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EXPERIMENTAL ALLERGY AND IMMUNOLOGY

Generation and functional analysis of human TNF- α /iNOS-producing dendritic cells (Tip-DC)

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Keywords

atopic dermatitis; dendritic cells; inducible NO synthase; psoriasis; TNF- α and iNOS-producing DC.

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Abstract

Background: A unique type of CD11c^{pos} dendritic cells (DC) is abundant in inflamed tissue, for example, in chronic inflammatory skin diseases. Due to their remarkable production of tumor necrosis factor (TNF)- α and inducible NO synthase (iNOS), these cells have been referred to as TNF and iNOS-producing DC (Tip-DC). While Tip-DC have been mainly characterized in murine models of infection, functional data about their human counterpart are lacking.

Objectives: We sought to generate human Tip-DC *in vitro* and thus provide a new model for the investigation of their phenotype and function.

Methods: We generated human Tip-DC from monocytic precursor cells of healthy individuals, atopic and psoriatic patients using human serum. Resting and stimulated cells were analyzed by flow cytometry, real-time PCR, and by ELISA. iNOS activity was measured by fluorometric detection of NO.

Results: Tip-DC closely resembled their *in vivo* counterparts by expressing CD11c, CD86, and CD40 while lacking CD1a, CD1c, or CD207/Langerin. Bacterial stimulation of Tip-DC from healthy donors, atopic dermatitis, or psoriasis patients resulted in a similar increase in iNOS activity and TNF- α production. In kinetic experiments, TNF- α , a putative activator of Tip-DC, could not induce NOS2. Upon bacterial stimulation, *TNFA*, *IL6*, *IL12B*, and *IL23A* mRNA appeared in a first wave, while *IL12A* and *NOS2* mRNA were up-regulated later on but not blocked by anti-TNF- α agents, implying a biphasic pro-inflammatory response.

Conclusions: We developed a new model for the study of human Tip-DC and provide the first evidence of their pro-inflammatory capacity.

Dendritic cells (DC) are the most effective inducers or silencers of T-cell responses and bridge the innate and adaptive immune system (1, 2). DC are subdivided into myeloid DC and plasmacytoid DC and are ubiquitously distributed. They contribute to allergic and tolerance mechanisms (3, 4). A newly identified DC subpopulation referred to as TNF- α and

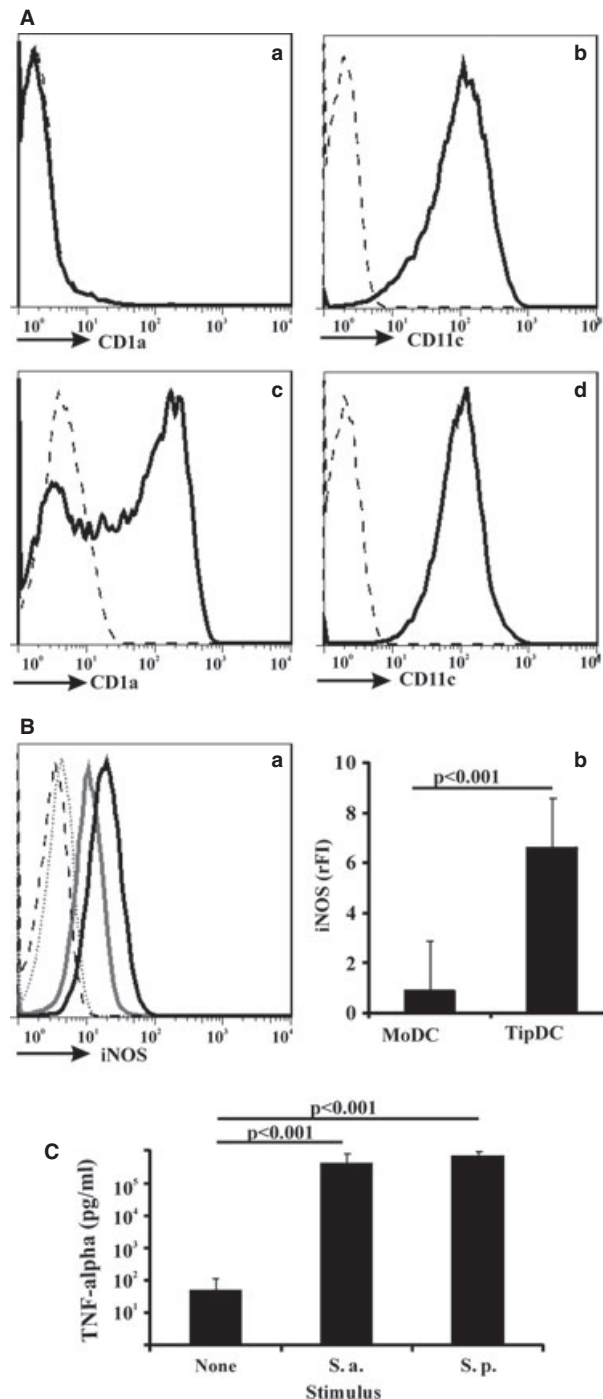
inducible NO synthase (iNOS)-producing DC (Tip-DC) has been reported to be critical in bacterial infections, as demonstrated in a model with *Listeria monocytogenes* (5). These cells were absent in chemokine receptor (CCR)-2-deficient mice, and the resulting lack of splenic Tip-DC led to extreme susceptibility to these gram-positive bacteria (5, 6). Although iNOS and TNF- α are known to be generally involved in antibacterial defenses, their role in inflammatory skin disease remains elusive. Evidence that TNF- α inhibitors are able to reverse disease activity in psoriasis suggests that Tip-DC may represent major inflammatory effector cells in psoriasis (7).

Nitric oxide synthase (NOS) is an enzyme that catalyzes the synthesis of NO and L-citrulline using oxygen and L-arginine as substrates. The enzyme exists in three isoforms encoded by distinct genes. Two constitutively expressed

Abbreviations

AD, atopic dermatitis; CCR, chemokine receptor; CCL, chemokine ligand; DAN, 2,3-diaminonaphthalene; DC, dendritic cell; FBS, fetal bovine serum; GM-CSF, granulocyte macrophage colony-stimulating factor; IFN, interferon; iNOS, inducible NO synthase; IL, interleukin; LPS, lipopolysaccharide; mAbs, monoclonal antibodies; MoDC, monocyte-derived DC; Tip-DC, TNF- α and iNOS-producing DC; TLR, toll-like receptor; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

isoforms, neuronal NOS (nNOS, type I) and endothelial NOS (eNOS, type III), produce small amounts of NO (picomolar range) for neuronal and cardiovascular regulatory processes. The third, calcium- and calmodulin-independent isoforms, inducible NOS (iNOS, type II), is induced by the cytokines TNF- α , IL-1, interferon (IFN)-gamma and by lipopolysaccharide (LPS) in a variety of cell types (8).



Tip-DC are a subtype of myeloid CD11c^{pos} DC that are increased in psoriasis compared with normal skin. On immunohistochemical studies of lesional psoriatic skin, these cells stain intensely for iNOS, as well as TNF. In uninvolved atopic dermatitis (AD) or psoriatic skin and normal skin, significantly fewer iNOS^{pos} cells are found (9,10). These CD11c^{pos} cells express HLA-DR, CD40, CD86, as well as maturation markers DC-Lamp and CD83 (11) but lack the CD207/Langerin and CD14 markers of Langerhans cells and monocytes. In addition, Tip-DC found in psoriasis produce the inflammatory mediators interleukin (IL)-8, IL-1, signal transducer and activator transcription 1, chemokine ligand (CCL) 20, IL-20, IL-23p19, and IL-12/IL-23p40, which mediate TH1 and TH17 responses (10, 12). While in healthy individuals, circulating monocytes, the precursors of DC, contain no functional iNOS, these cells are iNOS-positive in HIV infection, tuberculosis, Leishmania infection, sepsis, alcoholic hepatitis/cirrhosis, rheumatoid arthritis, and Graves' disease (13).

Here, we present an *in vitro* model for the generation of human Tip-DC from monocytic precursor cells of healthy individuals, atopic and psoriatic patients. We compared their phenotype to *in situ* described Tip-DC and investigated their function. This *in vitro* system provides a new and attractive immunopharmacological model for the functional investigation of human Tip-DC assumed to play a pivotal role as pro-inflammatory DC in psoriasis and possibly other chronic diseases.

Methods

Patients

Peripheral blood was taken from patients with AD or psoriasis or healthy control persons not receiving any immunosuppressive

Figure 1 Phenotype of Tip-DCs. (A) Tip-DCs cultured as described in the material and methods section (a, b) and conventional monocyte-derived DCs from the same donor cultured in the presence of FBS for 5 days (c, d) were stained for CD11a (left panels, heavy lines) and for CD11c (right panels, heavy lines). Thin dashed lines show staining with irrelevant antibodies of matched isotype. (B) iNOS expression by Tip-DCs. (a) Tip-DCs cultured as described in the material and methods section (black histogram and dashed line), and conventional monocyte-derived DCs (MoDCs) from the same donor cultured in the presence of FBS for 5 days (gray histogram and dotted line) were stained intracellularly for iNOS. Dashed/dotted lines show staining with irrelevant antibodies of matched isotype. (b) Comparison of rFI values of Tip-DCs and conventional MoDCs stained for iNOS as shown in A ($n = 8$ for Tip-DCs and $n = 19$ for MoDCs). Mean values $\pm$ SD are shown. (C) TNF- α expression in Tip-DCs. Tip-DCs were stimulated with *S. aureus* (S.a.) or with *S. pyogenes* (S.p.) for 72 h or left untreated (none) as described. TNF- α content in cell supernatants was determined by ELISA according to the manufacturer's instructions. Results are based on $n = 6/6/4$ donors (untreated/S.a./S.p.). Mean values $\pm$ SD are shown.

systemic therapy for more than four weeks. For further details, see online supplement.

Reagents

For the details of reagents used for isolation, culture, and stimulation of cells, flow cytometry and real-time PCR analysis, see online supplement.

Isolation of monocytes and generation of Tip-DC

Monocytes were isolated from peripheral blood of healthy adults and patients with AD or psoriasis by density gradient centrifugation (Nycoprep, Progen, Heidelberg, Germany) as described previously (14) or by negative selection using the monocyte isolation kit from Miltenyi Biotec, Bergisch-Gladbach, Germany. Further details on the protocols employed are found in the online supplement.

Flow cytometric analysis

After incubation with the indicated stimuli, cells were harvested, and further analyses were carried out using FACS analysis, real-time quantitative PCR, and ELISA. Further details are described in the online supplement.

Real-time PCR analysis

Gene expression was determined by SYBR Green-based real-time RT-PCR. Further details of the methods are given in the online supplement. The oligonucleotides used as primers are described in Table S1.

Enzyme-linked immunosorbent assay (ELISA)

TNF- α in culture supernatants of Tip-DC was determined by ELISA (R&D, Wiesbaden, Germany) according to the manufacturer's instructions.

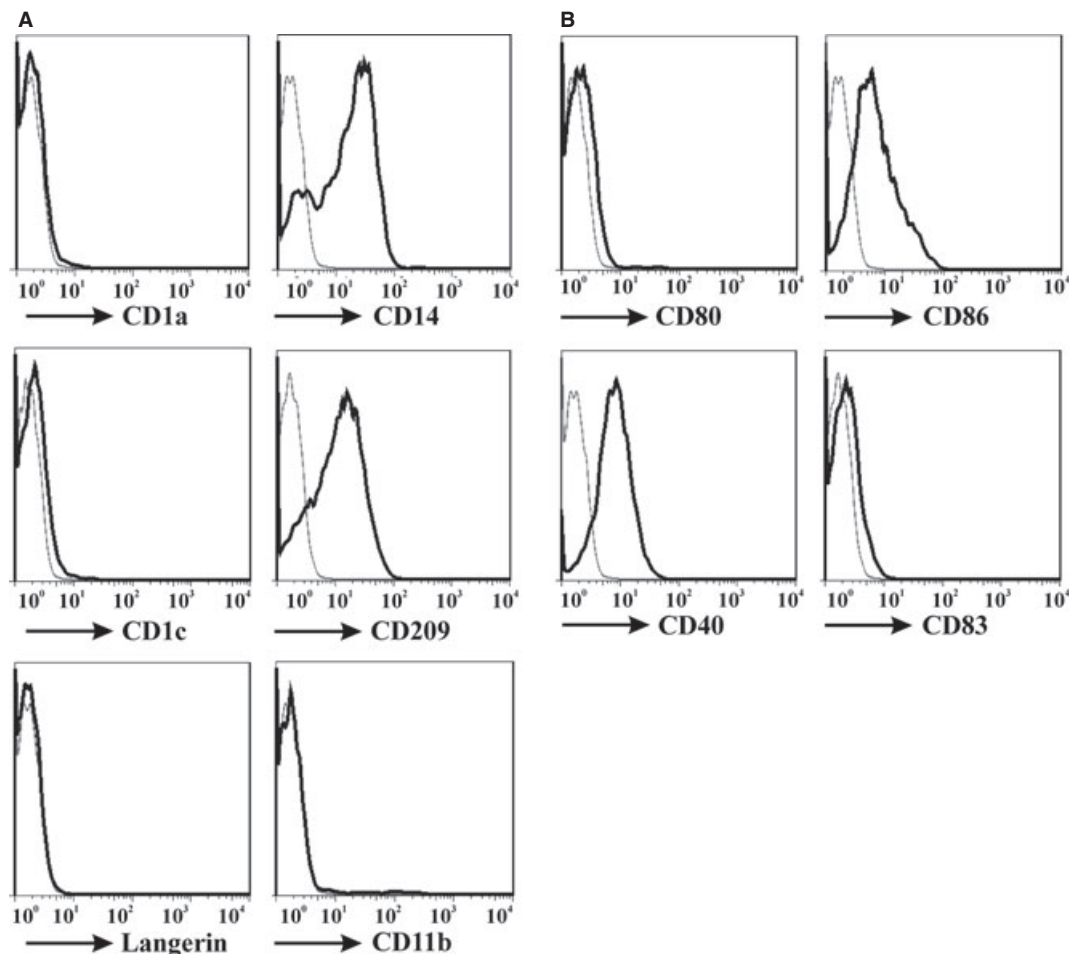


Figure 2 Phenotypic analysis of *in vitro* generated Tip-DC. (A–B) Tip-DC were cultured for five days as described in material and methods and subjected to flow cytometric analysis. Living CD11c^{pos} cells were analyzed with indicated antibodies specific for DC differentiation antigens (A) or maturation markers (B), as

indicated. Staining with specific antibodies is shown as bold black lines; staining with antibodies of irrelevant specificity and matched isotype is shown as fine gray lines. Results obtained are representative for three independent experiments with similar results.

Fluorometric detection of NO

For the detection of NO, the 2, 3-diaminonaphthalene method was used instead of the Griess reaction because it is much more sensitive and detects nitrite at a concentration of about 10 nM (15). The procedure is described in detail in the online supplement.

Statistical analysis

All pairwise comparisons between conditions were analyzed by a two-tailed Mann–Whitney *U* rank sum test. Correlation analysis was facilitated by SPSS 18.0.3.

Results

Expression of CCR 2 on monocytic precursor cells is higher in patients with atopic dermatitis or psoriasis than in healthy individuals

Chemokine receptor CCR2 has been reported to be mandatory for the recruitment of Tip-DC in a murine model (5). Therefore, we examined the expression of CCR2 on monocytic cells in whole blood from healthy volunteers and compared it with patients with psoriasis and AD. Cells were stained for CD14, CD16, and CCR2 and analyzed by flow cytometry. Special attention was focussed on CD14^{low}/CD16^{high} and CD14^{high}/CD16^{neg} monocyte subpopulations. Thereby, almost all CD14^{high}/CD16^{neg} monocytes expressed CCR2, whereas it was hardly detectable on CD14^{low}/CD16^{high} cells (Supplementary Fig. S1A), which represented a small population (2–5%) of the monocytic cells. Interestingly, CD14^{high}/CD16^{neg} monocytes in healthy controls expressed less CCR2 (median rFI = 21 ± 19.3) than in AD (median rFI = 39 ± 15.3) or psoriasis (median rFI = 34 ± 15.78) (Supplementary Fig. S1B). However, this trend did not reach statistical significance.

Human serum is mandatory for the *in vitro* generation of human Tip-DC from monocytic precursors

The most important hallmark of Tip-DC is their expression of TNF- α and iNOS while lacking CD1a (11). In preliminary experiments, we observed that fetal bovine serum (FBS) strongly supported the expression of CD1a on MoDC. Therefore, monocytes were cultured with GM-CSF and IL-4 in the presence of either FBS (*n* = 19) or human serum (*n* = 8). Cells were then subjected to surface staining for CD1a (Fig. 1A,a,c) and CD11c (Fig. 1A,b,d) and intracellular staining for iNOS (Fig. 1B,a,b) and analyzed by flow cytometry. MoDC cultured in the presence of FBS were strongly CD1a positive (Fig. 1A,c). In contrast, Tip-DC cultured in the presence of human serum remained negative (Fig. 1A,a). Thereby, iNOS expression of cells cultured in medium with human serum and without FBS (Fig. 1B,a,b) was significantly higher compared with MoDC cultured in FBS-containing medium. Titration experiments (data not shown) revealed that cells cultured with 5% serum had the

highest iNOS expression. In supernatants of stimulated Tip-DC cultures, high amounts of TNF- α were detectable (Fig. 1C). From this series of experiments, we conclude that human serum but not FBS is pivotal to generate CD1a^{neg} Tip-DC from circulating monocytic precursors.

In vitro generated Tip-DC are highly similar to Tip-DC *in situ*

Tip-DC identified in psoriatic lesions express CD11c and show a distinct phenotypic profile (11). We performed a detailed phenotypic analysis of *in vitro* generated Tip-DC to compare them with *in situ* Tip-DC. As described for *in situ* Tip-DC (10, 11, 16, 17), our *in vitro* generated Tip-DC expressed CD11c (Fig. 1A) but lacked CD1c, CD11b, or CD207/Langerin (Fig. 2A). Furthermore, as reported for *in situ* Tip-DC, they expressed CD86 and CD40, while both CD80 and CD83 were expressed at a very low level (Fig. 2B). Like *in situ* Tip-DC (18), *in vitro* generated Tip-DC expressed TRAIL and TLR 1,2 (Suppl. Fig. 2). TRAIL expression was more evident if cells stained with 7-AAD were not excluded from the analysis (Suppl. Fig. 2A, bottom panel). Although TLR1 cell surface expression could not be demonstrated (Suppl. Fig. 2C, upper panel), *TLR1* mRNA was detected (Suppl. Fig. 2D). Further on, functional TLR1 activity was revealed by treatment with the TLR1/TLR2 ligand Pam3Cys, which caused an increase of the maturation marker CD83 (Suppl. Fig. 2C, bottom panel). Taken together, this analysis showed that *in vitro* generated Tip-DC were highly similar to their *in vivo* counterparts (Table 1).

Table 1 Phenotypic characteristics of *in vitro* generated Tip-DC in comparison with *in situ* investigated Tip-DC or CD11c^{pos}CD1c^{neg} DC, respectively

Marker	Expression on <i>in vitro</i> generated Tip-DC (this report)	Expression on <i>in vivo</i> investigated human Tip-DC
CD1a	Negative	Negative (11)
CD1c/BDCA-1	Negative	Negative (17) [†]
CD11b	Negative	Negative (11)
CD11c	Positive	Positive (11)
CD14	Intermediate	Negative (11)
		Intermediate (17) [†]
CD40	Positive	Positive (11, 18) [†]
CD80	Intermediate	Unknown
CD83	Intermediate	Positive (11)
		Intermediate (18) [†]
CD86	Positive	Positive (11, 17) [†]
TRAIL	Intermediate	Positive (18) [†]
CD207/Langerin	Negative	Negative (11)
CD209/DC-SIGN	Positive	Positive (18) [†]
HLA-DR	Positive	Positive (11, 17) [†]
TLR1	Positive	Positive (18) [†]
TLR2	Positive	Positive (18) [†]

[†]Investigated in CD11c^{pos} CD1c^{neg} dendritic cells, of which Tip-DC might be a subpopulation.

Bacterial stimulation of Tip-DC activates iNOS and enhances NO production

Bacterial infections such as *S. pyogenes* are known as trigger factors of psoriasis, and it has been reported that the activity of iNOS in DC from patients with primary biliary cirrhosis is enhanced by *S. aureus* (19). Therefore, we investigated the expression and activity of iNOS by *in vitro* Tip-DC upon bacterial stimulation. Thereby, *S. aureus* led to significant increase of *NOS2* mRNA expression (Fig. 3A; $n = 5$). In order to confirm protein expression, cells were stained

intracellularly with two distinct iNOS-specific mAbs. While in the absence of stimulus, substantial iNOS protein was detected in permeabilized Tip-DC (Fig. 3B, left upper panel), we found enhanced expression of CD83 upon activation with *S. aureus* or *S. pyogenes* (Fig. 3B,C, right middle and bottom panels), but less iNOS protein as detected with both anti-iNOS antibodies (Fig. 3B,C, left middle and bottom panels). In contrast, there was clear increase in iNOS activity in stimulated cells as measured by the NO content in supernatants (Fig. 4A). Interestingly, an inverse correlation between iNOS protein expression and the amount of NO in the

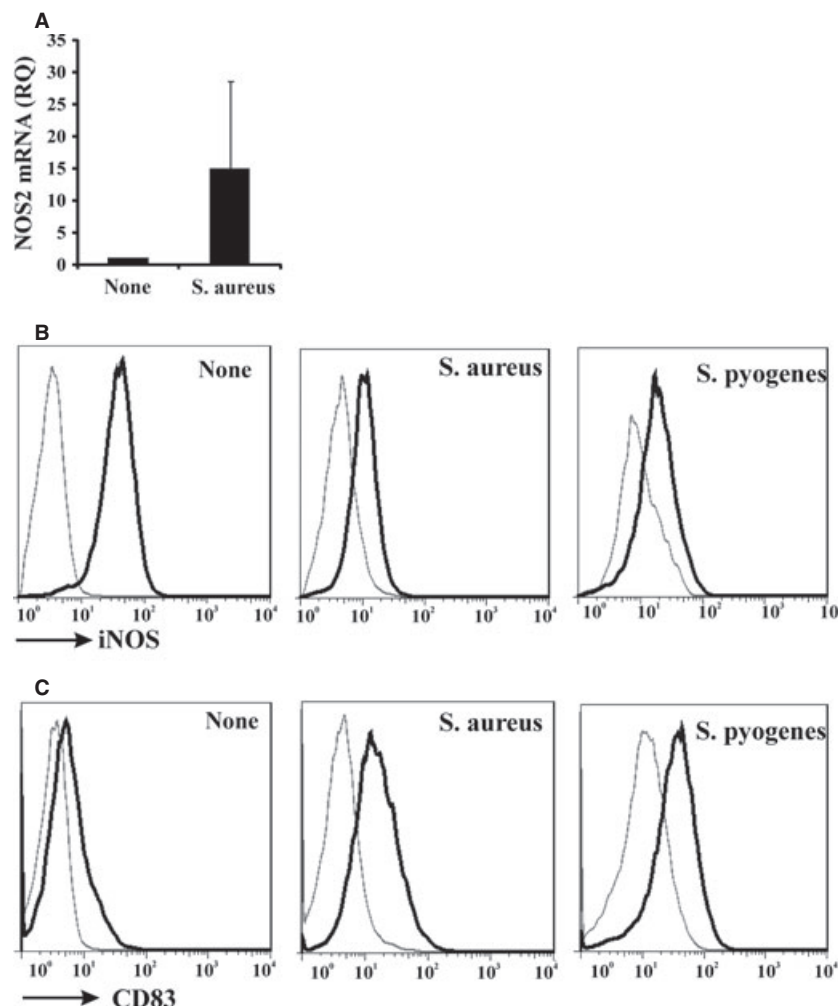


Figure 3 NOS2 mRNA and iNOS protein induction upon treatment of Tip-DCs with bacteria (A) Tip-DCs ($n = 5$) were stimulated with *S. aureus* or left untreated as described. RNA was harvested, and SYBR Green RT-qPCR was used to quantify *NOS2* transcript levels in triplicate relative to actin by the $\Delta\Delta C_T$ method. Relative quantity (RQ) is shown for *NOS2* relative to the untreated cells. Mean values $\pm$ SD are shown. (B) iNOS protein expression by Tip-DC stimulated with *S. aureus* or *S. pyogenes*. TipDCs cultured as described in the material and methods section stimulated for three days with *S. aureus*, *S. pyogenes* or left untreated (none), as indicated, were

stained intracellularly for iNOS (heavy black lines). Thin gray lines show staining with irrelevant antibodies of matched isotype. Data are representative for >30 experiments. (C) CD83 expression by Tip-DC stimulated with *S. aureus* or *S. pyogenes*. TipDCs cultured as described in the material and methods section stimulated for three days with *S. aureus*, *S. pyogenes* or left untreated (none), as indicated, were stained intracellularly for the DC maturation marker CD83 (heavy black lines). Thin gray lines show staining with irrelevant antibodies of matched isotype. Data are representative for >10 experiments.

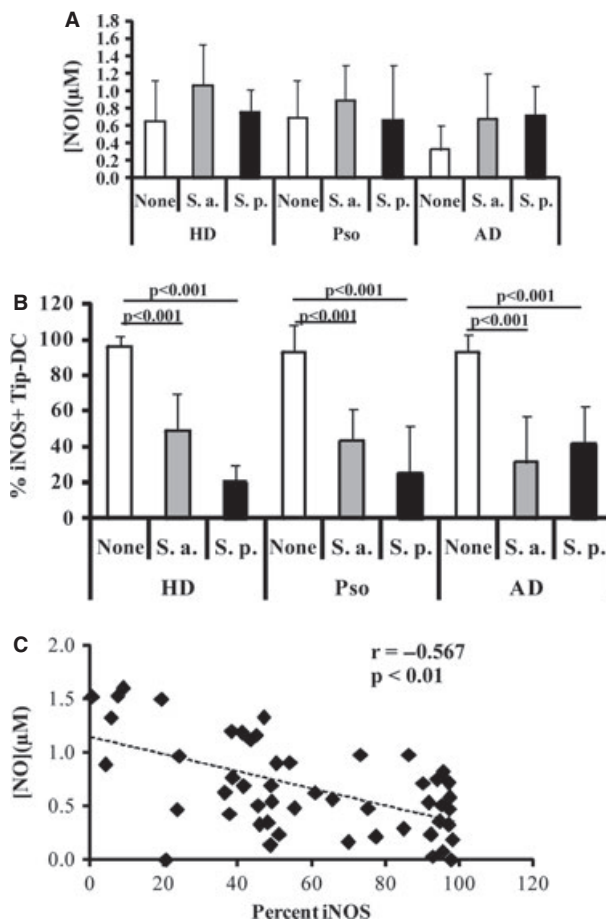


Figure 4 NO production and iNOS expression of Tip-DCs. (A) NO production of Tip-DC cultured from monocytes of patients with psoriasis (Pso, $n = 9/7/6$)* or atopic dermatitis (AD, $n = 10/10/8$)* and healthy donors (HD, $n = 10/9/7$)* under stimulated or untreated (none) conditions (* n = number of donors for Tip-DCs untreated/stimulated with S.a. = *S. aureus*/stimulated with S.p. = *S. pyogenes*). (A+B) Mean values $\pm$ SD are shown. (B) Percentages of iNOS^{pos} Tip-DC stimulated with S.a. or S.p. or untreated (none) cultured from monocytes of patients with psoriasis ($n = 10/8/7$)* or atopic dermatitis ($n = 11/8/10$)* and healthy donors ($n = 13/12/8$)* (* n = number of donors for Tip-DCs unstimulated/stimulated with S.a. = *S. aureus*/stimulated with S.p. = *S. pyogenes*) were determined by flow cytometry after staining with iNOS-specific mAb. (C) Percentages of iNOS^{pos} Tip-DC measured with FACS analysis by intracellular staining with iNOS antibodies in correlation with NO production of Tip-DCs. ($n = 50$). Data from all donors from Fig. 4A, B were included, for whom both analyses were available. Correlation analysis was facilitated by SPSS 18.0.3.

supernatants was found (Fig. 4B,C). This phenomenon was reproducible irrespective of the source of cells because it was also observed when investigating Tip-DC from AD patients ($n = 11$) and psoriasis patients ($n = 10$). There were no significant differences in the magnitude of response between the cells obtained from AD and psoriasis patients or healthy individuals (Fig. 4A,B).

Biphasic expression of inflammatory cytokines upon bacterial stimulation of Tip-DC

As it has been suggested from *in situ* investigations that Tip-DC may produce pivotal cytokines for the development of psoriasis such as IL-23 and IL-12 (10), we investigated cytokine mRNA expression after bacterial stimulation of *in vitro* generated Tip-DC. Besides high production of TNF- α (Fig. 1C), transcription of *IL6*, the common chain *IL12B*, *IL23A* and to a lesser extent *IL12A* mRNA was detectable as well (Fig. 5A).

In kinetic experiments, while most cytokines showed their peak between 6 and 24 h and decreased afterward, *NOS2* and *IL12A* mRNA were only increased after stimulation measured over 72 h (Fig. 5B). In order to clarify whether TNF- α plays a role as an autocrine factor in this biphasic behavior, we treated unstimulated Tip-DC on day 2 with TNF- α and measured expression of *NOS2* mRNA and *IL12A* mRNA after 72 h and *IL23A* mRNA after 6 h. None of these gene products could be shown upon treatment with TNF- α , whereas all were readily detectable after bacterial stimulation (Fig. 5C). Additionally, we were not able to block iNOS activity by adding either the humanized monoclonal anti-TNF mAb Adalimumab or the soluble p75 TNF receptor Etanercept (Fig. 5D). From these data, we conclude that bacterial stimulation induces Tip-DC to produce pro-inflammatory cytokines in a biphasic manner which is independent of TNF- α .

Discussion

Tip-DC have been suggested to be instrumental in the initiation and maintenance of the inflammatory reaction. Despite their paramount importance in the pathophysiology of infectious diseases and psoriasis, reports about the functional properties of human Tip-DC are still lacking. While the first report on human CD11c^{pos} Tip-DC was based on immunohistochemical analysis of skin samples (11), only few reports of *in vitro* models for the generation of human iNOS-producing DC are found so far in the literature (20, 21). We describe a simple protocol to generate any amount of Tip-DC *in vitro* enabling thorough phenotypic and functional investigations, thereby providing a new and important immunopharmacologic model to study these peculiar DC.

Own previous experiments as well as accumulating evidence from the literature suggest that the microenvironment determines the DC subtype and differentiation (22,23). Lack of CD1a expression on monocytes cultured in the presence of human serum has been reported previously (22, 24, 25). We show that the addition of human serum instead of FBS significantly contributes to the generation of CD1a^{neg} Tip-DC from circulating CD14^{high}CCR2^{pos} monocytic cells. Monocytes from AD and psoriasis patients showed an enhanced cell surface expression of CCR2 as compared with those from healthy individuals, as shown before (26). At least two mutually nonexclusive explanations can be considered: (i) the cutaneous inflammatory microenvironment of both AD and psoriasis produces yet-to-be-defined signals enhancing CCR2

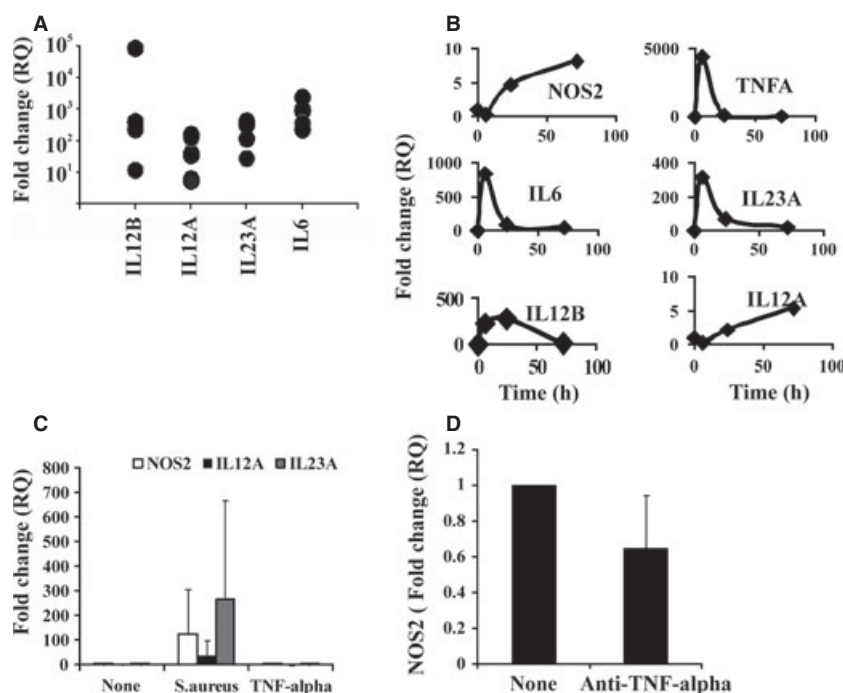


Figure 5 Cytokine gene expression of *in vitro* generated Tip-DCs. (A) Induction of cytokine gene expression upon treatment of Tip-DCs with *S. aureus*. Tip-DCs were stimulated with *S. aureus* for 6 h or for 72 h or left untreated as described. RNA was harvested, and SYBR Green RT-qPCR was used to quantify *IL-12p40*, *IL-12p35*, *IL-23p19*, *IL-6* transcript levels in triplicate relative to actin by the $\Delta\Delta C_T$ method. Fold change is shown relative to the untreated cells. *IL-12p40* (6 h): $n = 4$, *IL-12p35* (72 h): $n = 8$, *IL-23p19* (6 h): $n = 4$, *IL-6* (6 h): $n = 5$. (B) Induction of cytokine gene expression upon treatment of Tip-DCs with *S. aureus*. Tip-DCs were stimulated with *S. aureus* for the indicated time periods or left untreated as described. RNA was harvested, and SYBR Green RT-qPCR was used to quantify *TNF- α* , *IL-12p40*, *IL-12p35*, *IL-23p19*, *IL-6* transcript levels in triplicate relative to actin by the $\Delta\Delta C_T$ method. Fold change is shown relative to the untreated cells. In comparison, induction of *NOS2* gene expression is shown. Results shown here are representative for cells from three

independent donors. (C, D) *TNF- α* is not sufficient for induction of *NOS2*, *IL-12p35*, and *IL-23p19* in *in vitro* generated Tip-DCs. (C) Tip-DCs were left untreated (none) or treated with *S. aureus* or *TNF- α* for 6 or 72 h, as described. RNA was harvested, and SYBR Green RT-qPCR was used to quantify *NOS2*, *IL-12p35*, *IL-23p19* transcript levels in triplicate relative to actin by the $\Delta\Delta C_T$ method. Fold change is shown relative to the untreated cells. Results from Tip-DCs from three to four independent donors are shown. (D) Fold change of *NOS2* mRNA levels in the presence of anti-*TNF- α* mAb; $n = 3$. Tip-DCs were treated with *S. aureus* for 72 h, in the absence (none) or in the presence of *TNF- α* -specific mAbs. RNA was harvested, and SYBR Green RT-qPCR was used to quantify *NOS2* transcript levels in triplicate relative to actin by the $\Delta\Delta C_T$ method. Fold change is shown relative to cells treated with *S.a.* in the absence of anti-*TNF- α* mAbs. (C+D) Mean values $\pm$ SD are shown.

expression, and/or (ii) in both conditions, an intrinsic abnormality of the monocytic precursor cells leads to this phenomenon. In any case, this observation strongly supports the concept that both diseases should not be considered as purely local inflammatory conditions but as systemic diseases.

The pivotal role of Tip-DC in psoriasis has been further suggested by the observation of an increased expression of iNOS in lesional skin of psoriasis when compared with AD or normal skin (9). However, in the present study, the ability to generate these cells and the magnitude of their iNOS expression was not significantly different between these two groups of patients compared with healthy donors, suggesting that the development of Tip-DC in the skin and the magnitude of iNOS expression are dependent on the inflammatory microenvironment in the skin. Thus, a given disease-specific inflammatory milieu may drive the

differentiation of different types of inflammatory DC or plasmacytoid DC that contribute specifically to the disease process and ultimately to the clinical phenotype (9, 27). In the microenvironment of AD skin, TH2 cytokines-like IL-4 predominate and therefore the development of Tip-DC might be reduced in AD compared with psoriasis (28, 29), where specific T cells and their cytokines-like IL-12 and IL-23 predominate (30).

Recently, it has been proposed that IL-23 rather than IL-12 plays a critical pathogenic role in psoriasis (31,32). Although we detected a stronger increase of mRNA for the IL-12/IL-23 common p40 chain and IL-23p19 subunits in comparison with mRNA for the IL-12p35 subunit, we saw – regarding the kinetics of those mRNAs over 72 h after stimulation with *S. aureus* – that *IL12A* mRNA levels increased over time, whereas *IL23A* and to a lesser extent *IL12B*

mRNA transcription peaked at 6 h. Interestingly, in the same experiments, an increase of *NOS2* mRNA was noted, whereas the transcription of *TNFA* and *IL6* mRNA in Tip-DC after stimulation slowed down after 24 h. Immunohistochemical investigations suggested that TNF-blocking agents given to patients with psoriasis inhibit the pro-inflammatory pathways and reduce the production of iNOS of CD11c^{pos} DC in lesional skin (7, 33). From these observations, it was deduced that TNF secretion by Tip-DC may initiate an auto-crine loop leading to the amplification of Tip-DC-mediated inflammatory events in psoriasis. We tested this hypothesis and stimulated Tip-DC with TNF- α compared with *S. aureus*. As shown before, *S. aureus* stimulation increased *NOS2* mRNA transcription, whereas *NOS2* mRNA levels from TNF- α -treated cells were indistinguishable from untreated cells. Also, transcription of *IL12A* and *IL23A*, which were strongly enhanced upon *S. aureus* treatment, was not altered by TNF- α . Additionally, we were not able to block *S. aureus*-induced iNOS activity by TNF-blocking agents. From these results, we conclude that Tip-DC-derived TNF- α does not act as a direct activator for Tip-DC. Thus, the cytokine network acting on and resulting from Tip-DC needs further clarification, and our *in vitro* model provides a useful tool to answer these questions.

Finally, one methodological aspect deserves to be discussed with regard to the detection of iNOS expression. Our real-time PCR results clearly showed enhanced *NOS2* transcription levels in concordance with enhanced NO concentrations. However, flow cytometric analysis of iNOS protein after staining with different iNOS-specific mAbs displayed opposite results in that the iNOS-specific mAbs reacted more strongly in unstimulated Tip-DC than in stimulated ones. This inverse correlation between the percentage of the iNOS-positive stained cells and the NO production strongly suggests a special feature in the specificity of the iNOS-specific mAbs. We therefore hypothesize that iNOS activation leads to conformational change resulting in a decreased recognition of the antibodies' epitope. However, an increase in iNOS protein upon stimulation was reported for *in vitro* generated Tip-DC by Chong et al. (20) using one of the iNOS-specific mAbs used herein. This discrepancy may be explained by the different protocols used to raise the cells. Because these authors did not provide any information about the expression of CD11c and CD1a/c on their Tip-DC, we cannot interpret the similarities or differences between the cells obtained in these studies and consequently about the similarity in functional behavior.

In conclusion, we report the *in vitro* generation of Tip-DC by a simple protocol leading to cells which are phenotypically

very similar to their *in situ* counterparts. These cells respond to bacterial stimulation with an increased iNOS activity and the production of pro-inflammatory cytokines in a biphasic manner providing a suitable immunopharmacological model for studying their pathophysiological role in diseases such as psoriasis.

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Author contributions

Conceived and designed the experiments: DWT, SK, DvB, and TB. Performed the experiments: SK, CM, SB, HW, and SS. Analyzed the data: DWT, SK, TB. Wrote the paper: DWT, SK, and TB.

Conflict of interest

Besides support from Merck-Serono GmbH (Darmstadt, Germany) for part of this work, there are no commercial associations that might pose a conflict of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primers used for quantification of *NOS2*, *IL6*, *IL10*, *IL12B*, *IL12A*, *TNFA*, *IL23A* RNA expression via real time PCR.

Figure S1. CCR2 expression by monocyte subpopulations.

Figure S2. Phenotypic analysis of *in vitro* generated Tip-DC.

References

- Novak N, Koch S, Allam JP, Bieber T. Dendritic cells: bridging innate and adaptive immunity in atopic dermatitis. *J Allergy Clin Immunol* 2010;**125**:50–59.
- Hammad H, Lambrecht BN. Dendritic cells and airway epithelial cells at the interface between innate and adaptive immune responses. *Allergy* 2011;**66**: 579–587.
- Novak N, Allam JP. Mucosal dendritic cells in allergy and immunotherapy. *Allergy* 2011;**66**(Suppl. 95):22–24.
- Soyer OU, Akdis M, Ring J, Behrendt H, Cramer R, Lauener R et al. Mechanisms of peripheral tolerance to allergens. *Allergy* 2013;**68**:161–170.
- Serbina NV, Salazar-Mather TP, Biron CA, Kuziel WA, Pamer EG. TNF/iNOS-

- producing dendritic cells mediate innate immune defense against bacterial infection. *Immunity* 2003;**19**:59–70.
6. Engel D, Dobrindt U, Tittel A, Peters P, Maurer J, Gutgemann I et al. Tumor necrosis factor alpha- and inducible nitric oxide synthase-producing dendritic cells are rapidly recruited to the bladder in urinary tract infection but are dispensable for bacterial clearance. *Infect Immun* 2006;**74**: 6100–6107.
7. Gottlieb AB, Chamian F, Masud S, Cardinale I, Abello MV, Lowes MA et al. TNF inhibition rapidly down-regulates multiple proinflammatory pathways in psoriasis plaques. *J Immunol* 2005;**175**:2721–2729.
8. Alderton WK, Cooper CE, Knowles RG. Nitric oxide synthases: structure, function and inhibition. *Biochem J* 2001;**357**(Pt 3):593–615.
9. Guttman-Yassky E, Lowes MA, Fuentes-Duculan J, Whynot J, Novitskaya I, Cardinale I et al. Major differences in inflammatory dendritic cells and their products distinguish atopic dermatitis from psoriasis. *J Allergy Clin Immunol* 2007;**119**:1210–1217.
10. Guttman-Yassky E, Nograles KE, Krueger JG. Contrasting pathogenesis of atopic dermatitis and psoriasis—part II: immune cell subsets and therapeutic concepts. *J Allergy Clin Immunol* 2011;**127**:1420–1432.
11. Lowes MA, Chamian F, Abello MV, Fuentes-Duculan J, Lin SL, Nussbaum R et al. Increase in TNF- α and inducible nitric oxide synthase-expressing dendritic cells in psoriasis and reduction with efalizumab (anti-CD11a). *Proc Natl Acad Sci USA* 2005;**102**:19057–19062.
12. Lowes MA, Bowcock AM, Krueger JG. Pathogenesis and therapy of psoriasis. *Nature* 2007;**445**:866–873.
13. Dijkstra G, Zandvoort AJ, Kobold AC, de Jager-Krikken A, Heeringa P, van GH et al. Increased expression of inducible nitric oxide synthase in circulating monocytes from patients with active inflammatory bowel disease. *Scand J Gastroenterol* 2002;**37**:546–554.
14. Novak N, Tepel C, Koch S, Brix K, Bieber T, Kraft S. Evidence for a differential expression of the Fc ϵ RI γ chain in dendritic cells of atopic and nonatopic donors. *J Clin Invest* 2003;**111**:1047–1056.
15. Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorometric assay for the measurement of nitrite in biological samples. *Anal Biochem* 1993;**214**:11–16.
16. Johnson-Huang LM, McNutt NS, Krueger JG, Lowes MA. Cytokine-producing dendritic cells in the pathogenesis of inflammatory skin diseases. *J Clin Immunol* 2009;**29**:247–256.
17. Zaba LC, Krueger JG, Lowes MA. Resident and 'inflammatory' dendritic cells in human skin. *J Invest Dermatol* 2009;**129**:302–308.
18. Zaba LC, Fuentes-Duculan J, Eungdamrong NJ, Johnson-Huang LM, Nograles KE, White TR et al. Identification of TNF-related apoptosis-inducing ligand and other molecules that distinguish inflammatory from resident dendritic cells in patients with psoriasis. *J Allergy Clin Immunol* 2010;**125**:1261–1268.
19. Yamamoto K, Akbar SM, Masumoto T, Onji M. Increased nitric oxide (NO) production by antigen-presenting dendritic cells is responsible for low allogeneic mixed leucocyte reaction (MLR) in primary biliary cirrhosis (PBC). *Clin Exp Immunol* 1998;**114**:94–101.
20. Chong SZ, Wong KL, Lin G, Yang CM, Wong SC, Angeli V et al. Human CD8(+) T cells drive Th1 responses through the differentiation of TNF/iNOS-producing dendritic cells. *Eur J Immunol* 2011;**41**: 1639–1651.
21. Hansel A, Gunther C, Ingwersen J, Starke J, Schmitz M, Bachmann M et al. Human slan (6-sulfo LacNAc) dendritic cells are inflammatory dermal dendritic cells in psoriasis and drive strong TH17/TH1 T-cell responses. *J Allergy Clin Immunol* 2011;**127**:787–794.
22. Leslie DS, Dascher CC, Cembrola K, Townes MA, Hava DL, Hugendubler LC et al. Serum lipids regulate dendritic cell CD1 expression and function. *Immunology* 2008;**125**:289–301.
23. Jancic C, Chuluyan HE, Morelli A, Larregina A, Kolkowski E, Saracco M et al. Interactions of dendritic cells with fibronectin and endothelial cells. *Immunology* 1998;**95**:283–290.
24. Triozzi PL, Aldrich W. Phenotypic and functional differences between human dendritic cells derived in vitro from hematopoietic progenitors and from monocytes/macrophages. *J Leukoc Biol* 1997;**61**: 600–608.
25. Gogolak P, Rethi B, Szatmari I, Lanyi A, Dezso B, Nagy L et al. Differentiation of CD1a- and CD1a+ monocyte-derived dendritic cells is biased by lipid environment and PPARgamma. *Blood* 2007;**109**:643–652.
26. Vestergaard C, Just H, Baumgartner NJ, Thestrup-Pedersen K, Deleuran M. Expression of CCR2 on monocytes and macrophages in chronically inflamed skin in atopic dermatitis and psoriasis. *Acta Derm Venereol* 2004;**84**:353–358.
27. Bratke K, Klein C, Kuepper M, Lommatzsch M, Virchow JC. Differential development of plasmacytoid dendritic cells in Th1- and Th2-like cytokine milieu. *Allergy* 2011;**66**:386–395.
28. Guttman-Yassky E, Nograles KE, Krueger JG. Contrasting pathogenesis of atopic dermatitis and psoriasis—part I: clinical and pathologic concepts. *J Allergy Clin Immunol* 2011;**127**:1110–1118.
29. Nomura I, Gao B, Boguniewicz M, Darst MA, Travers JB, Leung DY. Distinct patterns of gene expression in the skin lesions of atopic dermatitis and psoriasis: a gene microarray analysis. *J Allergy Clin Immunol* 2003;**112**:1195–1202.
30. Alonso MN, Wong MT, Zhang AL, Winer D, Suhoski MM, Tolentino LL et al. T(H)1, T(H)2, and T(H)17 cells instruct monocytes to differentiate into specialized dendritic cell subsets. *Blood* 2011;**118**:3311–3320.
31. Chan JR, Blumenschein W, Murphy E, Diveu C, Wiekowski M, Abbondanzo S et al. IL-23 stimulates epidermal hyperplasia via TNF and IL-20R2-dependent mechanisms with implications for psoriasis pathogenesis. *J Exp Med* 2006;**203**:2577–2587.
32. Chen X, Tan Z, Yue Q, Liu H, Liu Z, Li J. The expression of interleukin-23 (p19/p40) and interleukin-12 (p35/p40) in psoriasis skin. *J Huazhong Univ Sci Technolog Med Sci* 2006;**26**:750–752.
33. Zaba LC, Cardinale I, Gilleaudeau P, Sullivan-Whalen M, Suarez-Farinas M, Fuentes-Duculan J et al. Amelioration of epidermal hyperplasia by TNF inhibition is associated with reduced Th17 responses. *J Exp Med* 2007;**204**:3183–3194.
34. Paludan SR, Ellermann-Eriksen S, Lovmand J, Mogensen SC. Interleukin-4-mediated inhibition of nitric oxide production in interferon-gamma-treated and virus-infected macrophages. *Scand J Immunol* 1999;**49**:169–176.
35. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C (T)) Method. *Methods* 2001;**25**:402–408.

ONLINE REPOSITORY

Original Article

Generation and functional analysis of human TNF-alpha/iNOS producing dendritic cells (Tip-DC)

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METHODS

Patients

The investigations were approved by the institutional review board of the Rheinische Friedrich-Wilhelms-University in Bonn, Germany. In accordance with the ethical principles stated in the declaration of Helsinki, blood was taken from patients with AD or psoriasis or healthy control persons not receiving any immunosuppressive systemic therapy for more than four weeks.

Reagents

PE-coupled monoclonal antibodies specific for CD11c, CD16, CD86, CD80, CD40, CD11b were all from BD Pharmingen (Heidelberg, Germany). Anti-toll-like receptor (TLR)1 and anti-TLR2 were from Imgenex (San Diego, CA). CD83-specific and CD14-specific monoclonal antibodies (mAbs) coupled to APC as well as unlabeled CD83, CD1a and CD1c mAbs were from Beckman-Coulter (Krefeld, Germany). CD207/Langerin- and CD14-specific mAbs were from Immunotech and iNOS/NOS Type II-specific mAbs were from Santa-Cruz Biotechnology (Heidelberg, Germany) or from BD Pharmingen. Anti- tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-PE antibodies and APC-coupled IgG2a were from Becton Dickinson, (San Jose, CA). Mouse IgG2b, IgG1 and 7-amino-actinomycin D (7-AAD) were all from Sigma Chemical (St. Louis, MO). CCR2- specific mAbs, IFN-gamma and anti-CD209 were from R&D Systems GmbH, (Wiesbaden, Germany).

Unlabeled mAbs were visualized by goat anti-mouse immunoglobulin (Ig)G-FITC (Dianova, Hamburg, Germany) or by goat anti-mouse IgG-PE (Sigma). HLA-DR antigens were identified using culture supernatant of L243 hybridoma cells. Normal mouse serum was from Dianova.

Dextran 500, FBS, human serum, NaNO₂ and DAN (2,3-Diaminonaphthalene) were from Sigma-Aldrich (München, Germany) and Nycoprep was from Progen (Heidelberg, Germany).

RPMI 1640 (Biochrom, Berlin, Germany) without phenol red was supplemented with heat-inactivated 5% human serum and with 1% antibiotics/antimycotics and 1% L-glutamine - both from Invitrogen (Darmstadt, Germany). Cytotfix/Cytoperm Fixation and Permeabilization Kit were from BD Pharmingen. HCl and NaOH were from Calbiochem Merck Biosciences (Darmstadt, Germany). GM-CSF (500 U/ ml) was from Berlex, Bayer Healthcare and IL-4 (500 U/ ml) was from Miltenyi Biotec (Bergisch Gladbach, Germany). For the stimulation experiments we used heat-inactivated *Staphylococcus aureus* (*S. aureus*) / Pansorbin from Calbiochem, Pam3Cys from EMC microcollections (Tübingen, Germany) and *Streptococcus pyogenes* (*S. pyogenes*), which was a kind gift from Prof. R. Lütticken (University of Aachen, Germany). LPS was from Sigma-Aldrich. TNF-alpha was from Bayer (Leverkusen, Germany) and a kind gift from Prof. D. Männel, Regensburg.

Isolation of monocytes and generation of Tip-DC

Monocytes were isolated from peripheral blood of healthy adults and patients with AD or psoriasis by density gradient centrifugation (Nycoprep) as described previously (14) or by negative selection using the monocyte isolation kit from Miltenyi Biotec. The purity of the cell preparation was evaluated by flow cytometric analysis via staining with anti-CD14-PE and was consistently greater than 70%. Viability (analyzed by 7-AAD staining) was found to be greater than 95%.

Monocytes were seeded in 24-well plates, each well containing 1×10^6 cells/ml medium RPMI 1640 without phenol red supplemented with 5% heat-inactivated human serum, 1% antibiotics/antimycotics and 1% L-glutamine in the presence of granulocyte macrophage colony-stimulating-factor (GM-CSF) (500U/ ml) and IL-4 (500U/ml). Alternatively in order to culture monocyte-derived DC (MoDC) we used fetal bovine serum (FBS) (10%) instead of human serum. On day 2 of culture, 500 U/ml GM-CSF were added to the medium. Since IL-4 inhibits the iNOS production (34) this cytokine was added only on day 0 to the culture.

Cells were stimulated at day 2 with Pansorbin (heat inactivated *S. aureus*, 1:100) or *S. pyogenes* (6.5×10^7 cells/ml), Pam 3Cys (1 μ g/ml) or TNF-alpha (10 ng/ml) before harvesting the cells for experiments on day 5. Stimulation schedules were optimized with regard to iNOS expression and activity. IL-4 was washed out during the stimulation step.

Flow cytometric analysis

Surface and intracellular staining

Expression of the surface markers CD1a, CD1c, CD11b, CD40, CD80, CD83, CD86, CD207/Langerin, CD14, DC-Sign(CD209), TLR1, TLR2, HLA-DR, and TRAIL was evaluated by staining with mAbs mentioned above visualized by FITC-conjugated goat-anti-mouse IgG. To block unspecific binding, the cells were incubated after washing with mouse serum for 30 min at 4°C. Finally, cells were counterstained by PE-conjugated CD11c-specific antibodies and 7-AAD and analyzed using a FACSCanto (Becton Dickinson, San Jose, CA) and FACSDiva software. Relative fluorescence index (rFI) was calculated as follows:
$$rFI = \frac{\text{Mean fluorescence intensity (MFI)}(\text{specific antibodies}) - \text{MFI}(\text{isotype control})}{\text{MFI}(\text{isotype control})}.$$

For staining of CCR2 on leukocytes in whole blood, incubation with primary antibodies was performed at room temperature. After the indicated time periods, aliquots of the cells were washed with FACS buffer, remaining cell membrane-bound antibodies were detected by FITC-labeled goat anti-mouse Ig and flow cytometric analysis. Monocytes were identified by counter-staining with CD14 and CD16 antibodies coupled to APC or PE, respectively. The rFI values were calculated as shown above. For intracellular staining, cells were fixed with the Cytofix/Cytoperm Fixation and Permeabilization Kit according to the manufacturer's instructions prior to the double staining procedure as described above.

Real-time PCR analysis

Gene expression was determined by SYBR Green-based real-time RT-PCR. RNA was reverse transcribed to cDNA and subjected to real-time PCR performed using the Applied Biosystems StepOnePlus Real-Time PCR System. The cycling conditions were 95°C for 15s, 60°C for 1 min, for 40 cycles, followed by a melting point determination. Melting curve analysis was performed to verify the presence of a single amplicon. Non template control (NTC) was used as a negative control. The expression level of each gene is indicated by the number of cycles needed for the cDNA amplification to reach a threshold, the results are normalized to beta-actin (ACTB) and evaluated using the $\Delta\Delta C_T$ method in order to calculate the fold increase (35). The oligonucleotides used as primers are described in Table S1.

TNF-alpha in culture supernatants of Tip-DC was determined by ELISA (R&D, Wiesbaden, Germany) according to the manufacturer's instructions.

Fluorometric detection of NO

For the detection of NO, the 2,3-diaminonaphtalene method can be used instead of the Griess-reaction because it is much more sensitive and detects nitrite at a concentration of about 10 nM (15). In this assay, 2,3-diaminonaphtalene reacts with the nitrite ions in the cell culture supernatant under acidic conditions to form the fluorescent product 1-(H)-dinaphthotriazole which is finally measured.

Only supernatants from cells cultured in medium without phenol red were used. A standard curve was prepared using NaNO₂ in culture medium. 100 µl sample or standard was added in triplicates to a black 96-well-plate as well as the blank and the nitrate standard. After that, freshly prepared DAN with a concentration of 0.05 mg/ml in 0.62 M HCl was added to each well immediately and incubated for 10 min at 20°C in the dark. The reaction was stopped with NaOH. The plate was read with a fluorescence plate reader with an excitation of 360 nm and 460 nm emission.

Table S1

Primers used for quantification of *NOS2*, *IL6*, *IL10*, *IL12B*, *IL12A*, *TNFA*, *IL23A* RNA expression via real time PCR.

Gene (polarity)	Sequence
NOS2+	CCCCTTCAATGGCTGGTACA
NOS2-	GCGCTGGACGTCACAGAA
ACTB+	GCGGATGTCCACGTCACACT
ACTB-	CCACTGGCATCGTGATGGAC
IL6+	AGGGCTCTTCGGCAAATGTA
IL6-	GAAGGAATGCCCATTAACAACAA
IL12B+	GCCCAGGACCGCTACTATAGC
IL12B-	CCTAACTGCAGGGCACAGATG
IL12A+	CCTTCACCACTCCCAAAACCT
IL12A-	TGTCTGGCCTTCTGGAGCAT
TNFA+	CCACTTCGAAACCTGGGATTC
TNFA-	TTAGTGGTTGCCAGCACTTCA
IL23A+	TTCTGCTTGCAAAGGATCCA
IL23A-	TCCGATCCTAGCAGCTTCTCA
TLR1+	TGTGCTGCCAATTGCTCATT
TLR1-	TTTTCCCCATAAGTCTCTCCTAAGAC

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Supplementary Figure S1

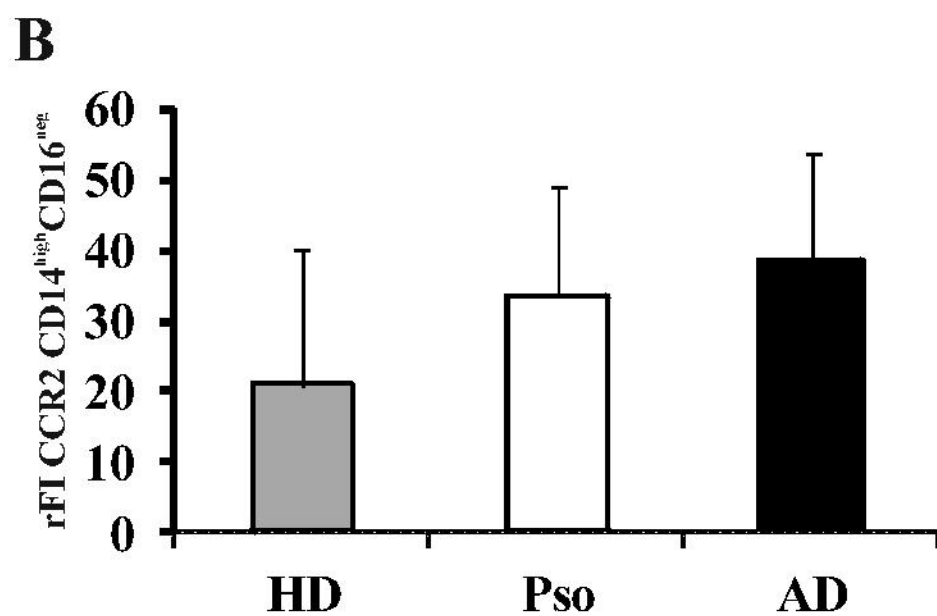
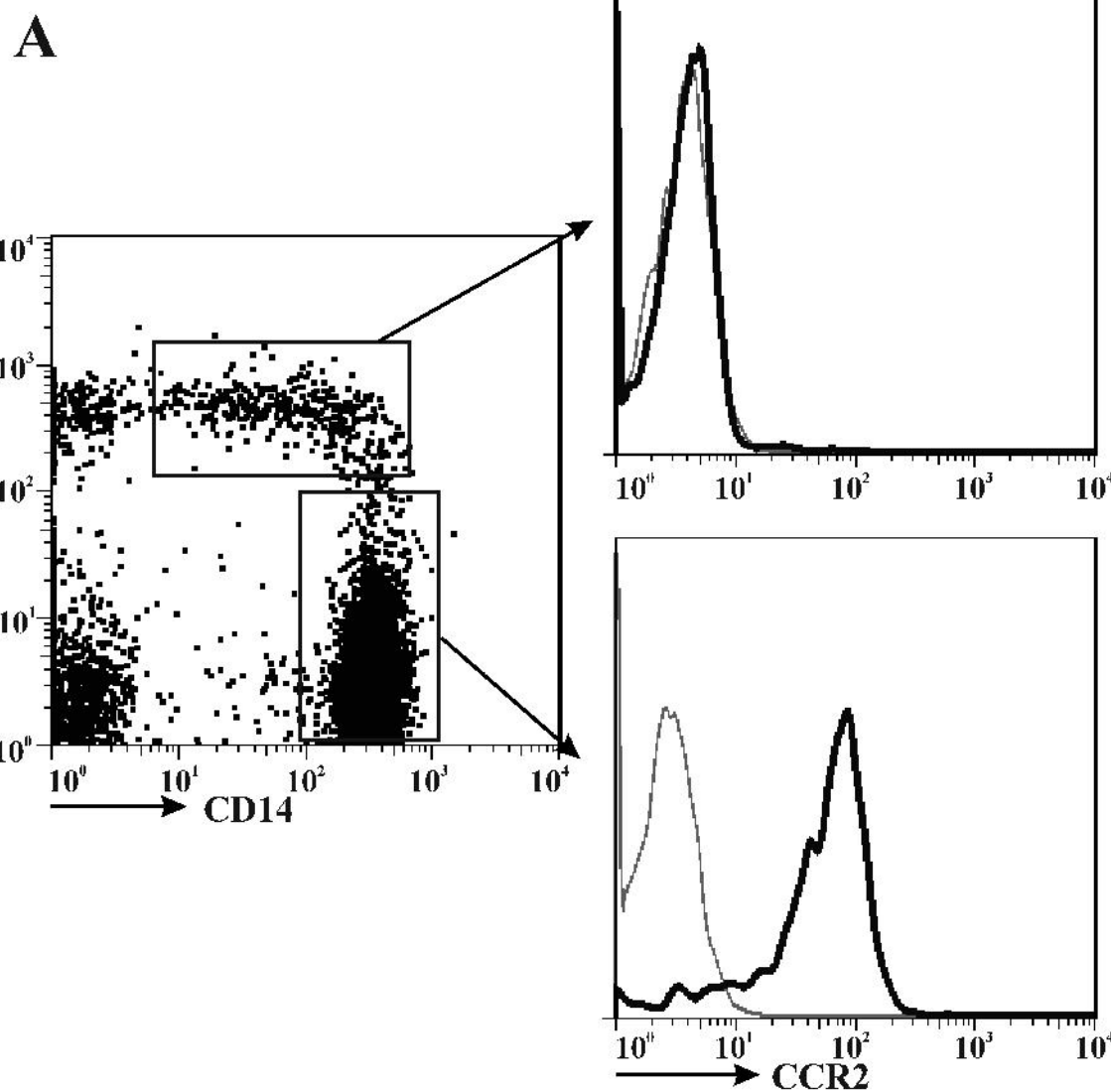
CCR2 expression by monocyte subpopulations. (A) Peripheral blood from healthy individuals or from patients was stained with CCR2-specific mAbs (black heavy lines) or with irrelevant Abs of matched isotype (grey thin lines) and counterstained with CD14- and CD16-specific mAbs. After exclusion of granulocytes by morphological criteria (FSC/SSC), CD14^{low}/CD16^{high} and CD14^{high}/CD16^{neg.} subpopulations were analyzed for CCR2 expression. (B) Expression of CCR2 on CD14^{high}/CD16^{neg.} monocytes isolated from healthy donors (n=12), donors with psoriasis (n=21) or atopic dermatitis (n=9) analyzed by flow cytometry. Relative fluorescence index (rFI) was calculated as follows: $rFI = [MFI(\text{specific antibodies}) - MFI(\text{isotype control})] / MFI(\text{isotype control})$. Mean values +/- SD are shown.

Supplementary Figure 2: Phenotypic analysis of *in vitro* generated Tip-DC.

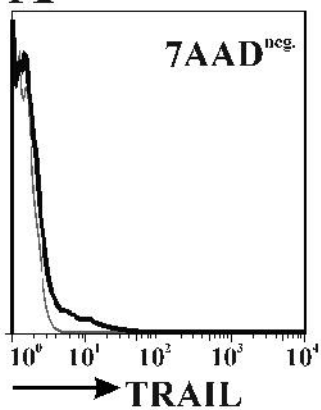
Tip-DC were cultured for five days as described in material and methods, stained with the indicated mAb and subjected to flow cytometric analysis of living CD11c^{pos} cells. (A) Since TRAIL expression is associated to apoptotic events, we compared expression of this receptor in the presence (top) and in the absence (bottom) of 7-AAD. (B) Tip-DC were stained with mAb specific for TLR2 (top, black heavy line) or HLA-DR (bottom, black heavy line) or with unspecific mAb with matched isotype and counterstained with CD11c-specific mAb. Histograms show results obtained for CD11c^{pos} cells. (C) In addition to flow cytometric analysis of TLR1 cell surface expression (top left panel), we analyzed Tip-DCs treated with the TLR1/TLR2 ligand Pam3Cys (bottom panel, bold line), which were stained with CD83-specific mAbs and analyzed by flow cytometry. Untreated cells are visualized by a fine line. Results shown are representative for experiments from three independent donors. (D) TLR1 mRNA from Tip-DC cells was quantified with real-time PCR. Absolute amounts of mRNA

154 molecules were quantified using global standard curves for TLR1 and actin derived from 6-10
155 experiments. n=5 samples were analyzed. Mean values +/- SD are shown.

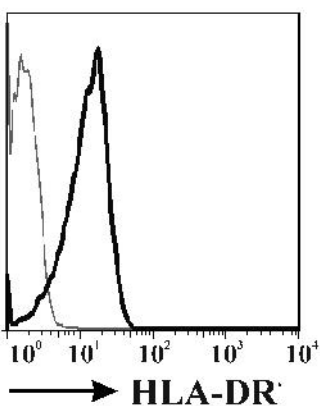
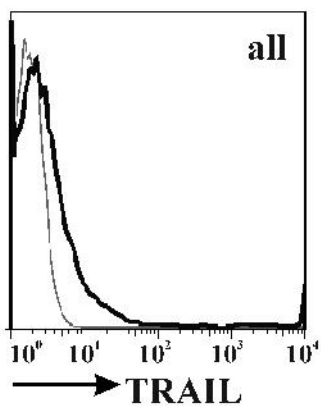
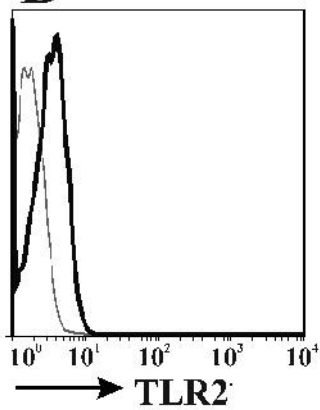
156



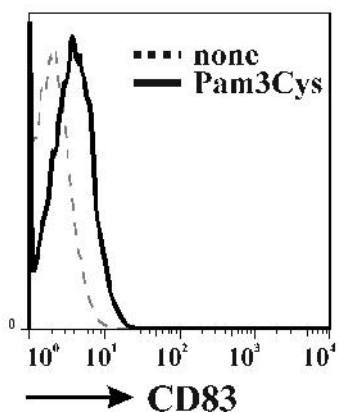
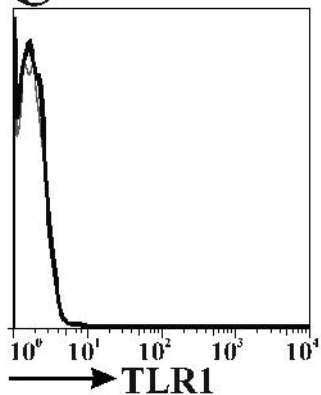
A



B



C



D

