

Infliximab induces downregulation of the IL-12/IL-23 axis in 6-sulfo-LacNac (slan)⁺ dendritic cells and macrophages

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Background: The spectrum of TNF- α -producing cells in patients with psoriasis, as well as their fate during treatment with TNF- α antagonists, is not clearly defined.

Objective: We sought to analyze the effects of anti-TNF- α treatment on TNF- α ⁺ cells in the skin and blood of patients with psoriasis.

Methods: Lesional psoriatic skin was analyzed by means of immunohistologic staining and quantitative RT-PCR, and peripheral blood cells were phenotypically characterized by means of multicolor immunofluorescence labeling.

Results: By using a tyramide-based signal amplification system, TNF- α was detected in dermal CD45⁺HLA-DR⁺ leukocytes consisting of CD11c⁺ dendritic cells and CD163⁺ macrophages. In peripheral blood we observed an increase in the TNF- α -producing myeloid subsets of CD14⁺ 6-sulfo-LacNac⁺ dendritic cells and CD14⁺CD16⁺ "intermediate" monocytes compared with healthy control subjects. Strikingly, we did not find detectable levels of TNF- α in other cells, including keratinocytes or T cells, making these cell types unlikely targets of TNF- α blockers.

Up to 48 hours after the intravenous administration of the TNF- α antagonist infliximab, we encountered no overt changes in numbers of TNF- α ⁺ cells or signs of apoptosis in lesional psoriatic skin. Yet we observed a rapid decrease in IL-12p40, IL-1 β , CCL20, and IL12RB1 mRNA levels. Consistently, TNF- α blockade during *in vitro* stimulation of 6-sulfo-LacNac DCs resulted in decreased production of IL-12 and IL-23 but not IL-6. In a mixed leukocyte reaction infliximab led to significantly decreased proliferation rates of T cells independent of the Fc antibody fragment.

Conclusion: The decrease in tissue inflammation during anti-TNF- α therapy is not due to immediate killing of

TNF- α -producing cells but rather results from a rapid downregulation of the pathogenic IL-12/IL-23-driven immune response. (J Allergy Clin Immunol 2013;■■■:■■■-■■■.)

Key words: Psoriasis, myeloid dendritic cells, 6-sulfo-LacNac, IL-12p40, TNF- α

Psoriasis is a chronic inflammatory skin disease affecting approximately 2% of the general population in the Western world.¹ Its most common form is the chronic plaque type, accounting for approximately 85% to 90% of all cases. Psoriasis is nowadays more and more regarded as part of a systemic inflammatory disease complex^{2,3} associated with other chronic inflammatory conditions, such as metabolic diseases (diabetes mellitus type 2, cardiovascular disease, and obesity), arthritis, and Crohn disease.⁴

One can safely assume that cells of both the adaptive and the innate immune system are pathogenetically relevant players,² and it is not unlikely that a vivid interplay exists between leukocytes/leukocyte products of the skin, the peripheral blood, and possibly other organ systems.⁵⁻⁷ TNF- α apparently plays a decisive role in this regard, as evidenced by the impressive clinical efficacy of TNF- α antagonists in patients suffering from psoriasis.⁸

Newly synthesized human TNF- α is only scarcely found in the cytoplasm⁹ but readily expressed as an integral 26-kDa transmembrane protein (ie, transmembrane TNF- α [tmTNF- α]) on the surface of TNF- α -producing cells. It is then cleaved off, among others, by TNF- α -converting enzyme and finally released as the 17-kDa soluble mediator soluble TNF- α (sTNF- α).¹⁰

The true nature of TNF- α -producing cells in the skin and blood of patients with psoriasis has long been a matter of conjecture and debate. Various cell types, such as mast cells,¹¹ activated T lymphocytes,¹² keratinocytes,¹³⁻¹⁵ endothelial cells,¹⁶ and certain myeloid cells,¹⁵ such as TNF- and inducible nitric oxide synthase (iNOS)-producing dendritic cells (DCs),¹⁷ 6-sulfo-LacNac (slan)⁺ DCs,⁶ and macrophages,¹⁸ have been held responsible in this regard. Identifying the spectrum of TNF- α -producing cells could be of major importance for understanding the role of TNF- α in disease pathogenesis and the mode of action of TNF- α antagonists in the treatment of psoriasis. In fact, current data suggest that anti-TNF- α antibodies are capable of directly modulating TNF- α -producing cells through binding to tmTNF- α , possibly mediating retrograde signaling or even apoptosis, antibody-dependent cellular cytotoxicity, and complement killing.^{10,19,20}

For the detection of TNF- α in tissue sections, it is critical to note that both TNF- α variants, tmTNF- α and sTNF- α , are capable of binding to the 2 known TNF- α receptors TNF receptor (TNFR) 1 and TNFR2.¹⁰ Consequently, sensitive staining for TNF- α might also visualize cells that display receptor-bound sTNF- α from exogenous sources on their surface. For this reason, we

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Abbreviations used

BDCA:	Blood dendritic cell antigen
DC:	Dendritic cell
iNOS:	Inducible nitric oxide synthase
MACS:	Magnetic cell sorting
mDC:	Myeloid dendritic cell
NK:	Natural killer
PASI:	Psoriasis Area and Severity Index
PMA:	Phorbol 12-myristate 13-acetate
slan:	6-Sulfo-LacNac
sTNF- α :	Soluble TNF- α (17 kDa)
tmTNF- α :	Transmembrane TNF- α (26 kDa)
TNFR:	TNF receptor
TUNEL:	Terminal deoxynucleotidyl transferase dUTP nick end labeling

used the anti-TNF- α antibody 52B83 for our *in situ* staining. This reagent does not detect receptor-bound TNF- α .²¹

In our study of peripheral blood and lesional skin of patients with psoriasis, we sought to determine the spectrum of TNF- α producers, as well as possible direct *in vitro* and *in vivo* effects of anti-TNF- α antibodies.

METHODS

For more information, see the [Methods](#) section and [Tables E1 to E3](#) in this article's Online Repository at www.jacionline.org.

RESULTS**TNF- α is produced by CD11c⁺ myeloid DCs and CD163⁺ macrophages in lesional psoriatic skin**

Using conventional immunohistochemistry (ie, horseradish peroxidase-mediated visualization of antibody-antigen binding with 3-amino-9-ethyl-carbazol as the chromogen), we detected tmTNF- α neither in sections from healthy (Fig 1, A) nor nonlesional psoriatic skin (data not shown). In lesional psoriatic skin we found a few anti-tmTNF- α -reactive cells scattered in the dermis (Fig 1, B, arrows), whereas keratinocytes remained uniformly negative (Fig 1, C) compared with the isotype control (Fig 1, D). By using a tyramide-based signal amplification method, we found tmTNF- α ⁺ cells in lesional psoriatic skin. They were located almost exclusively in the dermis, with essentially no signal in the epidermis (Fig 1, E). These tmTNF- α ⁺ dermal cells were all CD45⁺HLA-DR⁺ leukocytes coexpressing either CD11c or CD163, largely lacking the resident DC marker blood dendritic cell antigen (BDCA) 1 (Fig 1, F-J). This phenotype is consistent with that of inflammatory myeloid dendritic cells (mDCs) and macrophages, respectively.^{7,17,18,22} On quantification, 37.2% $\pm$ 10.3% ($\pm$ SD) of CD11c⁺ mDCs and 28.9% $\pm$ 6.1% of CD163⁺ macrophages were positive for tmTNF- α . Flow cytometric analysis of bulk leukocytes from lesional psoriatic skin confirmed that CD11c and CD163 are indeed nonoverlapping markers with distinct expression of HLA-DR (Fig 1, K), which is consistent with prior observations in both healthy and psoriatic skin.^{18,22} We did not find tmTNF- α colocalization on CD3⁺ T cells (Fig 1, L), plasmacytoid DCs (HLA-DR⁺BDCA-2⁺CD123⁺), endothelial cells (von Willebrand factor positive), mast cells (CD117⁺), or Langerhans cells (CD207/Langerin⁺; data not shown).

Nonlesional skin (Fig 1, M and N) and skin from healthy subjects (data not shown) was devoid of any tmTNF- α signal.

Slan⁺ mDC numbers are increased in the peripheral blood of patients with psoriasis compared with that of healthy control subjects

We next sought to determine whether TNF- α expression by slanDCs and macrophages is confined to lesional skin or, as a sign of systemic inflammation, also detectable in the circulation.

Results obtained showed that freshly isolated peripheral blood leukocytes and their subpopulations (both from patients with psoriasis and healthy subjects) are uniformly TNF- α ⁺, as determined by using both cell-surface and cytoplasmic staining (data not shown). In a next step we quantified peripheral blood mDCs (gated as lineage[−]HLA-DR⁺CD11c⁺ cells; Fig 2, A) by using the 3 subtype-specific, nonoverlapping human mDC markers BDCA-1 (CD1c), BDCA-3 (CD141), and CD16,²³ as well as slan, a carbohydrate structure defining a newly described subset of CD16⁺ mDCs (see Fig E1, A, in this article's Online Repository at www.jacionline.org).²⁴ We found that the proportion of slan⁺ mDCs was increased in the blood of patients with psoriasis compared with that of healthy control subjects, whereas BDCA-1⁺ mDC numbers were decreased (Fig 2, B). There was no difference in overall mDC counts between patients with psoriasis and healthy control subjects (data not shown). In patients successfully treated with the TNF- α blocker adalimumab (Psoriasis Area and Severity Index [PASI] score <1 for at least 12 weeks), levels of both BDCA-1⁺ and slan⁺ mDCs were no longer different from those found in healthy subjects, whereas the number of BDCA-3⁺ mDCs was significantly increased (Fig 2, B).

CD14⁺CD16⁺ "intermediate" monocytes are increased in the peripheral blood of patients with psoriasis

Peripheral blood monocytes can be divided into²⁵⁻²⁹ (1) CD14⁺CD16[−] "classical monocytes," accounting for the largest population; (2) CD14^{dim}CD16⁺ "nonclassical monocytes," partly coexpressing slan; and (3) and a third subset of "intermediate" CD14⁺CD16⁺ monocytes (Fig 3, A-C). Comparable with what was seen with mDCs, there was no difference in absolute and relative monocyte counts in the peripheral blood of healthy control subjects and patients with psoriasis (data not shown). However, on quantification of individual monocyte subtypes, we found an increase in the CD14⁺CD16⁺ subset accompanied by a concomitant decrease in the CD14⁺CD16[−] subset in patients with psoriasis compared with healthy control subjects (Fig 3, D). During successful treatment with adalimumab (ie, PASI score <1 for at least 12 weeks), these alterations in cell counts disappeared (Fig 3, D). Among CD14^{dim}slan⁺ monocytes, we did not see consistent differences between healthy control subjects and patients with psoriasis (Fig 3, E).

TNF- α expression by slanDCs and monocytes but not any other leukocyte subpopulation after *in vitro* culture of PBMCs

It has been shown *in vitro* that erythrocytes inhibit the phenotypic maturation of slanDCs, as well as their production of proinflammatory cytokines, including TNF- α .³⁰ To circumvent this

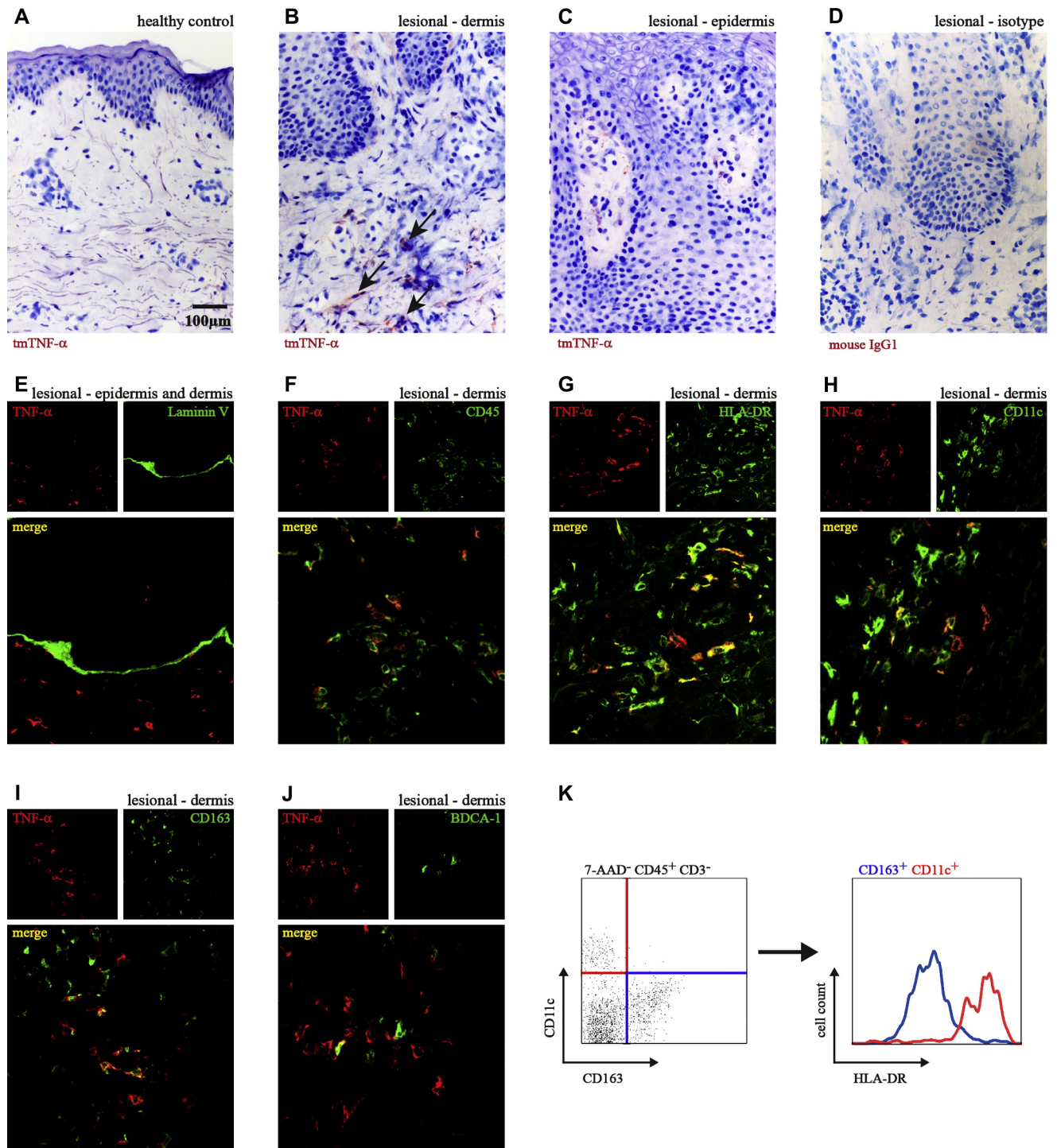


FIG 1. Characterization of tmTNF-α⁺ skin cells in healthy subjects and patients with psoriasis. **A-D**, Conventional immunohistochemistry for tmTNF-α was done on sections of healthy (Fig 1, A) and lesional psoriatic skin (Fig 1, B and C), including an isotype control (Fig 1, D). **E-J** and **L-N**, Tyramide signal amplification-based immunofluorescence was used to visualize tmTNF-α in lesional (Fig 1, E-J and L) and nonlesional (Fig 1, M and N) psoriatic skin counterstained for lineage markers of individual cell types. **K**, Flow cytometric analysis for CD11c, CD163, and HLA-DR surface expression of living (ie, 7-amino-actinomycin D⁻) bulk dermal CD3⁻ leukocytes from lesional psoriatic skin. Pictures are representative of at least 6 individual patients.

effect, we isolated PBMCs from patients with psoriasis and cultured them *in vitro* together with brefeldin A to increase staining sensitivity. After 4 hours of incubation and without further stimulus, we found positive TNF-α staining by

lineage⁻CD11c⁺HLA-DR⁺ mDCs (Fig 4, A) and CD14⁺ monocytes (Fig 4, B). Under the same conditions, plasmacytoid DCs (Fig 4, C), B cells (Fig 4, D), or natural killer (NK) cells (Fig 4, E) displayed no anti-TNF-α reactivity. T cells (Fig 4, F) produced

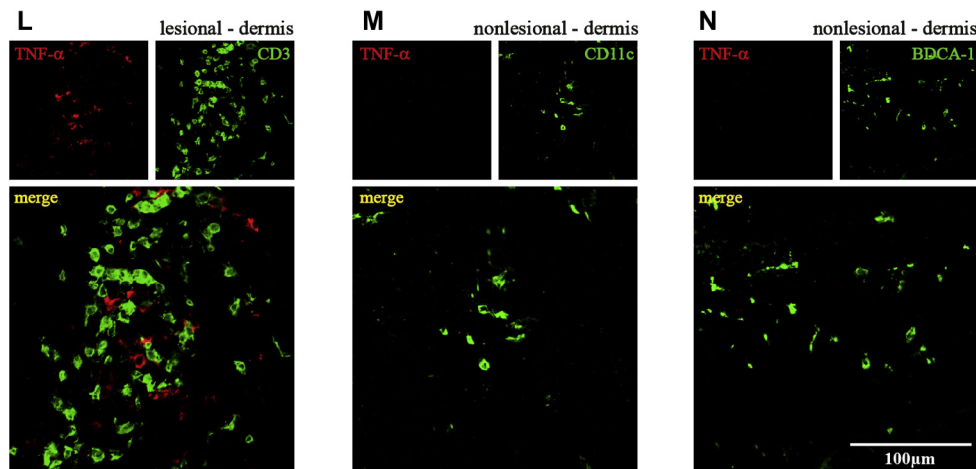


FIG 1. (Continued)

TNF- α only after stimulation with phorbol 12-myristate 13-acetate (PMA)/ionomycin (Fig 4, G). Although PBMCs from healthy subjects showed a similar pattern and staining intensity of overall TNF- α expression (data not shown), the proportion of TNF- α ⁺ mDCs was significantly higher in patients with psoriasis compared with that seen in healthy control subjects, and their numbers increased with disease severity (Fig 4, H). On further characterization of these cells (gating strategy depicted in Fig 4, I), we found that TNF- α ⁺ mDCs were largely positive for slan (Fig 4, J). Consistently, they were negative for BDCA-1 (CD1c) and BDCA-3 (CD141) but, surprisingly, also for CD16. This finding is most likely due to the (reported)³¹ rapid loss of DC-bound CD16 *in vitro*.

Because we could confirm that slan is also present on TNF- α ⁺ inflammatory DCs in the dermis of patients with lesional psoriasis (see Fig E1, B),⁶ we analyzed expression levels of surface molecules possibly involved in the recruitment of slanDCs into psoriatic skin.⁶ Among various receptors analyzed, CD54 (intercellular adhesion molecule 1) was upregulated in TNF- α ⁺ slanDCs compared with their TNF- α [−] counterparts (see Fig E1, C).

On stimulation with peptidoglycan or LPS, isolated peripheral blood slan⁺ mDCs produced much higher amounts of TNF- α (see Fig E1, D), IL-6, IL-12, IL-23, and IL-1 β (data not shown) compared with BDCA-1⁺ DCs, which is consistent with published data.^{6,24}

In contrast to mDCs, we did not find consistent differences in numbers of TNF- α ⁺ CD14⁺ monocytes between patients with psoriasis and healthy control subjects (data not shown). In addition, their allocation to an abovementioned monocyte subset was hampered by the fact that CD16 turned out to be an unstable marker because it was partly lost on stimulation with LPS *in vitro* both on cells from healthy control subjects and patients with psoriasis (see Fig E2 in this article's Online Repository at www.jacionline.org).

Infliximab modulates mRNA levels of proinflammatory molecules *in vitro* and *in vivo* without altering cell counts early after initiation of therapy

To determine the effect or effects of TNF- α antagonists on TNF- α -producing cells, we investigated lesional psoriatic skin

before and 16 and 48 hours after a single infusion of infliximab (5 mg/kg body weight). We did not detect a difference in the overall rate of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells (Fig 5, A) or any change in the number of TNF- α ⁺ cells (Fig 5, B), CD11c⁺ DCs (Fig 5, C), CD163⁺ macrophages (Fig 5, D), or, as a control, CD3⁺ T cells (Fig 5, E). Rates of PBMCs producing TNF- α *in vitro* were also not changed at these time points (data not shown). However, we saw significant reductions in mRNA levels of proinflammatory molecules, namely IL-12p40, IL-1 β , and CCL20 and, to a much lesser extent, TNF- α and IFN- γ , at the 16-hour time point (Fig 5, F). Additionally, we observed a slight decrease of IL12RB1 mRNA at 16 hours, encoding the common subunit of both the IL-12 and the IL-23 receptors. There were no significant changes in mRNA levels of IL-6, IL-10, TGF- β 1, IL-12p35, IL-23p19, IL-4, IL-17A, IL-22, IL-12RB2, IL-23R, IL-17RA, IL-17RC, TNFR1, TNFR2, iNOS, Langerin, forkhead box protein 3 (FOXP3), TNF-related apoptosis-inducing ligand (TRAIL), granzyme A, and granzyme B (data not shown).

In a next series of experiments, PBMC-isolated slanDCs were stimulated with LPS (Fig 5, G) or peptidoglycan (data not shown) to exhibit TNF- α (data not shown). In the presence of the TNF- α blocker infliximab, they produced less IL-12 and IL-23, whereas IL-6 levels were not altered. There was no effect of infliximab on cell survival (data not shown). Interestingly, we found that the proliferation rate of T cells toward allogeneic TNF- α ⁺ slan⁺ DCs was significantly decreased when TNF- α blockade (through infliximab or its Fab₁-fragment) was present (Fig 5, H). There was no effect of TNF- α blockade on anti-CD3/anti-CD28-driven T-cell proliferation, a process that is presumably independent of antigen-presenting cells (Fig 5, I).

To rule out a possible interaction of TNF- α blockers with receptor-bound TNF- α , we analyzed keratinocytes that had been incubated with sTNF- α *in vitro*. We did not find binding of infliximab and other clinically used TNF- α blockers to receptor-bound TNF- α on the surfaces of these cells (see Fig E3 in this article's Online Repository at www.jacionline.org).

DISCUSSION

TNF- α is of central importance in patients with psoriasis and other inflammatory diseases, as evidenced by the clinical efficacy

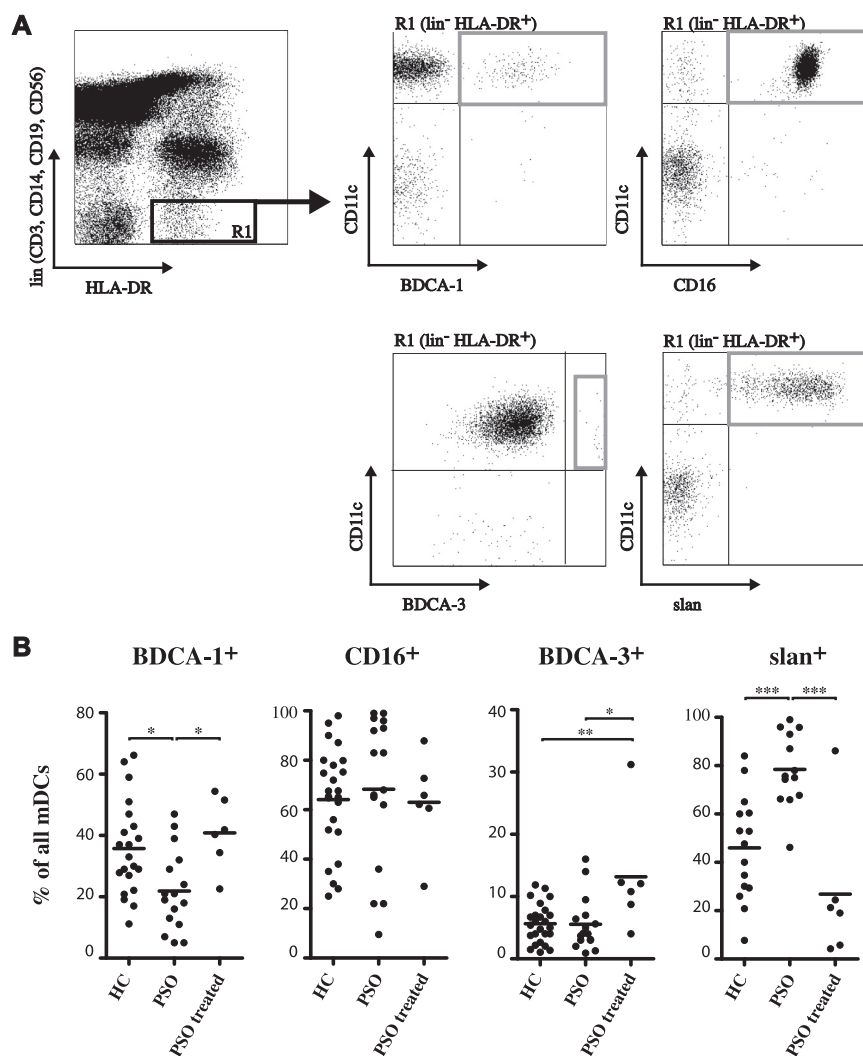


FIG 2. **A** and **B**, mDCs characterized by lineage negativity (ie, CD3⁻, CD14⁻, CD19⁻, and CD56⁻) and expression of HLA-DR and CD11c (Fig 2, **A**) were analyzed for the subset-specific surface markers BDCA-1, CD16, BDCA-3, and slan (Fig 2, **B**). **C**, Relative distribution of these mDC subsets of healthy control subjects (HC), untreated patients with psoriasis (PSO), and adalimumab-treated patients with a PASI score of less than 1 (PSO treated). Dot plots depicted are representative of at least 8 individual experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$, 2-tailed Student t test with the Bonferroni-Holm correction.

of TNF- α blockers.⁸ Therefore it is surprising that controversy still exists about the nature of the actual producers of this pathogenetically important cytokine and whether these cells are direct targets of anti-TNF- α antibodies.

In this study we have addressed this issue by using a tyramide-based amplification system for anti-TNF- α staining, which is described to lead to a 10- to 100-fold increase in staining efficiency.³² By doing so, the detection of tmTNF- α in lesional psoriatic skin was confined to myeloid cells of the “inflammatory” (ie, BDCA-1⁺) mDC lineage, as well as the monocyte/macrophage lineage. Confirming data from Hänsel et al,⁶ we also found slan to be expressed in lesional psoriatic skin, colocalizing, at least partly, with tmTNF- α . Consistently, CD11c⁺ slan⁺ DCs, which are absent in normal skin,³³ were recently reported as TNF- α ⁺iNOS⁺IL-23p19⁺BDCA-1⁻ dermal inflammatory DCs in lesional psoriatic skin.^{6,30} These markers are also characteristic of the previously described TNF- and iNOS-producing DC,¹⁷ and it might well be that these cell populations will ultimately turn out

to be closely related, if not identical. Other cell types, including keratinocytes and T cells, did not react with anti-TNF- α antibodies. Even though we could confirm that T cells produce large amounts of TNF- α on *in vitro* stimulation with, for example, PMA/ionomycin, we never found TNF- α to colocalize with CD3 in lesional psoriatic skin and thus challenge the notion of T cells being key producers of TNF- α , at least in chronic lesions,¹² or serving as direct targets of TNF- α blockers, especially because we could not find stable binding of TNF- α blockers to exogenous sTNF- α displayed on the surfaces of TNFR-bearing cells.

We are aware of the fact that other investigators detected TNF- α in keratinocytes of patients with psoriasis.¹³⁻¹⁵ The reason or reasons for the discrepancy to our results could be the type of antibodies used (polyclonal vs monoclonal³⁴), differences in staining protocols, and detection of exogenous TNF- α bound to TNFRs, which have been shown to be abundantly expressed throughout lesional psoriatic skin.¹⁴

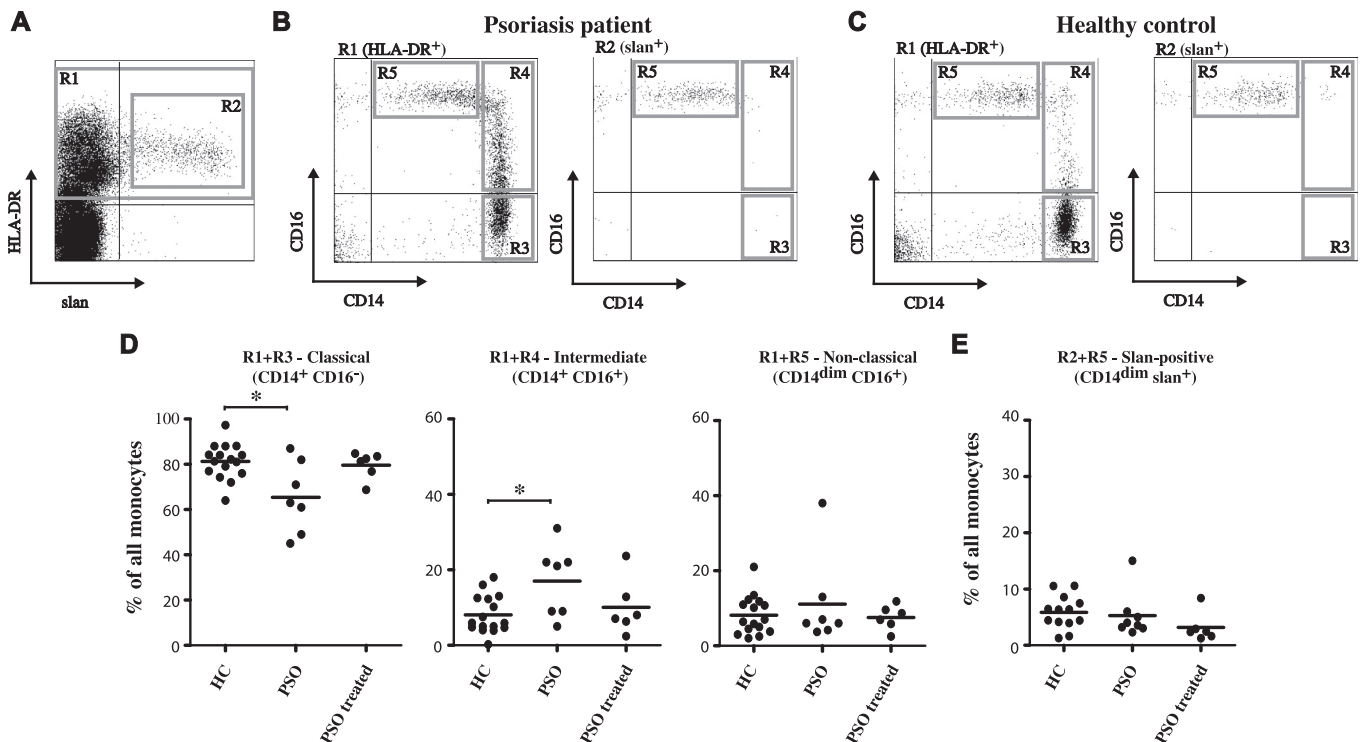


FIG 3. A-C, Distribution of monocyte subsets in PBMCs from healthy control subjects and patients with psoriasis. Gating strategy (Fig 3, A) for classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$), and nonclassical ($CD14^{dim}CD16^+$), as well as $CD14^{dim}slan^+$ monocyte subsets with representative dot plots for patients with psoriasis (Fig 3, B) and healthy control subjects (Fig 3, C). D and E, Percentage of monocyte subsets in healthy control subjects (HC), untreated patients with psoriasis (PSO), and adalimumab-treated patients with a PASI score of less than 1 (PSO treated). * $P < .05$, 2-tailed Student t test with the Bonferroni-Holm correction.

Certainly, we cannot definitively exclude the possibility that cell types other than those detected by our means do produce $TNF-\alpha$, given that this cytokine is quickly secreted into the extracellular space and thus not detectable by means of immunostaining. However, even if this were the case, the surface density of $tmTNF-\alpha$ would probably be too low to allow for direct cytotoxic effects of $TNF-\alpha$ blockers. This notion is supported by *in vitro* experiments showing that cytotoxicity is only observed in transfected cells expressing $TNF-\alpha$ at very high levels.^{10,35}

Compared with the skin, a similar pattern of $TNF-\alpha$ expression in cultured PBMCs was encountered, namely in mDCs and monocytes, with an increase in $TNF-\alpha^+$ mDC counts in patients with psoriasis compared with those seen in healthy control subjects. This “spontaneous” $TNF-\alpha$ production on *in vitro* culture was largely confined to the $slan^+$ subset, which, surprisingly, was negative for CD16, a marker previously described as present on all $slan^+$ DCs.^{24,36} Given the fact that CD16 is rapidly lost from the surface of mDCs *in vitro*,³¹ we would assume that $TNF-\alpha^+slan^+CD16^-$ lineage⁻ cells belong to the formerly CD16⁺ mDC subset.

Similar to what has been described by others, we found that on stimulation with peptidoglycan or LPS,²⁴ peripheral blood $slan^+$ mDCs produce much higher levels of $TNF-\alpha$ than BDCA-1⁺ mDCs. In unperturbed freshly isolated PBMCs, we observed a shift in the overall distribution of the mDC compartment toward an increase in $slan^+$ mDC counts and a concomitant decrease in BDCA-1⁺ mDC counts in the peripheral blood of patients with psoriasis compared with that of healthy control subjects. This

phenomenon was not observed by Hänsel et al,⁶ most likely because of different gating strategies because we included only $CD14^-$ cells in our DC populations, whereas $CD14^{dim}$ to $CD14^+$ cells were termed monocytes in line with prior publications.²⁵⁻²⁷ With this definition in mind, we did not find differences in the frequencies of $CD14^{dim}/CD14^+slan^+$ monocytes between healthy control subjects and patients with psoriasis. Therefore we believe that it is justified to distinguish between $CD14^-slan^+$ DCs and $CD14^{dim}/CD14^+$ (inflammatory) monocytes/macrophages. Admittedly, $CD14^{dim}slan^+$ cells have not always been sharply demarcated from their $CD14^-$ counterparts. For our experiments, we used fluorochromes that, in our hands, allowed a reasonably clear distinction of $CD14^-$, $CD14^{dim}$, and $CD14^+$ cells.

In the blood monocyte compartment of patients with psoriasis, we observed an increase in $CD14^+CD16^+$ cell counts compared with those seen in healthy control subjects. These cells have been demonstrated to be superior to classical monocytes in their capacity to produce $TNF-\alpha$ ^{25,37} and to strongly induce T_H17 cell expansion *in vitro*.³⁷ Apart from their numeric increase in several (auto) inflammatory disorders, increased counts of $CD14^+CD16^+$ cells independently predicted nonhemorrhagic stroke, myocardial infarction, and death from cardiovascular disease,²⁶ conditions all known to be linked to psoriasis.³⁸ Taken together, our results and those of other investigators demonstrate that in patients with psoriasis, $slan^+$ mDCs and inflammatory monocytes/macrophages are the major $TNF-\alpha$ -producing cells, and their reduction during remission of psoriasis underlines their critical role in

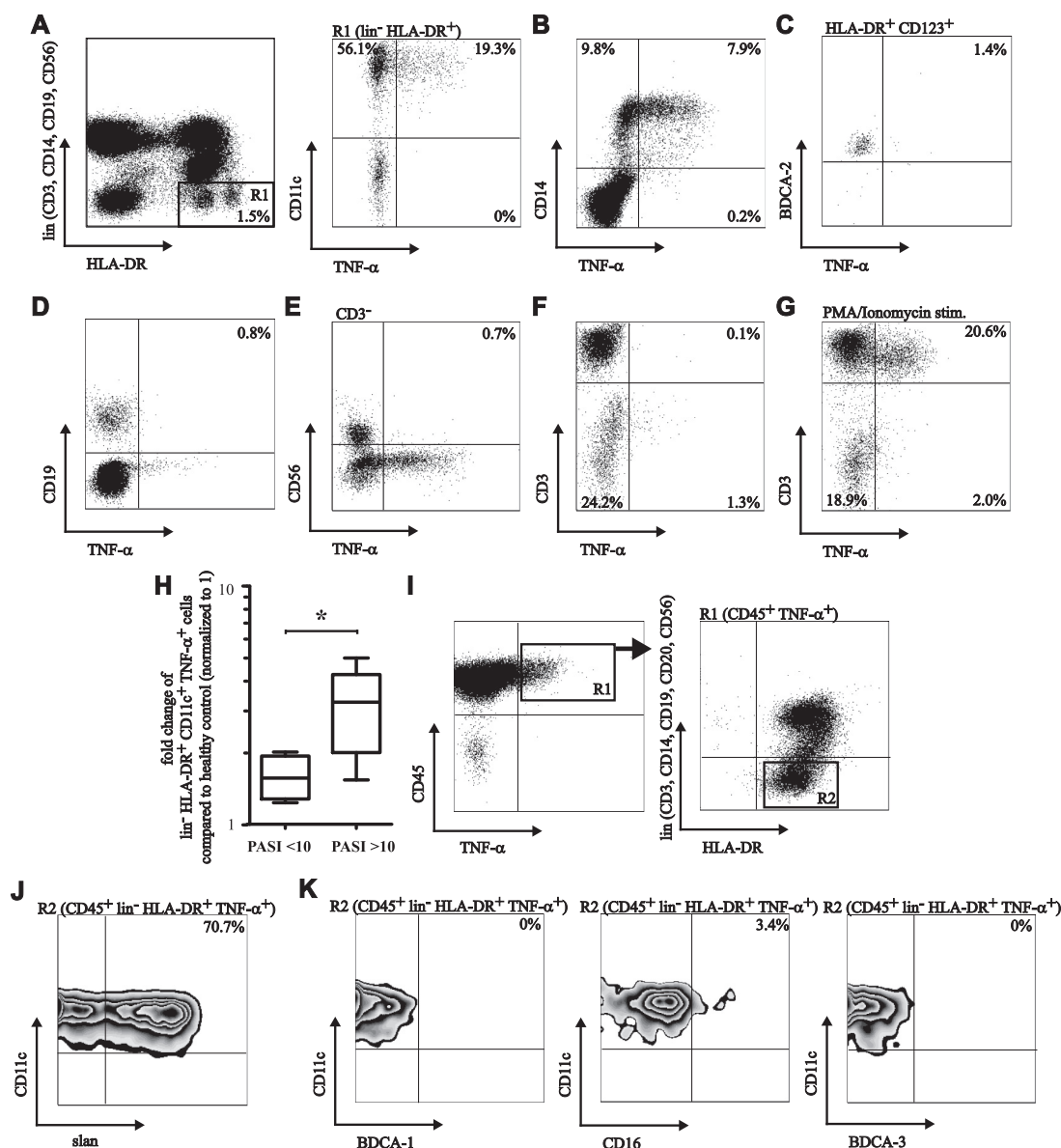
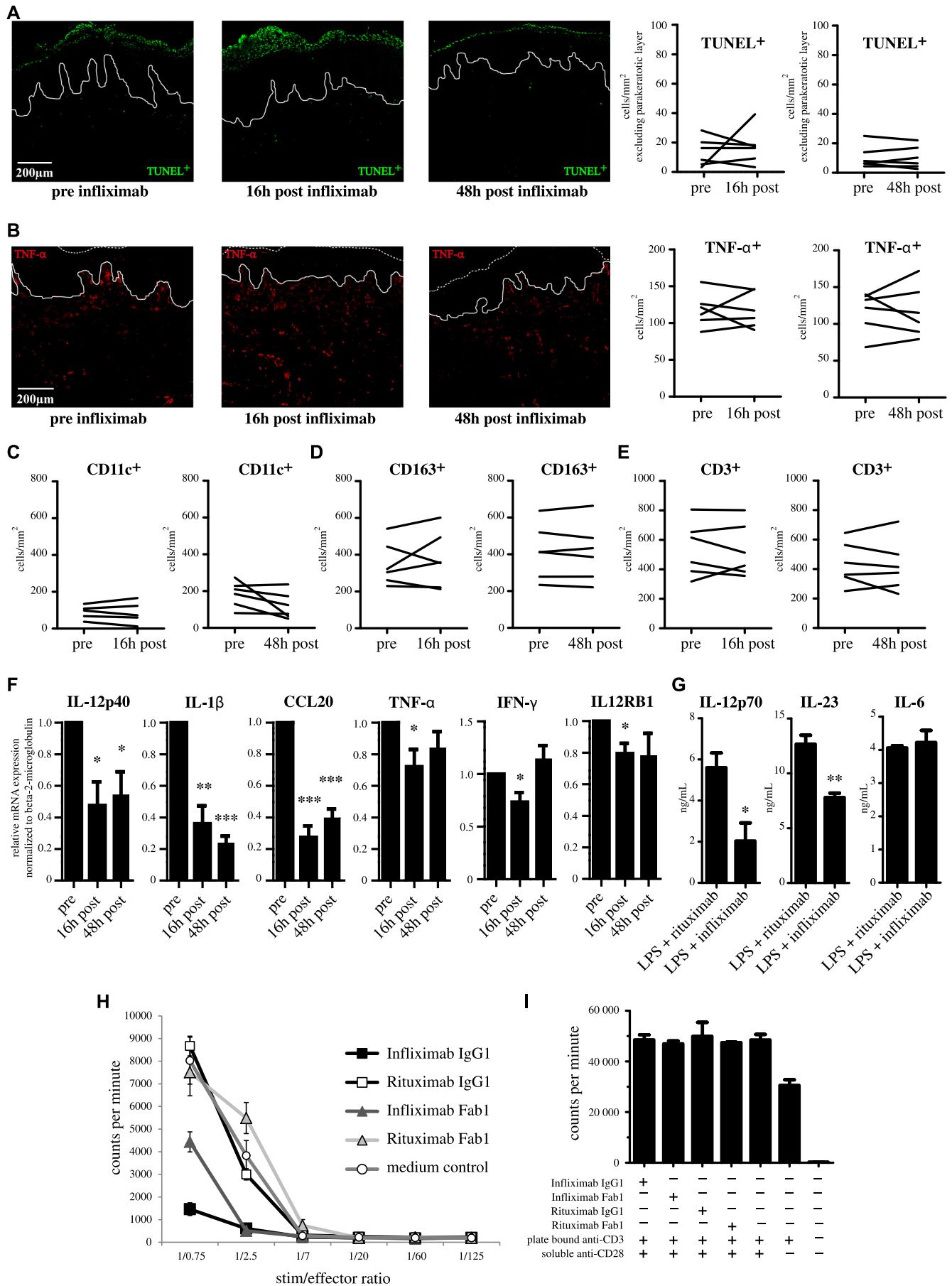


FIG 4. *In vitro* production of TNF- α in PBMCs from patients with psoriasis. **A-F**, mDCs (Fig 4, A) and monocytes (Fig 4, B), plasmacytoid DCs (Fig 4, C), B cells (Fig 4, D), NK cells (Fig 4, E), and T cells (Fig 4, F) were analyzed, and representative dot plots are shown. **G**, T cells produced TNF- α only after stimulation with PMA/ionomycin. **H**, Fold change of TNF- α ⁺ mDCs in patients with psoriasis compared with healthy control subjects depending on disease severity (healthy control population [n = 15] normalized to 1). PASI score less than 10 = mild-to-moderate disease activity (n = 5); PASI score greater than 10 = moderate-to-severe disease activity (n = 10). Whiskers, 5th-95th percentiles. **P* < .05, 2-tailed Student *t* test. **I-K**, These mDC subsets (alternative gating strategy depicted in Fig 4, I) were further characterized for the subset-specific surface markers BDCA-1, CD16, BDCA-3, and slan (Fig 4, J and K). Dot plots shown are representative for at least 8 individual experiments.

disease development, maintenance, or both. At the moment, it is not clear whether slan⁺ DCs and macrophages of lesional psoriatic skin acquire TNF- α expression in the skin or, alternatively, are recruited from a pool of TNF- α ⁺ peripheral blood leukocytes. Data by Hänsel et al,⁶ as well as our observation of intercellular adhesion molecule 1 upregulation and thus increased stickiness of TNF- α ⁺ slanDCs favors the latter possibility. Should this assumption be correct, it is also conceivable that circulating TNF- α ⁺ leukocytes have the potential to migrate also to tissues/organs other than the skin, thus contributing to psoriasis

comorbidities, such as psoriatic arthritis, cardiovascular disease, or obesity.

Ample evidence exists that TNF- α blockers are an effective treatment for psoriasis,⁸ but their mode of efficacy is not yet fully understood. Apart from merely neutralizing the soluble cytokine, there is *in vitro* evidence that anti-TNF- α antibodies have the capacity to mediate apoptosis, complement killing, antibody-dependent cellular cytotoxicity, and retrograde signaling in tmTNF- α -expressing cells.^{10,35,39} However, the *in vivo* relevance of these tmTNF- α -mediated effects has never been formally



proved.¹⁰ For this reason, we analyzed lesional psoriatic skin before and 16 and 48 hours after the first administration of the anti-TNF- α antibody infliximab. These time points were chosen to detect early lethal cell injury, potential later consequences of retrograde tmTNF- α signaling (ie, downregulation of inflammation and the immune response), or both.

Sixteen to 48 hours after the administration of infliximab, we did not see a change in the frequency of TUNEL⁺ cells or in the number of TNF- α ⁺ cells, CD11c⁺ DCs, or CD163⁺ macrophages in lesional psoriatic skin, which likely excludes relevant rates of direct cytotoxicity.

However, on the molecular level, we detected a profound change in mRNA expression of skin-derived proinflammatory mediators, namely a significant reduction in levels of (1) IL-1 β , a proinflammatory regulator of T_H17 cells both at priming and in the effector phase⁴⁰; (2) IL-12p40, the common subunit of IL-12 and IL-23, 2 key cytokines for the T_H1 and T_H17 axis, respectively; and (3) CCL20, a chemotactic factor for T_H17 cells,⁴¹ and, to a lesser extent at the 16-hour time point, TNF- α , IFN- γ , and IL12RB1. Levels of other cytokines, such as IL-6 and TGF- β , remained unchanged. In line with these findings, we found that PBMC-derived slanDCs stimulated with LPS or peptidoglycan produced less IL-12 and IL-23 in the presence of the TNF- α blocker infliximab, whereas IL-6 levels were not altered.

Our data suggest that infliximab has an immediate dampening effect not only on the T_H1 axis but also on the T_H17 axis in lesional psoriatic skin, even when IL-6 and TGF- β are still present. This notion is supported by the fact that ustekinumab, exclusively blocking IL12p40, profoundly improves psoriasis symptoms⁴² and that IL-1 β , but not TGF- β , is essential for the induction of IL-17-producing human T_H cells.⁴³ Moreover, T_H17 cells primed in the presence of IL-23 were shown to have a greater pathogenic potential to mediate the development of experimental autoimmune encephalomyelitis than those stimulated with TGF- β and IL-6 alone and that IL-6 by itself does not sufficiently generate T_H17 cells.⁴⁴

On a functional level, we demonstrated that proliferation of T cells was significantly decreased when infliximab or its Fab₁ fragment (the latter being incapable of mediating complement killing and antibody-dependent cellular cytotoxicity) was present. This effect was antigen-presenting cell dependent because infliximab did not have an effect on anti-CD3/anti-CD28-driven T-cell proliferation. Because DCs and T cells are often observed in close proximity in lesional psoriatic skin, it might well be that infliximab prevents antigen-presenting cell-dependent T-cell proliferation *in vivo*.

From these data, we conclude that the clinical effects of anti-TNF- α antibodies are not rooted in direct cytotoxicity of tmTNF- α -bearing cells but rather in the interference with either

TNFR-mediated mechanisms (paracrine, autocrine, and juxtacrine signaling), retrograde signaling through tmTNF- α ,¹⁰ or both. We therefore suggest that anti-TNF- α treatment not only results in decreased tissue inflammation but also in a rapid downregulation of the pathogenic IL-12/IL-23-driven immune response independent of direct killing of TNF- α -producing cells.

We thank Irene Klein and Sabine Kohlhofer for technical advice, Christopher Schuster for critically reading the manuscript, Peter Steinberger for the BW5147 cell line, and Knut Schäkel for providing us with the DD2 antibody.

Clinical implications: Efficacy of anti-TNF- α antibodies in patients with psoriasis is mediated through a rapid downregulation of key proinflammatory cytokines rather than direct cytotoxicity of TNF- α -producing cells (ie, mDCs and monocytes/macrophages).

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FIG 5. A-E, Before (*pre*) and 16 or 48 hours after (*post*) a single intravenous infusion of infliximab (5 mg/kg body weight), we quantified apoptotic cells with a TUNEL assay (Fig 5, A), TNF- α ⁺ cells through tyramide signal amplification-based immunofluorescence (*white line* denotes dermoepidermal junction, and *dotted line* indicates skin surface; Fig 5, B), and CD11c⁺ DCs, CD163⁺ macrophages, and CD3⁺ T cells by using conventional immunohistochemistry (Fig 5, C-E). **F,** Changes in mRNA levels at 16 (*n* = 7) and 48 (*n* = 6) hours were evaluated by using quantitative RT-PCR. **G,** Levels in supernatants of IL-12, IL-23, and IL-6 (intact cytokines) produced by LPS-stimulated slanDCs isolated from buffy coat preparations with or without TNF- α blockade by using infliximab. **H,** Tritiated thymidine uptake by T cells in an allogeneic mixed leukocyte reaction of irradiated slanDCs isolated from buffy coat preparations measured on day 6 of culture. **I,** Anti-CD3/anti-CD28-driven T-cell proliferation measured on day 4 of culture. Rituximab served as a control antibody. **P* < .05, ***P* < .01, and ****P* < .001, paired (Fig 5, F) and unpaired (Fig 5, G) Student *t* test with the Bonferroni-Holm correction.

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METHODS

Patient material

Skin and peripheral blood samples were obtained under an approved protocol (EK700/2009, Ethics Committee of the Medical University of Vienna), according to the Declaration of Helsinki. Patients with moderate-to-severe plaque-type psoriasis (mean age, 48.9 ± 12.5 years) and age-matched healthy volunteers were enrolled in the study after providing written informed consent. Six-millimeter punch biopsy specimens were taken from the abdomen after achievement of local anesthesia, embedded in the optimal cutting temperature compound OCT (Tissue-Tek; Sakura Finetek, Zoeterwoude, The Netherlands), and stored at -80°C until further processing.

Cell culture

All *in vitro* experiments were performed in RPMI 1640 medium (Invitrogen, Carlsbad, Calif) supplemented with 10% heat-inactivated AB Serum (Sigma-Aldrich, St Louis, Mo) and 1% penicillin/streptomycin (Invitrogen) at 37°C and 5% CO_2 , if not otherwise indicated.

Immunofluorescence and immunohistologic staining

Both immunohistologic and single and multicolor immunofluorescence staining procedures were performed on frozen sections of patients with chronic plaque-type psoriasis and healthy control subjects, as previously described.^{E1,E2} The mAbs used in this study are shown in Table E1.

Cytokine staining for TNF- α (52B83; Hycult Biotech, Plymouth Meeting, Pa) was performed at a final dilution of 1 $\mu\text{g}/\text{mL}$ either with conventional immunofluorescence (Dako REAL Detection Systems HRP/AEC, Dako AutostainerLink 48; Dako, Glostrup, Denmark) or by using tyramide-based signal amplification (TSA Biotin System; PerkinElmer, Waltham, Mass), as previously described by Hunyady et al.^{E2} Specificity was assessed by using the mouse thymoma cell line BW5147 overexpressing human tmTNF- α , a kind gift of Peter Steinberger (Institute of Immunology, Medical University of Vienna, Vienna, Austria). Negative controls were obtained in all staining experiments by substituting isotype-matched immunoglobulin for the primary antibody, as well as using preabsorbed anti-TNF- α antibodies, which had been preincubated for 45 minutes with TNF- α at room temperature (Pepro-Tech, Rocky Hill, NJ). Apoptosis was examined by using TUNEL staining. Cells were stained with fluorescein isothiocyanate-conjugated dUTP, according to the manufacturer's specifications (Roche Diagnostics, Risch, Switzerland). Immunofluorescence images were acquired with either a Zeiss Axiovert 200M confocal microscope (Carl Zeiss, Oberkochen, Germany) or a Zeiss Observer.Z1 microscope equipped with TissueFAXS and analyzed by using HistoQuest software (both from Tissue Gnostics GmbH, Vienna, Austria), as previously described.^{E3}

Flow cytometry

Flow cytometric analyses were performed, as previously described.^{E4} Briefly, blood samples were drawn with BD Vacutainer Cell Preparation Tubes with sodium heparin from patients with psoriasis with active disease (mean PASI score, 14.5 ± 5.5), patients in remission (PASI score < 1) because of treatment with the TNF- α blocker adalimumab (40 mg administered subcutaneously every other week), and healthy control subjects. The PASI score was assessed, as previously described,^{E5} by grading redness, thickness, and scaling of plaques, as well as the body surface involved, with higher scores indicating more severe disease. Samples were stained for the extracellular markers outlined in Table E2. For intracellular TNF- α staining, isolated PBMCs were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, penicillin (100 IU/mL), streptomycin (100 $\mu\text{g}/\text{mL}$, all from Invitrogen), and brefeldin A (10 $\mu\text{g}/\text{mL}$, Sigma-Aldrich) at 5 to 10×10^5 cells/mL in 6-well plates and cultured for 4 hours at 37°C in 5% CO_2 . T cells stimulated with PMA (25 ng/mL) and ionomycin (2 $\mu\text{g}/\text{mL}$, both from Sigma-Aldrich) served as a positive control. Intracellular staining for TNF- α (MAb11; BD PharMingen, San Diego, Calif) was performed with the Fix&Perm Cell Permeabilization Kit (An Der Grub BioResearch GmbH, Kaumberg, Austria) after extracellular staining of lineage markers.

Dermal single-cell suspensions from lesional psoriatic skin were prepared, as previously described.^{E6} Briefly, biopsy specimens were cut into small pieces and digested with collagenase IV (0.5 U/mL; Worthington Biochemical, Lakewood, NJ) for 30 minutes at 37°C . The resulting cell suspension was filtered (70 $\mu\text{mol}/\text{L}$ BD Falcon cell strainer, BD Biosciences), immunostained, and subjected to fluorescence-activated cell sorting (BD FACSARIA).

Quantitative RT-PCR

Snap-frozen biopsy specimens from lesional psoriatic skin taken before and 16 and 48 hours after a single administration of infliximab (Remicade; Centocor, Malvern, Pa; 5 mg/kg body weight, intravenous infusion over 2 hours) were thawed on ice. RNA isolation, its transcription to cDNA, and further quantification and analysis were performed, as described previously.^{E1} Results were normalized to the housekeeping gene human β_2 -microglobulin ($\beta_2\text{m}$). Primers and probes for the TaqMan RT-PCR assays were used from TaqMan Gene Expression Assays (all Applied Biosystems, Foster City, Calif), as outlined in Table E3.

Cell isolation, stimulation, and analysis of cytokine release

For *in vitro* stimulation assays with DCs, peripheral blood samples were purchased as buffy coats from the Vienna Red Cross Center and isolated, as previously described.^{E4} Briefly, PBMCs were obtained by using Ficoll-Paque density gradient centrifugation (Ficoll-Paque Plus; GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and depleted of T cells, B cells, and monocytes by means of anti-CD3/CD20/CD14 (3 $\mu\text{g}/\text{mL}$ each) immunolabeling and anti-mouse IgG immunomagnetic depletion (magnetic cell sorting [MACS]; Miltenyi Biotec, Bergisch Gladbach, Germany). DCs were positively selected by using anti-slan or anti-BDCA-1 microbeads (MACS). The purity of isolated DC populations was greater than 95%, as determined by using flow cytometric analysis (FACSCalibur; BD Biosciences, San Jose, Calif) with anti-CD11c/HLA-DR staining. None of the other leukocyte subpopulations tested for individually (T cells, B cells, NK cells, and monocytes) accounted for more than 1% of the sorted cell population.

Isolated slan⁺ mDCs and BDCA-1⁺ mDCs were washed and resuspended in cell-culture medium at 1×10^6 cells/mL in 0.1 mL in 96-well plates. After 6 hours of culture at 37°C and 5% CO_2 , cells were incubated with 100 ng/mL LPS (InvivoGen, San Diego, Calif) or 20 $\mu\text{g}/\text{mL}$ peptidoglycan (LEE Laboratories, BD Biosciences) with or without 0.5 $\mu\text{g}/\text{mL}$ infliximab or rituximab for another 12 to 24 hours. Media conditioned by these DCs were collected and stored at -20°C for further analysis. Supernatants were analyzed for cytokines with FlowCytomix Kits (eBioscience, Vienna, Austria), according to the manufacturer's instructions.

Isolation of CD16⁺ monocytes was performed with the Miltenyi CD16⁺ monocyte isolation kit, according to the manufacturer's instructions (Miltenyi Biotec). In brief, PBMCs from healthy control subjects and patients with psoriasis were obtained by using BD Vacutainer Cell Preparation Tubes with sodium heparin. After blocking of Fc receptors, granulocytes and NK cells were depleted with anti-CD15 and anti-CD56 microbeads. In a second step CD16⁺ monocytes were positively selected with anti-CD16 microbeads. Purity was greater than 97%, as determined based on results of anti-CD16 staining with flow cytometric analysis (FACSCalibur, BD Biosciences). Cells (10^5) were cultured in 100 μL of cell-culture medium with or without 100 ng/mL LPS (InvivoGen) for 24 hours and subsequently analyzed by means of flow cytometry.

For the isolation of T cells, PBMCs from buffy coat preparations were depleted from non-T cells (MACS, Miltenyi Biotec) by using antibodies against CD14 (Clone RM052), CD16 (3G8), CD19 (3-119), CD34 (581), CD56 (C218), CD41 (SZ22), CD235a (11E4B-7-6; all from Immunotech/Beckman Coulter, Marseille, France), and HLA-DR (G46-6, BD Biosciences). Purity was greater than 98%, as assessed based on anti-CD3 staining with flow cytometry.

Anti-CD3/CD28 stimulation of T cells

Ninety-six-well plates (Corning, Corning, NY) were coated for 2 hours at 37°C with 1 $\mu\text{g}/\text{mL}$ of the anti-CD3 antibody UCHT1 (Beckman Coulter) and

subsequently washed 4 times with PBS. In these plates 5×10^4 cells per well were incubated in cell-culture medium supplemented with 1 $\mu\text{g/mL}$ soluble anti-CD28 antibody (clone L293, BD Biosciences), as well as infliximab (Centocor) or its Fab₁ fragment. Rituximab (Roche) served as a control because CD20 is not expressed on T cells. On day 4 of culture, 20 μL of medium containing 1 μCi (37×10^3 [Bq]) of tritiated thymidine (PerkinElmer, Waltham, Mass) was added per well, and 24 hours later, cultures were harvested (Harvester 96; Tomtec, Hamden, Conn). Tritiated thymidine incorporation into DNA was measured by using the 1205 Betaplate Liquid Scintillation Counter (Wallac, PerkinElmer). Results of quadruplicate cultures are shown as mean counts per minute $\pm$ SEM.

Allogeneic mixed leukocyte reaction

For mixed leukocyte reaction experiments, 8×10^2 to 1.3×10^5 irradiated slanDCs (30 Gy, γ -irradiation/Cs-137) isolated from buffy coat preparations were admixed to 1×10^5 responder T cells with or without infliximab or its Fab₁ fragment. Rituximab served as a control antibody because CD20 is not expressed on slanDCs (data not shown) or T cells. On day 6 of cell culture, 20 μL of medium containing 1 μCi (37×10^3 [Bq]) of tritiated thymidine (PerkinElmer) was added per well, and 24 hours later, cultures were harvested (Harvester 96, Tomtec). Tritiated thymidine incorporation into DNA was measured with the 1205 Betaplate Liquid Scintillation Counter (Wallac, PerkinElmer). Results of quadruplicate cultures are shown as mean counts per minute $\pm$ SEM.

Assessment of antibody binding to tmTNF- α and receptor-bound TNF- α

Pooled primary human keratinocytes from juvenile foreskin (Promo-Cell, Heidelberg, Germany) were incubated with 1 $\mu\text{g/mL}$ sTNF- α (PeproTech, Rocky Hill, NJ) for 30 minutes and subsequently exposed to 1 $\mu\text{g/mL}$ biotinylated infliximab, adalimumab (AbbVie, Abbott Park, Ill), etanercept (Pfizer, New York, NY), human IgG₁ kappa (Sigma-Aldrich), and the anti-TNF- α antibody sc-52250 (clone 2C8; Santa Cruz Biotechnology, Santa Cruz, Calif). Binding was visualized by means of flow cytometry with Alexa Fluor 488-labeled streptavidin at 4 $\mu\text{g/mL}$ (Invitrogen). The mouse thymoma cell line BW5147 served as a negative (wild-type) and positive (TNF- α -overexpressing) control. All incubation steps were carried out at 4°C to avoid stimulation effects of sTNF- α or antibody binding.

Biotinylation of antibodies and etanercept

Antibody/etanercept (1 mg/mL) was incubated with Biotin-X-NHS (Calbiochem, La Jolla, Calif) dissolved in dimethylformamide for 3 hours at room temperature, and subsequently dialyzed overnight against PBC by using Slide-A-Lyzer Dialysis Cassettes, according to the manufacturer's suggestions (Thermo Scientific, Waltham, Mass).

Generation of Fab₁ fragments

Fab₁ fragments from infliximab and rituximab were generated with the Pierce Fab Preparation Kit (Thermo Scientific), according to the manufacturer's instructions, and purity was assessed by means of SDS-page and subsequent Coomassie blue staining. All antibodies used for stimulatory experiments were dialyzed in PBS by using Slide-A-Lyzer Dialysis Cassettes, according to the manufacturer's suggestions (Thermo Scientific) before being added to cell-culture dishes.

Statistics

Data are presented as means $\pm$ SEMs, if not otherwise indicated. Statistical differences were calculated by using the 2-tailed Student *t* test with the Bonferroni-Holm correction, and *P* values of less than .05 were considered statistically significant.

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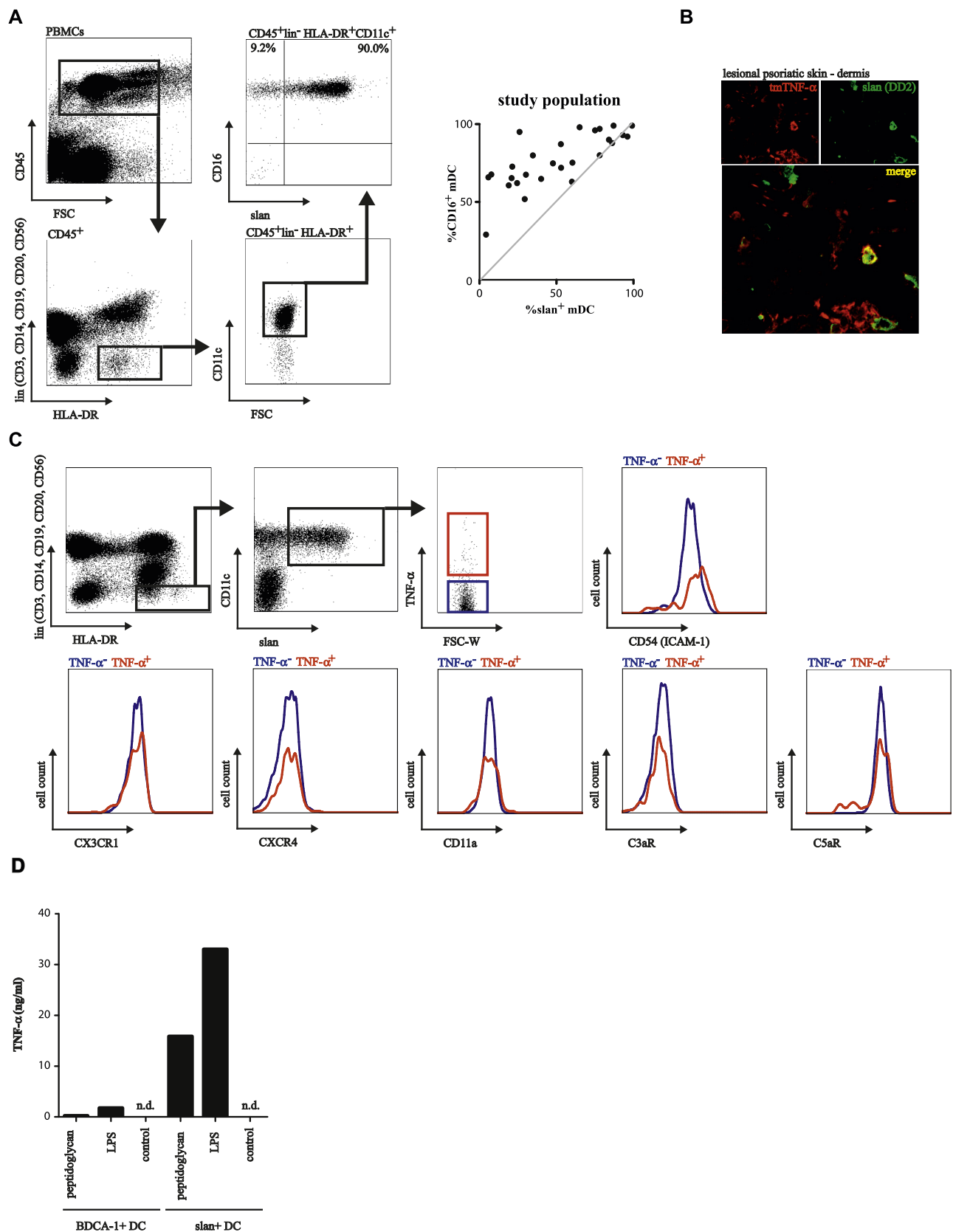


FIG E1. A, Coexpression of CD16 and slan on DCs from peripheral blood without prior incubation/stimulation (representative dot plots for gating strategy) and frequencies of CD16⁺ and slan⁺ DCs in PBMCs from our entire study population (ie, healthy control subjects and patients with psoriasis, with 1 dot representing each subject tested). **B,** Tyramide signal amplification–based immunofluorescence staining of tmTNF-α in

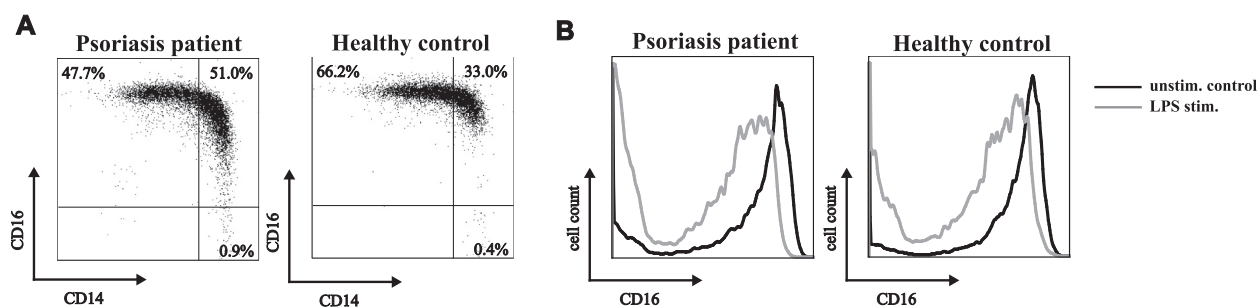


FIG E2. **A** and **B**, Purity of CD16⁺ monocytes isolated from PBMCs of patients with psoriasis and healthy control subjects (Fig E2, **A**) and their CD16 expression levels after 24 hours of culture with (gray line) or without (black line) 100 ng/mL LPS (Fig E2, **B**). Dot plots and histograms are representative of at least 3 independent experiments.

lesional psoriatic skin counterstained with slan (clone DD2). The picture is representative of at least 3 independent experiments. **C**, Expression levels (histograms) of homing receptors on DCs spontaneously producing TNF- α *in vitro* compared with their TNF- α ⁻ counterparts, both from patients with psoriasis. Histograms are representative for 5 individual experiments. **D**, MACS-isolated slan⁺ mDCs, as well as BDCA-1⁺ mDCs from buffy coat preparations of healthy control subjects, were stimulated with LPS or peptidoglycan for 12 hours, and the TNF- α level was measured in the cell-culture supernatant. The figure shown is representative of 5 independent experiments. *n.d.*, Not detected.

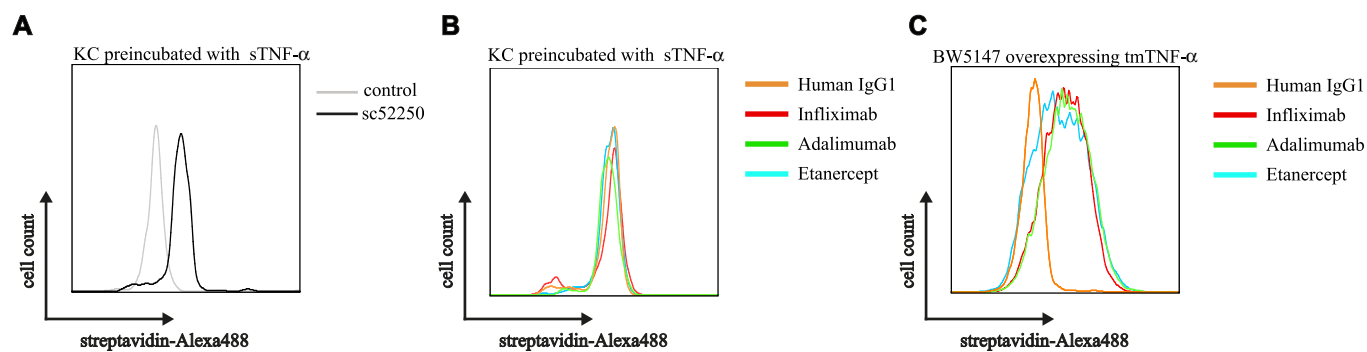


FIG E3. A-C. Biotinylated anti-TNF- α antibodies (infliximab, adalimumab, and sc52250) and etanercept were either incubated with keratinocytes preincubated with sTNF- α (Fig E3, A and B) or with the murine cell line BW5147 overexpressing tmTNF- α (Fig E3, C). Histograms shown are representative of at least 3 independent experiments.

TABLE E1. Antibodies used for immunofluorescence staining

Antigen	Clone	Company
Laminin V	D4B5	Millipore, Temecula, Calif
CD45	2D1	BD Biosciences, San Jose, Calif
HLA-DR	G46-6	BD Biosciences
CD11c	KB90	DAKO, Glostrup, Denmark
CD163	5C6-FAT	Acris, Herford, Germany
BDCA-1	M241	Ancell, Bayport, Minn
CD3	UCHT1	Immunotech, Marseille, France
Slan	DD2	Knut Schäkel, Heidelberg, Germany

TABLE E2. Antibodies used for flow cytometric analyses

Antigen-fluorophore	Clone	Company
CD3-FITC, -PE	SK7	BD Biosciences, San Jose, Calif
CD11c-PE, -APC	S-HCL-3	BD Biosciences
CD14-FITC, -PE	MφP9	BD Biosciences
CD16-PE	B73.1	BD Biosciences
CD16-APC	3G8	BioLegend, San Diego, Calif
CD19-FITC, -PE	4G7	BD Biosciences
HLA-DR-PerCP	L243	BD Biosciences
BDCA-1-APC	AD5-8E7	Miltenyi Biotec, Bergisch Gladbach, Germany
BDCA-2-APC	AC144	Miltenyi Biotec
BDCA-3-APC	AD5-14H12	Miltenyi Biotec
Slan-APC, -FITC	DD-1	Miltenyi Biotec
CD56-Alexa Fluor 488, -PE	HCD56	BioLegend
CD123-PE	7G3	BD PharMingen
CD163-FITC	5C6-FAT	Acris, Herford, Germany
CD45-PE-CF594	HI30	BD Biosciences
Lineage cocktail (CD3, CD14, CD19, CD20, CD56)-FITC	UCHT1, HCD14, HIB19, 2H7, HCD56	BioLegend
CD11c-PE/Cy7	Bu15	BioLegend
HLA-DR-Pacific Blue	L243	BioLegend
TNF-α-Brilliant Violet 605	MAB11	BioLegend
TNF-α-Alexa Fluor 488	MAB11	BD PharMingen
CD56-PE/Cy7	B159	BD PharMingen
CD54-PE	HA58	BioLegend
C3aR-PE	hC3aRZ8	BioLegend
C5aR-PE	S5/1	BioLegend
CX3CR1-PE	2A9-1	eBioscience, San Diego, Calif
CXCR4-PE	12G5	eBioscience
CD11a-PE	25.3	Immunotech

APC, Allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein complex.

TABLE E3. Primers used in the TaqMan RT-PCR assays

Gene	Reference no.
β_2 -Microglobulin	Hs00187842_m1
IL-12/IL-23p40	Hs01011518_m1
IL-12p35	Hs01073447_m1
IL-23p19	Hs00413259_m1
IFN- γ	Hs00174143_m1
IL-4	Hs00929862_m1
IL-17A	Hs00174383_m1
IL-22	Hs01574154_m1
IL-10	Hs00961622_m1
TGF- β 1	Hs00171257_m1
IL-6	Hs00985639_m1
IL-1 β	Hs01555410_m1
TNF- α	Hs00174128_m1
CCL20	Hs00355476_m1
IL-12RB1	Hs00538167_m1
IL-12RB2	Hs01548202_m1
IL-23R	Hs00332759_m1
IL-17RA	Hs01064648_m1
IL-17RC	Hs00994305_m1
TNFR1	Hs00533560_m1
TNFR2	Hs00153550_m1
iNOS	Hs01075529_m1
Langerin	Hs00210453_m1
FOXP3	Hs01085834_m1
TRAIL	Hs00234356_m1
Granzyme A	Hs00989184_m1
Granzyme B	Hs00188051_m1

All primers are from Applied Biosystems, Foster City, Calif.

FOXP3, Forkhead box protein 3; *TRAIL*, TNF-related apoptosis-inducing ligand.