor expressed on the surface of leukocytes, with a dominant role in leukocyte migration across blood-vessel walls into lymph nodes and tissues. Of major relevance, LFA-1 is also a key participant in the immunological synapse that forms between a lymphocyte and its target cell [15,16]. The mechanism of Efalizumab interference with immune cells is schematically presented in Figure 1A. Hence, the expected response from a therapy with an anti-LFA-1 blocker was a deep paralysis of the immune system [17–19]. Efalizumab was approved in the US (2003) and in the EU (2004) for the treatment of adult patients (age $\geq$18 years) with chronic moderate-to-severe plaque psoriasis in the absence of arthritis [20], who were candidates for systemic therapy or phototherapy or who have failed to respond to, have a contraindication to or are intolerant of other systemic therapies, including cyclosporine, methotrexate and PUVA (psoralen plus UVA photochemotherapy) [12,21]. Weekly subcutaneous injections of Efalizumab were effective and generally well tolerated in the treatment of adults with chronic moderate-to-severe plaque psoriasis, including high-need individuals (i.e. those for whom at least two currently available systemic therapies were unsuitable due to lack of efficacy, intolerance or contraindication) and patients with difficult-to-treat forms of the disease affecting the scalp, hands/feet or nails [22]. A number of independent phase III clinical trials have confirmed that the best clinical results at 12 weeks in these patients were obtained with weekly subcutaneous injections of Efalizumab [20,21].

Likewise to other biological treatments, not all patients responded to Efalizumab therapy [12,21]. Moreover, in a relevant portion of treated patients, a rather peculiar inflammatory exacerbation of psoriasis or even outbreak of psoriatic arthritis occurred. In a report by an English group [23], up to 20% of these patients developed mild, transient, papular flare localized on body folds and 5% of non-responders developed severe generalized inflammatory flare within 6–10 weeks. Up to now, there are no any reliable

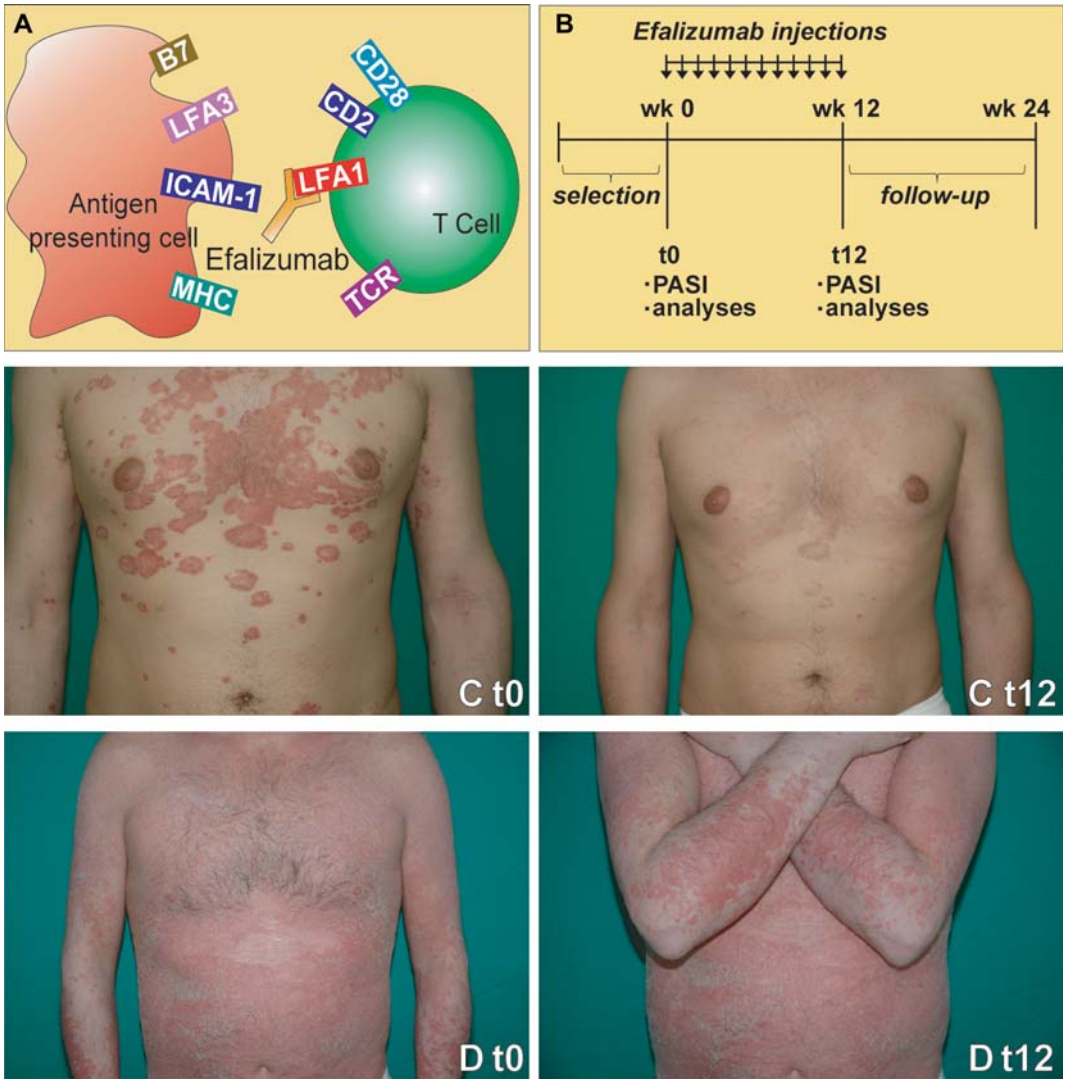

Figure 1. Mechanism of action of Efalizumab and clinical response of patients affected by psoriasis. (A) Efalizumab is a fusion protein, which specifically blocks the CD11a constituent of LFA-1, which is a receptor involved in the interaction of T lymphocytes and antigen-presenting cells; (B) Clinical study design; (C) Clinical example of positive clinical effect of Efalizumab therapy; (D) Clinical example of non-responder to Efalizumab therapy. t0, before therapy and t12, after therapy with subcutaneous injections of 1 mg/kg body weight once a week for 12 weeks.

predictive markers of the outcomes of the biological therapy of psoriasis [7,20].

Our final aim was to possibly find immunological and/or redox markers in circulating blood which correlate with clinical response to the drug, this last clinically measured as PASI.

Materials and methods

Study drug

Efalizumab was a gift from Merck Serono S.p.A–Medical Affairs Department (Rome, Italy). It is a humanized IgG1 version of the murine anti-human CD11a monoclonal antibody MHM24, generated by grafting the MHM24 complementarity determining regions into consensus human IgG1 k heavy and light chain sequences [24]. This drug has been prescribed to moderate-to-severe psoriasis patients in Europe and the US until 19 February 2009, when it was withdrawn from the European market following several reports on progressive multifocal leukoencephalopathy as a rare opportunistic infection associated with the therapy [14].

Patients, study design and the assessment of clinical response

Twenty-five patients (Table I) affected by moderate-to-severe psoriasis with resistance or intolerance to conventional systemic therapies were recruited in this study from June 2007 and December 2008. The clinical laboratory study has been carried out in strict compliance with the Helsinki Declaration on human experiments. None of the patients were receiving any systemic therapy for at least 2 weeks before testing

Table I. Demographic and clinical characteristics of psoriatic patients treated with Efalizumab.

n	Patient, sex, age (years)	PASI at baseline (t0)	PASI at week 12 (t12)	Disease remission	Partial response[a]	No response to therapy
1	F (46)	11	2		X	
2	M (31)	10	12			X
3	M (27)	18	2		X	
4	M (43)	11	10			X[b]
5	F (63)	10	10			X
6	M (39)	13	2		X	
7	M (59)	15.6	12			X
8	M (29)	14.8	10			X
9	M (47)	16.5	2.8		X	
10	M (50)	11	10			X[b]
11	M (34)	16	0	X		
12	F (59)	12	3		X	
13	F (31)	16	0	X		
14	M (62)	18.3	15			X
15[c]	M (33)	11.5	—	—	—	—
16	F (53)	15.6	0	X		
17	F (45)	11.5	3		X	
18	M (27)	13.5	0	X		
19	M (45)	16	4		X	
20	M (24)	10.6	0	X		
21	F (56)	11.8	0	X		
22	F (51)	16	0	X		
23	F (53)	12.4	1	X		
24	M (41)	10	2		X	
25	F (24)	15.4	0	X		

[a]Relapse occurred within 6 weeks of follow-up.
[b]Worsening of the disease was observed, such as first symptoms of psoriatic arthritis.
[c]Drop out before starting the therapy but after the first blood taking.

and the only medications allowed were limited amounts of topical corticosteroids. All patients agreed to participate into an observational study approved by the Ethical Committee of the Institute (IDI IRCCS, Rome, Italy, February 2007) and signed the informed consent. Participants of the study were adults (age ≥18 years) with chronic moderate-to-severe plaque psoriasis in the absence of arthritis, who were candidates for systemic therapy or phototherapy or who failed to respond to, had a contraindication to or were intolerant to other systemic therapies, including cyclosporine, methotrexate and PUVA (psoralen plus UVA photochemotherapy) [20]. The treatment with Efalizumab was performed according to the standard protocol for this drug, with weekly 1 mg/kg of body weight subcutaneous injections and assessment of the clinical response to the drug at week 12 [20,21]. The clinical assessment was performed by a dermatologist who applied PASI, a standard score to evaluate objectively skin lesions in psoriasis [25]. PASI was calculated twice at the moment of the inclusion in the study (t0) and right after the week 12 Efalizumab administration (t12). At these two time-points, the patients donated 20 ml of peripheral venous blood for biochemical and immune analyses. Of the 25 recruited patients, only one (N 15, Table I) dropped out of the study before starting the therapy. The follow-up period was 12 weeks after the last Efalizumab injection. The clinical study design is schematically presented in Figure 1B. Complete remission of the disease was considered when PASI, although decreased by 75% during therapy, returned to initial and even higher values within the first 6 weeks of the follow-up period. Succession of the trial occurred before the drug was removed from the market by safety concerns.

Blood sampling, processing and incubation with Efalizumab

Peripheral venous blood (20 mL) was drawn after overnight fasting into vacutainers with ethylene diamino tetra-acetic acid disodium salt (EDTA) as anti-coagulant. Donor's blood was obtained from the Blood Transfusion Centre in Rome the same days as the blood sampling from psoriasis patients was done. Both patient and donor blood samples were processed and analysed in parallel.

Circulating polymorphonuclear and mononuclear leukocytes were obtained from healthy controls ($n = 26$) and psoriatic patients ($n = 24$) by double density gradient centrifugation of total blood (Histopaque, $d = 1077$ and $d = 1119$). Peripheral blood mononuclear cells (PBMC, upper interface) were left to adhere (6×10^6 cells/ml) in Petri dishes in RPMI 1640 complemented with 2 mM glutamine, 1 mM sodium pyruvate, 1% non-essential amino acids,

0.05 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin (RPMI complete; all from Invitrogen, San Giuliano Milanese, Italy) and 5% human plasma (all reagents from Sigma Chemicals, Milano, Italy).

After 2 h at 37°C, the non-adherent cells, essentially composed of T-cells, were collected and CD4+ T cells were purified using magnetic cell sorting columns separators with anti-CD4 microbeads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany). The purity of the CD4-positive T-cell preparation (>99%) was verified by cytofluorimetric analysis using a fluorescein isothiocyanate-conjugated (FITC) anti-CD4 antibody (BD Biosciences, San Jose, CA). CD11a expression was analysed by means of flow cytometry using phycoerythrine-conjugated (PE) anti-CD11a Ab (BD Biosciences).

Polymorphonuclear leukocytes (PMN, lower interface) were re-suspended in phosphate buffer saline, centrifuged at 1650 rpm for 10 min and then aliquoted at 5×10^6/vial. Freshly isolated PMN were used in the chemiluminescence assay. The remaining PMN were frozen at −80°C until assayed for enzymatic activities.

Erythrocytes (at the bottom of the gradient centrifugation tube) were re-suspended in phosphate buffer saline and centrifuged at 1650 rpm for 10 min. After washing, aliquots of 500 μl of the cell pellet were prepared and stored at −80°C until fatty acid and enzymatic analyses.

In some experiments, normal peripheral mononuclear cells were incubated with Efalizumab (0.1 and 1 μg/mL) for 1 and 3 h and then analysed biochemically as well as by RT-PCR for gene expression.

Antigen presenting cell preparation

Monocytes were obtained from peripheral blood of healthy individuals ($n = 5$). First, PBMC were isolated as previously described and cultured at a density of 6×10^6 cells/ml in 6 ml of complete RPMI supplemented with 10% foetal calf serum (FCS; HyClone, Logan, UT). After 2 h incubation in a humidified atmosphere with 5% CO_2 at 37°C, supernatant containing non-adherent cells was removed and adherent cells were detached by further incubation with 0.2% EDTA for 30 min. The purity of monocytes (>90%) was verified by flow-cytometry analysis using a phycoerythrin-conjugated (PE) anti-CD14 antibody (BD Biosciences, San Jose, CA).

Mixed leukocyte reaction

For the primary mixed leukocyte reaction assay, donor monocytes were washed and then co-cultured in 96-well microculture plates in serial dilutions with 1×10^5 allogeneic psoriatic CD4+ T lymphocytes in complete RPMI supplemented with 5% human plasma. After 4 days of co-incubation, cell supernatants were collected and levels of TNF-α and IFN-γ were measured by ELISA (kits from R&D Systems, Minneapolis, MN). The co-cultures were then treated with 10 μM BrdU for 16 h at 37°C and cell proliferation was quantified using a BrdU cell proliferation ELISA (Roche Diagnostics, Monza, Italy). Samples were assayed in triplicate for each condition.

Oxidative stress parameters in peripheral blood components

Suspension of granulocytes (10 μL, 10^5 cells) was added to 1 mL of pre-heated Hanks' Balanced Salt Solution (pH 7.4) containing 0.2 mM luminol. Then, PMA (10 ng/mL) was added and the luminol-dependent chemiluminescence response was monitored for 5 min [26] with a Victor^2 (Wallac Oy) 1420 multi-label counter, equipped with Wallac 1420 Software (Perkin Elmer). Each measurement was repeated three–five times. Results were expressed in counts per second (cps) per 10^6 granulocytes.

The plasma levels of nitrites/nitrates (NO_2^-/NO_3^-) were determined by Griess reagent, following manufacturer's instructions (Cayman Chem. Co. Ann Arbor, MI).

Plasma levels of malonyl dialdehyde (MDA) were determined by slightly modified spectrophotometric analysis of thiobarbituric acid-reactive substances (TBARS) described elsewhere [27]. After a 15 min treatment of plasma (200 μL) with trichloracetic (1.22 M) + hydrochloric (0.6 M) acids, alkaline solution of TBA was added and the mixture was boiled for 30 min. TBARS were extracted with butanol and analysed spectrophotometrically at 535 nm. The results were expressed in μM of MDA using the calibration curve.

For enzymatic analyses, lysates of erythrocytes or polymorphonuclear cells (PMN) were prepared. Erythrocytes were lysed in hypotonic solution, granulocytes were destroyed by two freeze–thaw cycles and the post-spin cell lysates were analysed. Erythrocyte and granulocyte Cu, Zn superoxide dismutase (SOD) activity was measured spectrophotometrically at 505 nm using Cayman Chemical kits. Total glutathione S-transferase (GST) activity was measured spectrophotometrically by the methods described previously, using chloro-2,3-dinitrobenzene as substrate [28]. Catalase (CAT) activity was detected by the Aebi [29] method. GPx activity was determined using Cayman Chemical kits, according to the method [30]. Protein content was measured according to Bradford, using Bio-Rad microplate assay kit.

4-hydroxy nonenal (4-HNE)-protein and acrolein-protein adducts in peripheral blood plasma

The levels of adducts were detected as previously described [31]. Briefly, 60 μg of samples protein,

determined using BioRad protein assay (BioRad, Hercules, CA), were separated on 4–20% gradient sodium dodecyl sulphate-polyacrylamide SDS-PAGE gels (Lonza Group Ltd, Switzerland) and electro-transferred onto nitrocellulose membranes (Amersham). After blocking in 3% non-fat milk in phosphate buffered saline solution with Tween 20, the membranes were incubated with 1 μg/ml of specific polyclonal antibody against 4-HNE protein adducts (Millipore Corporation, Billerica, MA) or against acrolein protein adducts (a gift from Professor K. Uchida). The band densities between 100–220 kDa for HNE-protein adducts and higher than 170 kDa for acrolein-protein adducts were scanned and then quantified using NIH image software. Results of three independent experiments were expressed in arbitrary units, as mean values ± SD.

Assay for fatty acids esterified in the phospholipids of erythrocyte membranes

The fatty acid pattern in erythrocyte membranes was analysed by gas-chromatography coupled with mass spectrometry [32]. In brief, lipid extracts of purified erythrocyte membranes were trans-esterified by sodium methoxide. Resulting fatty acid methyl esters were extracted with hexane, concentrated by evaporation and finally analysed by a Shimadzu QP 5050 gas-chromatograph coupled with mass spectrometer, on a Hewlett Packard FFAP column (50 m × 0.32 mm × 0.52 μm). Results were expressed as a percentage of total fatty acid content.

Assay for cytokine levels in peripheral blood plasma

Cytokine levels in blood plasma were determined by multiplexed analysis using the panel within the Bio-Plex Suspension Array System (Bio-Rad Laboratories, Inc., Hercules, CA). The levels of 27 cytokines, chemokines and growth factors relevant to chronic inflammation were simultaneously detected with this panel: IL-1β, interleukin 1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, eotaxin, basic fibroblast growth factor (bFGF), granulocyte colony stimulating factor (G-CSF), granulocyte macrophage colony stimulating factor (GM-CSF), IFN-γ-induced protein of 10 kDa (IP-10), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory proteins (MIP-1α and MIP-1β), regulated upon activation, normal T-cell expressed and secreted (RANTES), the platelet derived growth factor disulphide-bonded B chain homodimer (PDGFbb), TNF-α and vascular endothelial growth factor (VEGF). The assay was performed according to the manufacturer's instructions. Cytokine concentrations were expressed in pg/mL of plasma and each factor was quantified in the linear range of its calibration curve using a Bio-Rad array reader.

Quantitative real-time-PCR in PBMC

Total RNA was isolated from frozen PBMC using the GenElute^{TM} Mammalian Total RNA Kit from Sigma (Milan, Italy), following the manufacturer's instructions. The amount of RNA was determined by absorbance at 260 nm. Total RNA (1 μg) was reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad), at 25°C for 5 min, then at 42°C for 30 min and, finally, at 85°C for 5 min, in a final reaction volume of 40 μl. Amplification of cDNA was performed with iQTM Supermix, using the MiniOpticon Real-Time PCR Detection System (Bio-Rad, Hercules, CA). All real time assays were carried out under the following conditions: 35 cycles of denaturation at 95°C for 15 s, annealing and extension at 60°C for 60 s. Melt curve analysis was performed to confirm the specificity of the amplified products. All samples were run in triplicate and relative expression was determined by normalizing samples to β-actin and 18S rRNA as housekeeping genes [33].

The following primer sets were designed using Primer-BLAST (NCBI) and synthesized by Eurofins MWG Operon (Ebersberg, Germany): β-actin fwd: 5′-AATCT GGCACCACACCTTCTAC-3′; β-actin rev: 5′-ATAG CACAGCCTGGATAGCAAC-3′; 18S rRNA fwd: 5′- TCCCCCAACTTCTTAGAGG-3′; 18S rRNA rev: 5′- GCTTATGACCCGCACTTAC-3′; Catalase frw: 5′-CGTCCTGAGTCTCTGCATCA -3′; Catalase rev: 5′- TTTGCAATAAACTGCCTCCC -3′; Gpx1 fwd: CGCTCCGCTGGCTTCTTGGA; Gpx1 rev: GCG CGCCGAGAAGGCATACA; Gpx2 fwd: GCCGC TCAGATGTGGCCTGG; Gpx2 rev: GGGAAGGT GCGGCTGTAGCG; Gpx3 fwd: GGCCCCAGCCT GGCACAAAT; Gpx3 rev: GATGGTACACATTC CCAGAAAGACACA; Gpx4 fwd: 5′-ACGCCCTCG GAGCCTTCCAC-3′; Gpx4 rev: 5′-CCAAGCCCA GCAGGCCATGG-3′

Statistics

All biochemical and molecular measurements were done in triplicate and data were statistically evaluated. Statistical analysis of clinical laboratory data was performed using the STATISTICA 6.0 program from StatSoft Inc. Reported values were treated as continuous. Normality of data was checked using the Shapiro-Wilk test. Since the distribution of the data was significantly different from normal, non-parametric statistics were used. Values of redox parameters in the blood components and the fatty acid content in the membranes of red blood cells were presented as mean, standard error of the mean, 95% confidence interval (1.96 × standard error). The Mann-Whitney U-test for independent samples was employed for

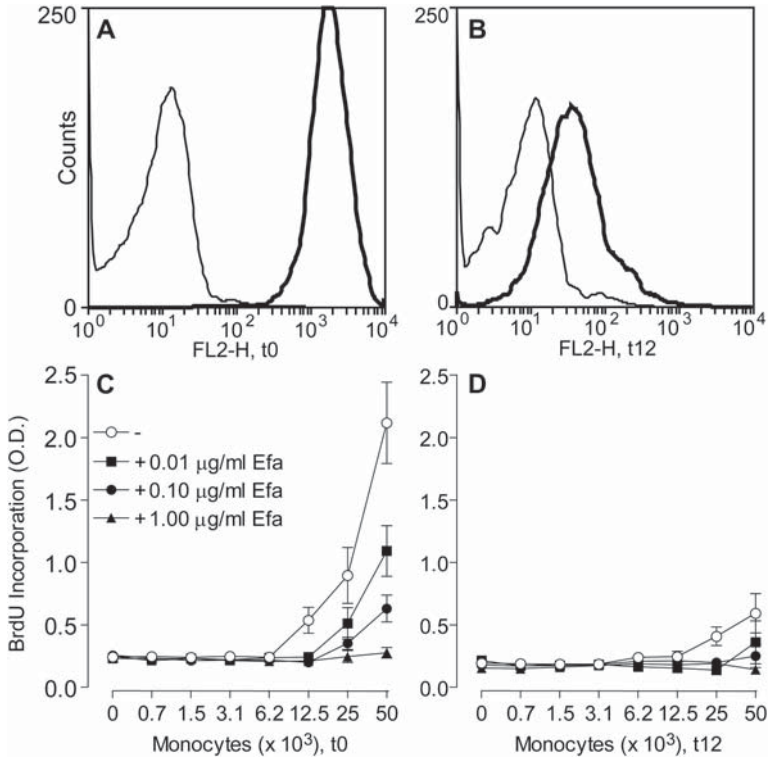

Figure 2. Treatment with Efalizumab abrogates CD11a expression on lymphocytes and suppresses the response of these cells in the mixed leukocyte reaction. (A) T-cell-associated CD11a expression in the group of psoriasis patients before Efalizumab therapy (t0); (B) T cell-associated CD11a expression in the group of psoriasis patients after 12 weeks of Efalizumab therapy (t12); bold line, CD11a levels; thin line, control with isotype-matched antibody; (C) Efalizumab exogenously added to co-culture of CD4 T cells of untreated psoriatic patients (t0) and monocytes from healthy donors inhibits CD4+ proliferation dose-dependently; (D) CD4+ lymphocytes isolated from psoriasis patients treated with Efalizumab for 12 weeks (t12) have decreased proliferative potential in the mixed leukocyte reaction. Exogenous Efalizumab further inhibits CD4+ cell proliferation.

comparison between patients and controls. The Mann-Whitney rank sum test was used to compare differences between the distinct groups of patients. The Wilkoxon signed-rank test was applied for within-group comparisons. Significance was assumed at a p-value < 0.05.

Results

Clinical outcomes of Efalizumab therapy range from complete remission or remission with relapse to no effect and exacerbation of psoriatic disease

Clinical outcomes of the treatment were assessed by PASI, a standard index for dermatological improvement/worsening in psoriasis. After 12 weeks of standard therapy with Efalizumab, eight patients responded with complete disease remission (Figure 1C, Table I), eight patients had a partial response followed by a fast (within 6 weeks) relapse of the disease and seven patients showed no improvement in their disease (no responders) or even exacerbation of skin symptoms of psoriasis (Figure 1D, Table I). Furthermore, two patients of the non-responder group developed symptoms of psoriatic arthritis during the Efalizumab therapy (Table I).

Efalizumab therapy induces a profound, dose-dependent suppression of T-cell-mediated immune functions in all patients independently on the clinical outcome

Despite the clear-cut evidence that patients were distributed in three distinct sub-groups in terms of their clinical response to the drug, we found that T-cell-associated CD11a expression was prominently suppressed in all the patients after 12 weeks of therapy with Efalizumab (t12, Figure 2B) when compared with the baseline condition (t0, Figure 2A), in keeping with the mechanism of action of this biological drug [8,17–19]. Accordingly, when exogenously added to the mixed leukocyte reaction performed by mixing CD4 T-cells from an untreated psoriatic patient as responding cells, and monocytes from a healthy donor as antigen presenting cells, Efalizumab inhibited the proliferative response dose-dependently (Figure 2C). Similar data were obtained in a mixed leukocyte reaction with CD4 T cells obtained from healthy donors (data not shown). In addition, proliferative capacity of CD4 T-cells obtained from all the 24 patients following 12 weeks of therapy with Efalizumab was significantly suppressed. Addition of exogenous Efalizumab to the mixed leukocyte reaction

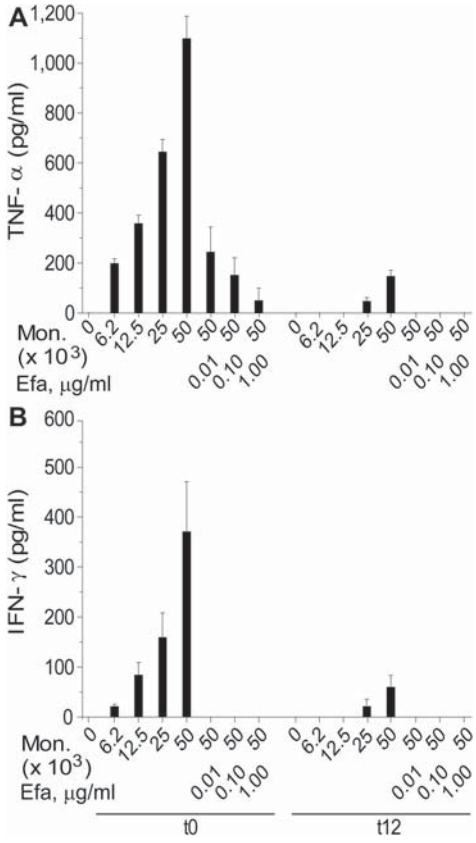

Figure 3. Efalizumab abrogates TNF-α and IFN-γ expression during the mixed leukocyte reaction. (A) TNF-α production by psoriatic T-lymphocytes isolated from the blood of patients before (t0) and after (t12) Efalizumab treatment and mixed with healthy donor's monocytes. Inhibitory effect of exogenously added Efalizumab; (B) IFN-γ production by psoriatic T-lymphocytes isolated before (T0) and after (T12) Efalizumab treatment and mixed with healthy donor's monocytes. Exogenous Efalizumab dose-dependently inhibits both TNF-α and IFN-γ release.

further and dose-dependently inhibited the proliferative response (Figure 2D). Efalizumab not only perturbed the proliferative response of CD4 T-cells dramatically, but also impaired their capacity to express pro-inflammatory cytokines in the mixed leukocyte reaction. CD4 T-cells from patients treated for 12 weeks with the drug (t12) expressed low levels of both cytokines. We observed also that, in untreated psoriatic patients (t0), exogenously added Efalizumab down-regulated the levels of both TNF-α and IFN-γ in the supernatant of the mixed leukocyte reaction (Figures 3A and B).

Psoriasis patients display characteristic plasma pro-inflammatory cytokine profile, which was not affected by Efalizumab therapy

Multiplex analysis of 27 cytokines, chemokines and growth factors in the plasma of psoriatic patients revealed that only three of them differed statistically significantly for the groups of healthy controls and untreated patients with psoriasis. Thus, expression of IL-8 and VEGF were enhanced whereas expression of RANTES was lower than normal (Figures 4A–C). Three other pathogenically involved cytokines such as IP-10, TNF-α and IFN-γ did not differ significantly for normal and psoriasis groups at baseline (Figures 4D–F). The treatment with Efalizumab did not result in significant changes in the plasma levels of any of these cytokines (Figure 4).

Fatty acids esterified in phospholipids of erythrocyte membranes of psoriasis patients are shifted to saturated compartment, and deficit of relevant-to-inflammation unsaturated ω-6 and ω-3 fatty acids was not corrected by Efalizumab therapy in both responders and non-responders

In order to elucidate whether or not the treatment with Efalizumab influenced the formation and/or levels of pro- and anti-inflammatory mediators of lipid origin, we analysed the relative membrane phospholipid contents of saturated and selected polyunsaturated fatty acids (PUFAs), such as arachidonic (AA, C20:4, ω6), eicosapentaenoic (EPA, C20:5, ω3) and docosahexaenoic (DHA, C22:6, ω3) acids. We found that the relative portion of saturated (mainly, C16:0 and C18:0) fatty acids in the erythrocyte membranes of psoriatic patients was much higher than in normal erythrocytes (Figure 5A). The parameters did not change after the course of biological therapy. Monounsaturated fatty acids (C16:1, C18:1 and C20:1) did not differ significantly for normal and psoriasis groups (data not shown). As expected, total content of PUFAs was remarkably decreased in the erythrocyte membranes of psoriatic patients (Figure 5B) and it was not affected by Efalizumab. Similar results were obtained for all three PUFAs: AA, EPA and DHA (Figures 5C, E and G). Our analysis of possible association between the PUFAs and responsiveness to Efalizumab therapy showed no difference between responders and non-responders (Figures 5D, F and H).

Plasma levels of the end lipid peroxidation products are increased at baseline and the Efalizumab therapy diminishes MDA independently of the clinical response

Figure 6 shows that neither PMA-activated luminol-dependent chemiluminescence (CL PMA) of PMN (Figure 6A) nor NO_2^-/NO_3^- levels (Figure 6B) were different in donors and psoriasis patients at baseline. Although production of reactive oxygen species by PMN assessed by CL remained unchanged after the course of Efalizumab, NO_2^-/NO_3^- plasma levels were found slightly but statistically significantly

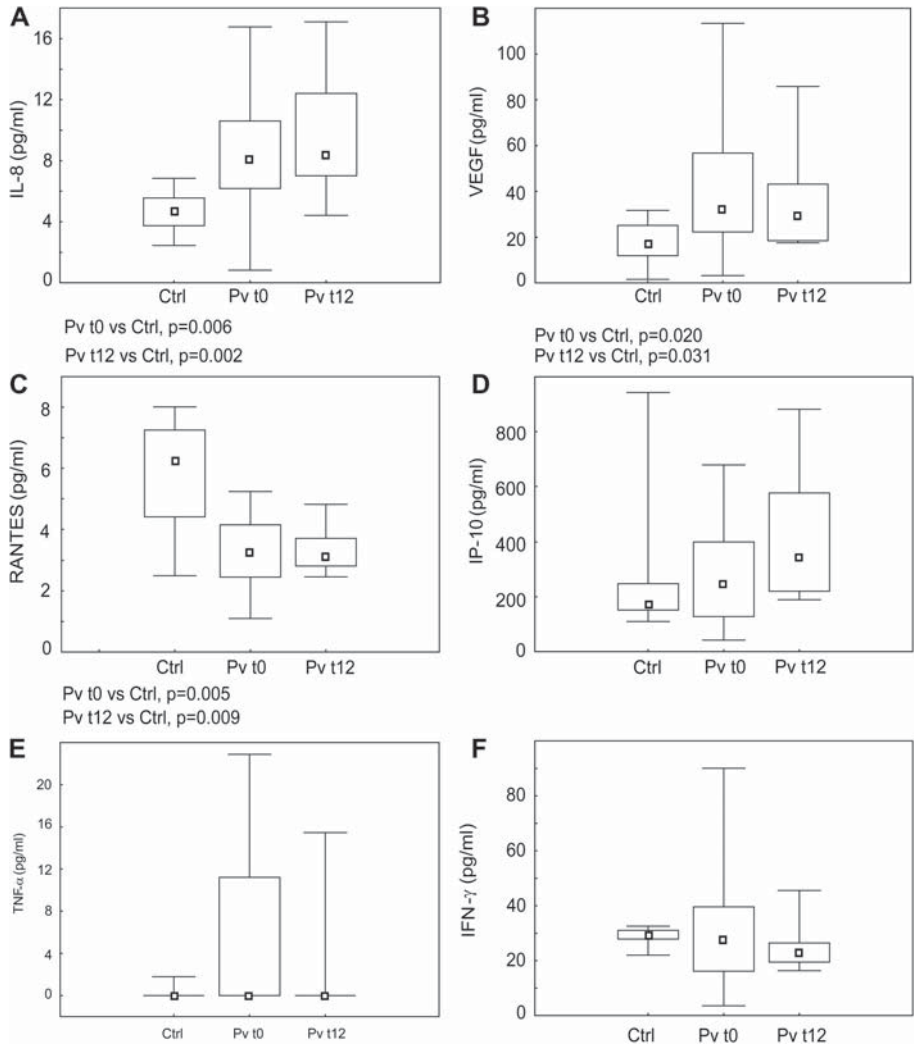

Figure 4. Efalizumab therapy does not perturb the plasma levels of pro-inflammatory cytokines involved in pathogenesis of psoriasis. (A) Interleukin 8 (IL-8) plasma levels in healthy donors (Ctrl) and psoriatic patients (Pv) before (t0) and after (t12) Efalizumab therapy; (B) Vascular endothelial growth factor (VEGF) plasma levels; (C) RANTES plasma levels; (D) IFN-γ-induced protein of 10 kDa (IP-10); (E) TNF-α plasma levels; (F) IFN-γ plasma levels.

diminished. There was no difference between the groups of responders and non-responders (data not shown). On the other hand, highly increased MDA levels were determined in psoriasis patients before the treatment and 12 week therapy with Efalizumab brought MDA back to the normal values (Figure 6C). However, the mean values of both initial and final levels of MDA were similar in the groups of responders, partial responders and non-responders (data not shown). The plasma content of both protein-HNE and protein-acrolein adducts were significantly increased in psoriasis patients at t0 (Figures 6D and E) independent of whether they did or did not respond to the therapy later on (data not shown). Due to an elevated cost and unavailability of anti-protein-acrolein antibodies, analytical determination of adducts was not carried out after the Efalizumab therapy.

Efalizumab therapy leads to distinct changes in the activities of both erythrocyte and PMN antioxidant enzymes: GPx, GST and CAT correlate with the clinical outcome

In our search for biochemical markers indicative for drug response, we investigated the activity of a cluster of enzymes involved in the antioxidant defence, namely GPx, CAT, GST and total SOD, analysed in PMN and erythrocytes. Notably, only the group of patients who did not respond to the drug selectively showed a peculiar up-regulation of PMN-associated GPx at t12, both when compared to GPx levels of the same group of patients at T0, and also when compared to GPx levels of the groups of responders at the same time-point (Figure 7A). Also GST activity in PMN tended to be quite higher in non-responders at t12 when compared to responders (Figure 7C). In addition, CAT

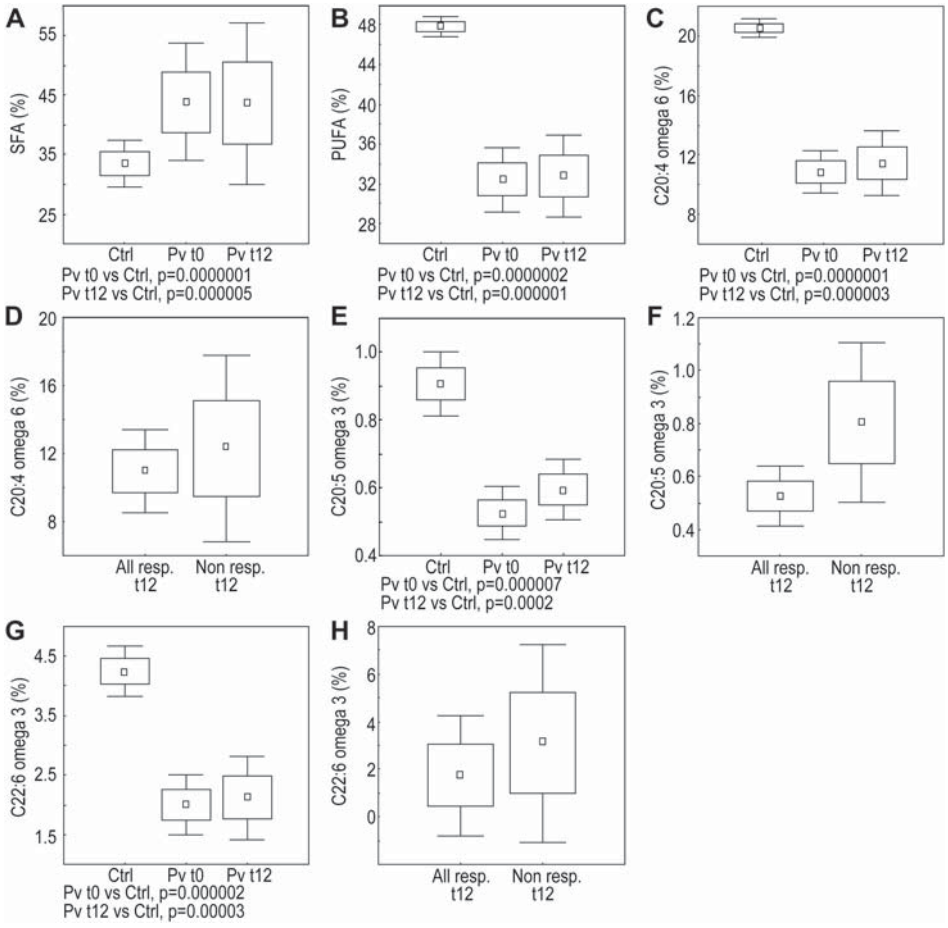

Figure 5. Fatty acids esterified in phospholipids of erythrocyte membranes are shifted to saturated compartment in psoriatic patients and the deficit of relevant-to-inflammation unsaturated ω-6 and ω-3 fatty acids is not corrected by Efalizumab therapy. (A) Percentage of saturated fatty acids in donors (Ctrl) and psoriasis (Pv) patients before (t0) and after (t12) the therapy; (B) Percentage of all polyunsaturated fatty acids (PUFAs) in donors (Ctrl) and psoriasis (Pv) patients before (t0) and after (t12) the therapy; (C) Arachidonic acid in donors (Ctrl) and psoriasis (Pv) patients before (t0) and after (t12) the therapy; (D) Arachidonic acid in responders and non-responders to Efalizumab at (t12); (E) Eicosapentaenoic acid in donors (Ctrl) and psoriasis (Pv) patients before (t0) and after (t12) the therapy; (F) Eicosapentaenoic acid in responders and non-responders to Efalizumab at t12; (G) Docosahexaenoic acid in donors (Ctrl) and psoriasis (Pv) patients before (t0) and after (t12) the therapy; (H) Docosahexaenoic acid in responders and non-responders to Efalizumab at t12.

activity in PMN was significantly lower in this group of patients at t12 when compared to responders at the same time-point (Figure 7B). No significant perturbation was found for SOD (Figure 7D). When examined in erythrocytes, GPx was found significantly reduced exclusively in the responding patients following a 12-week therapy (Figure 8A), but no relevant changes were found in the other antioxidant enzymes (Figures 8B–D).

In order to verify whether Efalizumab could directly affect activities and gene expression of antioxidant enzymes, we treated freshly isolated PBMC of healthy donors with therapeutically relevant concentrations of this drug (0.1 and 1.0 μg/10^6 cells) for 1 h and 3 h. Exogenous Efalizumab significantly increased exclusively catalase activity in a dose- and time-dependent manner (1 h: 100% (no Efalizumab); 330 ± 13%* (0.1 μg Efalizumab); 403 ± 28* (1.0 μg); 3 h: 100% (no Efalizumab); 480 ± 14%* (0.1 μg Efalizumab); 462 ± 35* (1.0 μg Efalizumab); $^*p < 0.01$). It did not, however, affect Gpx, GST and SOD activities in PBMC (data not shown). Also exogenous Efalizumab did not affect gene expression of catalase and different GPx isoforms (GPx1 through GPx4) in PBMC (data not shown).

Discussion

The tremendous success of molecular biology in the identification of pathways underlying chronic immune-mediated diseases and fast development of gene/cell engineering biotechnologies has brought up a number of targeted biological drugs, which revolutionized the therapy of these human pathologies. Designed and developed with the purpose to (i) target and interrupt molecular pathways leading to persistent inflammation, (ii) neutralize immune cells mediating inflammation or (iii) activate endogenous anti-inflammatory

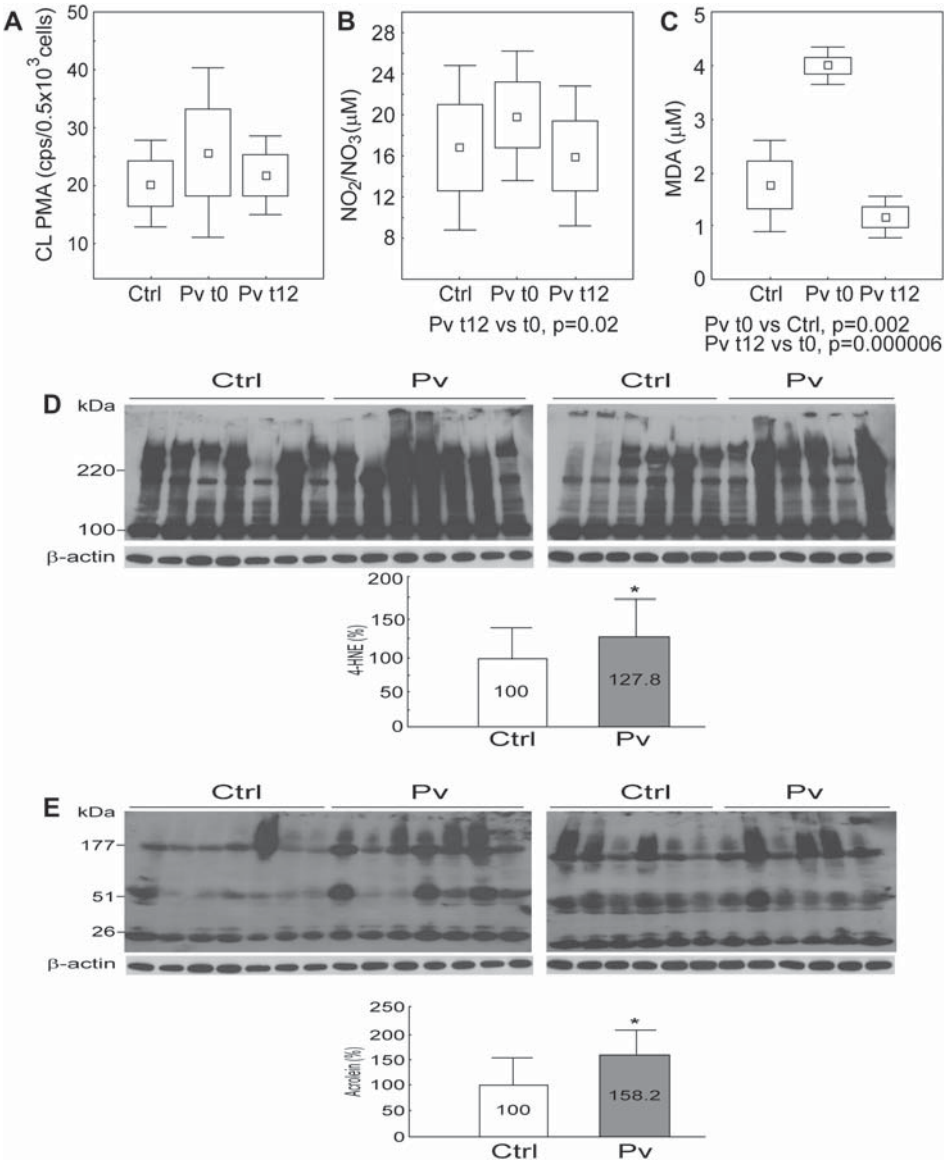

Figure 6. Plasma levels of the end lipid peroxidation products are increased in psoriatic patients at baseline and therapy with Efalizumab diminishes MDA independently of the clinical response. (A) Luminol-dependent PMA-triggered CL in circulating PMN of donors (Ctrl) and psoriatic patients (Pv) before (t0) and after (t12) the therapy; (B) Plasma NO_2^-/NO_3^- levels in donors and psoriatic patients before (t0) and after (t12) the therapy; (C) MDA plasma levels in donors and psoriatic patients before (t0) and after (t12) the therapy; (D) Plasma levels of HNE-protein adducts in donors and psoriatic patients before the treatment. Bands within the range of 100–220 kDa were quantified; (E) Plasma levels of acrolein-protein adducts in donors and psoriatic patients before the treatment. Band corresponding 177 kDa was quantified.

processes, biologics are extremely effective, sometimes the best known anti-inflammatory agents [12,21]. Despite the vast literature existing on the altered expression of soluble inflammatory mediators in the psoriatic disease both at the local level and in the systemic circulation [6,34,35], there are still no reliable biological markers that could be objective indicators of the severity/activity of the disease and/or of the pharmacological responses to therapeutic intervention.

Our clinical and laboratory data showed that the administration of biological drug Efalizumab, an antibody designed to block T-cell-to-antigen presenting cell contact and signal transduction through specific inhibition of CD11a expression on T-lymphocytes (Figure 1), led to profound inhibition of psoriatic CD4+ T-cell proliferation and cytokine release following activation by normal monocytes in the mixed leukocyte reaction. Moreover, exogenously added Efalizumab further inhibited all these parameters in a dose-dependent fashion (Figures 2 and 3). These results were well predicted from the mechanism of action of Efalizumab [17,36] and previous publications on the anti-CD11a-induced deep inhibition of immune cell interactions/functions in animal experiments [19,37]. What was surprising was the absence

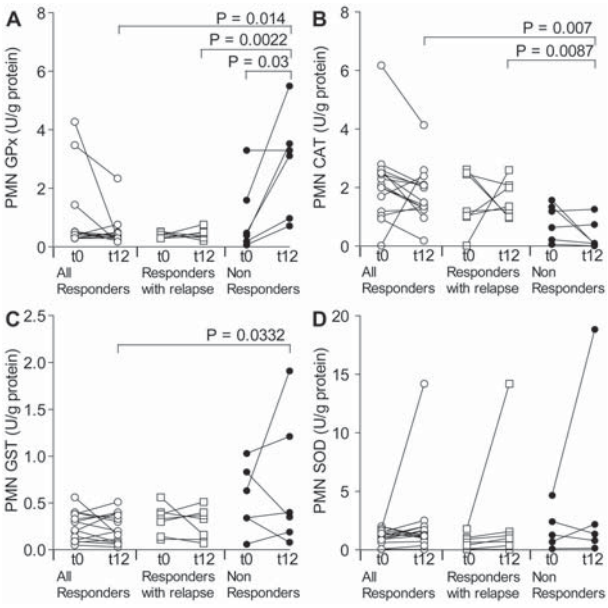

Figure 7. Efalizumab therapy associates to an increase in the activity of GPx and GST specifically in the PMN of non-responders. (A) Glutathione peroxidise (GPx) before (t0) and after (t12) the treatment in groups of all responders, responders with relapse and non-responders; (B) Catalase (CAT) (see A); (C) Glutathione S-transferase (GST) (see A); (D) Superoxide dismutase (SOD) (see A).

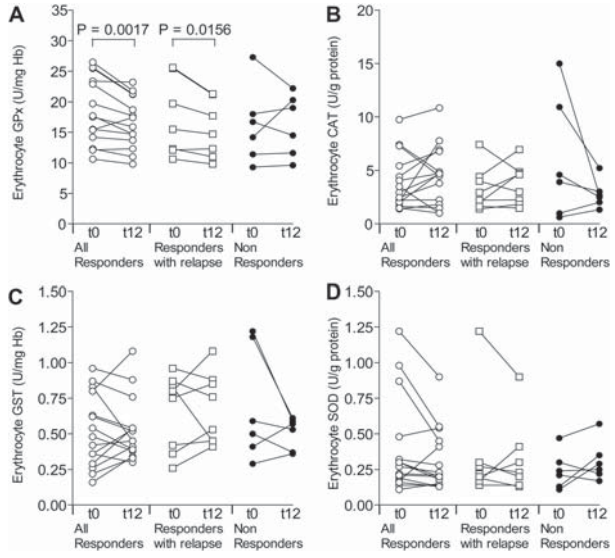

Figure 8. Efalizumab therapy associates to a decrease in the activity of GPx in the red blood cells of psoriasis patients who respond to this biological drug. (A) Glutathione peroxidase (GPx) before and after the treatment in groups of all responders, responders with relapse and non-responders; (B) Catalase (CAT) (see A); (C) Glutathione S-transferase (GST) (see A); (D) Superoxide dismutase (SOD) (see A).

of any association between the efficient immune suppression, as observed in 100% of psoriatic patients treated with Efalizumab and peculiar clinical efficacy of the drug. Only 37.5% of the treated patients responded with complete and durable (at least for 12 successive weeks) remission of skin symptoms of psoriasis, 33.5% of the patients had transient positive response to Efalizumab (the relapse occurred shortly after the interruption of the biological therapy) and the remaining portion (29%) did not respond to the therapy (Table I). Reflecting on the discrepancy of clinical and immunological results, one could speculate that the abrogation of the immune synapse and the deep impairment of leukocyte extravasation due to the anti-CD11a activity of Efalizumab [17,36] cannot be considered *the* crucial pathogenetic mechanisms invariably underlying the generation and/or maintenance of the psoriatic skin lesions. On the contrary, in a relevant portion of patients, Efalizumab-associated disease relapse and/or aggravation suggest that LFA-1 may be involved also in the recruitment and/or activation of regulatory T-cells, locally active in the suppression of the disease.

As an indirect result of interaction with immune cells, Efalizumab could seriously affect *de novo* synthesis of pro-inflammatory cytokines relevant for pathogenesis of psoriasis as we showed in the *ex vivo* and *in vitro* experiments (Figure 3). However, we observed that the psoriasis-associated increased circulating plasma levels of the chemokine IL-8 and angiogenic growth factor VEGF did not change after Efalizumab therapy independent on the clinical outcome (Figures 4A, B and D). Also, baseline levels of circulating TNF-α, IFN-γ and IP-10 were within the normal range and were not influenced by the therapy (Figures 4D–F). A wealth of previous publications confirm a relevant inconsistency in the circulating levels of chemokines and pro-inflammatory and angiogenic cytokines as well as in their correlation to severity of skin disease [35]. A number of factors may reasonably contribute to the lack of correlation with PASI, the clinical index which quantifies the affected skin surface and the intensity of local inflammation. This index does not, however, reflect important systemic co-morbidities—chaperons of psoriatic disease, such as autoimmune disorders and the metabolic syndrome, a low-level systemic inflammation, both possibly contributing to derangement of cytokine and chemokine expression [3,4,38].

Actually, an antibody specifically reacting with a membrane bound protein (LFA-1 in the case of Efalizumab) may also non-specifically influence structural and functional properties of the membrane lipids. Since LFA-1 is expressed on virtually all circulating and tissue leukocytes (T- and B-lymphocytes, granulocytes and macrophages) [17,36], an anti-LFA-1 antibody could react with all these cells, changing, for example, susceptibility to oxidation of phospholipids-components of their membranes. Much higher-than-normal susceptibility to oxidation has been shown for membrane phospholipids of psoriatic blood cells [34]. This fact, together with the increased production of superoxide and nitric oxide by leukocytes in psoriasis [39–42], has become a traditional explanation of increased lipid peroxidation in plasma and

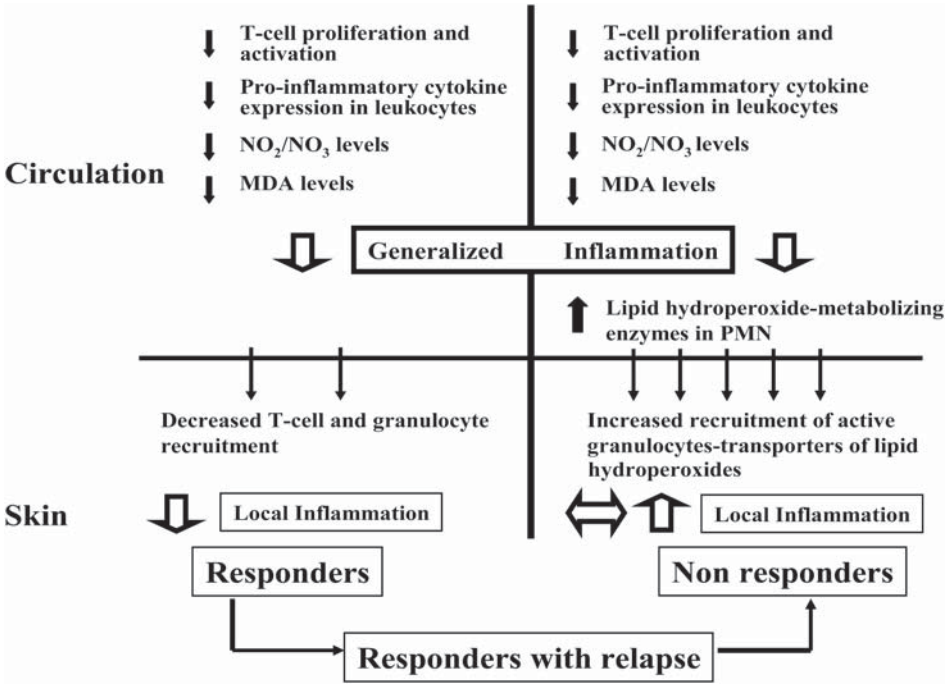

Figure 9. Hypothetic scheme of Efalizumab differential effects towards generalized and skin-localized inflammation in responders and non-responders. The upper parts of the figure show generalized (CIRCULATION), whereas the lower parts show local (SKIN) inflammatory processes taking place in 'Responders' (on the left) and in 'Non-Responders' (on the right) to Efalizumab therapy. Partial responders are considered as a transition state between responders and non-responders. Up arrows indicate stimulation, down arrows indicate inhibition and horizontal arrows indicate no changes in the processes. Thin arrows from the circulation to the skin represent inflammatory cells, which are recruited in much higher number in the skin of non-responders as compared to responders.

erythrocytes of patients with active psoriasis [1,6,10,34,42]. In a good consistence with previously published data, we observed abnormally high intensity of lipid peroxidation as assessed by MDA, HNE-protein and acrolein-protein plasma contents [27,31,43] in the group of psoriatic patients at baseline (Figures 6C–E). At the same time, the PMA-triggered release of reactive oxygen species from granulocytes (non-specifically determined by CL) was within normal range, as were the levels of NO_2^-/NO_3^-, an indirect measure of extracellular NO and superoxide production by circulating leukocytes and endotheliocytes (Figures 6A and B). Of note, both MDA and NO_2^-/NO_3^- levels were significantly diminished after the course of Efalizumab, although independent on the skin response. This fact makes us assume that Efalizumab non-specifically affects the redox parameters of generalized chronic inflammation, which, however, don't have an impact on the skin manifestations of psoriasis. Peculiarly highly increased lipid peroxidation in the group of psoriatic patients was also indirectly confirmed by the data on the profiles of fatty acids esterified in the phospholipids of red blood cell membranes. Thus, the contents of all PUFAs (Figure 5B) and selected PUFAs relevant to inflammatory/anti-inflammatory processes (AA, EPA and DHA) [44,45] were much lower-than-normal at baseline (Figures 5C, E and G), were not affected by Efalizumab and did not differ for responders and non-responders (Figures 5D, F and H). The normal pool of PUFAs esterified in the membrane phospholipids is subjected to intense enzymatic (12/15 lipoxygenase) and non-enzymatic (ROS and RNS) oxidation under certain pathological conditions such as inflammation, lung injury and atherosclerosis [44]. Both types of oxidation result in the formation of highly reactive lipid hydroperoxides, which are enzymatically neutralized by GPx (mainly GPx4) [46], GST [47], peroxireductase VI and aldo-keto-reductases [44]. In the present work, activities of GPx and GST were selectively increased in granulocytes (a major class of inflammatory cells recruited to psoriatic skin lesions [1]) of non-responders to Efalizumab (Figures 7A and C). In contrast, GPx in erythrocytes significantly decreased by the end of the trial exclusively in responders (Figure 8A). Efalizumab-associated modulation of GPx and catalase occurred, most probably, at post-translational level since the corresponding gene expressions did not change. Taken together, these data suggest that non-responders to Efalizumab have higher levels of lipid hydroperoxides (pro-inflammatory leukotrienes are among them) than responders or partial responders, maybe partly due to more profound inhibition of catalase in this sub-group of patients (Figure 7B). Lipid hydroperoxides [44,45] and small end-products of lipid peroxidation such as MDA, HNE and acrolein [33,48,49] transported to psoriatic skin lesions by activated granulocytes may facilitate and maintain local inflammation through

redox regulation of the pro-/anti-inflammatory molecular pathways in keratinocytes [42]. Moreover, lipid hydroperoxides together with HNE-protein and acrolein-protein adducts [44,49,50] could be recognized by the immune cells as a 'modified-self', hence inducing immune responses and aggravation of clinical conditions observed sometimes in the course of Efalizumab administration (Table I). Several serious limitations of the present study, including the small size of experimental group, the absence of redox analyses in the skin scales and direct analytical determinations of lipid hydroperoxides, did not allow us to further elucidate the mechanisms of responsiveness to Efalizumab.

To our knowledge, there have not been publications so far on the association of redox processes and clinical/metabolic responses to biological drugs in psoriasis and other immune mediated inflammatory diseases. Here, we showed for the first time that lipid hydroperoxides and their metabolizing enzymes could be redox markers of the responsiveness to Efalizumab. Being the antibody against the membrane-bound integrin LFA-1, Efalizumab might influence generalized and local inflammation by a specific block of immune cell-to-cell interaction or by non-specific effects on mediators of inflammation such as pro-inflammatory cytokines, NO, superoxide and lipid hydroperoxides. Our assumption on the pro- and anti-inflammatory effects of Efalizumab in psoriasis is schematically presented in Figure 9.

Declaration of interest

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