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Involvement of LEK1 in Dendritic Cell Regulation of T Cell Immunity against *Chlamydia*

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We investigated the hypothesis that the enhanced Ag-presenting function of IL-10-deficient dendritic cells (DCs) is related to specific immunoregulatory cytoskeletal molecules expressed when exposed to Ags. We analyzed the role of a prominent cytoskeletal protein, LEK1, in the immunoregulation of DC functions; specifically cytokine secretion, costimulatory molecule expression, and T cell activation against *Chlamydia*. Targeted knockdown of LEK1 expression using specific antisense oligonucleotides resulted in the rapid maturation of *Chlamydia*-exposed DCs as measured by FACS analysis of key activation markers (i.e., CD14, CD40, CD54, CD80, CD86, CD197, CD205, and MHC class II). The secretion of mostly Th1 cytokines and chemokines (IL-1α, IL-9, IL-12, MIP-1α, and GM-CSF but not IL-4 and IL-10) was also enhanced by blocking of LEK1. The function of LEK1 in DC regulation involves cytoskeletal changes, since the dynamics of expression of vimentin and actin, key proteins of the cellular cytoskeleton, were altered after exposure of LEK1 knockdown DCs to *Chlamydia*. Furthermore, targeted inhibition of LEK1 expression resulted in the enhancement of the immunostimulatory capacity of DCs for T cell activation against *Chlamydia*. Thus, LEK1 knockdown DCs activated immune T cells at least 10-fold over untreated DCs. These results suggest that the effect of IL-10 deficiency is mediated through LEK1-related events that lead to rapid maturation of DCs and acquisition of the capacity to activate an elevated T cell response. Targeted modulation of LEK1 expression provides a novel strategy for augmenting the immunostimulatory function of DCs for inducing an effective immunity against pathogens. *The Journal of Immunology*, 2008, 181: 4037–4042.

Efficacious vaccines are needed against the widespread diseases of several intracellular microbial pathogens, including the ocuogenital diseases caused by *Chlamydia trachomatis* whose control requires a prominent T cell immunity. A potentially efficacious chlamydial vaccine should induce an adequate T cell response and the accessory Abs (1–3). Dendritic cells (DCs) are primary APCs for inducing high levels of T cell immunity (4, 5) and have been established to play a major role in controlling chlamydial infection in both animals and humans (3, 6). DCs reside in an immature state in most nonlymphoid sentinel tissue sites and, upon encounter of an Ag, they undergo maturation which promotes their migration to the draining secondary lymphoid organs, where they initiate immune responses (7). During maturation, DCs strongly up-regulate MHC Ags, and costimulatory molecules such as CD40, CD80, and CD86, which are crucial for effective T cell activation. We previously reported that IL-10 deficiency rendered DCs to become highly potent APCs for activating a high level of predominantly Th1 response against *Chlamydia* in vitro and in vivo (8). It was hypothesized that IL-10-deficient DCs expressed specific immunostimulatory molecules that promote the ability to rapidly activate a high level of specific T cell response.

Subsequent proteomic analysis to identify immunoregulatory molecules associated with the potency of IL-10 deficient and DC established that IL-10 deficiency caused an early maturation and activation of DCs after exposure to *Chlamydia*. DC activation was marked by expression of high levels of activation markers and consequently an enhanced ability to process and present Ags for a rapid and robust T cell activation (8). Furthermore, a group of cytoskeletal proteins, namely, actin, vimentin, and LEK1 were identified to be differentially expressed in IL-10-deficient DCs that were exposed to *Chlamydia* but their role in Ag handling and T cell activation was unclear. The elucidation of the direct or indirect role of such molecules in Ag handling by DCs can lead to strategies to modulate their expression in vaccine delivery against *Chlamydia*. There is increasing evidence that the cytoskeleton is involved in DC uptake and handling of Ags, migration to lymphoid tissues and maturation, and leading to effective presentation for T cell activation. The functional changes occurring during DC maturation are associated with dynamic and specialized alterations in the configuration of the actin cytoskeleton, which involve Rho GTPases, Rac, Cdc42, Rho, and the Wiskott-Aldrich syndrome protein (7, 9, 10). Thus, appropriate and timed cytoskeletal reorganization may control the effector functions of DCs, including Ag handling and T cell activation (7, 11, 12). In this study, we analyze in greater detail one of the cytoskeletal molecules, LEK1, identified by proteomics to be differentially expressed by IL-10-deficient DCs, as compared with wild-type (WT) DCs. LEK1 is a member of the LEK family of proteins and known to play a role in cell  

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3 Abbreviations used in this paper: DC, dendritic cell; WT, wild type; EB, elementary body; MoPN, mouse pneumonitis; 2-DE, two-dimensional gel electrophoresis; MHC II, MHC class II; MOI, multiplicity of infection.

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demonstrated differentiation and microtubule function relating to cytoskeletal organization and cell shape (13, 14). With an estimated molecular mass of 200 kDa, LEK1 undergoes posttranslational cleavage that produces two peptides. The C-terminal fragment (nuLEK1) localizes to the nucleus and function in the regulation of cell division and differentiation; however, the N-terminal peptide (cytLEK1) remains in the cytoplasm and plays a role in the regulation of the cytoskeleton (15). The cytLEK1 consists of a spectrin repeat region and numerous leucine zippers that regulate pocket protein activity during developmental processes (13, 15). Since a spectrin repeat serves as a cytoskeletal and signal transduction docking region and leucine zippers participate in protein-protein interactions, we propose that LEK1 is involved in the cytoskeletal events that are related to Ag acquisition, APC maturation, processing, and presentation. The results from our study provide evidence for the involvement of LEK1 in regulating DC maturation and APC function for Th1 activation.

Materials and Methods

Animals
Female IL-10+/− (IL-10 knockout, IL-10K0) and the control IL-10+/+ mice on a C57BL/6 background (The Jackson Laboratory) were fed with food and water and libitum and maintained in laminar flow racks under pathogen-free conditions of 12 h of light and 12 h of darkness. The animal use protocols described in this proposal have been approved by the Institutional Animal Care and Use Committee of Morehouse School of Medicine and Centers for Disease Control and Prevention.

DC isolation and culture
DCs were isolated from the bone marrows of WT and IL-10K0 mice by the standard method and differentiated by in vitro culture with IL-4 and GM-CSF as previously described (8). DCs were characterized as loosely adherent monocellular cells and determined by FACS analysis to express high levels of MHC class II, CD54 (ICAM-1), and CD11c. After 5 days in culture, DCs were washed and used in the experiments as described.

Proteomic analysis
DCs from WT and IL-10K0 mice were pulsed with chlamydial elementary bodies (EBs) derived from the C. trachomatis agent of mouse pneumonitis (MoPn) for 2 or 8 h and lysed with a rehydration buffer containing 8 M urea, 10 mM DTT, 2 M CHAPS, 0.2% bio-Lyte (Bio-Rad), FOCUS-Pro tease Arrest and FOCUS-Pro tease Nuclease (EMD Chemicals) were added to a final concentration of 10 μg/ml. The amount of proteins was determined by using the Bio-Rad DC protein assay kit (Bio-Rad). Two hundred micrograms of each sample was separated by two-dimensional gel electrophoresis (2-DE). Selected spots corresponding to specific proteins on the SDS-PAGE were analyzed by MALDI-TOF-TOF as previously determined by using the Bio-Rad RC DC protein assay kit (Bio-Rad). Two-dimensional gel electrophoresis was performed with a Leica TCS SP5 confocal microscope. Triple-color images were acquired using a sequential acquisition mode to avoid cross-excitation, according to the supplier’s protocol, and photograph prints were processed in Adobe Photoshop.

Results

Expression pattern of LEK1 in Chlamydia-pulsed DCs
To identify and characterize specific proteins that may play a role in the enhanced T cell-activating capacity of IL-10- deficient DCs, combined 2-DE and MALD-TOF proteomics were used to identify differentially expressed proteins when WT and IL-10K0 DCs were cocultured with C. trachomatis. Supernatants were collected and assayed for IL-4, IL-10, IL-17, and IFN-γ expression by multiplex array according to the supplier’s instructions. The concentration of the cytokine in each sample was obtained by extrapolation from a standard calibration curve generated simultaneously. Data were calculated as the mean values (±SD) of triplicate cultures for each experiment. The results were derived from three independent experiments.

Effect of targeted inhibition of LEK1 expression on DC maturation and expression of cytokines
To assess the immunologic function of LEK-1 in DC activation when exposed to chlamydial Ags, we analyzed the effect of targeted inhibition of its expression on DC maturation and expression of cytokines. Fig. 2 revealed that blocking of LEK1 expression with antisense oligonucleotides enhances the production of IL-12p40, RANTES, MIP-1 α, MIP-1 β, and MCP-1 but inhibits
IL-10 expression by *Chlamydia*-pulsed WT DCs. The control oligomer with a scrambled sequence, as described in Materials and Methods, did not show any detectable biological activity.

Because these conditions facilitate rapid DC maturation and enhancement of T cell activation, we also measured the expression of key activation markers on antisense-treated WT DCs that were exposed to *Chlamydia*. Fig. 3 shows the results from FACS analysis of CD14, CD40, CD54, CD80, CD86, CD197, CD205, and MHC II expression by anti-LEK1-treated WT DCs that were exposed to MoPn, indicating an increase in the expression of these molecules after LEK1 knockdown. This result indicated that LEK1 knockdown results in the rapid maturation and activation of DCs.

**FIGURE 1.** Pattern of expression of LEK1 in *Chlamydia*-pulsed, WT, and IL-10KO DCs. MoPn-pulsed DCs (multiplicity of infection (MOI), 1:5) were analyzed with 2-DE and MALD-TOF proteomics to identify differentially expressed proteins as described in Materials and Methods. A and D, Control WT DCs and IL-10 KO DCs, respectively, that were not pulsed with MoPn. B and E, WT DCs and IL-10 KO DCs, respectively, that were pulsed with MoPn for 2 h. C and F, WT DCs and IL-10 KO DCs, respectively, that were pulsed with MoPn for 8 h. The differentially expressed proteins that were selected for MALDI-TOF identification are shown in numbers. Spots 1, 2, and 3 were identified as LEK1, vimentin, and actin, respectively.

**FIGURE 2.** Effect of targeted inhibition of LEK1 expression on DC expression of cytokines. WT DC and LEK1 knockdown DCs were pulsed with MoPn (MOI, 1:5) for 1 h and the supernatants were analyzed for cytokine expression (indicated in the figure) using Lumienx technology as described in Materials and Methods. Results represent the means of three independent experiments. The control oligomer with a scrambled sequence, as described in Materials and Methods, did not show any detectable biological activity.
when exposed to *Chlamydia*, suggesting that LEK1 is directly or indirectly involved in regulating DC function.

To investigate whether the function of LEK1 in DC regulation involves cytoskeletal changes, we analyzed the dynamics of expression of vimentin and actin, key proteins of the cellular cytoskeleton. Western blotting analysis of the kinetics of expression of actin and vimentin after exposure of LEK1 knockdown DCs to *Chlamydia* revealed that both proteins were significantly decreased, as compared with untreated DCs (Fig. 4). The results indicated that cellular cytoskeletal reorganization involving tubulin (actin) and intermediate filaments (vimentin) may partially be regulated by LEK1 action.

**Evidence for LEK1 regulation of Ag-processing events in DCs**

The late endosome (also called the MHC class II compartment, MIIC) is the composite lysosomal Golgi intracellular vesicle for peptide loading, where processed Ags interact with MHC II molecules from the trans-Golgi, and represents a critical stage in APC function (17, 18). We hypothesized that the cytoskeletal functions regulated by LEK1 are related to late endosome formation and function, such that the presence of LEK1 would affect the rate of formation and accumulation of the vesicles. To test this hypothesis, we compared the rate of late endosome formation and accumulation in WT and LEK1 knockdown DCs that were pulsed with
CSFE-labeled chlamydial EBs. Fig. 5 revealed that LEK1 knockdown results in a high accumulation of LysoTracker Yellow-HCK-123-stained late endosomes. In addition, unlike WT DCs, CSFE-labeled EBs could not be colocalized with the late endosomes in LEK1 knockdown, suggesting a rapid disappearance of the EBs in the absence of LEK1. The data may indicate that the cytoskeletal events regulated by LEK1 are related to the rate of Ag processing and late endosome formation.

**Effect of targeted inhibition of LEK1 expression in DCs on T cell activation against Chlamydia**

We hypothesized that the rapid maturation and activation of *Chlamydia*-pulsed DCs due to LEK1 knockdown will result in an enhancement of the immunostimulatory capacity of the DCs for T cell activation against *Chlamydia*. To test this hypothesis, antisense-treated and MoPn-pulsed DCs were used to restimulate T cells from MoPn-infected mice in a recall culture system in vitro. The results in Fig. 6 revealed that antisense LEK1 treatment enhanced the immunostimulatory action of DCs, such that they activated immune T cells at least 10-fold over untreated DCs. The increase in IFN-γ and IL-17 in these cultures would suggest that the protective CD4, Th1, and Th17 cells are activated against *Chlamydia* at the same time. Interestingly, the expression of the Th1-related chemokines and cytokines such as MIP-1α, GM-CSF, IL-9, and IL-12 was boosted in these cultures as well (data not shown). The results corroborate the data shown in Fig. 2 that the suppression of LEK1-related events in the absence of IL-10 leads to rapid maturation of DCs and acquisition of the capacity to activate an elevated T cell response.

**Discussion**

*Chlamydia*-pulsed IL-10-deficient DCs constitute an effective cellular vaccine capable of inducing a protective immune response against chlamydial genital infection in mice. The likely mechanism by which IL-10 deficiency enhances the APC function of DCs remains unclear, although the process appears to be related to cellular maturation and increased in the immunostimulatory ability of the DCs. We have proposed that IL-10-deficient DCs acquire increased immunostimulatory properties due at least partly to certain immunoregulatory molecules that affect Ag uptake, processing, and presentation to T cells (8). The identification and characterization of the immunostimulatory molecules that regulate the ability of IL-10-deficient DCs to activate a high level of protective immunity will facilitate vaccine design against certain intracellular pathogens and tumors.

Using a proteomic approach to analyze WT and IL-10KO DCs to identify differentially expressed molecules, we have identified LEK1, a prominent cytoskeletal protein whose expression is markedly suppressed when DCs acquired highly effective immunostimulatory function due to IL-10 deficiency. LEK1 is a member of the LEK family of proteins and known to play a role in cell differentiation and microtubule function (13, 14). LEK1 consists of a spectrin repeat region and numerous leucine zippers. A spectrin repeat serves as a cytoskeletal and signal transduction docking region and leucine zippers participate in protein-protein interactions. The disruption of LEK1 resulted in alteration of microtubule organization and cellular shape (13, 14). This would suggest that LEK1 is involved in cytoskeletal reorganization during the maturation of DCs, which may affect Ag processing and presentation. In
fact, it has been suggested that appropriate and timely actin rearrangement is vital for DC effector function, including TLR-mediated activation of Ag uptake (11) and DC-T cell interaction during Ag presentation for T cell activation (12). Our studies revealed that IL-10 deficiency resulted in LEK1 suppression and acquisition of an enhanced T cell activation by DCs. Similarly, targeted LEK1 knockdown in DCs also resulted in the suppression of IL-10 secretion and the increased immunostimulatory capacity of the DCs. Thus, IL-10 may suppress DC maturation and APC function for T cell activation through LEK1 action in the cytoskeletal network. This conclusion is supported by results showing that LEK1 is involved in the regulation of expression of key proteins of the cellular cytoskeleton of DCs, vimentin and actin that are involved in various cellular functions (18) (Fig. 4). Other corroborating reports have suggested that DC function is associated with dynamic and specialized alterations in the configuration of the actin cytoskeleton (7) and these processes involve the GTPases Cdc42, Rac, and Rho (9). In addition, the high rate of late endosome formation in LEK1 knockdown DCs would suggest that the absence of LEK1 leads to an enhanced Ag processing and presentation function. This would support the hypothesis that LEK1 regulates Ag-processing events in DCs and indicated that the cytoskeletal events regulated by LEK1 favor late endosome formation in the absence of LEK1. These events may include the vesicular fusion relating to the formation of late endosomes, as well as the intracellular trafficking of MHC molecules to the peptide-loading sites. Facilitation of these processes will likely culminate in an enhanced DC function associated with Ag processing and presentation for T activation.

As a direct confirmation of the role of LEK1 in the regulation of DC function for T cell activation, we have provided compelling results that Chlamydia-pulsed, LEK1 knockdown DCs activated T cells by ~10-fold compared with control DCs (Fig. 6). Ongoing experiments are assessing the effect of targeted inhibition of LEK1 expression in DCs on the induction of protective immunity against Chlamydia in vivo. Our prediction is that Chlamydia-pulsed, LEK1 knockdown DCs will also induce an enhanced T cell activation in vivo, as compared with WT DCs, which would result in a greater level of protective immunity against Chlamydia in vivo. Mechanistically, LEK1 appears to negatively regulate cytoskeletal reorganization that affects DC maturation, signaling events that promote cytokine and costimulatory molecule expression, efficient processing, and presentation of Ag for T cell activation. This effect appears to involve the regulation of expression of actin and vimentin which are components of the microtubule and microfilament network of the cell, as well as late endosome formation for peptide loading on MHC molecules. Thus, molecules that regulate the cytoskeleton are important modulators of the APC functions of DCs. Biochemically, the presence of IL-10 or LEK1 either delays or limits the cytoskeletal reorganization processes that culminate in efficient Ag handling and T cell activation by DCs. The known involvement of LEK1 in cell division and vesicular fusion associated with the endocytic pathway (14) would suggest that its presence causes cytoskeletal reorganization that promotes cell division and maintenance of DCs in the immature stage. Thus, the absence of LEK1 will lead to cytoskeletal events or reorganization that favor DC maturation as exhibited by IL-10-deficient or targeted LEK1 antisense-treated DCs. We propose that the action of LEK1 is exerted at multiple levels that include postinternalization events, such as endocytic maturation, Ag processing, and presentation since certain defects in cytoskeletal functions have been associated with suppression of these events (7, 10, 19, 20). Besides, the pharmacological manipulation of LEK1 expression in vivo may lead to an enhanced immune response to potential vaccines against Chlamydia, other intracellular microbial pathogens, and tumors that are controlled by robust T cell immunity.

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Disclosures
The authors have no financial conflict of interest.

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7. Burns, S., and A. Thrasher. 2004. Dendritic cell function for T cell activation, we have provided compelling evidence that LEK1 antisense-treated DCs. We propose that the action of LEK1 in the cytoskeletal network. This conclusion is supported by results showing that LEK1 is involved in the regulation of expression of key proteins of the cellular cytoskeleton of DCs, vimentin and actin that are involved in various cellular functions (18) (Fig. 4). Other corroborating reports have suggested that DC function is associated with dynamic and specialized alterations in the configuration of the actin cytoskeleton (7) and these processes involve the GTPases Cdc42, Rac, and Rho (9). In addition, the high rate of late endosome formation in LEK1 knockdown DCs would suggest that the absence of LEK1 leads to an enhanced Ag processing and presentation function. This would support the hypothesis that LEK1 regulates Ag-processing events in DCs and indicated that the cytoskeletal events regulated by LEK1 favor late endosome formation in the absence of LEK1. These events may include the vesicular fusion relating to the formation of late endosomes, as well as the intracellular trafficking of MHC molecules to the peptide-loading sites. Facilitation of these processes will likely culminate in an enhanced DC function associated with Ag processing and presentation for T activation.

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