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Molecular Basis for the Potency of IL-10-Deficient Dendritic Cells as a Highly Efficient APC System for Activating Th1 Response

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Identification and targeting of novel immunobiological factors that regulate the induction of Th1 cells are crucial for designing effective vaccines against certain intracellular pathogens, including *Chlamydia*. IL-10-deficient dendritic cells (DC) are potent APCs and effective cellular vaccines that activate a high frequency of specific Th1 cells. To elucidate the molecular basis for the potency of the IL-10-deficient APC system, we tested the hypothesis that *Chlamydia* Ag-primed IL-10 knockout (IL-10KO) DC are quantitatively and qualitatively distinct in their metabolic characteristics relating to T cell activation. Using a combination of RT-PCR, two-dimensional gel electrophoresis, and MALDI-TOF-based proteomics analyses, the transcriptional and translational activities of *Chlamydia*-pulsed DC from wild-type and IL-10KO mice were assessed. IL-10 deficiency caused early maturation and activation of pulsed DC (i.e., high CD11c, CD40, CD80, CD83, CD86, IL-1, IL-12, and the T cell-attracting chemokine CCL27/CTACK) and consequently an enhanced ability to process and present Ags for a rapid and robust T cell activation. Supporting comparative proteomics revealed further that IL-10 deficient DC possess specific immunobiological properties, e.g., the T cell-attracting chemokine CCL27/CTACK, calcium-dependent protein kinase, and the IL-1/IL-12 inducer, NKR-P1A (CD161), which differentiated them immunologically from wild-type DC that express molecules relating to anti-inflammatory, differentiative, and metabolic processes, e.g., the anti-IL-12 molecule peroxisome proliferator-activated receptor-α and thymidine kinase. Collectively, these results provide a molecular basis for the high Th1-activating capacity of IL-10KO APC and may provide unique immunomodulation targets when designing vaccines against pathogens controlled by T cell immunity. The *Journal of Immunology*, 2005, 174: 4860–4869.

A n efficacious vaccine is the best approach to protect the greatest number of people from the mucosal and systemic infections caused by several intracellular microbial pathogens that include *Chlamydia*. For several intracellular microbial pathogens, protective immunity in humans as well as animals is governed by the elicitation and maintenance of a high level of T cell-mediated immune response (1–7). The notable effectors of T cell-mediated immunity (CMI) are CTLs, which are mostly CD8 T cells; Th1 cells, commonly CD4 T cells; inflammatory cytokines such as IFN-γ, TNF-α, activated macrophages, NK cells; and perhaps other less characterized immune cells and molecules (7, 8). Thus, there is a high incidence of exacerbated disease caused by intracellular microbial pathogens in animals and humans deficient of T cells or Th1-like cytokines (2–5). Also, both Ab blocking and neutralization studies and analysis of specific gene knockout animals have established the requirement of effectors of CMI, especially IFN-γ derived from NK cells, macrophages, and Th1 cells of both CD4 and CD8 subsets, in the control of several intracellular pathogens, including *Chlamydia* (3–5), *mycobacteria* (9), *Listeria* (10), *Salmonella* (11), and *Rickettsia* (12). In particular, it has been unequivocally demonstrated in both clinical studies and experimentation in animal models of genital, ocular, and respiratory infections that *Chlamydia* immunity correlates with a strong Th1 response and a complementary Ab response that fosters a rapid and robust memory T cell immunity (3–5, 8, 13). Recent advances in human genetics and disease susceptibility have reinforced previous experimental findings that the induction of adequate CMI effectors, especially Th1-related cytokines such as IL-12 and IFN-γ, is obligatory for immunity against these intracellular microbes (2, 14–17).

Because CMI effectors are effective and required for protective immunity against several intracellular microbial pathogens, their induction is vital to vaccine efficacy against infections. Thus, a better knowledge of the factors that influence the generation of optimal T cell response against intracellular microbial pathogens is important for targeting those that will influence the designing of more effective vaccines to control the diseases. In this respect, it is

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3 Abbreviations used in this paper: CMI, T cell-mediated immunity; IL-10KO, IL-10 knockout; CTACK, cutaneous T cell-attracting chemokine; 2-DE, two-dimensional gel electrophoresis; DC, dendritic cell; EB, elementary body; WT, wild type; IFU, inclusion-forming unit; RT, reverse transcription; IEF, isoelectric focusing; pI, isoelectric point; MSP, macrophage-stimulating protein; RON, tyrosine kinase/recepteur d’origine nantais; PD-1, programmed cell death-1 ligand.

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0022-1767/05/$02.00
now better appreciated that the cytokine environment at the initiation of T cell response and the magnitude of the concurrent humoral immune response exert significant regulatory influence on the level and effectiveness of T cell immunity against intracellular microbial pathogens (8). Among the cytokines required, IL-12 derived from the APCs (18) is critical for Th1 response. TLRs, a family of microbial pattern recognition receptors expressed on APCs, play a major role in sensing the presence of microbial pathogens. They consequently stimulate the maturation/activation of APCs to acquire enhanced Ag processing and presentation capacity that includes IL-12 production (19–22). IL-1 and IL-18 are also potent inducers of Th1 response and are components of the cytokine environment that favor Th1 activation. IL-18 knockout mice are deficient in both NK cell function and Th1 development, and IL-18 is vital for antifungal immunity (7, 23). It is therefore conceivable that specific vaccine delivery systems can incorporate the concurrent administration of certain cytokines and vaccine to produce an ambient cytokine environment for optimal induction of protective Th1 response against microbial pathogens. Furthermore, the biological suppression of certain cytokines may promote Th1 induction and thereby enhance protective immunity against certain microbial pathogens. IL-10, an anti-Th1 cytokine (24) produced during genital chlamydial infection of experimental animals and humans (25, 26), is potentially an important local factor that would control Th1 response against Chlamydia. Thus, genetic differences in mouse strain susceptibility to Chlamydia trachomatis or Coccioides immitis was mapped to high IL-10 producers being more susceptible to infections (25, 27). Also, increased IL-10 levels in the endocervical secretion of women with chlamydial infection could predispose to enhanced HIV-1 transmission (26), possibly due to inadequate local T cell response. These observations would suggest that the temporary or inadequate immunity commonly induced against Chlamydia is at least partially due to the negative immunoregulation of IL-10, which could occur via IL-10-regulated production of a relatively low frequency of Chlamydia-specific Th1 cells. In such a case, genetic or biological suppression of IL-10 activity or expression during immune elicitation against Chlamydia should lead to the induction of high levels of specific Th1 response that could mediate long term antichlamydial immunity. Indeed, microbiological analysis of the role of IL-10 in resistance against Chlamydia using genetically engineered IL-10KO mice revealed that the animals were more resistant to genital and respiratory infection than wild types, which correlated with the high Th1 response (25, 28, 29). Also, the cellular and molecular basis for the high Th1 response in IL-10KO mice was shown to be due to the proclivity of IL-10KO APC for activating Th1 cells regardless of whether the T cells express IL-10, suggesting that the effect of IL-10 is exerted on the APC (28). In addition, Chlamydia-infected IL-10KO dendritic cells (DC) were efficient cellular vaccines, inducing high frequencies of systemic and mucosal Th1 response in recipients that acquired sterilizing, long term protective immunity against infection (28). These findings are corroborated by other reports that IL-10 affects DC maturation and APC function (30–35) and promotes tumor growth by suppressing T cell response (36, 37). In this study, we used transcriptional and proteomics techniques to analyze the immunobiochemical features of Chlamydia-exposed IL-10-deficient DC to determine the molecular and biological basis of the high T cell activating capacity of this efficient APC system. The results from these studies could lead to the identification of ligand-sensitive pathways that can be rationally manipulated during vaccine delivery to obtain an optimal protective immunity against intracellular microbial pathogens.

Materials and Methods

Chlamydia stocks and Ags

Stocks of C. trachomatis serovar D and Chlamydia muridarum (the agent of mouse pneumonitis) used for infections were prepared by propagating elementary bodies (EBs) in McCoy or HeLa cells, according to standard procedures. Chlamydial stock titers were expressed as inclusion-forming units (IFU) per milliliter. Chlamydial Ags were prepared by growing the agent in HeLa cells and purifying EBs over Renograin gradients, followed by inactivation under UV light for 3 h.

Knockout and wild-type (WT) mice

Female IL-10−/−, IFN-γR−/−, and the respective control IL-10+/+ and IFN-γR+/+ mice, on a C57BL/6 background and 5–8 wk old, were obtained from The Jackson Laboratory. All animals were fed with food and water ad libitum and maintained in laminar flow racks under pathogen-free conditions of 12 h light:12 h darkness.

DC isolation and culture

DCs were isolated from the bone marrows of normal and IL-10KO mice by the standard method and differentiated by in vitro culture with IL-4 and GM-CSF, as previously described (28). The cells were characterized as loosely adherent mononuclear cells and determined by FACS to express high levels of MHC class II, CD54 (ICAM-1), and CD11c. After 5 days in culture, DCs were washed and pulsed with UV-inactivated Chlamydia EBs.

Adoptive immunotherapeutic vaccination with Chlamydia-pulsed DCs

DCs isolated from IL-10−/− and IL-10+/+ mice were pulsed with UV-inactivated C. trachomatis serovar D EBs for 12 h and adoptively transferred into 4-wk-old homogeneic, female IFN-γR−/− and the control IFN-γR+/+ mice (2.5 × 105 cells/mouse), by i.v. infusion into the retro-orbital sinus in 0.2 ml of PBS. Treated mice were maintained in a laminar flow hood, fed, and maintained under a 12-h light:12-h dark cycle. After 1 week, the mice were infected intravaginally with 104 IFU/mouse of live C. trachomatis serovar D. The status of the infection was monitored by periodic cervical swabbing of individual animal and isolation of chlamydiae in tissue culture (38). Experiments were repeated twice to give 10–12 mice/group.

Measurement of frequency of Chlamydia-specific Th1 cells (Th1 frequency) after vaccination with Chlamydia-pulsed DC

A modified procedure of the limiting dilution technique (39) was used to assess Th1 frequency in infected mice. Briefly, T cells were isolated from the genital tracts of infected mice at the indicated times, as previously described (40). Limiting dilution cultures were established by seeding T cells in a serial doubling dilution into 96-well round-bottom tissue culture plates at 12–24 wells/dilution. Typically, four and eight dilutions were established for each T cell responder in the range of 1 × 104 through 8 × 106 cells/well. The T cells were stimulated with APCs from WT mice (2 × 105 cells/well) and chlamydial Ag (10 μg/ml). Background cultures contained 24 wells with APCs and Ag. After 5 days of incubation, the supernatants were assayed for IFN-γ by a sensitive ELISA (40). The mean and SD of all 24 replicates of background cultures were calculated. Three times the value of the SD was added to the mean value, and the sum was the baseline for positive experimental wells. After determination of the number of positive and negative wells per dilution of each T cell preparation, the data were analyzed by a limiting dilution computer program (LIDIA) (39, 41), which provided both the Th1 frequency and the conformity of the input data with a single-hit Poisson model. Genital tract T cells from naive IL-10−/− mice have Th1 frequency of 15 (range, 9–21).

Measurement of the rate of T cell activation by DC from IL-10−/− and IL-10+/+ mice

The efficiency of Ag presentation by DCs from IL-10KO mice and WT mice was compared by assessing the rate of Th1 activation by γ-irradiated DC from IL-10KO and WT mice. Splenic cells from chlamydial-infected IL-10KO and WT mice were enriched for T cells by the nylon wool adherence method (42, 43). Purified splenic cells contained at least 97% CD3+ cells, as determined by FACS. To assess the Ag-presenting function of γ-irradiated DCs from either IL-10KO or control mice, 1 × 105 or 1 × 106 cells were cocultured with 2 × 105 nylon wool-purified T cells in the presence or absence of chlamydial Ag (i.e., UV-inactivated mouse pneumonitis EBs at 10 μg/ml) in 96-well tissue culture plates for 24, 48, 72, 96,
or 120 h. At the end of each incubation period, the supernatants were collected and assayed for IL-2 and/or IFN-γ content by a quantitative ELISA (Cytoscreen Immunoassay Kit; BioSource) 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 at least three independent experiments.

**Semiquantitative RT-PCR analysis of mRNAs from Chlamydia-pulsed bone marrow DCs**

To measure changes in gene expression of specific transcripts in Chlamydia-exposed IL-10KO and WT DC by semiquantitative RT-PCR, cultured DC were pulsed with Chlamydia (multiplicity of infection, 10) at different time intervals representing early (8 h) and late (72 h) exposure. For total RNA isolation using Trizol reagent (Life Technologies), cultured DC were lysed and subsequently homogenized in Trizol (1 ml per 3.5-cm-diameter culture dish). RNA was precipitated, pelleted, and resuspended in diethyl pyrocarboxylate-treated, nuclease-free water and stored at 80°C. For reverse transcription (RT), 4 μl of random hexamer (50 μM) were added to each reaction tube containing 5 μg of total RNA in 14.5 μl of sterile water, incubated for 5 min at 70°C, and then quickly chilled on ice. RNA-50.5 μl, 5× RT buffer (10 μl), dNTPs (20 μl), and Moloney murine leukemia virus RT (1 μl) were added, and the reaction tubes were incubated at 37°C for 1 h. The reaction was terminated by heating at 95°C for 10 min followed by quick chill on ice. Sterile water (50 μl) was added to each reaction tube. Subsequently, the cDNAs were amplified using gene-specific primers for IL-12, IL-10, IL-12/p40, ICAM-1, CD11c, and B7.1 (CD80), and B7.2 (CD86), because these molecules are known to be associated with APC function in Ag presentation for T cell activation (44).

PCR amplification was conducted in a PerkinElmer Gene Amp 2400 thermocycler. Each tube contained a total volume of 50 μl: 5 μl of cDNA from RT reaction; 2 μl of dNTP mixture (100 μM); 5 μl of 10× PCR buffer; 0.5 μl of Taq polymerase (3.5 μg/ml); and 5 μg/ml primer (forward and reverse). Sterile water was used to adjust volume in thin-walled reaction tubes. Conditions for DNA amplifications were set as follows: heating at 94°C for 3 min followed by 35 cycles of DNA denaturation at 94°C for 1 min; annealing at 58°C for 1 min; and an extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. The housekeeping gene GAPDH was used as the internal standard for the expression of genes of interest. Equivalent amounts of cDNA template were used in reactions with primers specific to GAPDH as well as negative controls containing no cDNA. A volume of 10 μl of each PCR product was combined with 2 μl of loading buffer and 2 μl of sterile water and electrophoresed on a 1.5% agarose (Invitrogen) gel containing 0.5 μg/ml ethidium bromide (Sigma-Aldrich) in 1× Tris-acetate-EDTA buffer (Life Technologies). The levels of the PCR products for the primers in gels with separate bands were photographed, and the ratio of band intensity was analyzed using GelExpert software by Nucleovision (Nucleotech), and the values were normalized to GAPDH for each sample tested (the relative density of the band for each gene at each time point was divided by the GAPDH density). Because IL-10KO mice were used, transcript levels of this cytokine were measured as a negative control. Statistical analysis using a t test was performed to determine whether there was a significant difference in the expression of each gene of interest in infected WT and IL-10KO DCs at the time periods evaluated.

**Proteomic analysis of Chlamydia-pulsed DC using two-dimensional gel electrophoresis (2-DE)**

DCs from WT and IL-10KO mice were pulsed with chlamydial EBs (serovar D) for 2 or 8 h, washed three times with cold sterile PBS, and lysed with a rehydration buffer containing 8 M urea, 10 mM DTT, 2 M CHAPS, 0.2% bio-Lyte (Bio-Rad Laboratories). FOCUS-Protease Arrest and FOCUS-Protease Nuclease (Calbiochem) were added to a final concentration of 10 μg/ml. The lysate was subjected to sonication at 4°C for 15 min. The sample was then centrifuged using a Sorvall Ultra Pro 80 ultracentrifuge at 14,000 rpm for 30 min at 4°C to remove any insoluble cell debris. The amount of proteins in the supernatants was determined by using the BioRad DC RC protein assay kit (Bio-Rad Laboratories). The 2-DE analysis was performed essentially on the basis of protocols optimized for 2-DE analysis of cell lines, as supplied by Bio-Rad Laboratories. Brieﬂy, the first-dimensional isoelectric focusing (IEF) was conducted by loading 10 μg of protein lysate on 11-cm immobilized pH gradient strips (Bio-Rad) at 20°C with a maximum current setting of 50 μA/strip using a Bio-Rad Laboratories IEF cell. The strips were rehydrated at 50 V for 12–16 h, and the IEF run was conducted using Bio-Rad focusing conditions. The second-dimensional SDS-PAGE (4–20%) was conducted according to Bio-Rad protocols as well.

**SYPRO Ruby staining and image analysis.** Gels were ﬁxed for 30 min in a mixture of 10% methanol and 7% acetic acid and stained with SYPRO Ruby (Molecular Probes) overnight. Before the gels were imaged, the stained gels were washed again for 2 h in a mixture of 10% methanol and 7% acetic acid. Gel imaging was performed on a Bio-Rad Molecular Imager FX Pro Plus and analyzed by visual inspection and PDQuest (version 7.1) software (Bio-Rad). Three well-resolved 2-DE gels were used to create a master image and the resulting master gel was used as the experimental condition with the PDQuest software. The intensities of the spots in the different gels were normalized using the normalize function in the image analysis software. These normalized synthetic master gels were used for the differential comparison to identify differentially expressed protein spots with PDQuest.

**Enzymatic digestion and MALDI-TOF mass spectrometry.** Selected spots corresponding to specific proteins on the SDS-PAGE were excised and washed three times with 10 mM ammonium bicarbonate, 50% acetonitrile to remove the stain and SDS and then dried in a Savant Speed Vac for 30 min. Enzymatic digestion was performed by the addition of 5 μl of 100 μg/ml trypsin (sequencing grade; Promega) in 10 mM ammonium bicarbonate to each gel piece and incubated at 37°C for 40–30 min to allow fluid to be absorbed. Approximately 30 μl of 10 mM ammonium bicarbonate were used to cover the gel pieces, and the gel pieces were washed at 37°C with shaking overnight to facilitate rehydration. Peptides in the selected spots were extracted using three 20-min incubations in 10 mM ammonium bicarbonate, 60% acetonitrile solution. The different extractions were pooled, and the volume was reduced to ~10 μl in a Savant Speed Vac. A volume of 2 μl of this mixture was mixed with 8 μl of α-cyano-4-hydroxycinnamic acid (10 mg/ml in 30% methanol, 50% acetonitrile, 0.1% TFA, water), and 2 μl were spotted on a MALDI target. Mass spectra for the different spots were acquired on a Bruker Reflex IV MALDI-TOF MS equipped with pulsed ion extraction and a nitrogen laser. The peaks corresponding to specifically identiﬁed peptides were picked using XMass software (Bruker Reflex IV). All the mass spectra were internally calibrated with trypsin autolysis peaks. The resulting peptide mass fingerprints were searched against a local copy of the nonredundant database NCBInr (http://www.ncbi.nlm.nih.gov) using the Mascot protein identiﬁcation search program adjusted for murine proteins. The parameters used in the search were as follows: peptide mass, isoelectric point (pI), tolerance 50 ppm, 1 missed cleavage, carbamylated cysteine, and N-terminal hydroxyl.

**Statistical analysis**

The levels of cytokines in samples from different experiments were analyzed and compared by performing a one- or two-tailed t test, and the relationship between different experimental groupings was assessed by ANOVA. Minimal statistical significance was judged at p < 0.05.

**Results**

Protective Th1 cells have obligatory requirement for IFN-γ to clear chlamydial infection

Initial studies extended previous findings that IL-10-deﬁcient APC have a proclivity for activating a rapid and protective anti-chlamydial Th1 response. We tested the hypothesis that protective Chlamydia-specific Th1 cells induced by IL-10KO DC cleared chlamydial infection in vivo via IFN-γ-induced antimicrobial processes. Chlamydia-pulsed IL-10KO DC were adoptively transferred into female IFN-γ receptor knockout (IFN-γR−/−) and the control IFN-γR−/− mice. The mice were infected intravaginally with the live chlamydial agent after 1 wk of adoptive transfer, and the course of the infection was monitored by isolation of chlamydiae from cervicovaginal swabs. Fig. 1 shows that on day 3 postchallenge, only 1 of 12 control IFN-γR−/− mice that received Chlamydia-pulsed IL-10KO DC revealed evidence of a suspicious low grade (2.0 ± 2 IFU/ml) infection, which was resolved by day 6 (Fig. 1, A). Thus, 11 of 12 IFN-γR−/− mice that received Chlamydia-pulsed IL-10KO DC were immune to challenge infection, and all these mice cleared their infections within 1 week. The speciﬁcity of the protective immunity achieved is evident in the results, showing that the course of the genital infection in IFN-γR−/− mice that received non-Chlamydia-pulsed IL-10KO DC...
independent experiments. The genital mucosa was monitored by determining the frequency of specific Th1 cells in the genital tracts of infected mice, as described in Materials and Methods. Results represent the mean and SD of three independent experiments involving 25 mice.

(Fig. 1, □) was similar to that of a natural (nonimmune) genital chlamydial infection in an immunocompetent WT mouse, as previously reported (28). In addition, results represented in Fig. 1 further verified the working hypothesis that Th1 cells control Chlamydia infection in mice by IFN-γ-induced mechanisms, because IFN-γR−/− recipients of the protective Chlamydia-pulsed IL-10KO DC could not resolve their infections (Fig. 1, ▼).

A rapid induction and maintenance of a high level of genital mucosal Th1 response is required for clearance of chlamydial infection and establishment of protective immunity

We experimentally confirmed that the rapid clearance of the genital infection by the IFN-γR−/− mice that received Chlamydia-pulsed IL-10KO DC was due to the timely induction of an elevated genital mucosal Th1 response. Thus, the kinetics of induction of chlamydia-specific Th1 cells was monitored by determining the frequency of specific local mucosal Th1 cells in the genital tracts of infected mice. As revealed in Fig. 2, recipients of Chlamydia-pulsed IL-10KO DC mounted a strong local genital tract Th1 response within 1 wk (with specific T cell frequency of 686 ± 38 Th1 cells/10^6 genital tract cells) that surpassed (by almost 3-fold) the response of unvaccinated WT mice when the latter resolved the infection by 4–6 wk (specific T cell frequency of 286 ± 24 Th1 cells/10^9 genital tract cells). Overall, there was ~15-fold difference in the frequency of Th1 cells induced by the pulsed IL-10KO DC compared with the natural host response during the course of the infection. When the IFN-γR−/− mice that received Chlamydia-pulsed IL-10KO DC (vaccinated group) and the recipients of DC only (control group) were rechallenged after 99 days of the primary infection, the former enjoyed a sterilizing immunity against Chlamydia, whereas 100% of the latter were successfully infected during the first 10 days after challenge (0 of 12 mice infected in the vaccinated group and 10 of 10 mice infected in the control group). Evaluation of the frequency of Chlamydia-specific Th1 cells in the genital mucosa of these reinfected mice revealed that the IFN-γR−/− mice that previously received Chlamydia-pulsed IL-10KO DC had 1256 ± 85 Th1 cell/10^6 genital tract cells, whereas the control group had 16-fold lower levels (76 ± 8). The results corroborate previous findings that the susceptibility to chlamydial re-infection is due to the time-dependent waning of local T cell effectors after the resolution of the initial infection (43). Also, long term immunity is due to the retention of a high frequency of immune effectors in the mucosal site of infection. In addition, these data support the need for an efficacious vaccine regimen that induces robust immune effectors beyond the level provided by the natural host response to an infection to maintain adequate and effective residual local immune effectors to control chlamydial infection and reinfections. Because the level of Th1 response measured did not appear to cause any discernible overt pathological consequences for vaccinated mice, the results would suggest that the level of T cell response attained was safe for the animals. However, the optimal Th1 response that will be safe for vaccinated host would have to be established in preclinical evaluation of any vaccine regimen against Chlamydia.

IL-10 deficiency enhances the rate of Th1 activation in vitro

The general hypothesis derived from the foregoing studies is that IL-10 deficiency leads to a rapid activation of an elevated level of Th1 response but the cellular targets of IL-10 effects must be clarified. Also, previous studies indicated that the endogenous IL-10 deficiency at the level of the APC, not T cells, is crucial for the elevated Th1 activation (28); however, it is unclear whether IL-10 deficiency at the level of the T cells has a role at any stage during the initiation and progression of T cell activation. In the current study, the rate of Th1 development was assessed in the presence or absence of IL-10 at the level of the APC or T cells during the course of T cell activation in vitro. The data shown in Fig. 3 reveal that the differential rate of T cell activation by IL-10KO and WT DC manifests within 24 h of stimulation when there is ~19-fold enhancement in the absence of IL-10. By 48, 72, 96, and 120 h of culture, there were ~12-, 8-, 6-, and 4-fold increases in T cell activation by IL-10KO DC over WT DC, respectively. These results indicated that IL-10KO APC activate a high frequency of T cells very rapidly and reaffirm previous findings that the endogenous IL-10 produced by the APC is a crucial regulatory factor in the rate and magnitude of T cell activation; however, the IL-10 contributed by T cells do not play a discernible role in the regulation of T cell activation by Ag and APC. Thus, further analysis of IL-10 deficient DC could furnish a model APC system for defining the molecular and biochemical determinants for inducing an enhanced T cell response, and the modulation of these factors can lead to more effective vaccines against agents controlled by T cell immunity.
Two strategies were adopted to analyze the molecular basis for the potency of IL-10-deficient DC as a highly efficient Th1-activating APC system. The first strategy (i.e., Ag-induced gene expression analysis) involves exposing WT and IL-10KO DC to Chlamydia Ags and measuring the early transcriptional activities of genes encoding molecules that are known to be involved in APC-T cell interaction during Ag presentation and T cell activation. The second strategy (i.e., Ag-induced proteomics) involves exposing WT and IL-10KO DC to Chlamydia Ags, the separation and analysis of the proteome by 2-DE, and the identification of differentially expressed molecules by MALDI-TOF. The second strategy could reveal novel molecular entities that may play a role in the enhanced Th1 activation capacity of IL-10-deficient APCs. We have previously shown that the enhanced APC function of IL-10-deficient DC is evident within 24 h of exposure of T cells to Ag-pulsed DC (Fig. 3 and Ref. 28). Besides, the kinetics of activation of T cells in vitro with Ag-pulsed WT DC indicated that a pulse time of 8 h produced the optimal immunostimulatory DC (45). Thus, the time periods selected for transcriptional and translational analyses reflected these established reports.

When WT and IL-10KO DCs were exposed to Chlamydia Ags and analyzed by RT-PCR for the expression of specific immunoregulatory molecules, the results revealed that there was a more rapid induction of high levels of CD11c, IL-1β, IL-12, CD40, and CD80 by IL-10KO DCs when compared with WT DCs (Fig. 4, A–E) except CD86 and ICAM-1 (Fig. 4, F and G). Immunoassay analysis of secreted or cell-associated gene products confirmed the differential expression of these immunostimulatory molecules by IL-10KO and WT DCs (data not shown). Immunoassay evaluation also confirmed that IL-10KO DC did not secret significant IL-10 after exposure to Chlamydia although the WT DC secreted elevated levels of the cytokine (Fig. 4H). CD11c is a component of the leukocyte integrin α5β2 (CD11c/CD18 or a surface receptor CR4) on mature DC that function in cell adhesion, particle uptake, and phagocytic responses (46–48). There was at least a 9-fold increase in the levels of CD11c mRNA expression (8 h) in Chlamydia-pulsed IL-10KO DCs as compared with WT DCs. Thus, the rapid and early expression of the marker of DC maturation and activation (CD11c) would suggest that DCs are more rapidly activated as functional APCs by Ags in the absence of IL-10. The results indicated that IL-10 deficiency caused an early maturation of APC such as DC, leading to an enhanced capacity to express crucial costimulatory molecules such as IL-1β and CD40, and IL-12 that are important for the differentiative activation of T cells along the Th1 pathway.

Proteomic analysis of the differential translational activities in Ag-pulsed IL-10KO and WT DC involves the used of 2-DE and MALDI-TOF techniques. The study objective was to confirm the differential expression of the costimulatory molecules detected by transcriptional analysis described previously (Fig. 4) and to explore the possibility of identifying novel molecular targets for modulating T cell response during vaccine delivery. The time of DC exposure to Ag for proteomic analysis was limited to the first 8 h because this time period was previously shown to sufficiently prime DC for efficient Ag presentation and T cell activation (45). When IL-10KO and WT DC were pulsed with Chlamydia EBs for 2 or 8 h, then analyzed by 2-DE, Fig. 5 shows a representative protein profile map revealing that a number of proteins were differentially expressed by IL-10KO DC compared with WT DC. Table I provides the biological profiles of the more visibly identified and MALDI-TOF-characterized spots in Fig. 5. At least six notable categories of differentially expressed proteins were identified: 1) proteins that were expressed rapidly early (2 h) in IL-10KO DC but delayed (8 h) in WT DC (i.e., B7.1; the T cell-attracting chemokine CCL27/CTACK; macrophage-stimulating protein [MSP]) receptor tyrosine kinase/receptor d’origine natale (RON) involved in leukocyte spreading, migration, and particle uptake; prolyl endopeptidase; and a certain selenoprotein); 2) proteins that were expressed rapidly early in WT DC but delayed in IL-10KO DC (G protein γ2, CAMP-dependent protein kinase regulatory subunit RI-α, and calgranulin B S100A9); 3) proteins that were expressed only (early or delayed) in IL-10KO DC but not in WT DC (i.e., α cardiac actin; sodium channel; calcium/calmodulin-dependent protein kinase that increase CD83 and costimulation; C-kinesis, and NKR-P1 the engagement of which enhances DC activation for IL-12 production); 4) proteins that were expressed exclusively (early or late) in WT DCs but not in IL-10KO DCs (i.e., anti-inflammatory programmed cell death-1 ligand (PD-1) and CCR4-NOT complex; vimentin related to macrophage function; MLCK involved in cellular interaction; ribonucleoside-diphosphate reductase prominin-1 T3 isoform; purine nucleoside phosphorylase; caveolin-1; intersectin 6 associated with cell division and marker of immature DC; keratinocyte lipid-binding protein [that suppresses inflammation via binding to pepsin-resistant prolinator-activated receptor (PPAR)-α]; carboxyembryonic Ag-7; β3-adrenergic receptor (which is antiactivatory); calcium-activated chloride channel-2 associated with cell secretion and division; anti-inflammatory sterol 1; and the transcription factor Sox-10 involved in cell differentiation, glucose-6-phosphate deaminase, an unknown protein (mKIAA1759, molecular mass, 100+ kDa/pf 5.6; lymphocyte Ag 6 complex involved in differentiation; Elac 1 protein; thymidine kinase; and extracellular matrix protein 1 precursor); 5) proteins that were expressed early at higher (i.e., STAT1, latent transforming growth factor β, Bcl-2, and the GTP-binding protein rho) or lower (the anti-IL-12 molecule PPAR-α) levels in IL-10KO DC than WT DC; and 6) proteins that were expressed at equal levels in IL-10KO and WT DC (i.e., Janus kinase (STAT3); and IFN-γ-class II cytokine receptor, an unknown mKIAA1775 protein; the tripartite motif protein, calmodulin; CLIP-associating protein-1). A better understanding of the functional aspects of the differentially expressed proteins, through analysis of genetically engineered specific gene knockout systems and the use of immunological and biochemical blockers, may furnish targets for
modulating Ag presentation for an enhanced T cell response against microbial pathogens and tumors that require rapid and elevated T cell response for clearance and establishment of long term immunity.

Discussion
A better understanding of the cellular and molecular immunological factors that govern the induction of a robust T cell response against microbes is a prerequisite for designing efficacious vaccines to protect the population against certain intracellular microbial pathogens that include *Chlamydia, Listeria,* and *Mycobacterium.* For several of these pathogens, results from analysis of experimental animal models of the diseases and clinical observation from infected patients have established that: 1) a relatively elevated T cell response is crucial for clearance of infection and maintenance of protective immunity against...
reinfections; and 2) the immunobiological basis of the partial and transient protective immunity observed after the clearance of an infection or the administration of several experimental vaccines appears to be due to the induction of an inadequate or a low frequency of CMI effectors at the mucosal sites of infection.

In the case of the animal models used for study of the immunobiology of C. trachomatis genital infection and vaccine evaluation, immunization regimens that include the use of ex vivo Ag-pulsed DC to deliver and present Chlamydia Ags in vivo have produced some of the most promising experimental protection studies to date (28, 49). Chlamydia-pulsed DC appear to possess the necessary antigenic, costimulatory, and immunomodulatory machinery for inducing high levels of Th1 response and the accessory IgA and IgG effectors required for boosting protective immunity against Chlamydia (28, 49). Although the use of wild-type DC produced a partial but significant protection against genital chlamydial infection, the application of IL-10KO DC achieved a sterilizing, long term protective immunity that correlated with the capacity to induce a high frequency of specific Th1 cells and elevated titers of the CMI-associated IgG2a and IgA Abs (3, 28).

The present study has confirmed the proposed correlation between rapid resolution of genital chlamydial infection, acquisition of protective immunity, and a rapid induction of a high frequency of genital mucosal Th1 response by Chlamydia-pulsed IL-10KO DC. In addition, IFN-γ produced by the activated Th1 cells was crucial for chlamydial clearance in this model system.

Table I. Biological profiles of the more visibly identified and MALD-TOF-characterized spots in Fig 5

<table>
<thead>
<tr>
<th>Spot</th>
<th>Molecular Mass (kDa)</th>
<th>pI</th>
<th>Name of candidate proteins</th>
<th>IL-10+/− 2 h</th>
<th>IL-10+/− 8 h</th>
<th>IL-10−/− 2 h</th>
<th>IL-10−/− 8 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>150–250</td>
<td>5.3</td>
<td>MSP receptor (RON): macrophage spreading, migration, and phagocytosis</td>
<td>0</td>
<td>422</td>
<td>3205</td>
<td>2981</td>
</tr>
<tr>
<td>2</td>
<td>32.1–39.7</td>
<td>5.4</td>
<td>Programmed cell death 1 ligand 1(PD-1): a new member of the CD28/CTLA-4 family and is undetectable on activated macrophages or DCs</td>
<td>2943</td>
<td>3988</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>7.1–17.5</td>
<td>5.3</td>
<td>1.NKR-P1: increases intracellular calcium and IL-1 β and IL-12 production by DCs. Thus help to present Ags to T cell</td>
<td>0</td>
<td>0</td>
<td>1279</td>
<td>2551</td>
</tr>
<tr>
<td>4</td>
<td>32.1–39.7</td>
<td>4.7</td>
<td>CCR4-NOT complex</td>
<td>2890</td>
<td>4095</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>&lt;7.1</td>
<td>9.7</td>
<td>B7.1: Costimulatory molecule of mature DCs</td>
<td>0</td>
<td>2273</td>
<td>4095</td>
<td>4095</td>
</tr>
</tbody>
</table>
basis of the antimicrobial action of IFN-γ has been recently reviewed (see recent review in Ref. 8).

It has been suggested that the phenomenon efficacy of the DC-based cellular vaccines makes them natural adjuvants or preeminent delivery vehicles, useful as tools to guide the design of effective delivery systems that mimic the potent immunostimulatory action of DC; such DC-like delivery systems will be useful in vaccines to induce protective immunity against intracellular pathogens and to unravel the necessary vaccine machinery in terms of Ags, delivery, costimulation, immunomodulation, and homing requirements (50, 51). In fact, DC-based cellular vaccines have shown that, given an effective delivery vehicle, inactivated chlamydial elementary bodies possess sufficient immunogenic epitopes to elicit a protective immunity against Chlamydia. The challenge for vaccinology therefore is to develop a delivery system that mimics the superior immunostimulatory properties of DC to achieve an effective chlamydial vaccine. In particular, the ability of IL-10-deficient APC to rapidly activate a high frequency of protective specific T cells suggested that they could furnish a model APC system for defining the molecular and biochemical determinants for induction of an enhanced T cell response, and the modulation of these factors can lead to more effective vaccines against agents controlled by T cell immunity.

We have used a combination of gene expression analysis of transcriptional activities and proteomics techniques based on 2-DE and MALDI-TOF to investigate the molecular basis for the potency of IL-10-deficient DC as a highly efficient Th1-activating APC system. The objective was to define and identify the determinants of T cell augmentation against intracellular microbial pathogens, which can be applied in vaccine design against specific pathogen, including Chlamydia and mycobacteria. The RT-PCR studies measured the early transcriptional activities of genes encoding molecules that are involved in APC-T cell interaction during Ag presentation and T cell activation. These results revealed that unlike WT DC, Chlamydia-pulsed IL-10KO DCs rapidly acquired markers (CD11c, CD40, and CD83) that are associated with matured DCs, suggesting that early maturation of DCs is an important factor in the enhanced T cell activation. In fact, early maturation of DCs leads to the rapid acquisition of the capacity to express crucial costimulatory molecules such as IL-12, CD40, and IL-12 that are important for the differentiative activation of T cells along the Th1 pathway. Our results are further corroborated by the fact that under normal physiological conditions of the mammalian host, DC exist as relatively immature mononuclear cells scattered in various mucosal and nonmucosal tissues in the body. On encounter of an antigenic signal (e.g., through the TLR-mediated signals delivered by recognition of pathogen-associated molecular patterns such as CpG, LPS, dsRNA), DC undergo a differentiative maturation from the immature (highly endocytic cells) to mature and activated cells with up-regulated costimulatory molecules that promote enhanced Ag presentation and T cell activation (52). The DC maturational process controls their capacity to process and generate MHC-peptide complexes, their migration toward secondary lymphoid tissues such as lymph nodes, acquisition of costimulatory molecules, and efficient Ag presentation for T cell activation. Thus, whereas Ag handling by fully mature DC can activate T cells and prime immune responses, immature DC may induce tolerance (53) or suboptimal response. In addition, T cells differentiate into specific phenotypes depending on the TCR-epitope combination, the costimulatory stimuli they receive, and the soluble factors present in the inflammatory milieu during activation. Specifically, the presence of IL-12 delivered by APCs is important for signaling via STAT4, which stimulates IFN-γ gene expression and activation-induced differentiation of naïve T cells to the Th1 phenotypes (54, 55). In fact, the efficacy of a DC-based cellular vaccine at inducing protective antichlamydial immunity in mice was suppressed by functional IL-12 elimination, which resulted in lack of Th1 activation (56), and the superiority and preferential induction of protective Th1 response by live over inactivated pathogens, including chlamydiae (57–60), was associated with early induction of GM-CSF which promotes DC maturation and production of IL-12 (61). Moreover, the enhanced T cell activation by Ab-mediated delivery of Ag via Fc receptors on DCs (62) was attributed in part to the effective targeting of Ags to critical cytoplasmic compartments for rapid processing, as well as the induction of DC maturation by initiating specific signaling events that may involve the Syk protein tyrosine kinase, leading to DC activation and enhancement of Ag-presenting function (63, 64). Also, ligation of the costimulatory molecule CD40 on DCs cells triggers production of high levels of IL-12 and enhances T cell-stimulatory capacity via APC activation (65). Finally, immunosuppressive agents such as IL-10 and trichosanthin which inhibit surface costimulatory molecule expression (including B7.1) APCs also inhibit T cell activation and IL-2 production (66). Thus, agents that will foster the rapid maturation of DC and support T cell differentiation will be useful vaccine adjuvants for inducing elevated T cell response.

The comparative proteomic results revealed that IL-10-deficient DC expressed specific metabolic properties that differentiated them immunologically from WT DC and therefore explain their potency in Th1 activation. For instance, the costimulatory molecule B7.1 (CD80) and the T cell-attracting chemokine CCL2/CTACK were among the proteins expressed rapidly and earlier in IL-10KO than WT DCs, whereas the anti-IL-12 molecule PPAR-α had limited expression in IL-10KO DC. Cutaneous T cell-attracting chemo-kine (CTACK; CCL27) is a member of the CC chemokine family and a functional ligand for CCR10. It is important for rapid recruitment of T cells to the vicinity of Ag-bearing DC, which enhances DC-T cell interaction and Ag presentation. Chlamydia-pulsed IL-10KO DC (but not WT DC) also expressed calcium-dependent protein kinase, sodium channel, and NKR-P1A (CD161) that promote IL-1/IL-12 expression. However, they did not express the anti-inflammatory programmed cell death 1 ligand (PD-1), CCR4-NOT complex, and keratinocyte lipid-binding protein. The results provide a molecular basis for the high Th1-activating capacity of IL-10KO APC and could furnish unique immunomodulation targets when designing vaccines against pathogens controlled by T cell immunity. The immunological analysis of an infections disease system in a compatible and relevant animal model has allowed us to answer fundamental questions in basic immunology and host defense as they relate to the immunoregulatory determinants of T cell immunity. Although possible immunological differences may be encountered in extending murine results to humans, such subtle immunobiological differences can usually be adequately adjusted to maximize the utility and significance of the experimental finding. Finally, a detailed analysis of these molecules involved in the Th1-enhancing ability of IL-10KO APCs and the molecular mechanisms by which they exert their effect could lead to the designing of more effective vaccines against Chlamydia and other microbial pathogens as well as tumors that are controlled primarily by a robust T cell response.

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Disclosures
The authors have no financial conflict of interest.
References


