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LPS activation is required for migratory activity and antigen presentation by tolerogenic dendritic cells

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Abstract: Autoimmune pathologies are caused by a breakdown in self-tolerance. Tolerogenic dendritic cells (tolDC) are a promising immunotherapeutic tool for restoring self-tolerance in an antigen-specific manner. Studies about tolDC have focused largely on generating stable maturation-resistant DC, but few have fully addressed questions about the antigen-presenting and migratory capacities of these cells, prerequisites for successful immunotherapy. Here, we investigated whether human tolDC, generated with dexamethasone and the active form of vitamin D3, maintained their tolerogenic function upon activation with LPS (LPS-tolDC), while acquiring the ability to present exogenous autoantigen and to migrate in response to the CCR7 ligand CCL19. LPS activation led to important changes in the tolDC phenotype and function. LPS-tolDC, but not tolDC, expressed the chemokine receptor CCR7 and migrated in response to CCL19. Furthermore, LPS-tolDC were superior to tolDC in their ability to present type II collagen, a candidate autoantigen in rheumatoid arthritis. tolDC and LPS-tolDC had low stimulatory capacity for allogeneic, naïve T cells and skewed T cell polarization toward an anti-inflammatory phenotype, although LPS-tolDC induced significantly higher levels of IL-10 production by T cells. Our finding that LPS activation is essential for inducing migratory and antigen-presenting activity in tolDC is important for optimizing their therapeutic potential. *J. Leukoc. Biol.* **85: 243–250; 2009.**

Key Words: *tolerance · migration · CCR7 · naïve T cells · immunotherapy*

INTRODUCTION

The optimal treatment for autoimmunity and transplant rejection should be the reinstatement of tolerance in an antigen-specific manner, leaving immunogenic responses to pathogen-derived antigens and cancer immunity intact. One potential immunotherapeutic tool for achieving antigen-specific self-tolerance is tolerogenic dendritic cells (tolDC). DC are professional APC, which display inherent plasticity. Thus, depending on their maturation state, they can initiate and modulate immune responses to effector immunity (mature state) or tolerance (immature state) [1–4].

Numerous studies using rodent models of transplantation and disease have demonstrated that tolDC have great therapeutic potential: They can inhibit rejection and prolong survival of allografts [5–10], prevent graft-versus-host disease [11], and inhibit various autoimmune diseases [12–17]. We have shown recently that "alternatively activated" DC [DC modulated by dexamethasone (Dex), vitamin D3 (VitD3), and LPS] have regulatory effects on T cells and can migrate in a CCR7-dependent manner [18]. However, we did not address the role that LPS activation played in the induction of these functional characteristics.

Studies about DC vaccines for the induction of cancer immunity have highlighted the fact that DC maturation is essential for effective antigen-specific immunotherapy [19, 20]. Immature DC (immDC) capture and process antigen but do not have the abilities to migrate from peripheral tissues to draining lymph nodes and present the antigen to T cells: DC only acquire these abilities after maturation by inflammatory stimuli, such as LPS.

Although our aim is to induce tolerance and not immunity, it is necessary that tolDC have the abilities to migrate to lymph nodes, process and present the antigen to which tolerance is to be induced, and promote a tolerogenic effect on T cells. There are numerous strategies available to generate stable tolDC in vitro. These include modification of DC with immunological agents, such as IL-10 [21, 22] or vasoactive intestinal peptide [23, 24]; with drugs, such as Dex [25, 26], rapamycin [27, 28], or aspirin [29]; via gene modification, for example, transduction of DC with IL-10 [30, 31] or TGF-β [32, 33]; and through gene silencing using NF-κB decoy oligodeoxyribonucleotides [34, 35] or small

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interfering RNA [36, 37]. However, most of these strategies impair DC maturation, retaining them in an immature state.

Here, we hypothesize that LPS activation of tolDC is essential for the induction of characteristics necessary for optimal immunotherapeutic potential: the ability to migrate in a CCR7-dependent manner and to present exogenous antigens. We examined the chemokine receptor profiles of tolDC and LPS-activated tolDC and their abilities to migrate in response to the CCR7 ligand CCL19 and to present exogenous autoantigen in the context of MHC class II molecules (MHC II). Importantly, we also tested whether LPS activation of tolDC affected their regulatory activity on naïve T cells. An important finding for the development of tolDC therapies is that LPS activation is essential for the promotion of migratory activity and antigen presentation by tolDC and does not alter their tolerogenic function.

MATERIALS AND METHODS

Isolation of cells from peripheral blood

Human samples were obtained with informed consent after approval by the North Tyneside Research Ethics Committee (Newcastle upon Tyne, UK). PBMC were isolated from fresh blood or buffy coats by density gradient centrifugation on Lymphoprep (Axis-Shield Diagnostics, Dundee, UK). CD14$^+$ monocytes were isolated by positive magnetic selection using anti-CD14 magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). CD45RA$^+$/RO$^-$ naïve T cells were isolated by negative magnetic selection using RoboSep (StemCell, Vancouver, Canada). The purity of naive T cells was routinely >95%.

Generation of DC populations

Monocytes were cultured at 0.5×10^6 cells/ml in the presence of IL-4 and GM-CSF (50 ng/ml each, Immunotools, Friesoythe, Germany) for 6 days to generate immDC. Medium supplemented with cytokines was refreshed at day 3. tolDC were generated by addition of Dex (10^{-6} M, Sigma, Poole, UK) at day 3 and Dex plus the active form of VitD3, 1α,25-dihydroxyvitamin D3 (10^{-10} M, Leo-Pharma, Ballerup, Denmark), at day 6. On day 6, immDC and tolDC were left untreated for another 24 h or were treated with LPS (0.1 μg/ml, Sigma) for 24 h to generate mature DC (matDC) and LPS-tolDC, respectively. All DC populations were washed extensively before using them in functional assays. Cells were cultured in RPMI 1640, supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO$_2$.

DC cytokine production

DC were washed and stimulated at 1.5×10^5 DC/ml with CD40 ligand (CD40L)-transfected J558L mouse cells (kindly provided by Peter Lane, Birmingham University, UK) at a 1:1 ratio. After 24 h, supernatants were harvested and stored at –20°C. Cytokines (IL-10, TNF-α, and IL-12p70) were quantified using sandwich ELISA (BD PharMingen, San Jose, CA, USA).

Flow cytometry

The following antibodies were used for cell-surface phenotype analysis: CD3-PE (UCHT1), CD4-FITC (RPA-T4), CD40-PE (5C3), CD80-PE (L307.4), and CD86-FITC (2331; all from BD PharMingen); HLA-DR-FITC (B8.12.2; Immunotech, Marseille, France); CD83-FITC (HB15a; Immunotech); and CCR7-allophycocyanin (150503; R&D Systems, Abingdon, UK). Briefly, cells were centrifuged and resuspended in staining buffer (PBS supplemented with 3% FCS, 2 mM EDTA, and 0.01% sodium azide). Human IgG (Grifols, Los Angeles, CA, USA) was added with antibodies to prevent FcR binding. Cells were incubated on ice for 30 min, centrifuged, and resuspended in staining buffer. To assess IL-10 production, cells were stimulated with T cell CD3/CD28 expander beads (1:1, Dynal, Invitrogen, Paisley, UK) for 18 h, and IL-10 secretion was measured using an IL-10 secretion assay (Miltenyi Biotec). Cells

were also surface-stained for anti-human CD3 and anti-human CD4. Cell viability was assessed using Via-Probe (Becton Dickinson, San Diego, CA, USA). Data were collected on a Beckton Dickinson FACScan and analyzed using FlowJo (Treestar, Ashland, OR, USA).

Micro Fluidic Cards

RNA was extracted from DC using an RNeasy kit (Qiagen, Crawley, UK). RNA was reverse-transcribed to cDNA using random hexamers and SuperScript II RT (Invitrogen). cDNA samples were run on a custom Micro Fluidic Card (Applied Biosystems, Foster City, CA, USA) using an ABI Prism 7900HT system (Applied Biosystems). mRNA expression was normalized to that of human GAPDH by subtracting the comparative threshold (C$_T$) value of the gene of interest from the C$_T$ value of the human GAPDH gene (ΔC$_T$). Results are expressed as $2^{-\Delta CT}$.

DC migration

DC migration was assessed in a transwell system (pore size 8 μm, Corning Life Sciences, UK). DC (2×10^5; input DC) were added in the upper chamber, and medium, with or without CCL19 (250 ng/ml, R&D Systems), was added to the lower chamber. Migration of DC was assessed after a 2 h incubation period at 37°C by harvesting the DC in the lower chamber and counting using a hemocytometer. DC migration is expressed as the percentage of input DC that had migrated (migration efficiency). To assess whether migration was CCR7-dependent, DC were incubated with 5 μg/ml neutralizing anti-human CCR7 antibody (150503; R&D Systems) or 5 μg/ml matched isotype control (mouse IgG$_{2A}$: 20102; R&D Systems) for 30 min prior to their addition to the upper chamber of the transwell system.

Antigen-presentation assay

DC were generated from a HLA-DR1$^+$ donor and were loaded with 20 μg/ml human type II collagen (hCII) or 20 μg/ml nonglycosylated peptide pCII$^{259-273}$ (GenScript, Piscataway, NJ, USA) on Day 6 along with the addition of LPS and/or Dex and VitD3. After 24 h, loaded or unloaded DC were harvested and washed thoroughly. DC were incubated with the transgenic murine HLA-DR1-restricted T cell hybridoma, HCII-9.1, at the indicated ratio (see Fig. 4). The T cell hybridoma HCII-9.1 was developed against the nonglycosylated version of the immunodominant T cell epitope of hCII (CII$^{259-273}$; GIAGFKGEQGP-KGET), as described previously [38]. Upon recognition of hCII, the T cell hybridoma secretes IL-2. The IL-2 content of hybridoma supernatants was measured by bioassay as the proliferative response of the cytotoxic T cell line-2 (CTLL-2; American Type Culture Collection, Manassas, VA, USA; 3×10^4/well) during 20 h incubation. CTLL-2 proliferation was assessed by incorporation of ^{3}H-thymidine for the last 18 h of culture by scintillation counting (Microbeta TriLux, Perkin Elmer, Wellesley, MA, USA).

DC-T cell cocultures

DC (1×10^4) were cultured with 1×10^5 allogeneic, naïve T cells in 200 μl cultures. Supernatants were harvested after 6 days and assayed for IFN-γ by sandwich ELISA (BD PharMingen). Proliferation was determined during the last 18 h of culture by incorporation of ^{3}H-thymidine.

T cell restimulation

Allogeneic, naïve T cells were primed with DC (one DC:10 T cells) for 6 days and rested for 4 days with 0.1 ng/ml recombinant (r)IL-2. Primed T cells were washed and restimulated with matDC from the original DC donor (one DC:10 T cells) or T cell CD3/CD28 expander beads (1:1, Dynal, Invitrogen). No residual DC were observed in the T cell lines. Supernatants were harvested after 72 h and assayed for IL-10 and IFN-γ by sandwich ELISA (BD PharMingen). Cultures were pulsed with ^{3}H-thymidine for a further 8 h to determine proliferation. IL-10 secretion was assessed using an IL-10 secretion assay (Miltenyi Biotec) after 18 h incubation with CD3/CD28 expander beads.

Statistics

Two-tailed paired t-tests were performed using Prism 4.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

LPS activation induces a semi-mature phenotype in tolDC with retention of an anti-inflammatory cytokine profile

We first compared the cell-surface phenotype of tolDC and LPS-tolDC (**Fig. 1A**). tolDC displayed an immature phenotype with moderate levels of HLA-DR and low levels of CD40, the costimulatory molecules CD80/CD86, and the DC maturation marker CD83. Upon LPS activation, tolDC switched to a semi-mature phenotype with high levels of HLA-DR and CD80 and intermediate levels of CD40 and CD86. CD83 was expressed only at a low level by LPS-tolDC. These changes reflect those seen upon maturation of immDC (Fig. 1A).

tolDC and LPS-tolDC had similar anti-inflammatory cytokine profiles upon restimulation with CD40L, although LPS-tolDC produced lower levels of all cytokines measured (Fig. 1B). Compared with matDC, tolDC and LPS-tolDC produced high levels of IL-10 and low levels of IL-12p70. tolDC produced high levels of TNF-α but upon LPS activation levels, reduced to those seen in matDC.

### tolDC and LPS-tolDC have different chemokine receptor profiles

As DC function is closely associated with their migratory ability, we measured chemokine receptor expression in the tolDC populations. We found that a number of chemokine receptor genes were expressed differentially by tolDC and LPS-tolDC (**Fig. 2A**). tolDC expressed chemokine receptors characteristic of an immature phenotype: *CCR1*, *CCR2*, *CCR5*, *CXCR1*, and *CXCR2*. LPS activation of tolDC induced a down-regulation of these chemokine receptor genes and an up-regulation of *CCR7* and *CXCR4*, although the levels of expression were lower than in matDC.

To confirm that *CCR7* transcripts were translated, we used flow cytometry to assess CCR7 protein expression on the cell surface. CCR7 was up-regulated moderately following LPS activation of tolDC (Fig. 2B), although levels remained lower than on matDC. Together, these data show that tolDC expressed a chemokine receptor profile associated with immDC, whereas LPS-tolDC displayed a profile more consistent with matDC, although to a lower degree (semi-mature phenotype).

LPS activation of tolDC enables them to migrate in response to the CCR7 ligand CCL19

CCR7 directs DC migration to T cell areas in secondary lymph nodes in response to the chemokine CCL19. As tolDC and LPS-tolDC displayed differential expression of CCR7, we assessed the functional capacity of these DC populations to migrate in response to CCL19. As expected, tolDC, which expressed low levels of CCR7 (Fig. 2B), did not migrate in response to CCL19 (**Fig. 3A**). LPS-tolDC were able to migrate in response to CCL19 (Fig. 3A), consistent with their higher expression of CCR7 (Fig. 2B). Migration of LPS-tolDC and matDC was CCR7-dependent, as neutralization of CCR7 bioactivity resulted in a decrease in migration of DC toward CCL19 (Fig. 3B). These data suggest that activation of tolDC by LPS is vital for the induction of migratory capacity. LPS-tolDC migrated with 25% of the efficiency of matDC in response to CCL19 (Fig. 3A), most likely reflecting their lower levels of CCR7 expression (Fig. 2).
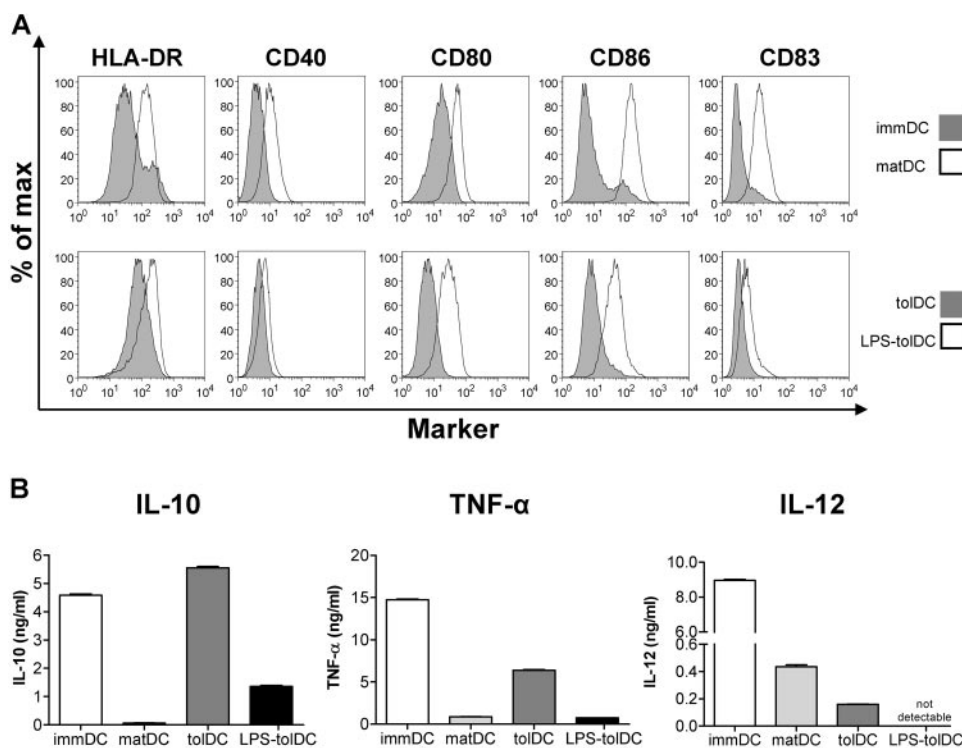
Fig. 1. LPS activation induces a semi-mature phenotype in tolDC with retention of an anti-inflammatory cytokine profile. (A) immDC, matDC, tolDC, and LPS-tolDC were stained with antibodies against cell-surface molecules, as indicated, and were assessed by flow cytometry. Debris and dead cells were excluded on the basis of forward-scatter and side-scatter, and viability was detected with Via-Probe. One representative experiment of 12 independent donors is shown. (B) DC populations were washed, cultured at 1.5×10^5 DC/ml, and activated with CD40L-expressing cells (1.5×10^5/ml) for 24 h. Cytokines in supernatants were quantified by sandwich ELISA. Error bars represent SEM of triplicates. One representative experiment of five independent donors is shown. Detection level of IL-12p70 ELISA was 30 pg/ml.
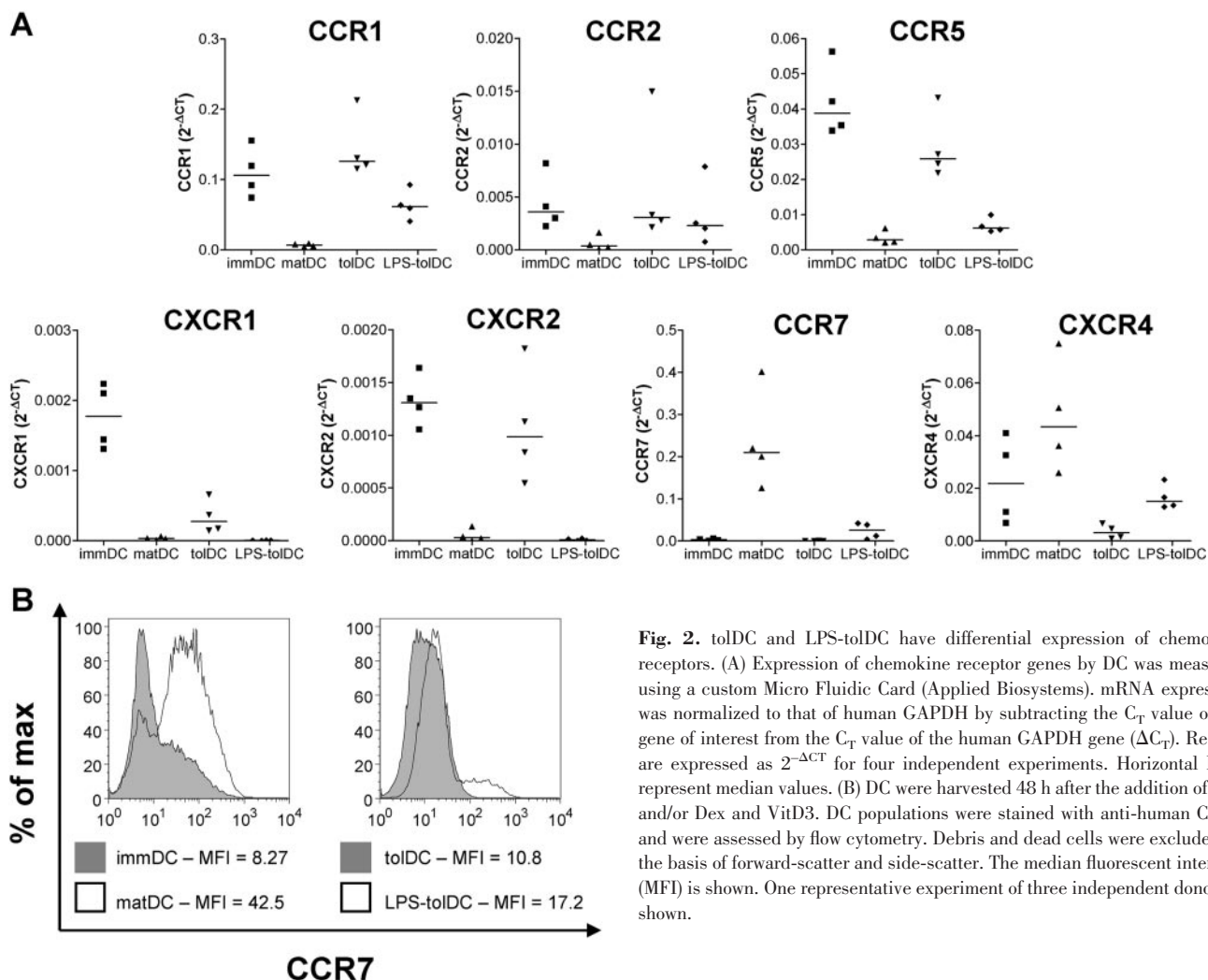
Fig. 2. tolDC and LPS-tolDC have differential expression of chemokine receptors. (A) Expression of chemokine receptor genes by DC was measured using a custom Micro Fluidic Card (Applied Biosystems). mRNA expression was normalized to that of human GAPDH by subtracting the C_T value of the gene of interest from the C_T value of the human GAPDH gene (ΔC_T). Results are expressed as $2^{-\Delta CT}$ for four independent experiments. Horizontal lines represent median values. (B) DC were harvested 48 h after the addition of LPS and/or Dex and VitD3. DC populations were stained with anti-human CCR7 and were assessed by flow cytometry. Debris and dead cells were excluded on the basis of forward-scatter and side-scatter. The median fluorescent intensity (MFI) is shown. One representative experiment of three independent donors is shown.
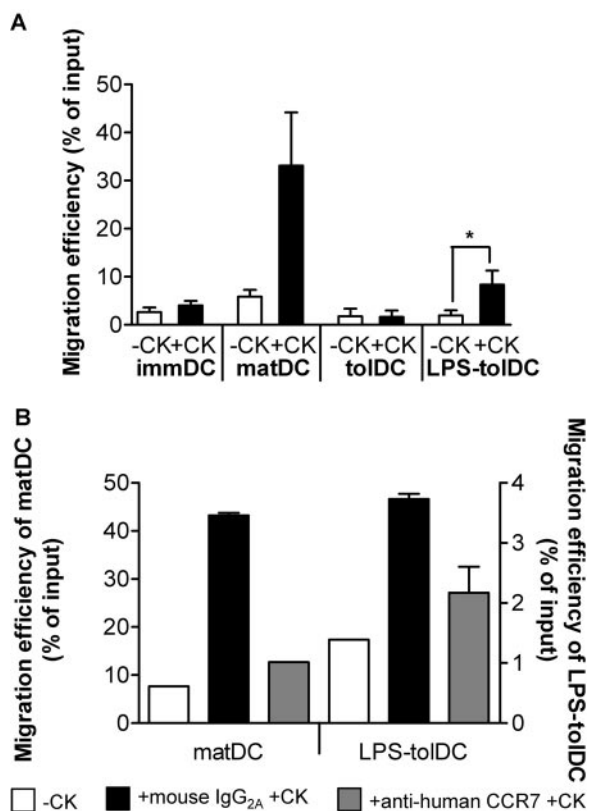
LPS activation of tolDC promotes antigen presentation

To restore self-tolerance efficiently and safely, in vivo tolDC therapy should be tailored toward a specific antigen(s) to avoid inducing nonspecific immunosuppression. To determine the antigen-presenting ability of the various DC populations, we used a hCII-specific murine T cell hybridoma derived from HLA-DR1 transgenic mice, HCII-9.1. In this system, antigen is presented independently of costimulation and cytokine activation, thus the ability to present exogenous antigen is the sole determinant of the response. Similar to immDC, tolDC presented hCII inefficiently (**Fig. 4A**). As it is not clear if this inefficiency to present antigen reflects reduced antigen processing or reduced antigen presentation by tolDC, we assessed presentation of the nonglycosylated peptide pCII$^{259-273}$, which is loaded directly onto HLA-DR1 without antigen processing. Similarly to hCII, tolDC presented pCII$^{259-273}$ inefficiently (Fig. 4B), suggesting a reduced ability to present antigen. LPS activation of tolDC, however, enhanced their ability to present hCII and pCII$^{259-273}$ to the same level as matDC (Fig. 4). These data suggest that LPS activation of tolDC is essential for efficient antigen presentation in a MHC II-restricted manner.

tolDC and LPS-tolDC have low stimulatory capacity and similar immunomodulatory effects on allogeneic, naïve T cells

In this study, we have shown that LPS activation of tolDC modulates their migratory and antigen-presenting capabilities. To test whether LPS activation of tolDC had an impact on their tolerogenic function, we assessed the T cell-stimulatory capacity of the tolDC populations. Compared with matDC, tolDC and LPS-tolDC had a reduced ability to induce proliferation and IFN-γ production in allogeneic, naïve T cells (**Fig. 5A**).

To further investigate the regulatory effects of these tolDC populations on naïve T cell differentiation, primed T cells were recovered from primary DC-T cell cocultures and were restimulated with matDC or CD3/CD28 beads. Upon restimulation, proliferative responses of naïve T cells primed by tolDC (T_{tol}) and LPS-tolDC ($T_{LPS-tol}$) were comparable with those of T cells primed by matDC (T_{mat}), indicating that tolDC subsets had not anergized the T (T_{tol}) cells (Fig. 5, B and C). However, T_{tol} and $T_{LPS-tol}$ were skewed toward lower IFN-γ production (Fig. 5B). $T_{LPS-tol}$ produced significantly higher levels of IL-10 than T_{mat}, and the higher levels produced by T_{tol} did not reach significance (Fig. 5B). To confirm that the enhanced IL-10 levels in T_{tol} and $T_{LPS-tol}$ cultures were T cell-derived, cytokines were

ligand CCL19 and enhanced ability to present the candidate autoantigen hCII. Importantly, activation of tolDC with LPS did not alter their regulatory action: tolDC and LPS-tolDC skewed naïve T cell differentiation toward an anti-inflammatory cytokine profile.

The maturation state of DC has been shown previously to be an important determinant for the efficacy of DC-based immunotherapy in cancer [19, 20]. DC maturation stimuli, such as LPS, enhance the antigen-presenting ability and migratory activity of DC, both of which are required for the induction of effective immunity [39–42]. Studies in the tolerance field have focused largely on DC that are resistant to maturation stimuli, such as DC that have been treated with the immunosuppressive drugs Dex and VitD3. Both of these drugs have inhibitory effects on DC maturation by down-regulating components of the NF-κB signaling pathway [43, 44]. Treatment of human monocyte-derived DC with Dex and/or VitD3 leads to reduced expression of costimulatory molecules and inhibition of proinflammatory cytokine production [18, 43, 45–50], making these tolDC ideal candidates for the induction of tolerance. However, the abilities to present autoantigens on MHC II and to migrate in a CCR7-dependent manner are also critical for successful therapy with tolDC. The question of how these capabilities can be enhanced in tolDC while retaining their tolerogenic capacity

Fig. 3. LPS activation of tolDC induces migratory activity in response to the CCR7 ligand CCL19. DC populations were left untreated (A) or were treated with 5 μg/ml anti-human CCR7 antibody or mouse IgG_{2A} isotype control for 30 min prior to assessment of migration. (B) Migration of DC was measured over a 2 h period in a transwell system. −CK, No chemokine; +CK, CCL19 (250 ng/ml) in the lower compartment. Migration efficiency was calculated as the percentage of the input DC that had migrated. Data are shown as the mean ± SEM of five independent experiments (A) or are representative of three independent experiments; mean ± SEM of duplicates are shown (B). * $P < 0.05$.
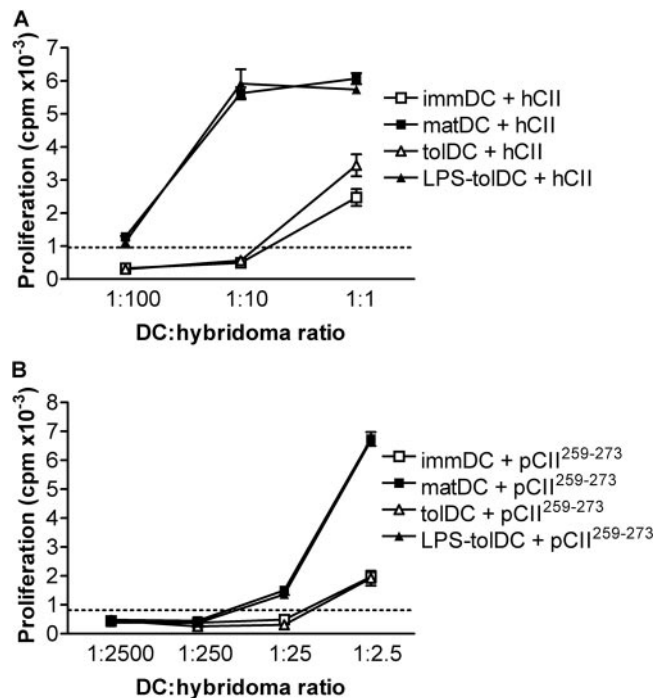
also measured after restimulation with CD3/CD28 beads in the absence of DC, by ELISA and flow cytometry (Fig. 5, C and D). Under these conditions, T_{tol} and $T_{LPS-tol}$ produced significantly higher amounts of IL-10 than T_{mat} (Fig. 5C). The $CD4^{high}$ population displayed in Figure 5D represents the blasting cell population, whereas the $CD4^{low}$ population corresponds to the nonblasting cell population (data not shown). It should be noted that $T_{LPS-tol}$ also secreted significantly higher amounts of IFN-γ than T_{tol} but that the IL-10/IFN-γ ratios of these T cell populations were similarly enhanced compared with T_{mat} (Fig. 5E).

DISCUSSION

This study demonstrates for the first time that LPS activation of human tolDC, generated with Dex and VitD3, is essential for efficient induction of CCR7-directed migration and antigen presentation. We found that LPS activation of tolDC induced a semi-mature phenotype, including up-regulated CCR7 expression, while leaving the anti-inflammatory cytokine profile unaffected. Compared with unactivated tolDC, LPS-tolDC displayed increased migratory activity in response to the CCR7

Fig. 4. LPS activation of tolDC promotes antigen presentation. DC, generated from a $HLA-DR1^+$ donor, were loaded with 20 μg/ml hCII (A) or 20 μg/ml nonglycosylated peptide $pCII^{259-273}$ (B) on day 6, along with the addition of LPS and/or Dex and VitD3, or were left unloaded. After 24 h, DC were washed and cocultured with the T cell hybridoma HCII-9.1, which is specific for $hCII^{259-273}$ (ratios are indicated in the figure). IL-2 production by the T cell hybridomas was measured by bioassay as the proliferative response of the IL-2-responsive cell line, CTLL-2. Proliferation was determined during the last 18 h of culture by incorporation of ^{3}H-thymidine. Results are representative of two independent experiments. The dotted line shows the maximum background proliferation induced by unloaded DC. Error bars represent SEM of triplicates.
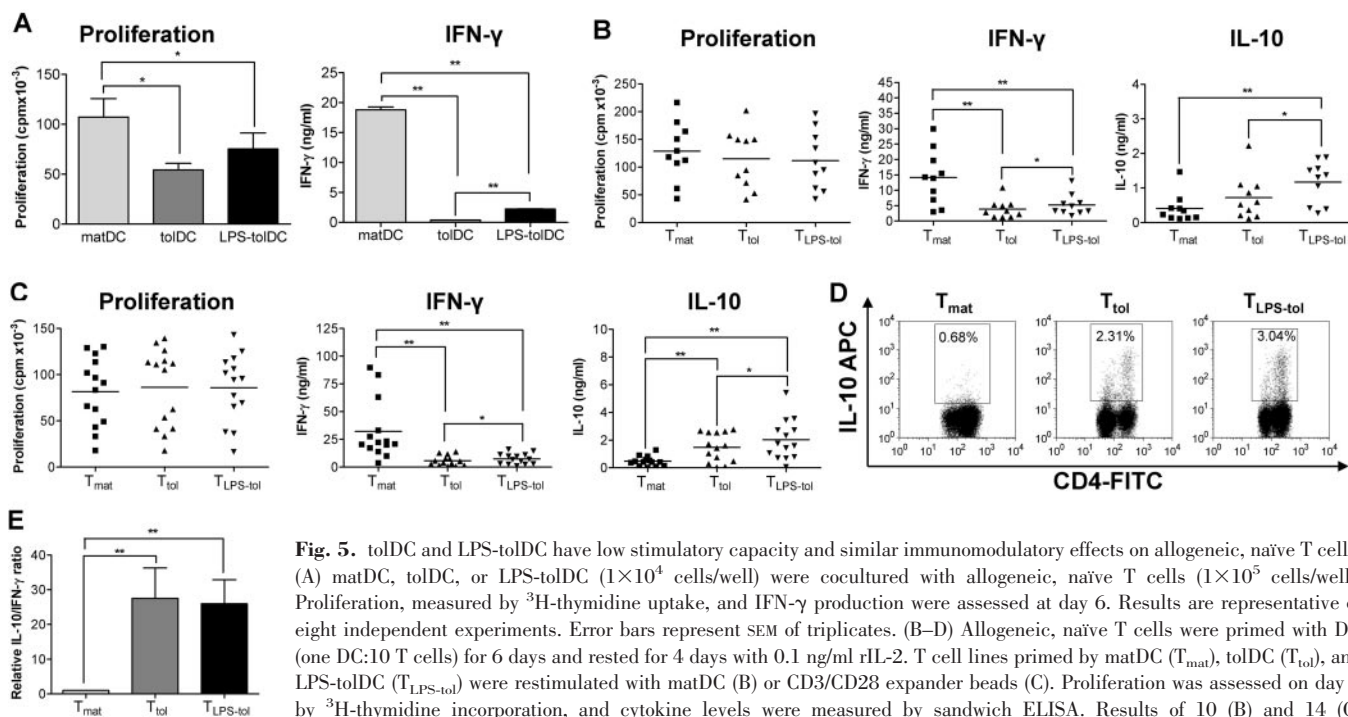
Fig. 5. tolDC and LPS-tolDC have low stimulatory capacity and similar immunomodulatory effects on allogeneic, naïve T cells. (A) matDC, tolDC, or LPS-tolDC (1×10^4 cells/well) were cocultured with allogeneic, naïve T cells (1×10^5 cells/well). Proliferation, measured by ^{3}H-thymidine uptake, and IFN-γ production were assessed at day 6. Results are representative of eight independent experiments. Error bars represent SEM of triplicates. (B–D) Allogeneic, naïve T cells were primed with DC (one DC:10 T cells) for 6 days and rested for 4 days with 0.1 ng/ml rIL-2. T cell lines primed by matDC (T_{mat}), tolDC (T_{tol}), and LPS-tolDC ($T_{LPS-tol}$) were restimulated with matDC (B) or CD3/CD28 expander beads (C). Proliferation was assessed on day 3 by ^{3}H-thymidine incorporation, and cytokine levels were measured by sandwich ELISA. Results of 10 (B) and 14 (C) independent experiments are shown. Horizontal lines represent mean values. (D) Primed T cells were restimulated with CD3/CD28 expander beads for 18 h, and IL-10 production was assessed using an IL-10 secretion assay. A CD3$^+$CD4$^+$ gate, identifying T cells, was used to exclude DC from subsequent analysis. The cytokine-positive gates shown were determined using unstimulated T_{mat}, T_{tol}, and $T_{LPS-tol}$ populations. Results are representative of two independent experiments. IL-10 APC, IL-10 allophycocyanin. (E) The mean production of IL-10 was divided by the mean production of IFN-γ ($n = 14$; data from Fig. 4D), and the ratio was normalized to the IL-10:IFN-γ ratio of T_{mat}, which was set to 1. *, $P < 0.05$; **, $P < 0.01$.

is therefore important and has not been formally addressed by previous studies.

The DC chemokine receptor profile determines, to a large extent, their migratory capacity and is associated with their maturation state [51]. Upon antigen uptake and maturation, DC down-regulate chemokine receptors involved in recruitment to and retention in peripheral tissue and up-regulate expression of CCR7 and CXCR4 [52]. CCR7 is particularly important, as it binds the chemokines CCL19 and CCL21, which direct the DC to draining lymph nodes, facilitating interactions with T cells and induction of immunity. Interestingly, it has been shown that CCR7 is also required for the induction of tolerance and it is thought that CCR7-dependent migration of tolDC plays a pivotal role in this respect [53, 54].

The chemokine receptor profile of our human monocyte-derived tolDC, generated with Dex and VitD3, is similar to those in an immature state, and this is reflected by their inability to migrate in response to CCL19. Using these tolDC for therapy would most likely be ineffective, as the tolDC would be unable to migrate from the site of injection to draining lymph nodes and would therefore be unable to tolerize naïve T cells. We have demonstrated that our tolDC acquire the ability to migrate in response to CCL19 after LPS activation. Our results are similar to a previous murine tolDC study showing that bone marrow-derived DC, genetically modified to express the soluble TNF-α receptor (TNFR) and stimulated with LPS, have the ability to migrate in vitro in response to CCL19, although with lower efficiency than mature DC [55]. It was not clear from that study whether their tolDC migration required LPS activation, however, as unactivated tolDC were not tested.

In contrast, our findings are dissimilar to another study demonstrating the inability of migration of a murine DC cell line treated with Dex and LPS [56]. The differences in DC source (human monocyte-derived vs. murine bone marrow-derived vs. murine DC cell line), treatment regimes (Dex/VitD3 vs. TNFR vs. Dex), and migration read-outs (in vitro vs. in vivo) make it difficult to compare these studies but emphasize the need to assess the migratory ability for every type of tolDC prior to clinical application.

Another requirement for successful tolDC therapy, particularly in autoimmune diseases, is the ability of tolDC to present autoantigen(s) on MHC II, but most studies on tolDC therapy have not addressed this issue. Although peptide loading could be considered for autoantigens, where the immunodominant epitope(s) and the patient's MHC haplotype are known, this is not always the case nor practical, and loading with whole protein antigens may be the preferred option. Here, we demonstrate that LPS activation of tolDC is required for efficient antigen presentation of a candidate autoantigen to levels comparable with matDC. In apparent contrast to our data, two previous studies using human monocyte-derived DC treated with Dex or VitD3 showed that these tolDC displayed low efficiency in presenting tetanus toxoid (TT) to autologous T cell lines, even after treatment with a DC maturation stimulus [45, 48]. However, whether the low stimulation of TT-specific T cells in these studies was due to a low antigen presentation capacity per se or to other features of tolDC (e.g., lack or low expression of costimulatory molecules, low production of proinflammatory cytokines, or expression of inhibitory molecules) cannot be distinguished. Our antigen-presentation model using

T cell hybridomas generated from human HLA-DR1 transgenic mice that recognize hCII in a DR1-restricted manner [38, 57] is independent of costimulation and DC cytokine production and is therefore a useful tool to assess the antigen-presenting capacity of tolDC.

The importance of LPS activation for effective tolDC therapy in vivo has been demonstrated in a murine model of transplantation [58]. tolDC generated with Dex and LPS but not those generated with Dex alone prolonged the survival of a fully MHC-mismatched heart allograft. The reason why LPS activation of Dex-treated tolDC was required for tolerance induction in vivo was unclear, but our data provide a possible explanation. This would be consistent with another in vivo study, which demonstrated that CCR7 expression by DC transduced with viral IL-10 was essential for the prolongation of cardiac allograft survival in a murine model [53].

Importantly, we have shown that LPS activation of tolDC improves their ability to present exogenous antigen without diminishing their tolerogenic function. tolDC and LPS-tolDC had a reduced ability to stimulate allogeneic, naïve T cells as compared with mature DC. Although the T cells were not anergized, their responses were skewed toward higher IL-10 and lower IFN-γ production upon restimulation. Immune deviation of T cells from a harmful proinflammatory to a protective, anti-inflammatory cytokine profile may be beneficial for the treatment of a variety of diseases, including various autoimmune disorders [59, 60] and allograft rejection [61].

In conclusion, LPS activation of tolDC is required for the acquisition of migratory activity and enhancement of exogenous antigen presentation but does not affect their tolerogenic function. Migratory capacity and antigen-presenting ability will be essential for the induction of safe, effective antigen-specific T cell tolerization in vivo. These findings are important for optimizing the therapeutic potential of tolDC for the use in diseases such as autoimmunity.

ACKNOWLEDGMENTS

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