

Lactoferrin, a major defense protein of innate immunity, is a novel maturation factor for human dendritic cells

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ABSTRACT Lactoferrin (LF) is an important protein component of the innate immune system that is broadly distributed within the body fluids. LF is endowed with multiple biological activities. Talactoferrin (TLF), a recombinant human LF, is in clinical development as an anticancer agent and is entering Phase III clinical trials. Here, we show that TLF induces the maturation of human dendritic cells (DCs) derived from monocytes. TLF, at physiologically relevant concentrations (100 µg/ml) up-regulates the expression of human leukocyte antigen (HLA) class II, CD83, CD80, and CD86 costimulatory molecule and CXCR4 and CCR7 chemokine receptors, acting primarily through the p38 MAPK signaling pathway. DCs matured by TLF displayed an enhanced release of IL-8 and CXCL10, as well as a significantly reduced production of IL-6, IL-10, and CCL20. They also display a reduced ability to take up antigen and increased capacity to trigger proliferation and release IFN-γ in the presence of allogeneic human T cells. TLF-matured DCs are able to prime naive T cells to respond to KLH antigen and display a significantly increased capacity to present Flu-MA_{58–66} peptide to HLA-A2-matched T cells. These data suggest that a key immunomodulatory function that may be mediated by TLF is to link the innate with adaptive immunity through DC maturation.—Spadaro, M., Caorsi, C., Ceruti, P., Varadhachary, A., Forni, G., Pericle, F., Giovarelli, M. Lactoferrin, a major defense protein of innate immunity, is a novel maturation factor for human dendritic cells. *FASEB J.* 22, 000–000 (2008)

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LACTOFERRIN (LF), AN IMPORTANT immunomodulatory protein, and a known component of the innate immune system present in neutrophil-specific granules (1) and broadly distributed within the body fluids and exocrine secretions (2), enhances host protection against infections, cancer (3), and inflammation in both adults and infants (4). It also improves the resolution of symptoms or survival rate, reduces pathogen level, and maintains homeostasis in animals, and humans or cell lines infected with bacteria, fungi, protozoa, or viruses. In mice, talactoferrin (TLF), a recom-

binant human form of LF, inhibits the growth of implanted tumors at distant sites, potentiates the anti-tumor activity of cisplatin (5), and inhibits rat *neu*-oncogene-driven mammary carcinogenesis in transgenic mice (6).

Iron sequestration is involved in some of LF's biological activities, including reduction of the free iron required for bacterial growth, biofilm formation, and free radical-mediated inflammatory damage (7). LF immunomodulatory effects, however, do not appear to be related to iron sequestration. It has been demonstrated that LF enhances Th1 cytokine responses in spleen and lymph node cells (8–10), activates macrophages and NK cells (11), and induces the expansion of leukocytes in blood and lymphoid tissues (12).

Dendritic cells (DCs) play a pivotal role in triggering T-cell responses that lead to the secretion of Th1 cytokines (13). It has also been shown that β-defensin 2, another key molecule of innate immunity acts directly on immature DCs (iDCs) to induce their functional maturation and enables them to elicit a robust Th1 response (14). Thus, we assessed the ability of LF to induce the maturation of DCs and the ability of the LF-induced DCs to trigger a Th1 response.

In the present study we provide the first evidence that TLF is able to induce the maturation of human DCs derived from monocytes (MoDCs). Furthermore, we demonstrate the capacity of TLF-matured MoDCs to trigger a robust type I polarized T-cell response. These results suggest that DC maturation is one of LF's key immunomodulatory functions that links the innate with the adaptive immunity.

MATERIALS AND METHODS

Generation and maturation of MoDCs

Human peripheral blood mononuclear cells (PBMCs) were obtained from buffy coat preparations of healthy donors by

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sedimentation over Ficoll-Hystopaque (Amersham Pharmacia, Uppsala, Sweden). CD14⁺ cells were isolated from fresh PBMCs by positive immunoselection with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The resulting preparations were consistently >90% CD14⁺, as determined by FACSCalibur (BD Biosciences, San Diego, CA, USA). iDCs were generated in 6-well plates; 5×10^6 CD14⁺ cells in 3 ml RPMI 1640 medium (Bio Whittaker Europe, Vervies, Belgium) supplemented with 10% heat-inactivated certified FBS (Invitrogen Life Technologies, Grand Island, NY, USA), 100 ng/ml of GM-CSF, and 50 ng/ml of IL-4 (both from PeproTech, Rocky Hill, NY, USA). On day 3, two-thirds of the medium was replaced by fresh medium containing GM-CSF and IL-4. On day 6, cells were harvested and resuspended as 1×10^6 cells/ml of RPMI medium with 10% FBS alone or supplemented with 50 ng/ml tumor necrosis factor (TNF)- α and 50 ng/ml IL-1 β (both from PeproTech) or with 1, 10, 100, 1000 μ g/ml TLF (Agennix, Houston, TX, USA). These experiments were performed with three distinct TLF batches (batches 995767, 4567890, and 87522; provided at a concentration of 100 ± 10 mg/ml and stored frozen at -20°C before using). Their endotoxin level was <0.03 EU/ μ l, as determined by the Limulus Amebocyte Lysate assay (Bio Whittaker Europe) performed according to manufacturer's protocol. In some experiments, 100 μ g/ml TLF preparations were preboiled for 15 min before incubation with iDCs to rule out the possibility of LPS causing DC maturation. After 24 h, cells were harvested and analyzed. The phenotype of each batch of newly generated DCs was always verified on day 6 prior to their use in experiments, to confirm that the cells had lost their monocyte features. Cells were evaluated for complete down-regulation of CD14 and high expression of CD1a by FACS analysis. No reversion to monocyte/macrophage type was observed when 6-day iDCs were cultured for 24 h with medium alone as unstimulated control cells in the experiments.

FACS analysis

Cells recovered from 24-h cultures in the presence of medium only, TNF- α and IL-1 β , or TLF were washed and resuspended in PBS (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 0.2% BSA and 0.01% sodium azide. Nonspecific sites were blocked with rabbit IgG (Sigma-Aldrich). Cells were then incubated with fluorochrome-conjugated monoclonal antibodies and isotype-matched negative controls for 20 min at 4°C . The following monoclonal antibodies were used: anti-human CD83 PE, anti-human CD80 PE, anti-human CD86 PE (all from BD Pharmingen, San Diego, CA, USA), anti-human myosin heavy chain II fluorescein isothiocyanate (FITC) (Ancell Corporation, Bayport, MN, USA), anti-human CCR5 PE, anti-human CCR6 PE, anti-human CCR7 PE, and anti-human CXCR4 PE (all from R&D Systems, Minneapolis, MN, USA). Isotype-matched monoclonal antibodies were from DakoCytomation (Heverlee, Belgium). Samples were collected and analyzed with a CyAn ADP and Summit 4.3 software (DakoCytomation). Cells were gated according to their light-scattering properties to exclude cell debris and contaminating lymphocytes, and according to their propidium iodide negativity (Sigma-Aldrich) to exclude dead cells.

To determine the signaling pathway effective in the TLF-induced maturation of MoDCs, iDCs were pretreated with inhibitors, namely pertussis toxin (PTX) from *Bordetella pertussis* (100 ng/ml; Sigma-Aldrich), LY294002 (25 μ M; Sigma-Aldrich), SB203580 (20 μ M; Sigma-Aldrich), U73122 (1 μ M; Sigma-Aldrich), PD98059 (50 μ M; Calbiochem, San Diego, CA, USA), BAY 11-7082 (10 μ M; Alexis, San Diego, CA, USA), SP600125 (20 μ M; Calbiochem), DMAT (10 μ M;

Calbiochem), and PP2 (1 μ M; Calbiochem) or 0.1% dimethyl sulfoxide (DMSO) as a control, for 45 min at 37°C and, without washing, incubated in the presence or not of TLF. After 24 h, supernatants were harvested to evaluate the amount of CXCL10 by ELISA, and cells were processed as indicated above, to evaluate the percentage of expression of CD83, CD86, CXCR4, and CCR7. The percentage of inhibition was calculated as $100 - [(data\ from\ TLF-treated\ DCs\ in\ the\ presence\ of\ inhibitors)/(data\ from\ TLF-treated\ DCs\ without\ inhibitors)] \times 100$.

Cytokine detection

Production of cytokines in the supernatants harvested after 24 h incubation in the presence of medium only, TNF- α and IL-1 β , or TLF was evaluated by human Th1/Th2 11plex FlowCytomix kit (Bender MedSystem, San Bruno, CA, USA) or standard ELISA for IL-10, IL-8, IL-12 p70, CCL20, CXCL10 (R&D Systems), and IL-12 total (Mabtech AB, Nacka Strand, Sweden). For kinetics experiments, supernatants were harvested after 6, 12, and 24 h incubation to evaluate the production of IL-12. In these last experiments, iDCs were also stimulated with 1 μ g/ml LPS (from *Escherichia coli* 055:B5; Sigma-Aldrich), a ligand of Toll-like receptor (TLR) 4, and with 50 μ g/ml poly (I:C) (Amersham Biosciences, Piscataway, NJ, USA), a ligand of TLR3.

Antigen uptake assay

After 24 h culture in the presence of medium only, TNF- α and IL-1 β , or TLF, differentially matured DCs were incubated at 1×10^6 /ml in RPMI 1640 supplemented with 1% heat-inactivated FBS for 20 min at 4°C (negative control) or 37°C . FITC-labeled dextran (MW 70,000; Sigma-Aldrich) was added at the final concentration of 1 mg/ml, and the cells were incubated for 30 and 90 min to allow its capture. After thorough washing with cold PBS, fluorescence was measured by FACSCalibur CellQuest analysis to reveal dextran uptake.

Mixed lymphocyte reaction

T cells were purified on a nylon-wool column (Robbins Scientific Corporation, Sunnyvale, CA, USA) and placed in 96-well round-bottomed culture plates at 1×10^5 cells/well with allogeneic iDCs and differentially mature DCs in decreasing concentrations (10,000–1250 cells). After 5 days, 1 μ Ci of [^3H]thymidine (TdR) (Amersham Biosciences, Uppsala, Sweden) was added to each well, and incubation was prolonged for 16 h. Cells were directly collected with a CellHarvester (Packard Biosciences B.V., Groningen, The Netherlands) in UNIfilter plates (Packard Biosciences) and [^3H]TdR uptake was quantitated (TopCount microplate scintillation counter; Packard Biosciences). Proliferation was determined as counts per minute of quadruplicate determinations.

Supernatants from T cells cultured with differentially matured DCs at a ratio of 20:1 T:DC were harvested after 48 h and analyzed by ELISA for IFN- γ and IL-4 production (both R&D Systems), in accordance with the manufacturer's protocol.

Ag-specific T-cell response

To assess whether maturation of MoDCs in the presence of TLF modulates their ability to present human leukocyte antigen (HLA)-A2-restricted peptides to T cells, differentially mature DCs from HLA-A2⁺ donor were resuspended at 1×10^6 /ml in serum-free RPMI 1640 and pulsed with the influ-

enza matrix Flu-MA_{58–66} (GILGFVFTL; Primm, Milan, Italy) (3 µg/ml) for 4 h at 37°C. They were then washed 2× in serum-free RPMI 1640, resuspended in RPMI medium supplemented with 10% heat-inactivated FBS, and placed in a 6-well plate at 1×10^6 cells/well with autologous T cells at a 1:10 DC:T. On day 7, the recovered cells were restimulated in the same conditions by Flu-MA_{58–66} peptide pulsed DCs and 1 ng/ml of IL-2 (PeproTech) was added to the wells. After another 7 days, their specific activation against Flu-MA_{58–66} peptide was evaluated by ELISpot assay. Tyrosinase_{1–9} (ML-LAVLYCL; Primm) was used as an unrelated control peptide.

To investigate the ability of TLF100/mDCs to prime naive T cells to respond to a foreign antigen, iDCs prepared as described above were resuspended at 1×10^6 /ml in serum-free RPMI 1640 and incubated with 10 µg/ml keyhole limpet hemocyanin (KLH) (Calbiochem) for 6 h at 37°C. They were then washed 2× in serum-free RPMI 1640, resuspended in RPMI 1640 supplemented with 5% heat-inactivated AB human serum (BioWhittaker Europe), and matured for 24 h with 50 ng/ml TNF-α and 50 ng/ml IL-1β or with 100 µg/ml TLF. Then, differentially mature DCs were cocultured with autologous CD45RA⁺ T cells at a ratio of 1:10 DC:T. No exogenous cytokines were added. CD45RA⁺ T cells were previously isolated from thawed PBMCs from the same donor by positive immunoselection with magnetic beads (Miltenyi Biotec), performed according to manufacturer's protocol. The resulting preparation was 100% CD45RA⁺, as determined by FACS analysis. On day 0 at 12 h, and day 7, IFN-γ production by activated T cells was evaluated by ELISpot assays.

ELISpot assay

Five $\times 10^4$ recovered cells were cultured 10:1 with peptide-pulsed HLA-A2⁺ T2 cells or 30:1 with unpulsed or KLH-pulsed DCs in a final volume of 100 µl of AIM-V medium (Gibco, Invitrogen Life Technologies) in 96-well plates for the ELISpot assay (Millipore, Bedford, MA, USA). These wells were previously coated with 100 µl of human primary anti-IFN-γ mAb solution (5 µg/ml in PBS) (Endogen, Woburn, MA, USA), incubated overnight at 4°C, and washed 4× with PBS, and 200 µl of PBS supplemented with 4% BSA (blocking buffer) was added for 1 h to block nonspecific binding. The assay was performed in duplicate wells for each experimental condition. The cultures were then incubated in a humidified atmosphere of 5% CO₂ in air at 37°C for 24 h. Cells were removed by washing the plates 4× with PBS and 4× with PBS supplemented with 0.05% Tween 20 (Sigma-Aldrich) (wash buffer). Then 100 µl of biotinylated secondary anti-IFN-γ mAb (1 µg/ml in PBS/4% BSA) (Endogen) was added to each well. The plates were again incubated in a humidified atmosphere of 5% CO₂ in air at 37°C for 2 h. The washing steps were repeated, and after 30 min incubation at room temperature with horseradish peroxidase (HRP)-streptavidin (100 µl/well of 1:1000 dilution in wash buffer) (Endogen), the plates were washed again 4× with wash buffer. Substrate (100 µl) (AEC Staining Kit, Sigma-Aldrich) was added to each well to develop the spots. The reaction was stopped after 5–10 min under running tap water, and the membrane was left to dry in the dark. The spots were counted by computer-assisted image analysis (Transtec 1300 ELISpot Reader, AMI Bioline, Buttigliera Alta, Turin, Italy). The background values were subtracted. As positive controls, some of the T cells were incubated with phorbol 12-myristate 13-acetate (PMA; 10 ng/ml) (Sigma-Aldrich) and ionomycin (500 ng/ml) (Sigma-Aldrich) in the ELISpot plate.

Statistical analysis

The significance of differences in counts per minute obtained from the [³H]TdR uptake assay, in cytokine secretion, and in the number of spots was evaluated with an unpaired two-tailed Student's *t* test (GraphPad Prism 4; GraphPad Software, San Diego, CA, USA). Values of *P* < 0.05 were considered statistically significant.

RESULTS

TLF induces MoDC maturation

Control iDCs had undetectable levels of the maturation marker CD83 after 24-h culture with medium alone. By contrast, CD83 was strongly expressed by control mature DCs (mDCs) recovered from medium supplemented with TNF-α and IL-1β and increased progressively when the medium was supplemented with 1, 10, 100, and 1000 µg/ml of TLF (TLF1/mDCs, TLF10/mDCs, TLF100/mDCs, TLF1000/mDCs, respectively) (Fig. 1A). Expression of HLA II, CD80, and CD86 costimulatory molecules also increased in dose-dependent manner. TLF at both 100 and 1000 µg/ml induced expression of the markers of DC maturation at levels comparable to those observed with DCs matured using the standard TNF-α and IL-1β treatment. TLF at 100 µg/ml was, therefore, used for further DC maturation assays.

To rule out the possibility that DC maturation was induced by LPS contamination, 100 µg/ml TLF preparations were preboiled for 15 min before incubation. Boiled TLF was unable to elicit significant changes in the expression of DC maturation markers (data not shown).

Because MoDC maturation is associated with a differential regulation of chemokine receptors (15), we further evaluated TLF's ability to modify the expression profile of chemokine receptors. Consistent with results observed with mDCs, the expression of CXCR4 and CCR7 by TLF100/mDCs was up-regulated (Fig. 1B), while CCR5 was down-modulated as compared to iDCs and mDCs. CCR6 was not expressed by either TLF100/mDCs or mDCs.

To identify signal transduction pathways involved in the TLF-induced MoDC maturation, we pretreated iDCs with inhibitors for G protein, PI3K, ERK, p38 MAPK, JNK, phospholipase C (PLC), Src, and NF-κB pathways at 37°C, followed by incubation in the presence or absence of TLF for an additional 24 h. Medium-0.1% DMSO was used as the stimulation background. In each case, we evaluated the expression of CD83, CD86, CXCR4, and CCR7 (Table 1). Inhibitors to the G protein (PTX), ERK1/2 (PD98059), and PLC (U73122) had no effect, suggesting that these pathways are not involved in TLF-induced MoDC maturation. The other inhibitors differentially reduced the TLF-induced expression of CD83, CD86, CXCR4, and CCR7. CD83 expression was strongly inhibited by SB203580 (p38 inhibitor), and moderately by SP600125 (JNK inhibitor) and by PI3K inhibitor Ly294002. Up-

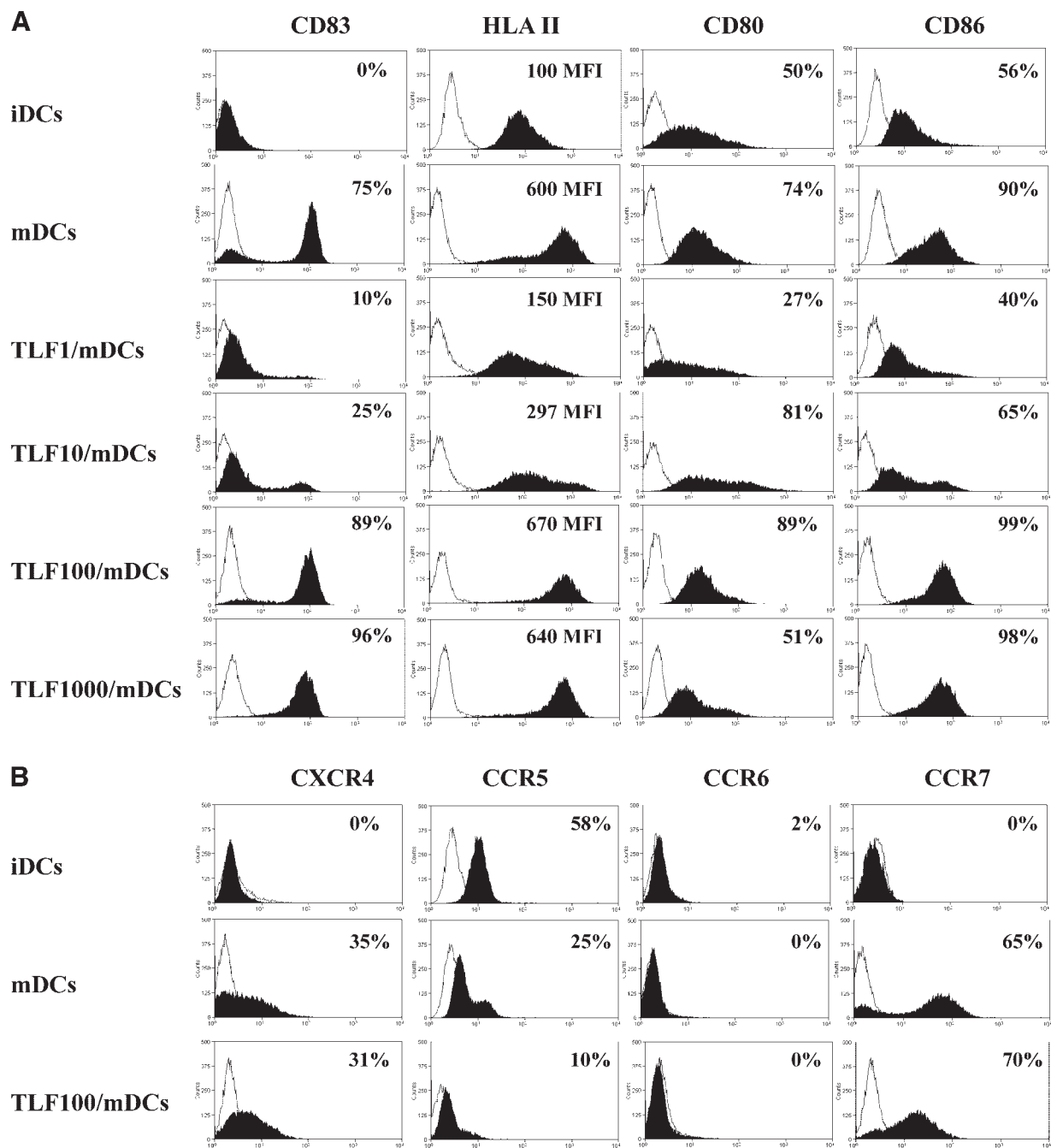


Figure 1. Flow cytometric analysis showing that iDCs cultured with TLF acquire a mature DC phenotype. *A*) Dose-response of TLF-induced iDC maturation ($CD83^+$, $HLA II^{high}$, $CD80^{high}$, $CD86^{high}$). iDCs were cultured for 24 h in medium only, medium supplemented with $TNF-\alpha$ and $IL-1\beta$ (mDCs), or medium supplemented with 1, 10, 100, or 1000 $\mu g/ml$ TLF (TLF1/mDCs, TLF10/mDCs, TLF100/mDCs, TLF1000/mDCs, respectively). *B*) Expression of chemokine receptors (CXCR4, CCR5, CCR6, CCR7) by iDCs cultured in medium only (iDCs) or medium supplemented with $TNF-\alpha$ and $IL-1\beta$ (mDCs) or 100 $\mu g/ml$ TLF (TLF100/mDCs). Shaded curves show specific antibody binding; unshaded curves show isotype control Ig staining. Values are percentages of specific antibody binding or mean fluorescence intensity (MFI). Data are representative of one of five independent experiments performed with different donors.

modulation of CD86 was marginally affected by the blockade of several pathways: p38, $NF-\kappa B$, and Src kinase. CXCR4 expression was strongly inhibited by SB203580 and moderately reduced by $NF-\kappa B$ (BAY) and Src kinase inhibitors. Finally, CCR7 expression was moderately inhibited by the blockade of JNK, and strongly by the inhibition of p38. SB203580, the p38 inhibitor, was the strongest and most consistent inhib-

itor. It greatly reduced the expression of CD83, CXCR4, and CCR7, but had a marginal effect on CD86.

Cytokine production

Conditioned medium from MoDCs cultured for 24 h with medium only, medium supplemented with $TNF-\alpha$

TABLE 1. *Signal pathways involved in the TLF-induced maturation of human MoDCs*

Inhibitor	Percentage of inhibition			
	CD83	CD86	CXCR4	CCR7
PTX	0	0	0	0
LY294002	38	0	0	0
PD98059	0	0	0	0
SP600125	50	0	0	33
SB203580	76	3	83	83
BAY	7	0	31	3
DMAT	3	7	0	0
U-71312	0	0	0	0
PP2	0	3	30	0

iDCs were incubated with inhibitors for 45 min at 37°C and then in the presence or absence of TLF for 24 h. Results are expressed as percentage of inhibition for each marker increased or induced by TLF. Inhibition up to 25% is considered low, up to 50% moderate and over 50% high. One of two experiments is represented.

and IL-1 β , or 100 μ g/ml of TLF was analyzed with the human Th1/Th2 cytokine FlowCytomix multiplex kit to detect the presence of 11 different cytokines (IL-12p70, IL-10, IL-8, IL-4, IL-2, IL-5, IL-6, IFN- γ , TNF- α , TNF β , IL-1 β) (**Fig. 2A**). Production of IL-1 β and TNF- α by mDCs was not measured because they were included in the standard medium. iDCs produced very small amounts of these 11 cytokines, whereas a distinctive repertoire was secreted by mDCs and TLF100/mDCs. Interestingly, TLF100/mDCs displayed an enhanced release of IL-8, and a significant inhibition of IL-6 ($P=0.008$) and IL-10 ($P=0.0213$) production compared to mDCs. There was no significant difference in the secretion of the other Th1 and Th2 cytokines. IL-12, TNF β , and IFN- γ were never detected. The multiplex data were repeated with ELISA assays for IL-10 and IL-8 using the same supernatants and provided similar results (data not shown). To assess whether the absence of IL-12 production could be due to low sensitivity of the multiplex kit or to a different kinetics of production, we performed subsequent experiments and evaluated both IL-12p70 and IL-12p40 concentrations in supernatants after 6, 12, and 24 h culture with more sensitive ELISA assays. In these experiments, we also used as positive controls iDCs stimulated with LPS, a ligand of TLR4, and poly (I:C), a ligand of TLR3. Poly (I:C) was the strongest inducer of IL-12p70, as known, displaying highest levels around 12 h. TLF100/mDCs released smaller amounts of IL-12 p70 and similar levels of IL-12 p40, in comparison to mDCs, LPS/mDCs, and poly I:C/mDCs, in a time-dependent manner (**Fig. 2B**).

CXCL10 and CCL20 chemokines were also quantified by ELISA in these supernatants. TLF100/DCs released higher amounts of CXCL10 and lower levels of CCL20 than with mDCs (**Fig. 2C**).

We investigated whether the pathways involved in the TLF-induced MoDC maturation were also involved in the increased production of CXCL10 by MoDCs. Supernatants from iDCs pretreated with inhibitors for G protein, PI3K, ERK, p38 MAPK, JNK, PLC, NF- κ B, and Src pathways at 37°C and then incubated in the pres-

ence of TLF for 24 h were evaluated for the presence of CXCL10 by ELISA. Medium-0.1% DMSO was used as the stimulation background. LY294002 and SB203580 strongly inhibited the release of CXCL10 induced by TLF (78 ± 5 and $87\pm1\%$, respectively), whereas the other inhibitors had little or no effect (**Fig. 2D**), suggesting that TLF enhancement of CXCL10 release was mainly achieved *via* the p38 and PI3K signaling pathways.

Antigen uptake by TLF100/mDCs

In view of the fact that MoDCs take up antigen efficiently and lose this ability on maturation (16, 17), we compared antigen uptake by iDCs, mDCs, and TLF100/mDCs by flow cytometry (**Fig. 3**). At 4°C, cell metabolism was inhibited and DCs were unable to capture antigen. However, when warmed to 37°C, the control iDCs showed a significant uptake of FITC-labeled dextran after 30 min, which progressively increased until 90 min (**Fig. 3A, D**). By contrast, both mDCs and TLF100/mDCs displayed a reduced uptake (**Fig. 3B–D**), consistent with the behavior expected with true mature DCs.

Ability of TLF100/mDCs to activate a T-cell response

We also examined the ability of the DCs to induce a T-cell response. The capacity of mDCs and TLF100/mDCs to induce the proliferation of allogeneic T cells was similar to each other and markedly higher than that of iDCs (**Fig. 4A**). Both types of mature DCs also triggered a significantly higher production of IFN- γ than iDCs (**Fig. 4B**). In contrast IL-4 release triggered by mDCs and TLF100/mDCs, as well as iDCs, was barely detected (**Fig. 4C**). These data suggest that both mDCs and TLF100/mDCs promote a comparable Th1-directed response.

We also assessed whether the maturation of MoDCs in the presence of TLF modulates their ability to present HLA-A2-restricted peptides to T cells. TLF100/mDCs or mDCs generated from the same HLA-A2⁺ donors were loaded with the influenza matrix Flu-MA_{58–66} peptide and cocultured for 7 days with autologous nylon-wool-purified T cells. Recovered cells were restimulated in the same conditions by Flu-MA_{58–66} peptide-loaded DCs for additional 7 days, and their ability to release IFN- γ following Flu-MA_{58–66} peptide presentation by HLA-A2-matched T2 cells was tested by an ELISpot assay. T cells from cultures activated by TLF100/mDCs expressed a higher number of spots than mDCs (**Fig. 5A**). This enhanced peptide presentation was consistently observed with T cells from three distinct HLA-A2⁺ donors, suggesting that TLF100/mDCs may be more efficient antigen-presenting cells than mDCs, as they induce a better T-cell activation against a specific peptide.

Because the Flu model may give an indication of the capacity to reactivate a memory CD8 response, we also investigated whether the maturation of MoDCs in the presence of TLF modulates their ability to prime T-cell responses to new antigens. We cocultured CD45RA⁺ T

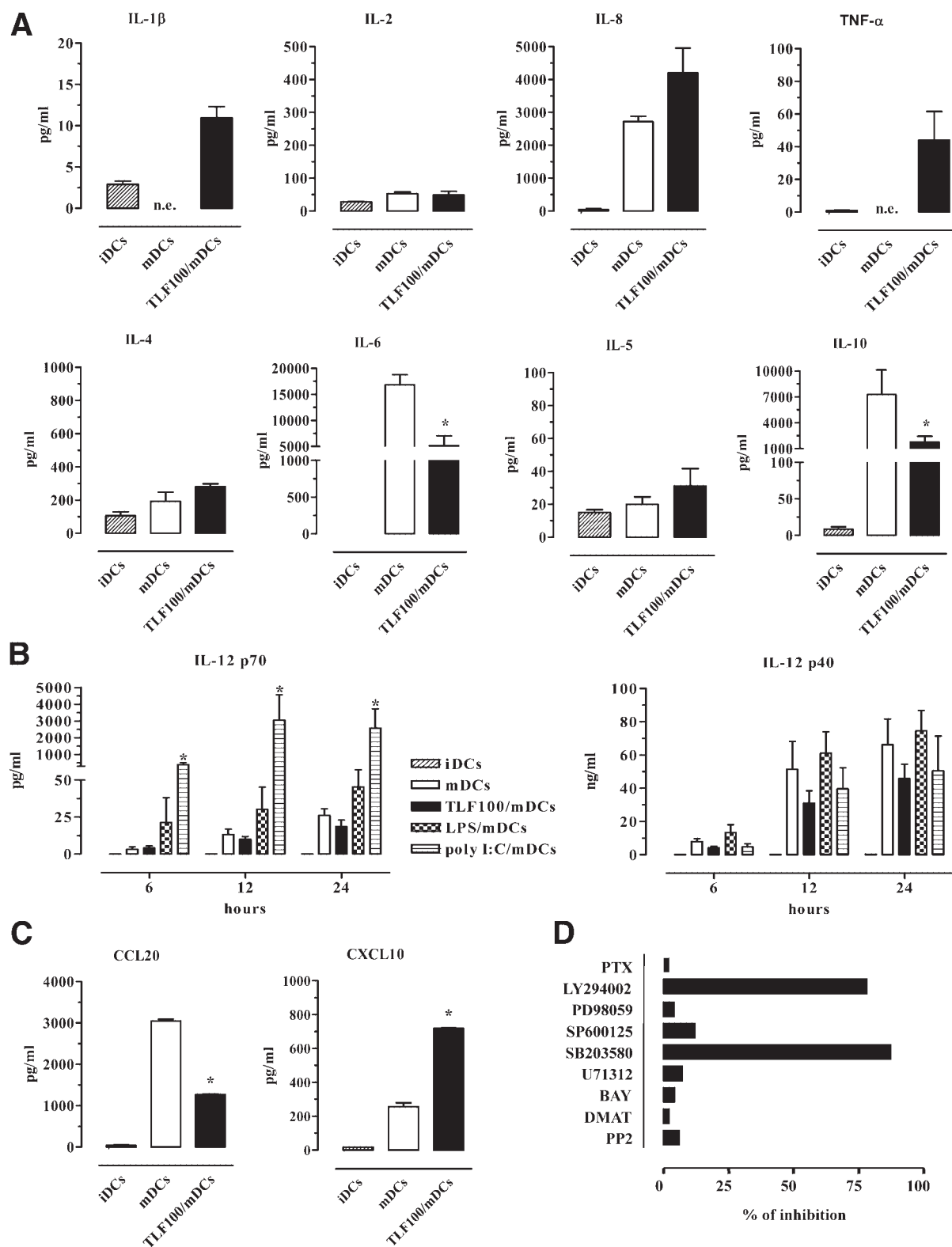


Figure 2. TLF/mDCs produce a distinctive repertoire of cytokines and chemokines. A) Conditioned medium from iDCs incubated for 24 h with medium only (iDCs, hatched bars), medium supplemented with TNF- α and IL-1 β (mDCs, white bars), or medium supplemented with 100 μ g/ml TLF (TLF100/mDCs, black bars) was measured for the presence of Th1 cytokines (top panels) or Th2 cytokines (bottom panels) by a human Th1/Th2 cytokine FlowCytomix multiplex kit. IL-1 β and TNF- α were not measured for the mDC supernatant because the cytokines were exogenously added to the medium. IL-12p70, TNF- β and IFN- γ were not detectable and are not shown. Values are means $\pm$ SE of three independent experiments performed with different donors. * $P < 0.05$ vs. mDCs. B) The production of IL-12p70 and IL-12p40 was measured by ELISA kit in conditioned medium from iDCs incubated for 6, 12, and 24 h with medium only (iDCs) or medium supplemented with TNF- α and IL-1 β (mDCs), 100 μ g/ml TLF (TLF100/mDCs), 1 μ g/ml LPS (LPS/mDCs), or 50 μ g/ml poly (I:C) (poly I:C/mDCs). Values are

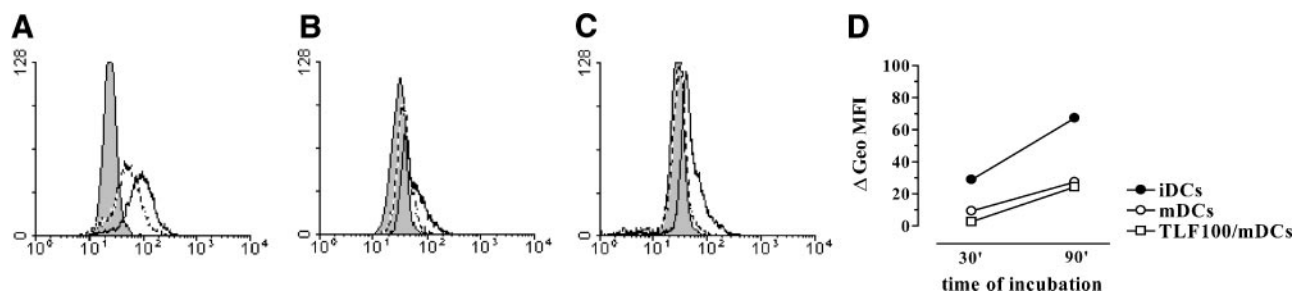


Figure 3. Decrease in endocytosis of FITC-dextran by TLF100/mDCs. A–C) iDCs cultured for 24 h in medium only (A) or medium supplemented with TNF- α and IL-1 β (B) or 100 μ g/ml TLF (C) were further incubated for 30 and 90 min with FITC-dextran at 37°C and then analyzed by flow cytometry. The fluorescence intensity of cells after 30 min (dotted line) and 90 min (bold line) is shown. The filled peak shows a parallel sample maintained at 4°C with FITC-dextran as a negative control. D) Difference between the geometric MFI of the sample incubated at 37°C or maintained at 4°C, calculated for each time of incubation: iDCs (●); mDCs (○); TLF100/mDCs (□). Data are representative of one of three independent experiments performed with different donors.

cells with autologous KLH-pulsed DCs matured with TLF or TNF- α + IL-1 β , and tested their ability to release specifically IFN- γ . On the first day of coculture, only the positive control group of T cells, restimulated with PMA and ionomycin, produced any IFN- γ secretion, and no KLH-specific responses were seen with either maturation group (data not shown). By day 7 of coculture, without the addition of exogenous cytokines, similar numbers of KLH-specific, IFN- γ producing spots were detected in both groups of T cells activated by TLF100/mDCs or mDCs (Fig. 5B). Thus, TLF100/mDCs and mDCs appear to be equally efficient as antigen-presenting cells in priming naive T cells.

DISCUSSION

We have shown that LF, at physiologically relevant concentrations, induces maturation of immature DCs as measured by FACS analysis (cell surface maturation and cytokine markers) and by function (cytokine production, loss of antigen internalization, and induction of a T-cell response). This novel finding expands our understanding of how the innate immunity crosstalks with adaptive immunity. The significance of this link rests on the fact that LF is one of the principal components of innate immunity present where a pathogen invasion is most likely to take place. It is found in mucosal surfaces exposed to the external environment and in inflammatory settings where it is released from neutrophil-specific granules (1). Moreover, the pharmacological activity of LF is under evaluation in several clinical indications (18–20), in which MoDC maturation may have an important, and so far unnoticed, role. In particular, TLF is in development as an anticancer drug, has demonstrated promising anticancer activity

in human clinical trials (21–23) and is now entering Phase III clinical development.

The TLF concentrations that led to the full MoDC maturation *in vitro* (≥ 100 μ g/ml) are close to the physiological concentrations of LF *in vivo*. LF concentrations have been reported for normal human secretions (24), including bronchial mucus (35 μ g/ml), synovial fluid (46 μ g/ml), tear fluid (2.2 mg/ml), mature milk (1–2 mg/ml), and colostrum milk (5–7 mg/ml), while endogenous LF levels increase substantially in inflammatory settings following neutrophilic degranulation (25).

Although the TLF/mDCs are similar to standard mDCs in most of the ways examined, there are some very interesting differences (Fig. 6). Secretion of IL-6 and IL-10, two cytokines that often play a negative regulatory role, is markedly impaired in the TLF-matured DCs, and could contribute to the stronger induction of T-cell response observed with the TLF-matured DCs. In contrast, the TLF/mDCs appear to produce greater amounts of IL-8 than mDCs. This may make the TLF-DCs better suited to orchestrate a neutrophil-dominated immune reaction at their activation site (26), consistent with LF's presence in the neutrophil-specific granules. TLF induces iDCs to release TNF- α and IL-1 β , but in small amounts, which suggest that these two cytokines should not have a major role in the TLF-induced DC maturation.

TLF/mDCs display a high expression level of several membrane molecules with distinct but integrated roles in T-cell activation. CD83 is an immunoglobulin family glycoprotein whose expression by mDCs directly correlates with their ability to prime T cells (27), driving their antigen-specific expansion and supporting their long-term survival (28). CD80 and CD86 are two co-stimulatory molecules of the B7 family with a key role in

means $\pm$ SE of six independent experiments performed with different donors. * P < 0.05 *vs.* mDCs, TLF100/mDCs, and LPS/mDCs. C) CCL20 and CXCL10 were measured by ELISA kit in the same conditioned media evaluated in A. * P < 0.05 *vs.* mDCs. D) Signal pathways involved in the TLF-induced production of CXCL10 of human MoDCs. Supernatants from iDCs incubated with inhibitors for 45 min at 37°C and then in the presence of TLF for 24 h were measured for the presence of CXCL10 by ELISA. Results are expressed as percentage of inhibition of TLF-induced production of CXCL10 by the presence of various inhibitors. Data are representative of one of two independent experiments performed with different donors.

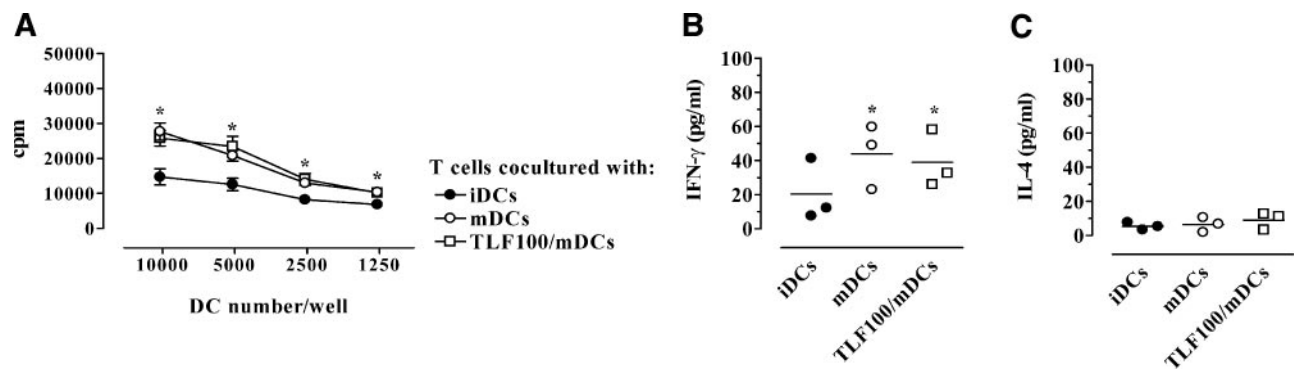


Figure 4. Ability of TLF/mDCs to trigger proliferation and IFN- γ release by allogeneic T cells. **A)** Proliferative response elicited by decreasing numbers of irradiated iDCs cultured for 24 h in medium only (iDCs, ●) or medium supplemented with TNF- α and IL-1 β (mDCs, ○) or 100 μ g/ml TLF (TLF100/mDCs, □), cultured with 1×10^5 nylon-wool-purified allogeneic T cells. After 5 days culture, T-cell proliferation was measured by uptake of [^{3} H]TdR. Values are mean $\pm$ SE cpm triggered by the same DC preparations in three allogeneic T-cell preparations. **B, C)** IFN- γ (**B**) and IL-4 (**C**) production triggered by iDCs (●), mDCs (○), and TLF100/mDCs (□). The supernatant of the mixed lymphocyte culture of **A** was recovered after 48 h culture and evaluated by ELISA. Both mDCs and TLF100/mDCs trigger a stronger proliferation (**A**) and IFN- γ release (**B**) as compared to iDCs; * $P < 0.05$. Data were obtained with DCs from one donor tested against three distinct allogeneic donors. DC preparations from four different donors triggered a similar response pattern in allogeneic T cells from nine donors.

antigen-specific activation of naive T cells (29) that act in concert with CD83 (28). Their expression may fully account for the ability of TLF/mDCs to efficiently trigger the proliferative response of allogeneic human T cells in a mixed-lymphocyte reaction and to prime naive T cells to a neoantigen, the foreign protein KLH. KLH-pulsed, TLF-matured DCs successfully primed autologous CD45RA $^+$ T cells to develop into IFN- γ -producing cells after 7 days coculture. Moreover, TLF-matured DCs were strikingly more effective at stimulating an influenza antigen-specific CD8 $^+$ T-cell response, which may suggest a differential ability of TLF/mDCs to activate memory CD8 $^+$ lymphocytes. TLF/mDCs release lower amounts of bioactive IL-12p70, but similar high levels of IL-12p40 in comparison to mDCs, which may predict a similar ability to produce IL-23. We also compared the capacity of TLF to induce IL-12 production with that of two well-known TLR ligands, LPS and poly (I:C). Indeed, both are stronger stimuli to trigger the release of IL-12p70, especially poly (I:C). However, a too strong stimulus may induce “exhaustion” of DCs. It has been reported that after activation by LPS, DCs produced IL-12 only transiently and became refractory to further stimulation (30). The exhaustion of cytokine production may affect the T-cell polarizing process. DCs matured by TLF (they display all characteristic features of mature DCs), indeed, release small amounts of IL-12p70, in a reproducible manner, but could be induced to release more IL-12p70 on encounter with T cells. The high expression of costimulatory molecules along with the overexpression of HLA-II glycoproteins and the release of CXCL10 may work together in triggering an antigen-specific Th1-polarized response (31). In addition to the production of CXCL10, TLF/mDCs may stimulate natural killer cells and T-cell cytotoxicity and display antiangiogenic properties (32). Lastly, the *de novo* expression of CXCR4 and CCR7 chemokine receptors guides the directional migration of TLF/mDCs toward draining secondary lymphoid organs (33).

Immune cells, like virtually any other cells in the organism, can bind LF (34–37). The cationic nature of the LF protein moiety accounts for its propensity to bind to anionic molecules (38). This results in massive binding to cells and makes identification of receptors contributing to the immunoregulatory roles of LF difficult. A specific receptor responsible for LF endocytosis has been identified and characterized on the surface of intestinal cells, but it was not found on immune cells (39, 40). Other important LF receptors, namely the low density lipoprotein receptor-related proteins (LPRs) have been found on cells such as hepatocytes, fibroblasts, osteoblasts, and brain endothelial cells (41–46). These molecules are multiligand receptors (47) widely expressed on several cell types (48). LF binding to LPR on immune cells has not yet been demonstrated, but the presence of LPRs on mononuclear cells (48) makes its binding to such cells likely. Bovine LF has been shown to bind the mannose receptor of immature antigen-presenting skin cells (49). Such binding was confirmed in a study showing that the binding of bovine LF to the DC-SIGN receptor on dendritic cells blocks its interaction with HIV glycoprotein 120 and subsequent virus transmission (50). Human LF was, however, less efficient. Bovine LF has also been shown to activate murine macrophages *via* TLR4-dependent and -independent signaling pathways (51).

The receptor mediating TLF-induced MoDC maturation has not been identified. In the present study, we observed that the specific inhibition of G proteins, ERK, and PLC transduction pathways does not affect the TLF-induced expression of CD83, CD86, CXCR4, and CCR7, whereas the blockage of p38 MAPK inhibits the expression of most TLF-induced markers, with the exception of CD86. The PI3K signaling pathways seem also involved in the TLF-induced expression of DC surface maturation markers and the increased production of CXCL10, and the NF- κ B pathway appears to be part of the *de novo* expression of CXCR4. Thus, as

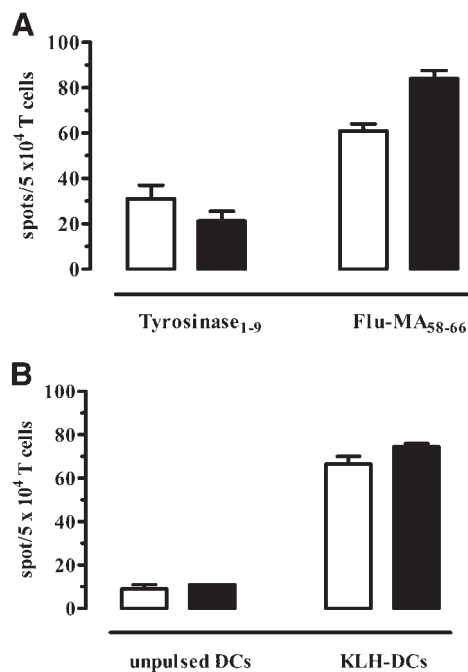


Figure 5. Comparative ability of TLF100/mDCs and mDCs to induce T-cell antigen-specific activation. *A*) T cells from an HLA-A2⁺ donor were cultured twice for 7 days at 10:1 ratio with autologous mDCs (white bars) or TLF100/mDCs (black bars) pulsed with Flu-MA₅₈₋₆₆. Antigen-specific, IFN- γ -producing cells were quantified by an ELISpot assay after 24 h culture in the presence of 5×10^4 responder T cells with 5×10^3 stimulator T2 cells (HLA-A2⁺) pulsed with Flu-MA₅₈₋₆₆ or tyrosinase₁₋₉ as unrelated control peptide. The spots were counted by computer-assisted image analysis (Transtec 1300 ELISpot Reader). The number of spots formed by T cells stimulated by TLF100/mDCs was significantly higher ($P < 0.05$) than those formed by T cells stimulated by mDCs. Data are representative of results from three independent experiments performed with distinct HLA-A2⁺ donors. *B*) CD45RA⁺ T cells were cultured for 7 days at 10:1 ratio with autologous mDCs (white bars) or TLF100/mDCs (black bars) previously pulsed with KLH. Antigen-specific, IFN- γ -producing cells were quantified by an ELISpot assay after 24 h culture in the presence of stimulator autologous unpulsed or KLH-pulsed DCs at 30:1 T:DC ratio. Data are representative of one of three independent experiments performed with different donors.

observed with inflammatory cytokines and LPS, the central p38 MAPK pathway (52–54) appears to require accessory pathways to modulate some specific aspects of the DC phenotype (53). Although LPS is known to induce MoDC maturation (55), it is unlikely that contaminating LPS played a significant role in TLF-induced MoDC maturation, since TLF's ability to elicit MoDC maturation was completely lost by boiling (data not shown).

Much as DCs play a crucial role in the initiation and regulation of both innate and acquired immune responses, LF may have a key role in the induction and control of both (56). Its increased concentrations in an inflammatory microenvironment following neutrophil degranulation may accelerate MoDC maturation prior to the production of proinflammatory cytokines and directly “condition” MoDCs for the acquisition of a

complete mature phenotype. β -Defensin, another important antimicrobial molecule of innate immunity, has also been shown to directly induce the full maturation of iDCs (14). Although monocytes form a large pool of potential myeloid DC precursors, agents that can induce their differentiation into DCs and their maturation could be of assistance in the quick induction of effective immune responses. Th1-polarized T-cell responses elicited by both β -defensin and TLF-matured MoDCs indicate that various molecules typically ascribed to innate immunity may have an important role in triggering antigen-specific adaptive responses. Th1 T-cell responses triggered by MoDCs matured in the presence of components of the innate immunity may result in an effective T-cell and antibody-mediated reaction. Moreover, these responses could participate and facilitate a bidirectional regulatory crosstalk between natural and acquired immunity, resulting in an enhancement of both adaptive and innate reaction mechanisms (57).

We have previously shown that oral TLF in wild-type BALB/c mice bearing implanted *neu+* mammary adenocarcinomas triggered significant tumor inhibition, initiating an immune activation in the gut and the gut-associated lymphoid tissue (5). This resulted in an IFN- γ -dependent increase in number of NKT cells and CD8⁺ T lymphocytes in small intestinal Peyer's patches and systemic CTL activity (6). TLF has also been successfully tested in Phase II human clinical trials in

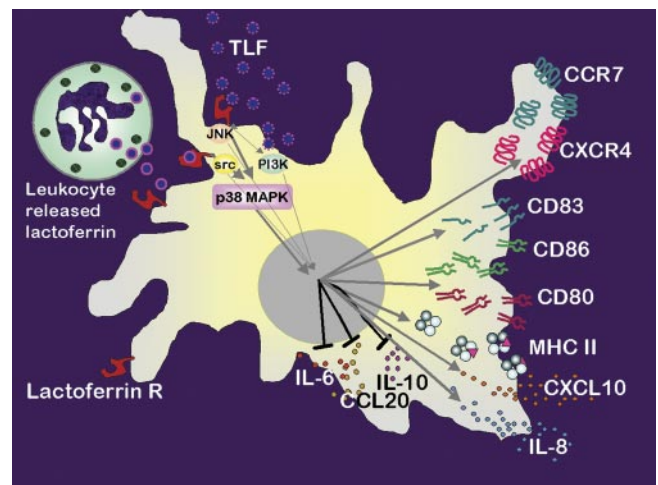


Figure 6. Model of the events activated by TLF—and hypothetically by neutrophil-released LF—in MoDCs. On MoDCs, TLF binds membrane receptors that act mostly through the activation of p38 MAPK and to a lesser extent through JNK, src, and PI3K. The result of these transduction pathways is maturation of MoDCs in a characteristic subset of fully mature DCs characterized by a hampered release of IL-6, IL-10, and CCL20. Their enhanced release of IL-8 appears to make these cells able to recruit and orchestrate neutrophil reactivity. The concurrence of CXCL10 secretion, high membrane expression of MHC class II glycoproteins (MHC II), CD80, CD86, and CD83 costimulatory molecules builds the perfect conditions for an efficient T-cell activation deflection of their response toward a Th1-type reaction. Lastly, expression of CCR7 and CXCR4 chemokine receptors enables them able to migrate to the local lymph node, where the antigen-specific response can be activated efficiently.

patients with nonsmall cell lung cancer (22) and renal cell cancer (23), which met their primary efficacy endpoints and will soon enter Phase III clinical trials in cancer. DCs are distributed along the gastrointestinal tract lying between epithelial cells and protruding through the mucosal barrier (58). Thus, even if oral TLF doses (administered at 100 mg/ml) are diluted by gastric and intestinal juices, mucosal DCs will likely be exposed to TLF levels well in excess of those that induce their maturation in our study. The postulated ability of TLF to induce the maturation of gastrointestinal iDCs and blood MoDCs is a new and important immunomodulatory function. In addition to its direct anticancer activity, oral TLF could play an important role when combined with vaccines to induce a more effective immune response. GM-CSF plus IL-4 have been used to generate DCs from monocytes, and proinflammatory cytokines plus PGE2 to induce their maturation *in vitro* (59). Their use as vaccine adjuvants, however, is limited. Identification of new agents able to trigger MoDC differentiation may provide a new way to trigger more effective Th1 response from vaccines. Thus, in addition to its development as an anticancer agent, evaluation of TLF as a vaccine adjuvant deserves consideration, given that TLF is administered orally (21, 60).

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