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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 ocukogenital 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 potential APCs for inducing high levels of T cell immunity (4, 5) and have been well-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, 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 timely 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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§ 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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on SDS-PAGE were analyzed by MALDI-TOF-TOF as previously described. Hundred micrograms of each sample was separated by two-dimensional gel electrophoresis and protein activity during developmental processes has been determined by using the Bio-Rad DC protein assay kit (Bio-Rad). Two spectrin repeat regions and numerous leucine zippers that regulate pocket protein and transcriptional activity that plays a role in the regulation of the cytoskeleton (15). The cytoskeleton consists of a spectrin repeat region and regions that 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-10KO) and the control IL-10 knockout mice on a C57BL/6 background (The Jackson Laboratory) were fed with food and water ad libitum and maintained in laminar flow rooms under pathogen-free conditions of 12 h of light and 12 h of darkness. The animals used 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-10KO 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 mononuclear 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-10KO 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, 100 mM CHAPS, 0.2 M bio-Lyte (Bio-Rad), FOCUS-Protease Arrest and FOCUS-Protease Nuclease (EMD Chemicals) were added to a final concentration of 10 μg/ml. The amount of proteins was determined using the Bio-Rad DC protein assay kit (Bio-Rad). Two hundred microliters 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 described (8).

Morpholino-based antisense oligomer

The antisense morpholino oligomers of LEK1 were supplied by Gene Tools. The sequence of the antisense oligomer was 5'-AGCTCCTCACA GAACCTGGCTCCG-3' (LEK1-ASMor-50), which is an effective inhibitor of LEK1 expression (13). The standard control oligomer (Gene Tools) had an inept sequence 5'-CCTCTCACCAGTGATACATTTATA-3' with no cellular gene target and no detectable biological activity. The oligomers were prepared according to the supplier’s protocol and administered to cultured cells using the suggested Endo-Poter delivery system. The final concentration of LEK1 morpholino oligomers was 10 μM/10⁶ cells and the treatment did not cause any phenotypic changes on the cells as verified by microscopy.

FACS analysis

WT DCs were cultured in 24-well plates and treated with either standard control oligonucleotide or LEK1-ASMor-50 for 48 h. Nontreated or oligo-treated cells were then pulsed with UV-inactivated MoPn EBs for 1 h and the supernatants were collected for cytokines and chemokines analysis. Cells were harvested and washed with FACS buffer at 4°C and stained with FITC- or PE-conjugated Abs against CD11c, CD14, CD40, CD54, CD80, CD86, CD197, CD205, and MHC II. The effect of LEK1 knockdown on the expression of these molecules on chlamydial-pulsed DC was measured on a FACSscan flow cytometer (BD Biosciences) using CellQuest software (BD Biosciences). Cytokines and chemokines were measured by the Lumex assay (Bio-Rad).

Western blotting

WT and LEK1 knockdown DCs were harvested, adjusted to a concentration of 1 × 10⁶/ml and pulsed with MoPn EBs that were stained with CFSE for 60 min at 37°C. The cells were washed three times and fixed for 15 min in 1% paraformaldehyde at room temperature. For cell surface staining, DCs were treated with a PE-conjugated goat anti-mouse CD11c Ab (BD Biosciences), washed, and stained with Lysotracker Yellow-HCK-123 ( Molecular Probes) for 30 min on ice for late endosomes (16). Confocal microscopy 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 photographed prints were processed in Adobe Photoshop.

T cell stimulation in vitro

Spleen cells from chlamydial-infected WT mice were enriched for T cells by the nylon wool adherence method and analyzed for CD3+ cells by FACS. One × 10⁶ WT DCs or LEK1 knockdown DCs were cocultured with 2 × 10⁵ purified T cells in the presence or absence of chlamydial Ag in 96-well tissue culture plates for 5 days as previously described (8). The 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.

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-10KO DCs were exposed to Chlamydia in culture. Fig. 1 shows that LEK1 is one of the differentially expressed proteins identified on a 2-D gel, with an estimated molecular mass of 200 kDa. LEK1 was down-regulated in Chlamydia-pulsed IL-10KO DCs at 2 and 8 h of exposure. Since DCs undergo maturation by expression of activation markers during these time periods following exposure to chlamydiae (8), the results would suggest that the regulation of LEK1 is a maturation-dependent process. Thus, as DC matured, the expression of LEK1 decreases. It was interesting that certain other cytoskeletal proteins, specifically vimentin and actin, were also similarly and differentially expressed as LEK1. The prominence of LEK1 among the differentially expressed proteins led us to further characterize its role in DC functions in immune regulation.

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-1α, MIP-1β, 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 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 1. Pattern of expression of LEK1 in Chlamydia-pulsed, WT, and IL-10 KO 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 MALD-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 Luminex 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...
Role of LEK1 in late endosome activity in chlamydial-pulsed DCs. WT and LEK1 knockdown DCs were pulsed with CSFE-labeled chlamydial EBs and treated with Lysotracker Yellow-HCK-123 (Molecular Probes) to stain for late endosomes as described in Materials and Methods (16). Confocal microscopy was performed and triple-color images (red = CD11c⁺ DCs; blue = CSFE-labeled chlamydial EBs; yellow = late endosomes; and merging of colors colocalizes endosomes and EBs in DCs) were acquired according to the supplier’s protocol. Merged images (green) show a high level of late endosomes and absence of EBs in LEK1 knockdown DCs; however, WT DCs showed numerous EBs and a limited level of late endosomal activity.

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

We hypothesized that the rapid maturation and activation of chlamydial-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 cytokotosome 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 cytokotosome (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 cytokotosomal 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 cytokotosomal 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 cytokotosome are important modulators of the APC functions of DCs. Biochemically, the presence of IL-10 or LEK1 either delays or limits the cytokotosomal 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 cytokotosomal reorganization that promotes cell division and maintenance of DCs in the immature stage. Thus, the absence of LEK1 will lead to cytokotosomal 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 cytokotosomal 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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