IL-4 blocks T_H1-polarizing/inflammatory cytokine gene expression during monocyte-derived dendritic cell differentiation through histone hypoacetylation

María López-Bravo, PhD‡ María Minguito de la Escalera, MSc‡ Pilar M. Domínguez, PhD‡ Leticia González-Cintado, BSc, Carlos del Fresno, PhD* Pilar Martín, PhD* Gloria Martínez del Hoyo, PhD* and Carlos Ardavín, PhD *Madrid, Spain*

Background: Whereas recent research has characterized the mechanism by which dendritic cells (DCs) induce T_H1/T_H17 responses, the functional specialization enabling DCs to polarize T_H2 responses remains undefined. Because IL-4 is essential during T_H2 responses not only by acting on $CD4^+$ T cells through the activation of GATA-3 but also by regulating IgE class-switching, epithelial cell permeability, and muscle contractility, we hypothesized that IL-4 could also have a role in the conditioning of DCs during T_H2 responses.

Objective: We sought to analyze whether IL-4 exerts an immunomodulatory function on DCs during their differentiation, leading to their functional specialization for the induction of T_H2 responses.

Methods: Monocyte-derived DCs (moDCs) conditioned by IL-4 during their differentiation (IL-4–conditioned moDCs [IL-4–moDCs]) were analyzed for T_H1-polarizing/inflammatory cytokine production in response to Toll-like receptor stimulation. The acetylation level of the promoters of the genes encoding these cytokines was analyzed by using chromatin immunoprecipitation. Gene expression profiling of IL-4–moDCs was defined by using mouse genome microarrays. IL-4–moDCs were tested for their capacity to induce house dust mite–mediated allergic reactions.

Results: Our data suggest that IL-4 inhibits T_H1-polarizing/inflammatory cytokine gene expression on IL-4–moDCs through the deacetylation of the promoters of these genes, leading to their transcriptional repression. Microarray analyses confirmed that IL-4 upregulated T_H2-related genes as eosinophil-associated ribonucleases, eosinophil/basophil chemokines, and M2 genes. IL-4 licensed moDCs for the induction of T_H2 responses, causing house dust mite–mediated allergic airway inflammation.

Conclusion: This study describes a new role for IL-4 by demonstrating that moDCs are conditioned by IL-4 for the induction of T_H2 responses by blocking T_H1-polarizing/inflammatory cytokine production through histone hypoacetylation and upregulating T_H2-related genes. (J Allergy Clin Immunol 2013;132:1409-19.)

Key words: *T_H2 responses, IL-4, dendritic cells, monocyte-derived dendritic cells, house dust mite–induced allergic reactions*

T_H2 responses are crucial for defense against infections by helminths and trigger allergic reactions that can lead to severe clinical disorders, such as asthma or anaphylaxis, and ultimately to death. The induction of T_H2 responses relies on specialized dendritic cells (DCs) that present peptides from pathogen- or allergen-derived antigens to unpolarized $CD4^+$ T cells, leading to the T_H2 polarization of these antigen-specific $CD4^+$ T cells. This process is dependent on the transcription factor GATA-3, controlling the production by T_H2-polarized $CD4^+$ T cells of T_H2 cytokines that are responsible for the initiation and regulation of T_H2 responses.[1]

Whereas recent research has allowed us to define the mechanism by which DCs induce T_H1/T_H17 responses, the mechanistic basis for the functional specialization enabling DCs to polarize T_H2 responses has remained elusive. In contrast to T_H1/T_H17 polarization, induction of T_H2 differentiation does not appear to depend on the production of T_H2-polarizing cytokines by DCs. Recent data support that promoting a T_H2 genetic program involves a specific antigen presentation process by T_H2-polarizing DCs (T_H2-DCs) that requires the presence of IL-4 to activate GATA-3, the expression of costimulatory molecules by T_H2-DCs for a productive $CD4^+$ T-cell activation, and the blockade of T_H1-polarizing cytokine production by T_H2-DCs.

The blockade of T_H1-polarizing cytokine production by T_H2-DCs appears essential for T_H2 response induction because recent evidence supports that helminth-derived compounds can activate Toll-like receptors (TLRs) on DCs and thus induce T_H1-polarizing cytokine production[2] and that T_H1-polarizing TLR ligands, such as LPS, can be present in allergenic microscopic arthropods or pollen grains.[3] However, the mechanism by which T_H1-polarizing cytokine production is blocked in T_H2-DCs during T_H2 responses is largely unknown.

Because IL-4 functions as a key mediator of T_H2 responses not only at the $CD4^+$ T-cell level but also by regulating IgE class-switching, epithelial cell permeability, and muscle cell contractility,[4] we hypothesized that IL-4 could also exert a role on the licensing of DCs for the induction of T_H2 responses. Our data demonstrate that the presence of IL-4 during monocyte-derived DC (moDC) differentiation blocked the potential of LPS-stimulated moDCs to produce T_H1-polarizing cytokines and inflammatory mediators while allowing a correct costimulatory molecule upregulation. Our results support that IL-4 blocks T_H1-polarizing/inflammatory cytokine gene expression

From Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología (CNB/CSIC).

*These authors are currently affiliated with Centro Nacional de Investigaciones Cardiovasculares Carlos III, Madrid, Spain.

‡These authors contributed equally to this work.

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Corresponding author: Carlos Ardavín, PhD, Departamento de Inmunología y Oncología, Centro Nacional de Biotecnología/CSIC, Darwin 3, 28049 Madrid, Spain. E-mail: ardavin@cnb.csic.es.

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Abbreviations used

AAMΦ: Alternatively activated macrophage
BMDC: Bone marrow–derived dendritic cell
BMMΦ: Bone marrow macrophage
cDC: Conventional dendritic cell
ChIP: Chromatin immunoprecipitation
cM-LN: Caudal mediastinal lymph node
C-moDC: Control moDC
C-moMΦ: Control moMΦ
CTL: Cytotoxic T lymphocyte
DC: Dendritic cell
Ear: Eosinophil-associated ribonuclease
HDAC: Histone deacetylase
HDM: House dust mite
IL-4–DC: IL-4–conditioned DC
IL-4–moDC: IL-4–conditioned moDC
IL-4–moMΦ: IL-4–conditioned moMΦ
iNOS: Inducible nitric oxide synthase
moDC: Monocyte-derived DC
moMΦ: Monocyte-derived macrophage
NO: Nitric oxide
PPARγ: Peroxisome proliferator-activated receptor γ
qPCR: Quantitative PCR
Stat: Signal transducer and activator of transcription
T_H2-DC: T_H2-polarizing DC
TLR: Toll-like receptor
TSA: Trichostatin A

by causing the deacetylation of the promoters of these genes, leading to their transcriptional repression. In addition, microarray analyses revealed that IL-4 promoted the upregulation on moDCs of genes related to T_H2 responses, such as eosinophil-associated ribonucleases (Ears), eosinophil/basophil attractants chemokines, and M2 genes. Finally, *in vivo* experiments revealed the potential of IL-4 to license DCs for the induction of T_H2-polarized immune responses *in vivo* during allergic airway inflammation reactions induced by house dust mite (HDM) allergens.

METHODS

Mice

C57BL/6 mice were purchased from Harlan (Bicester, United Kingdom). Bone marrow from LXR-deficient mice (*Nr1h3*$^{-/-}$, *Nr1h2*$^{-/-}$ mice generated by Dr Mangelsdorf) was provided by A. Castrillo (Universidad de Las Palmas, Gran Canaria, Spain), from LysM-Cre-PPARγ-deficient mice by L. Nagy (University of Debrecen, Hungary), and from signal transducer and activator of transcription (Stat) 6–deficient mice by P. Murray (St Jude Children's Research Hospital, Memphis, Tenn). All the experiments were approved by the Animal Care Committee of the Centro Nacional de Biotecnología (CNB/CSIC).

Monocyte isolation and *in vitro* moDC and monocyte-derived macrophage differentiation

Monocytes were isolated and differentiated into moDCs and monocyte-derived macrophages (moMΦs), as described in the Methods section in this article's Online Repository at www.jacionline.org.

Differentiation of alternatively activated macrophages

Bone marrow cells were cultured for 7 days in nontreated, cultured 60-mm Petri dishes in complete Dulbecco modified Eagle medium supplemented with 20% FCS and 20 ng/mL M-CSF (PeproTech, London, United Kingdom) at 37°C and 5% CO_2; these cultures contained more than 95% CD11b$^+$F4/80$^+$ bone marrow macrophages (BMMΦs). Alternatively activated macrophages (AAMΦs) were obtained after BMMΦ culture for 24 hours in the presence of 20 ng/mL IL-4 and subsequently stimulated with 1 μg/mL LPS for the indicated times. AAMΦs were analyzed by means of flow cytometry with a FACSCalibur flow cytometer (BD Biosciences, San José, Calif) after double staining with fluorescein-conjugated anti-CD11b and phycoerythrin-conjugated anti-F4/80.

Analysis of cytokine production

Cytokine production by moDCs, moMΦs, and AAMΦs was analyzed at the mRNA level, protein level, or both by using quantitative PCR (qPCR) and ELISA, respectively, as described in the Methods section and Table E1 in this article's Online Repository at www.jacionline.org.

Analysis of histone acetylation

Histone acetylation was analyzed by means of chromatin immunoprecipitation (ChIP), as described in the Methods section this article's Online Repository.

Gene expression profiling

The gene expression profiling of moDCs and IL-4–conditioned moDCs (IL-4–moDCs) was performed by using mouse whole-genome microarrays, as described in the Methods section in this article's Online Repository.

Induction of moDC-mediated allergic airway responses against HDM

Induction of moDC-mediated allergic airway inflammation was performed according to a protocol modified from that reported by Lambrecht's group.[5] Control moDCs (C-moDCs; 5×10^4) or IL-4–moDCs (5×10^4) incubated for 16 hours with 30 μg/mL HDM extracts (Greer Laboratories, Lenoir, NC) were transferred intraperitoneally into C57BL/6 mice. On days 7 and 11, mice were anesthetized with ketamine/xylazine and challenged intranasally with 10 μg of HDM in a total volume of 40 μL of PBS. On day 14, mice were killed, and the lungs and caudal mediastinal lymph nodes (cM-LNs) were collected. Bronchoalveolar lavage was performed with 3×1 mL EDTA-containing PBS, and eosinophil infiltration was analyzed by using fluorescence-activated cell sorting after staining with phycoerythrin-conjugated anti–Siglec-F (BD PharMingen, San Diego, Calif). cM-LN cells were restimulated with 15 μg/mL HDM for 96 hours in 24-well plates at 1×10^6 cells/well, and cytokine production was measured in the supernatants with BD OptEIA ELISA kits.

RESULTS

Blockade of LPS-triggered T_H1-polarizing cytokine production on moDCs differentiated in the presence of IL-4

To explore whether IL-4 has a role in blocking the production of T_H1-polarizing cytokines, we analyzed the responsiveness to LPS of moDCs differentiated in the presence of IL-4. Culture of bone marrow Ly-6C^{high} monocytes with GM-CSF for 24 hours led to their differentiation into CD11c$^+$/MHCII$^+$ C-moDCs, expressing intermediate levels of the costimulatory molecules CD86 and CD40. Monocyte differentiation with GM-CSF in the presence of IL-4 for 24 hours generated IL-4–moDCs, which expressed higher levels of CD86 and CD40 than C-moDCs (Fig 1, *A*). Compared with C-moDCs, IL-4–moDCs displayed similar forward scatter and side scatter values, lower levels of F4/80 and CD11b, and more complex dendritic-like cytoplasmic

extensions than C-moDCs (see Fig E1 in this article's Online Repository at www.jacionline.org). After LPS stimulation, C-moDCs and IL-4–moDCs upregulated CD86 and CD40 similarly (Fig 1, *A*) but differed in their capacity to produce T_H1-polarizing/inflammatory cytokines. The production of the T_H1-polarizing cytokine IL-12 and the proinflammatory cytokines IL-1β, IL-6, and TNF-α was strongly reduced in IL-4–moDCs compared with C-moDCs (Fig 1, *B*). Nitric oxide (NO) production, which is crucial for defense against intracellular pathogens, was also notably reduced in LPS-stimulated IL-4–moDCs (Fig 1, *D*). Similar results were obtained after stimulation of IL-4–moDCs with the TLR9 ligand CpG (see Fig E2 in this article's Online Repository at www.jacionline.org) and with moDCs differentiated with GM-CSF in the presence of IL-13 (see Fig E3 in this article's Online Repository at www.jacionline.org).

To ascertain whether this was due to a blockade at the transcriptional level, we analyzed the expression of the mRNAs specific for these cytokines using qPCR 1 and 5 hours after LPS stimulation. The mRNAs for the 2 IL-12 chains (IL-12/p35 and IL-12/p40), IL-1β, IL-6, and TNF-α were expressed at significantly lower levels in IL-4–moDCs (Fig 1, *C*). Correspondingly, mRNA for the enzyme inducible nitric oxide synthase (iNOS), which catalyzes the synthesis of NO, was also expressed at lower levels in IL-4–moDCs (Fig 1, *E*). Interestingly, expression of the anti-inflammatory cytokine IL-10 was completely inhibited in IL-4–moDCs at both the protein and mRNA levels (see Fig E4 in this article's Online Repository at www.jacionline.org).

According to our flow cytometry data, no reduction was observed in mRNA expression for CD86 and CD40 by LPS-stimulated IL-4–moDCs (Fig 1, *F*). Analysis using microarrays of the gene expression profile of 5-hour LPS-stimulated IL-4–moDCs and C-moDCs confirmed our qPCR data on mRNA expression for IL-12, IL-1β, IL-6, TNF-α, and iNOS (Table I). In line with these data, the expression of mRNA for other inflammatory cytokines (IL-1α, IL-18, IL-23, GM-CSF, and neurotensin) and chemokines (CXCL1 and CCL7) was also reduced in LPS-stimulated IL-4–moDCs. Therefore the presence of IL-4 during GM-CSF–driven differentiation of monocytes altered the responsiveness to LPS of IL-4–moDCs, which showed a notably reduced capacity to produce T_H1-polarizing/inflammatory cytokines but retained the ability to upregulate costimulatory molecules.

Engagement of IL-4 receptor activates a Stat6-dependent signaling, leading to the transcription of IL-4– and IL-13–responsive genes and to a Stat6-independent, insulin-receptor substrate (IRS)-1/2–dependent pathway, leading to phosphoinositide 3-kinase and extracellular signal-regulated kinase activation.[6] The blockade of LPS-induced T_H1-polarizing/inflammatory cytokines observed in IL-4–moDCs was dependent on Stat6 signaling because no defect in cytokine production was detected in IL-4–moDCs from *Stat6*$^{-/-}$ mice (see Fig E5 in this article's Online Repository at www.jacionline.org).

IL-4 has been reported to have a synergistic effect with LPS on IL-12 production when IL-4 and LPS acted simultaneously on differentiated bone marrow–derived dendritic cells (BMDCs).[7,8] Accordingly, C-moDCs produced higher amounts of IL-12 when stimulated simultaneously with LPS and IL-4 than after stimulation with LPS alone (see Fig E6 in this article's Online Repository at www.jacionline.org). Thus IL-4 exerted opposite effects on IL-12 production when acting during DC differentiation or stimulation. Interestingly, IL-4 treatment did not block IL-12 production when performed on already differentiated moDCs before LPS stimulation (see Fig E7 in this article's Online Repository at www.jacionline.org).

Reversal of T_H1-polarizing/inflammatory cytokine production blockade in LPS-stimulated IL-4–moDCs by histone deacetylase inhibitors

Histone deacetylase (HDAC)–containing corepressor complexes, such as nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) complexes, are recruited to the promoters of inflammation-associated genes that are regulated by the transcription factors nuclear factor κB (NFκB) and activator protein 1 (AP-1).[9] TLR activation triggers the release of these corepressor complexes, allowing the transcription of their target genes. Because IL-4–moDCs were blocked in their capacity to produce T_H1-polarizing/inflammatory cytokines that are regulated by the transcription factors NFκB and AP-1 we hypothesized that IL-4 could exert an inhibitory function on the responsiveness of IL-4–moDCs to LPS by preventing the release of corepressor complexes from the promoter regions of genes encoding T_H1-polarizing/inflammatory cytokines. Because the retention of corepressor complexes would lead to a process of HDAC-mediated histone deacetylation of these promoters and consequently to their transcriptional repression,[10] we tested whether the inhibition of mRNA expression for T_H1-polarizing/inflammatory cytokines in LPS-stimulated IL-4–moDCs could be reverted by treatment with the HDAC inhibitor trichostatin A (TSA). In IL-4–moDCs differentiated in the presence of TSA and stimulated by LPS, mRNAs for T_H1-polarizing/inflammatory molecules were transcribed at levels comparable with (for IL-12/p40, IL-6, and iNOS) or even higher than (for IL-12/p35 and IL-1β) those observed for LPS-stimulated C-moDCs, whereas TNF-α mRNA inhibition was only partially reverted (Fig 2, *A* and *B*).

These data suggest that in IL-4–moDCs inhibition of the transcription of the IL-12/p35, IL-12/p40, IL-1β, IL-6, TNF-α, and iNOS genes resulted from a process of deacetylation in the promoter region of these genes mediated by the action of HDACs. TSA-mediated reversal of T_H1-polarizing/inflammatory cytokine mRNA expression by IL-4–moDCs was paralleled by the reversal of the inhibition of the production at the protein level of IL-12, IL-1β, and IL-6 (Fig 2, *C*). TSA partially reverted TNF-α production at the protein level. Similar results were obtained when IL-4–moDCs were differentiated in the presence of valproic acid, another HDAC inhibitor (see Fig E8 in this article's Online Repository at www.jacionline.org).

Whereas TSA treatment did not affect the differentiation of IL-4–moDCs, C-moDC differentiation was severely impaired at TSA concentrations greater than 6 nmol/L (see Fig E9 in this article's Online Repository at www.jacionline.org), which is in line with previous data describing that BMDC differentiation was blocked by TSA-mediated inhibition of STAT5 target genes.[11] Consequently, the effect of TSA on LPS-induced cytokine production by C-moDCs cannot be addressed.

Analysis of histone acetylation of T_H1-polarizing/inflammatory cytokine genes in LPS-stimulated IL-4–moDCs

To confirm that T_H1-polarizing/inflammatory cytokine production blockade in LPS-stimulated IL-4–moDCs involved a

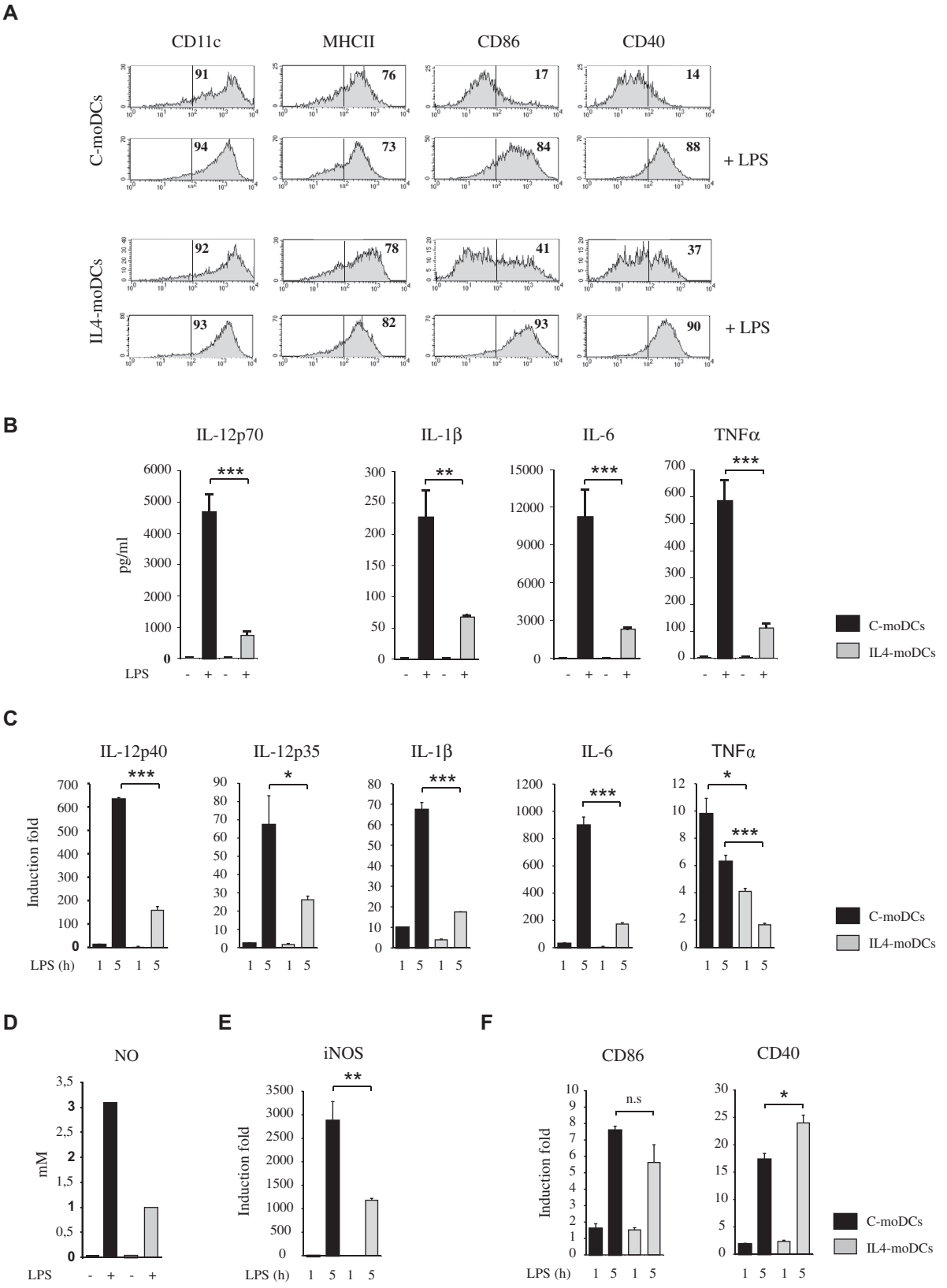

FIG 1. Analysis of the responsiveness of IL-4–moDCs to LPS stimulation. **A,** Phenotypic characterization of unstimulated or LPS-stimulated C-moDCs and IL-4–moDCs analyzed by using flow cytometry. The percentage of cells positive for CD11c, MHCII, CD86, or CD40 (cells with a fluorescence intensity over that seen with control staining, which is defined by a *vertical line*) is indicated. **B,** Production of the indicated cytokines by C-moDCs or IL-4–moDCs 16 hours after LPS stimulation analyzed by means of ELISA. Data are expressed as

TABLE I. Genes with a higher expression in LPS-stimulated C-moDCs than in LPS-stimulated IL-4–moDCs

Gene	Description	Fold change
Il10	Interleukin 10	23.08
Il1a	Interleukin 1 alpha	10.39
Plat	Plasminogen activator, tissue	7.19
Il1b	Interleukin 1 beta	5.67
Saa3	Serum amyloid A3	5.19
Ptx3	Pentraxin-related gene	5.03
Il12b	Interleukin 12b	4.88
Il17rd	Interleukin 17 receptor D	4.44
Cxcl1	Chemokine (C-X-C motif) ligand 1	4.26
Nts	Neurotensin	4.21
Kdr	Kinase insert domain protein receptor	4.08
TNF	Tumor necrosis factor	3.98
Ifng	Interferon gamma	3.26
Dusp16	Dual-specificity phosphatase 16	3.22
Il12a	Interleukin 12a	3.18
Il6	Interleukin 6	3.08
Nupr1	Nuclear protein 1	2.84
Serping1	Serine (or cysteine) peptidase inhibitor, clade G, member 1	2.84
Il23	Interleukin 23	2.83
Btla	B and T lymphocyte associated	2.79
Pdzk1ip1	PDZK1-interacting protein 1	2.78
Cdc42ep1	CDC42 effector protein/Rho GTPase binding 1	2.78
Sorbs1	Sorbin and SH3 domain containing 1	2.70
Il18	Interleukin 18	2.69
Niacr1	Niacin receptor 1	2.66
Ccl7	Chemokine (C-C motif) ligand 7	2.61
Csf2	Colony-stimulating factor 2 (granulocyte-macrophage)	2.60
Gstt2	Glutathione-S-transferase, theta 2	2.57
Tnfsf9	Tumor necrosis factor (ligand) superfamily, member 9	2.52
B3gnt5	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 5	2.51
Irf8	Interferon regulatory factor 8	2.47
Rgag4	Retrotransposon gag domain containing 4	2.47
Rsad2	Radical S-adenosyl methionine domain containing 2	2.46
Socs3	Suppressor of cytokine signaling 3	2.34
Nos2	Nitric oxide synthase 2	2.25

The gene expression profiles of 5-hour LPS-stimulated C-moDCs and LPS-stimulated IL-4–moDCs were compared by using Whole Mouse Genome Microarrays from Agilent Technologies.

process of histone deacetylation, we analyzed the level of acetylation of histone H3 at the promoter regions of these genes by using ChIP. Our results revealed that for all the genes analyzed (*Il12a, Il12b, Il1b, TNFa,* and *Nos2*), LPS stimulation led to a significant increase in the level of H3 acetylation of their promoter regions in C-moDCs, but not in IL-4–moDCs, and therefore the H3 acetylation level was significantly lower in LPS-stimulated IL-4–moDCs (Fig 3, *A*).

Involvement of the nuclear receptors LXR and peroxisome proliferator-activated receptor γ on the blockade of T_H1-polarizing/inflammatory cytokine production by LPS-stimulated IL-4–moDCs

In the presence of their specific ligands, the nuclear receptors liver X receptor (LXR) and peroxisome proliferator-activated receptor γ (PPARγ) have been described to exert a function of transrepression of proinflammatory genes by binding to NCoR and SMRT corepressor complexes, a process that inhibits LPS-induced clearance of these corepressors.[12] Therefore we sought to explore whether LXR or PPARγ nuclear receptors were involved in the blockade of T_H1-polarizing/inflammatory gene transcription affecting LPS-stimulated IL-4–moDCs. For this purpose, C-moDCs and IL-4–moDCs were generated from LXR-deficient mice or LysM-Cre-PPARγ–deficient mice and analyzed for their responsiveness to LPS stimulation. Our data revealed that neither LXR nor PPARγ deficiency reverted the blockade of LPS-induced cytokine production in IL-4–moDCs (Fig 3, *B* and *C*), suggesting that neither of these nuclear receptors were involved in this process.

Gene expression profiling of IL-4–moDCs versus C-moDCs

Our results demonstrate that IL-4 resulted in a strong blockade of T_H1-polarizing cytokine production by IL-4–moDCs after TLR engagement, a process that, as discussed above, appears to be crucial for the induction of T_H2 responses. Therefore during infections by helminths or allergic reactions, the *de novo* differentiation of DCs in the presence of IL-4 would lead to the generation of DCs endowed with the ability to efficiently polarize T_H2 responses. In this regard microarray analyses were performed to compare the gene expression profiles of unstimulated IL-4–moDCs and C-moDCs by using Whole Mouse Genome microarrays from Agilent Technologies (Santa Clara, Calif; Table II) to explore in depth the physiologic properties of IL-4–moDCs and the functional relevance of their altered responsiveness to LPS stimulation. Our data revealed that IL-4–moDCs overexpressed a number of genes, such as those encoding members of the Ear family (Ear1, Ear2, Ear10, Ear11, and Ear12); chemokines acting as chemoattractants for eosinophils, basophils, neutrophils, and monocytes (Ccl2, Ccl8, Ccl24, Cxcl3, and Cxcl5); and M2 markers, such as chitinases (Chi3l3/Ym1, Chi3l4/Ym2), Fizz 1 (Retnla), and arginase 1 (Arg1), which are highly induced during T_H2 responses triggered by helminth infections or allergic reactions. In line with these observations, BMDCs treated with IL-4 have also been reported to upregulate Ym1/2 and Fizz 1 but not Arg1.[8] Finally, IL-4–moDCs displayed a higher expression of mRNA specific for molecules related to macrophage alternative activation (eg, PPARγ and CD36) and for molecules involved in retinoic acid metabolism (eg, RBP4 and Cyp1b1) that have been reported to regulate T_H2 responses.[13]

means ± SDs of duplicates. **C, E,** and **F,** Expression of mRNA for the indicated molecules by C-moDCs or IL-4–moDCs 1 and 5 hours after LPS stimulation analyzed by using qRT-PCR and normalized to β-actin. Data are expressed as induction fold relative to unstimulated C-moDCs (means ± SDs of triplicates). **D,** Production of NO by C-moDCs and IL-4–moDCs 16 hours after LPS stimulation analyzed by using the Griess reaction. Significant differences, as determined by using the unpaired *t* test, are indicated as follows: *n.s.*, not significant; $*P < .05$; $**P < .01$; $***P < .001$. Data are representative of 4 (Fig 1, *A* and *B*), 3 (Fig 1, *C, E,* and *F*), and 2 (Fig 1, *D*) independent experiments with similar results.

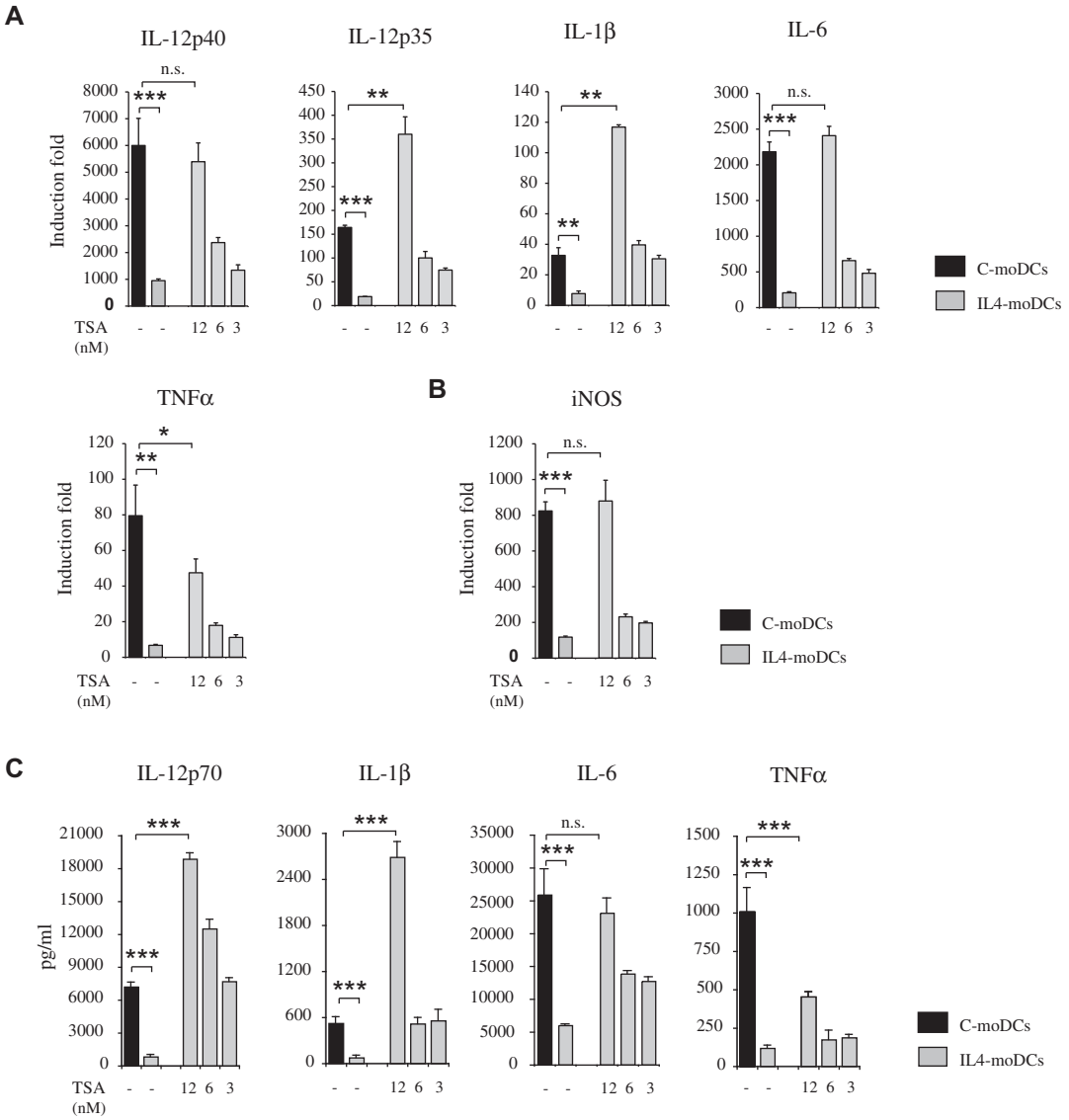

FIG 2. Reversal of T_H1-polarizing/inflammatory cytokine production blockade on LPS-stimulated IL-4–moDCs by the HDAC inhibitor TSA. **A** and **B,** Expression of mRNA for the indicated molecules by untreated C-moDCs or IL-4–moDCs untreated or treated with TSA analyzed after 5-hour LPS stimulation by means of qRT-PCR and normalized to β-actin. Data are expressed as induction fold relative to unstimulated C-moDCs (means ± SDs of triplicates). **C,** Production of the indicated cytokines by untreated C-moDCs or IL-4–moDCs untreated or treated with TSA at the indicated concentrations and analyzed 16 hours after LPS stimulation by means of ELISA. Data are expressed as means ± SDs of duplicates. Significant differences, as determined by using the unpaired *t* test, are indicated as follows: *n.s.*, not significant; $*P < .05$; $**P < .01$; $***P < .001$. Data are representative of at least 3 independent experiments with similar results.

Responsiveness to LPS of IL-4–moDCs versus AAMΦs

Our microarray data revealed that IL-4–moDCs expressed prototypical M2 genes that characterize AAMΦs. Because macrophage alternative activation is triggered by IL-4 receptor engagement, we sought to confirm whether IL-4 signaling also leads to blockade of T_H1-polarizing/inflammatory cytokine production after LPS stimulation in AAMΦs. AAMΦs generated from BMMΦs, as described in Fig 4, *A*, according to previous reports,[14] were stimulated with LPS and analyzed for cytokine production. In contrast to IL-4–moDCs, IL-12, IL-6, and TNF-α production by LPS-stimulated AAMΦs was not reduced but increased with regard to that seen in LPS-stimulated BMMΦs (Fig 4, *B*). moMΦs were generated with M-CSF in the absence (C-moMΦs) or presence of IL-4 (IL-4–conditioned moMΦs [IL-4–moMΦs]) to further explore the effect of IL-4 on the responsiveness of macrophages to LPS stimulation, as described in Fig 4, *C*. In contrast to IL-4–moDCs, IL-4–moMΦs produced higher amounts of IL-12 and IL-6 than C-moMΦs in response to LPS, whereas TNF-α levels were reduced approximately 40% (Fig 4, *D*). These data confirmed that IL-4 differentially affects moDCs and macrophages regarding their responsiveness to LPS and consequently their ability to produce T_H1-polarizing/inflammatory mediators.

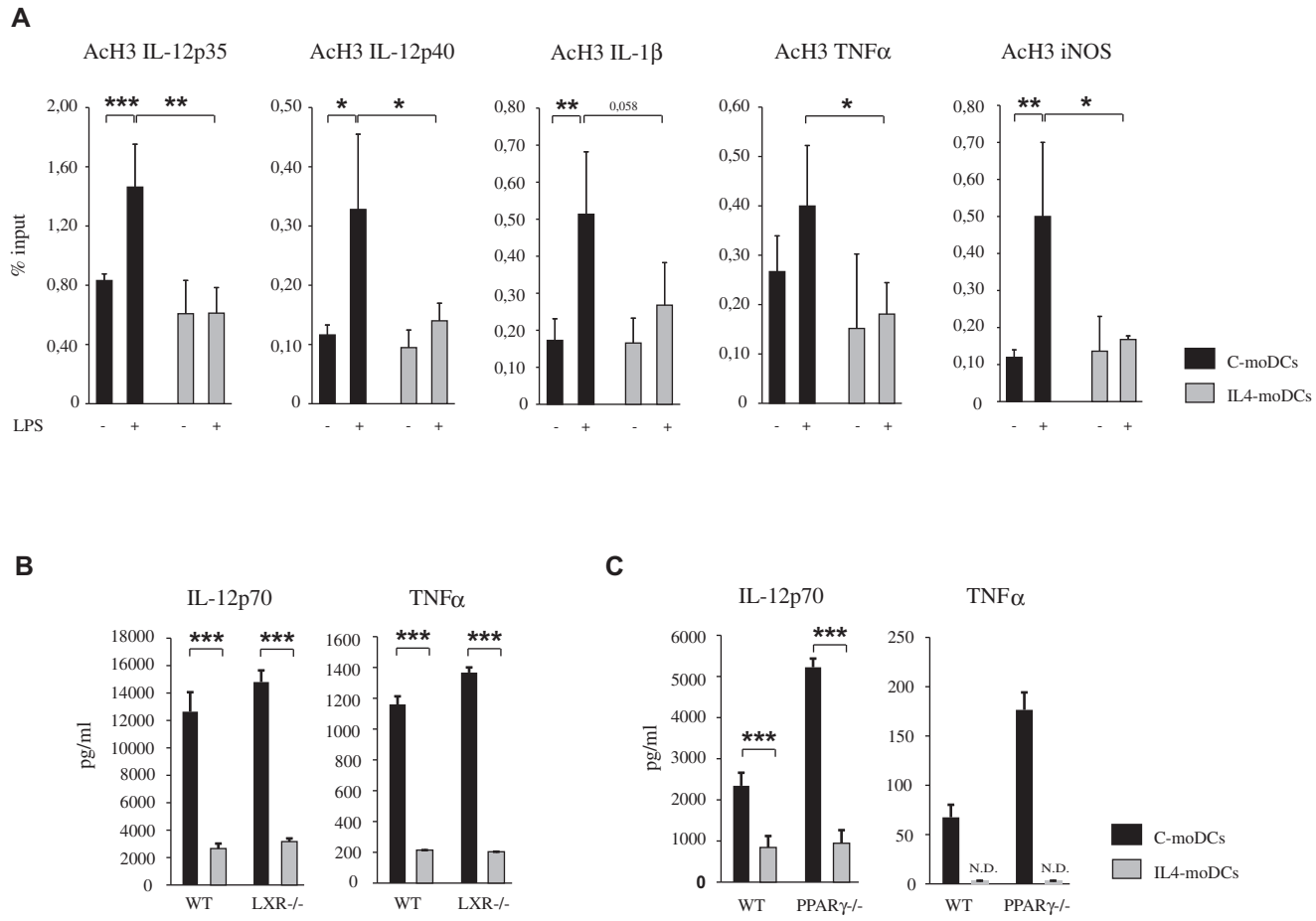

FIG 3. Analysis of the process of T_H1-polarizing/inflammatory cytokine blockade. **A,** Analysis of histone H3 deacetylation of the promoter regions of indicated genes in unstimulated or 5-hour LPS-stimulated C-moDCs and IL-4–moDCs analyzed by using ChIP and qPCR. **B,** Production of the indicated cytokines by C-moDCs or IL-4–moDCs from wild-type *(WT)* and LXR-deficient *(LXR−/−)* mice analyzed 16 hours after LPS stimulation by means of ELISA. **C,** Production of the indicated cytokines by C-moDCs or IL-4–moDCs from WT and LysM-Cre-PPARγ–deficient *(PPARγ−/−)* mice analyzed 16 hours after LPS stimulation by means of ELISA. Data are expressed as means ± SDs of duplicates. Significant differences, as determined by using the unpaired *t* test, are indicated as follows: **P* < .05, ****P* < .001. *N.D.*, Not detectable. Data are representative of 4 (Fig 3, *A*), 3 (Fig 3, *B*), and 2 (Fig 3, *C*) independent experiments with similar results.

IL-4–mediated licensing of DCs for the induction of T_H2 responses *in vivo* during allergic airway inflammation

C-moDCs and IL-4–moDCs were analyzed for their capacity to induce a T_H2 response during allergic airway inflammation caused by HDM allergens to test the potential of IL-4 to license DCs for the polarization of T_H2 responses *in vivo*. For this purpose, HDM-pulsed C-moDCs or IL-4–moDCs were injected intraperitoneally into C57BL/6 mice that were subsequently challenged intranasally with HDM according to the protocol described in Fig 5, *A* and *E*. We previously confirmed that both C-moDCs and IL-4–moDCs injected intraperitoneally had a comparable capacity to induce the proliferation of antigen-specific $CD4^+$ T cells in the cM-LNs (see Fig E10 in this article's Online Repository at www.jacionline.org). Analysis of bronchoalveolar lavage fluid at day 14 after transfer of either HDM-pulsed IL-4–moDCs or C-moDCs revealed strong airway inflammation characterized by potent eosinophil infiltration of the bronchoalveolar space that was approximately 20% greater in mice immunized with IL-4–moDCs (64.31% vs 79.87%, n = 4, $P < .05$; Fig 5, *B*, *C*, *F*, and *G*). Interestingly, IL-4–moDCs induced an effective anti-HDM T_H2-polarized response in the cM-LNs characterized by a robust secretion of IL-4 and IL-5, paralleled by low levels of IFN-γ, compared with those characterizing a T_H1-polarized lung immune response induced by LPS-stimulated moDCs (see Fig E11 in this article's Online Repository at www.jacionline.org). In contrast, C-moDCs were weak inducers of anti-HDM T_H2 responses and consequently promoted the production of lower levels of IL-4 and IL-5 but also IFN-γ (Fig 5, *D* and *H*). These data reflect that IL-4 had the potential to license moDCs for the induction of an effective T_H2 response during allergic airway inflammation.

DISCUSSION

The present study has been undertaken with the aim of exploring whether the cytokine IL-4 has the ability to license

TABLE II. Genes with a higher expression in IL-4–moDCs than in C-moDCs

Gene	Description	Fold change
RBP4	Retinol binding protein 4, plasma 2	103.84
Ear11	Eosinophil-associated, ribonuclease A family, member 11	70.13
Chi3l3	Chitinase 3-like 3 (Chi3l3)	60.97
Ccl24	Chemokine (C-C motif) ligand 24	48.48
Ear10	Eosinophil-associated, ribonuclease A family, member 10	37.59
Chi3l4	Chitinase 3-like 4 (Chi3l4)	29.41
Retnla	Resistin like alpha	26
CD36	CD36 antigen	23.4
Ear2	Eosinophil-associated, ribonuclease A family, member 2	22.15
Flt1	FMS-like tyrosine kinase 1	21.87
Chst7	Carbohydrate (N-acetylglucosamino) sulfotransferase 7	14.65
Bex6	Brain expressed gene 6	14.62
Arg1	Arginase, liver	14.5
Ear12	Eosinophil-associated, ribonuclease A family, member 12	11.53
Atp6v0d2	ATPase, H+ transporting, lysosomal V0 subunit D2	11.29
Vcam1	Vascular cell adhesion molecule 1	11.22
Cyp1b1	Cytochrome P450, family 1, subfamily b, polipeptide 1	10.46
Ccl8	Chemokine (C-C motif) ligand 8	10.03
Gfi1b	Growth factor independent 1B	9.23
Cxcl5	Chemokine (C-X-C motif) ligand 5	8.73
Arhgap6	Rho GTPase activating protein 6	8.41
Pparg	Peroxisome proliferator activated receptor gamma	8.07
Lpl	Lipoprotein lipase	7.66
Igf1	Insulin-like growth factor 1	7.47
Edn1	Endothelin 1	7.45
Cxcl3	Chemokine (C-X-C motif) ligand 3	7.04
Pbpb	Pro-platelet basic protein	7.01
Cttn	Cortactin	6.78
Ear1	Eosinophil-associated, ribonuclease A family	6.48
Rras2	Related RAS viral (r-ras) oncogene homolog 2	6.39
Ccl2	Chemokine (C-C motif) ligand 2	6.34
Tmtc2	Transmembrane and tetratricopeptide repeat containing 2	6.19
Soat2	Sterol-acyltranferase 2	6.01

The gene expression profiles of IL-4–moDCs and C-moDCs (after 24-hour culture with GM-CSF in the presence or absence of IL-4, respectively) were compared by using Whole Mouse Genome Microarrays from Agilent Technologies.

DCs for the induction of T_H2-polarized immune responses. IL-4, which is produced by basophils and mast cells during the first phases of defense against helminth infection or allergic reactions, has a crucial role on T_H2 responses by inducing in $CD4^+$ T cells the expression of the transcription factor GATA3 and also by modulating the function of different cell types in the context of T_H2 immunity, such as B cells, epithelial cells, and muscle cells.[4] In addition, IL-4 triggers the alternative activation of macrophages, a process dependent on the nuclear receptor PPARγ, which drives the generation of the specialized subset of AAMΦs.[15] AAMΦs play a crucial role during T_H2 responses by promoting the recruitment and activation of eosinophils, basophils, mast cells, and neutrophils and by fulfilling essential scavenging and tissue repair functions.[16]

The data presented in the present report have unveiled a new role for IL-4 by showing that IL-4 has the ability to modulate the function of moDCs for the induction of T_H2 responses by acting during their differentiation. Our results revealed that the presence of IL-4 during *in vitro* moDC differentiation deeply influenced the functional properties of the resulting IL-4–moDCs, leading to blockade of their potential to produce T_H1-polarizing and inflammatory cytokines in response to TLR ligands, such as LPS or CpG. In addition, in IL-4–moDCs IL-4 induced the upregulation of genes involved in the recruitment of cells playing a key role in T_H2 responses (eg, eosinophils, basophils, and monocytes), and IL-4 promoted the licensing of IL-4–moDCs for the induction of T_H2-polarized $CD4^+$ T-cell responses during *in vivo* allergic airway inflammation reactions induced by HDM allergens. Interestingly, the generation of human moDCs for the induction of tumor-specific T_H1-polarized cytotoxic T-lymphocyte (CTL) responses in DC-based cancer immunotherapeutic trials generally relies on the culture of monocytes with GM-CSF and IL-4, suggesting that IL-4 does not have a blocking effect on the ability of human IL-4–moDCs to produce T_H1-polarizing cytokines in response to activation stimuli. However, to our knowledge, human moDCs differentiated with GM-CSF in the absence or presence of IL-4 have not been analyzed for their efficiency to induce T_H1 responses in clinical trials, although interestingly, human moDCs differentiated with GM-CSF in the presence of IL-15 were reported to be significantly more efficient in producing T_H1/inflammatory cytokines and inducing CTL responses *in vitro* than those differentiated in the presence of IL-4.[17] On the basis of these data, it can be hypothesized that IL-4 also reduces T_H1-polarizing cytokine production in human moDCs, although they can still induce T_H1-polarized CTL responses in patients after DC-based cancer immunotherapy. This can be explained by the fact that the powerful combination of cytokines and TLR ligands used to activate human IL-4–moDCs for immunotherapeutic purposes counteracts the negative regulatory action of IL-4. In support of this hypothesis, mouse IL-4–moDCs can be induced to produce T_H1/inflammatory cytokines at levels comparable with those triggered by LPS-stimulated C-moDCs when activated by a combination of LPS, IFN-γ, and anti-CD40 antibodies (López-Bravo and Ardavín, unpublished data).

Both IL-4–moDCs and AAMΦs expressed M2 genes and produced chemokines controlling leukocyte recruitment during T_H2 responses. However, our results support that IL-4 did not block T_H1-polarizing/inflammatory cytokine production in response to LPS in AAMΦs or moMΦs, suggesting that IL-4 differentially modulates the function of moDCs and macrophages, which is in line with previous reports demonstrating that DCs and macrophages can differ in the signaling processes triggered after engagement of defined activating receptors.[18,19] Interestingly, whereas macrophage alternative activation is dependent on PPARγ,[14] conditioning of IL-4–moDCs relied on neither PPARγ nor LXR. In contrast, as described for tolerizable genes encoding proinflammatory cytokines during endotoxin tolerance,[10] our results, based on ChIP assays and experiments with HDAC inhibitors, suggest that the blockade of IL-4–moDCs to produce T_H1-polarizing/inflammatory cytokines in response to LPS was the consequence of the hypoacetylation of the promoters of the genes encoding these cytokines. Most likely, this phenomenon resulted from an IL-4–mediated process, preventing the release from these promoters of HDAC-containing corepressors, such as NCoR and SMRT,

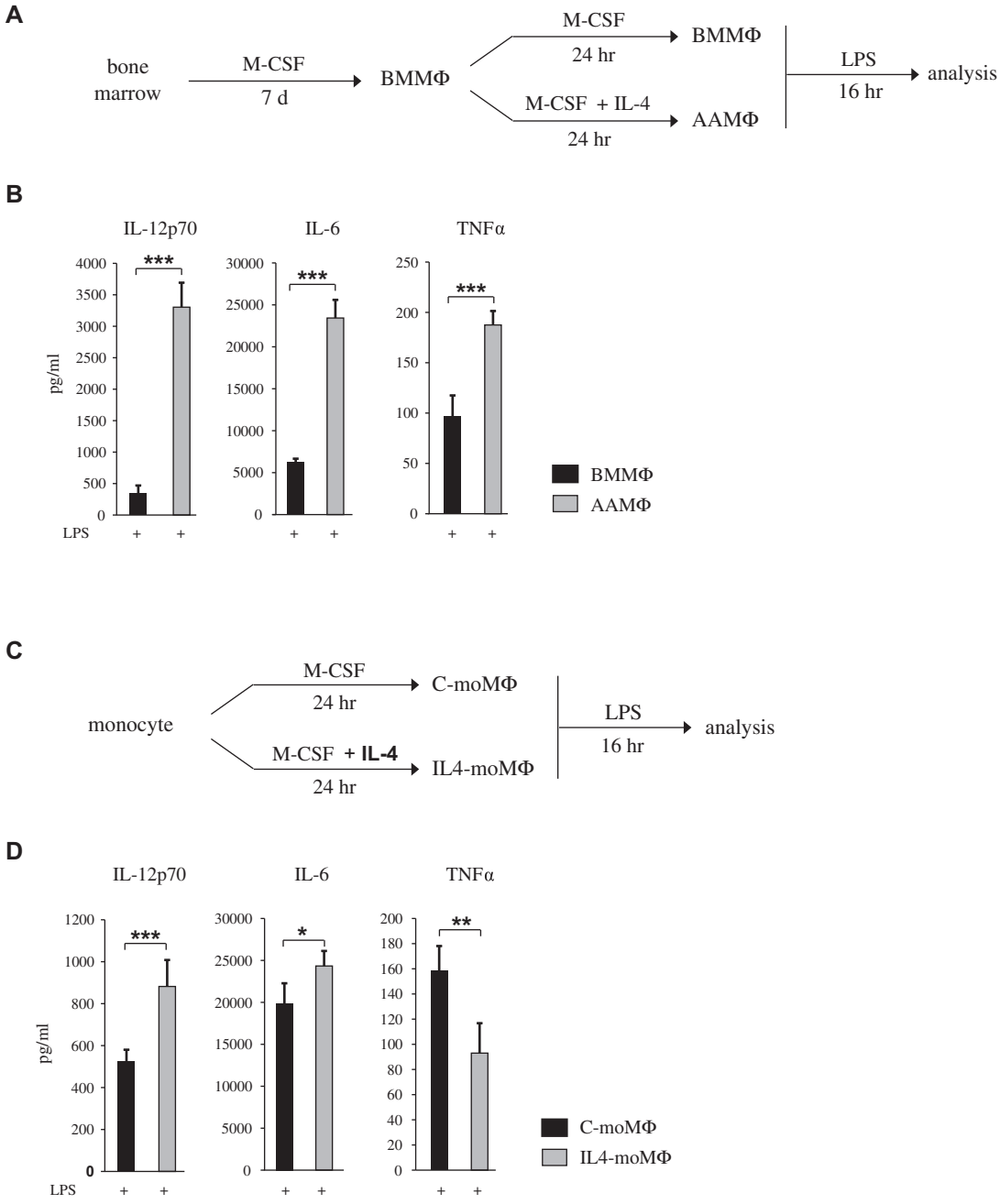

FIG 4. Analysis of the responsiveness of AAMΦs and IL-4–moMΦs to LPS stimulation. **A,** Protocol for the generation and LPS stimulation of BMMΦs and AAMΦs from bone marrow cell suspensions. **B,** Responsiveness of BMMΦs versus AAMΦs to LPS stimulation. Production of the indicated cytokines by BMMΦs and AAMΦs was analyzed 16 hours after LPS stimulation by means of ELISA. Data are expressed as means ± SDs of duplicates. **C,** Protocol for the generation and LPS stimulation of C-moMΦs and IL-4–moMΦs from bone marrow monocytes. **D,** Responsiveness of C-moMΦs and IL-4–moMΦs to LPS stimulation. The production of the indicated cytokines by C-moMΦs and IL-4–moMΦs was analyzed 16 hours after LPS stimulation by means of ELISA. Data are expressed as means ± SDs of duplicates. Significant differences, as determined by using the unpaired *t* test, are indicated as follows: **P* < .05, ***P* < .01, ****P* < .001. Data are representative of 2 independent experiments with similar results.

which are known to play a crucial role in negative regulation of the transcription of inflammatory genes.[9] The mechanism by which IL-4 promotes hypoacetylation at the promoter regions of T_H1-polarizing/inflammatory genes is currently being investigated in our laboratory. Alternative epigenetic mechanisms could also be involved in the transcriptional repression of these genes. In this regard the trimethylation of H3 on lysine 9 mediated by the histone methylase SUV39H1 has been recently reported to cause the transcriptional silencing of the IFN-γ gene in T_H2-polarized $CD4^+$ T cells,[20] a process that could also participate in the silencing of T_H1-polarizing/inflammatory genes in IL-4–moDCs.

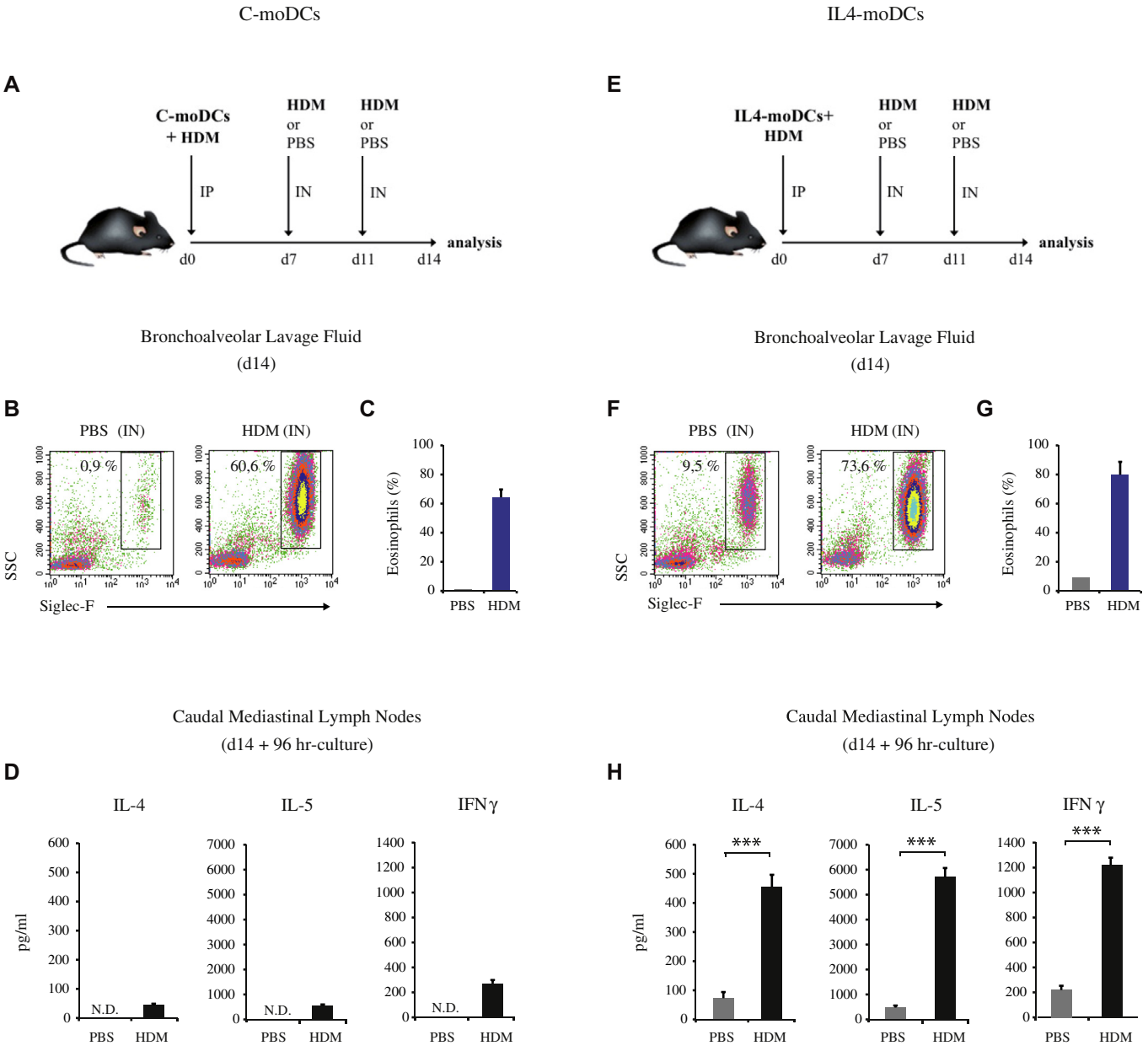

FIG 5. IL-4–mediated licensing of DCs for the induction of T_H2 responses during allergic airway inflammation. **A** and **E**, Protocol for the induction of an allergic response to HDM after intraperitoneal *(IP)* transfer of HDM-loaded C-moDCs and IL-4–moDCs, respectively, followed by intranasal *(IN)* administration of HDM or PBS. **B** and **F**, Flow cytometric analysis of eosinophils (defined by their side scatter vs Siglec-F expression profile) in the bronchoalveolar lavage fluid at day 14 after transfer of HDM-loaded C-moDCs or IL-4–moDCs. The eosinophil percentage is indicated. **C** and **G**, Quantitation of eosinophil infiltration in the bronchoalveolar lavage fluid (means ± SDs of 4 mice per condition) analyzed as described in Fig 5, *B* and *F*. **D** and **H**, Analysis by means of ELISA of the production of the indicated cytokines by cM-LNs at day 14 after culture for 96 hours in the absence or presence of HDM. Data are expressed as means ± SDs of duplicates. Significant differences, as determined by using the unpaired *t* test, are indicated as follows: ***$P < .001$. Data are representative of 4 independent experiments with similar results. *N.D.*, Not detectable.

It can be hypothesized that in the course of helminth infections or allergic reactions, newly formed DCs derived from monocytes or nonmonocytic DC precursors (pre-DCs) could be licensed for the induction of T_H2 responses by IL-4 during their differentiation. In this regard the identity of the DCs responsible for $CD4^+$ T-cell T_H2 polarization under physiologic conditions is still a matter of controversy. Whereas Nakano et al[21] reported that $CD103^+$ conventional dendritic cells (cDCs) were responsible for the induction of allergy to HDM, Plantinga et al[22] found that both $CD11b^+$ cDCs and moDCs were involved in the induction of allergic reactions after administration of high HDM doses, but only $CD11b^+$ cDCs were efficient in promoting allergy at low HDM doses. On the basis of our data, DCs conditioned by IL-4 during their differentiation would be endowed with a reduced

capacity to release T_H1-polarizing/inflammatory mediators in the presence of helminth- or allergen-related TLR ligands. More importantly, IL-4–DCs would not produce T_H1-polarizing cytokines, such as IL-12, a process that, as mentioned above, appears to be of particular relevance in relation to the functional specialization of T_H2-DCs, as demonstrated during *in vivo* infection by T_H2-polarizing pathogens[23-25] and during allergic reactions.[26-28] Finally, IL-4–DCs would promote the recruitment of eosinophils, basophils, and monocytes during T_H2 responses triggered by helminths or allergens and, more importantly, would be licensed to polarize T_H2 $CD4^+$ T cells. In conclusion, the data presented in this report suggest that IL-4 produced during helminth infections or allergic reactions fulfills an important regulatory function on DCs contributing to their conditioning for the induction of T_H2 polarization.

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Key messages

- **IL-4 blocks T_H1-polarizing/inflammatory cytokine gene expression on moDCs through the deacetylation of the promoters of these genes through a mechanism independent on the nuclear receptors PPARγ or LXR.**
- **IL-4 induces the upregulation on moDCs of T_H2-related genes, such as Ears, eosinophil/basophil chemokines, and M2 genes.**
- **IL-4 licenses moDCs for the induction of T_H2-polarized immune responses *in vivo* during allergic airway inflammation reactions induced by HDM allergens.**

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METHODS

Monocyte isolation and *in vitro* moDC and moMΦ differentiation

Monocytes were isolated from lysis buffer–treated bone marrow cell suspensions by using immunomagnetic depletion of T cells, B cells, DCs, and granulocyte natural killer cells with anti-rat immunoglobulin-coated magnetic beads (Dynal-Invitrogen, Karlsruhe, Germany) at a 7:1 bead-to-cell ratio after incubation with the mAbs anti–Thy-1.2, B220, MHC-II, CD43, and CD24. After immunomagnetic depletion, monocyte preparations had a purity of greater than 95%. Differentiation of C-moDCs or IL-4–moDCs was achieved by culturing monocytes in 24-well plates (or in nontreated, cultured 60-mm Petri dishes for ChIP assays) in RPMI supplemented with 10% FCS and 20 ng/mL GM-CSF (PeproTech) for 24 hours at 37°C and 5% CO_2 in the absence or presence of 20 ng/mL IL-4 (PeproTech); these cultures contained approximately 90% moDCs. For qPCR analyses, and experiments involving the use of HDAC inhibitors, C-moDCs or IL-4–moDCs were further purified by using immunomagnetic positive selection after incubation with biotin-conjugated anti-CD11c and streptavidin-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany); C-moDC and IL-4–moDC preparations had a purity of greater than 97%. Phenotypic analysis of C-moDC or IL-4–moDC cultures was performed by using flow cytometry after quadruple immunofluorescence staining with fluorescein-conjugated anti–MHC class II, phycoerythrin-conjugated anti-CD86, allophycocyanin-conjugated anti-CD11c, and biotin-conjugated anti-CD40, anti-F4/80, or anti-CD11b antibodies, followed by Streptavidin-Peridinin Chlorophyll-a (PerCP; BD PharMingen). Data were acquired on a FACSCalibur flow cytometer (BD Biosciences). When indicated, C-moDC or IL-4–moDC cultures were performed in the presence of the HDAC inhibitors TSA or valproic acid (Sigma, St Louis, Mo), which where added 30 minutes before IL-4. Differentiation of moMΦs was achieved by culturing monocytes in 24-well plates in RPMI medium supplemented with 10% FCS and 20 ng/mL M-CSF (PeproTech) for 24 hours at 37°C and 5% CO_2. Activation of moDC or moMΦ cultures was achieved after treatment with 1 μg/mL LPS from *Escherichia coli* (Sigma) or 1 mmol/L CpG ODN1826 (InvivoGen, San Diego, Calif) for the indicated times in 24-well plates at 2×10^5 cells per well.

Confocal microscopy

Confocal microscopy studies were performed on C-moDCs or IL-4–moDCs cultured on 12-mm cover slips in 24-well plates, fixed with 2% formaldehyde, permeabilized with Triton X-100, and stained with rat anti–MHC II, followed by Alexa Fluor 488–conjugated anti-rat IgG (Molecular Probes, Eugene, Ore). Nuclei were stained with the DNA binding fluorescent dye 4′-6-diamidino-2-phenylindole dihydrochloride. Images were acquired with a Zeiss LSM-510 META microscope (Carl Zeiss, Jena, Germany).

ELISA

Production of cytokines and NO was analyzed 16 hours after stimulation of C-moDCs or IL-4–moDCs with 1 mg/mL LPS or 1 mmol/L CpG ODN1826. Cytokines were analyzed with BD OptEIA ELISA kits (BD Biosciences). NO was analyzed by using the Griess reaction, measuring nitrite concentration with a nitrate/nitrite Colorimetric Assay Kit (Fluka, Buchs, Switzerland).

RNA extraction and qPCR

RNA was extracted with the High Pure RNA Isolation kit (Roche, Mannheim, Germany) and retrotranscribed by using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, Calif). qPCR was performed with a FluoCycle SYBR Green mix (EuroClone, Milano, Italy) on an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Primer sequences are listed in Table E1.

ChIP

Unstimulated or 5-hour LPS-stimulated moDCs (2×10^6) were fixed with 1% formaldehyde for 10 minutes at 37°C, washed twice with 6 mL of cold PBS supplemented with 1 mmol/L of the serine protease inhibitor phenylmethylsulfonyl fluoride and 6 μL of the protease inhibitor cocktail (Sigma), and treated with 200 μL of 1% SDS lysis buffer supplemented with 1 mmol/L phenylmethylsulfonyl fluoride and 0.2 μL of protease inhibitor cocktail (Sigma) for 10 minutes at 4°C. Lysates were sonicated for 20 minutes (30 seconds on/30 seconds off) at high intensity to obtain fragments ranging from 200 to 700 bp in size by using a Bioruptor Standard water bath sonicator (Diagenode, Liège, Belgium). Sonicates were diluted 10× and incubated with antibodies against acetyl-Histone H3 (Usptate Biotechnology, Lake Placid, NY) with overnight rotation. Protein A/G PLUS-Agarose Immunoprecipitation beads (Santa Cruz Biotechnology, Santa Cruz, Calif) were added for 3 hours, and collected beads were washed extensively. Protein-DNA complexes were eluted from the beads and treated with 200 mmol/L NaCl to reverse cross-links and proteinase K to digest proteins. Recovered DNA was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Immunoprecipitated DNA and input DNA were amplified by means of qPCR with primers specific for the promoter regions of the analyzed cytokines with input DNA to generate a standard curve. ChIP data are represented as a percentage of input.

Microarrays

RNA was extracted with the RNeasy Mini Kit (Qiagen); amplified by using the MessageAmp II aRNA amplification kit from Ambion, Life Technologies (Paisley, United Kingdom); labeled with Cy3 or Hy5 by using the SuperScript indirect cDNA labeling system (Invitrogen, Life Technologies); and hybridized at 65°C for 17 hours to a Mouse Whole Genome Microarray 4 × 44K (Agilent Technologies), according to the manufacturer's instructions. In all cases 2-channel hybridizations were performed with Cy3 and Hy5, and 2 independent replicates were analyzed. Slides corresponding to the analysis of 5-hour LPS-stimulated C-moDCs versus 5-hour LPS-stimulated IL-4–moDCs were scanned with a GenePix 4000B (Axon Instruments, Foster City, Calif), and spots were quantified with GenePix Pro 5.1 software (Axon Instruments). Slides corresponding to the analysis of IL-4–moDCs versus C-moDCs were scanned with a DNA Microarray Scanner (Agilent), and spots were quantified by using Feature Extraction Software (Agilent). Background correction and normalization of expression data were performed with LIMMA. Log-ratio values were scaled by using the median absolute value as a scale estimator to produce similar distribution across arrays and to achieve consistency among arrays. Linear model methods were used for determining differentially expressed genes. Each probe was tested for changes in expression over replicates by using empiric Bayes-moderated t statistics. *P* values were corrected by using the Benjamani-Hochberg method to control the false discovery rate. The expected false discovery rate was controlled to be less than 5%. FIESTA viewer (http://bioinfogp.cnb.csic.es/tools/FIESTA) was used to analyze all microarray results and to evaluate the numeric thresholds applied for selecting differentially expressed genes. Hybridizations and statistical analysis were performed by using the Genomics Facility at Centro Nacional de Biotecnologia/CSIC. The entire data set for all microarray experiments is available on the NCBI-GEO database (accession no. GSE39863, http://www.ncbi.nlm.nih.gov/geo/).

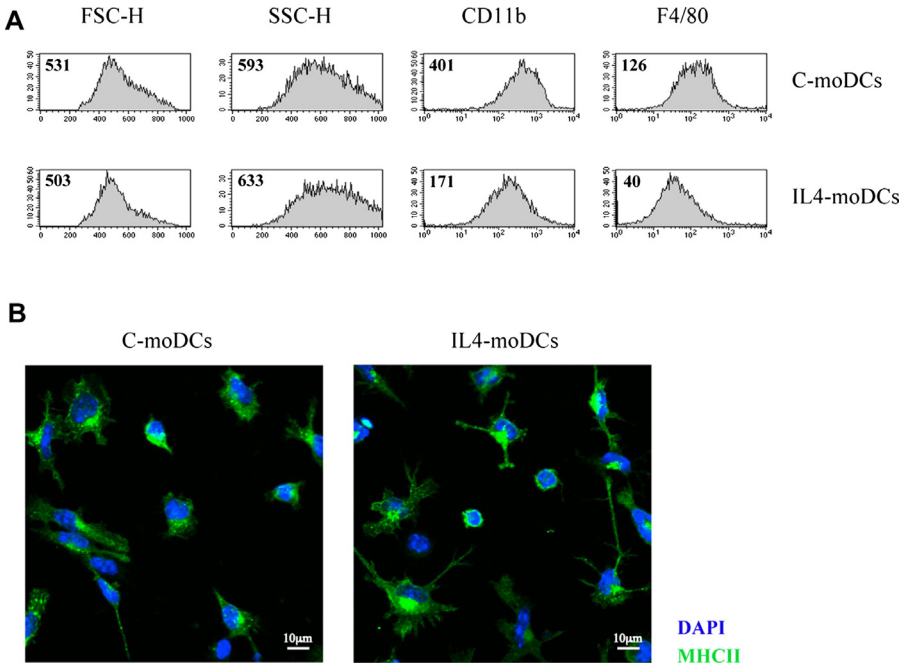

FIG E1. Phenotypic and morphologic characterization of IL-4–moDCs. **A,** Phenotypic characterization of unstimulated C-moDCs and IL-4–moDCs analyzed by means of flow cytometry. The forward scatter *(FSC)* and side scatter *(SSC)* values or mean fluorescence intensity are indicated. **B,** MHC class II expression *(green)* by C-moDCs and IL-4–moDCs analyzed by using confocal microscopy after staining with anti–MHC II/Alexa Fluor 488 *(green)*; nuclei were stained with the DNA-binding fluorescent dye 4′-6-diamidino-2-phenylindole dihydrochloride (*DAPI*; *blue*). *Scale bar* = 10 μm.

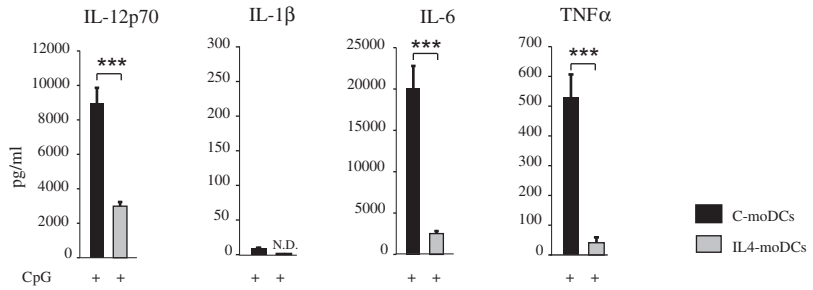

FIG E2. Analysis of the responsiveness of IL-4–moDCs to CpG stimulation. Production of the indicated cytokines by C-moDCs *(black bars)* or IL-4–moDCs *(gray bars)* 16 hours after CpG stimulation analyzed by means of ELISA. Significant differences, as determined by using the unpaired *t* test, are indicated as follows: ***$P < .001$. *N.D.*, Not detectable. Data are expressed as means ± SDs of duplicates and are representative of 2 independent experiments with similar results.

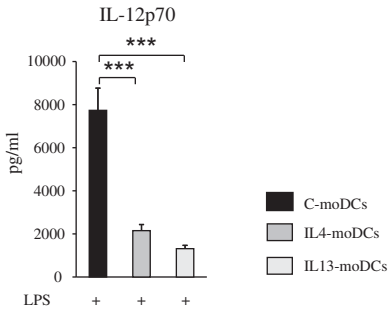

FIG E3. Analysis of the responsiveness of IL-13–moDCs to LPS stimulation. Production of the indicated cytokines by C-moDCs *(black bars)*, IL-4–moDCs *(dark gray bars)*, or IL-13–moDCs *(light gray bars)* 16 hours after LPS stimulation analyzed by means of ELISA. Significant differences, as determined by using the unpaired *t* test, are indicated as follows: ***$P < .001$. Data are expressed as means ± SDs of duplicates and are representative of 2 independent experiments with similar results.

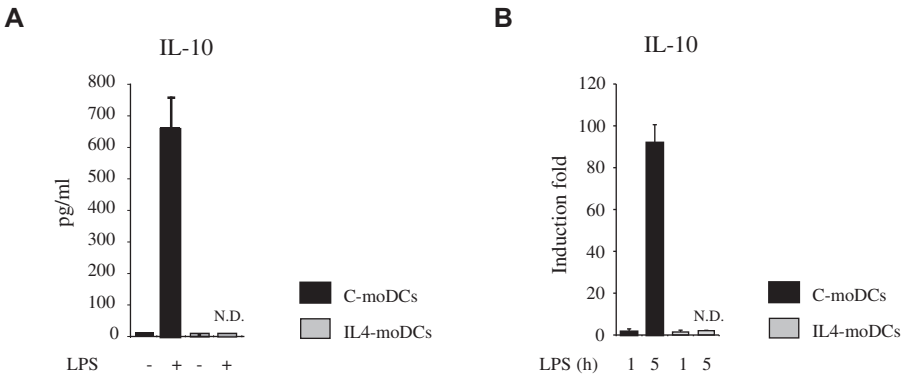

FIG E4. IL-10 production by C-moDCs and IL-4–moDCs. **A,** Production of IL-10 by C-moDCs or IL-4–moDCs 16 hours after LPS stimulation analyzed by means of ELISA. Data are expressed as means ± SDs of duplicates. **B,** Expression of mRNA for IL-10 by C-moDCs or IL-4–moDCs 1 and 5 hours after LPS stimulation analyzed by using qRT-PCR and normalized to β-actin. Data are expressed as induction fold relative to unstimulated C-moDCs (means ± SDs of triplicates). Data are representative of 4 independent experiments with similar results. *N.D.*, Not detectable.

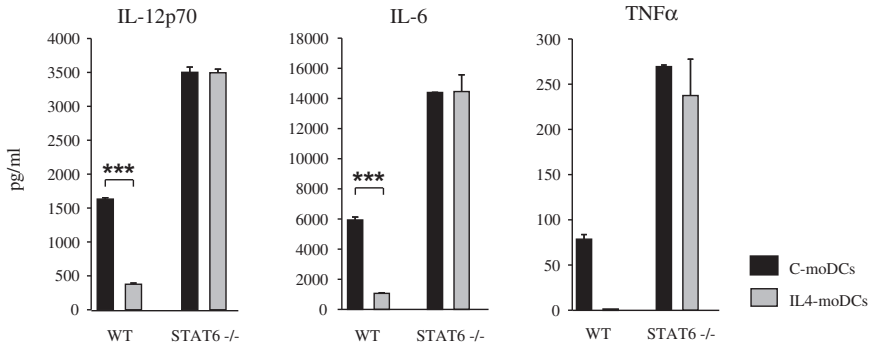

FIG E5. Analysis of the effect of Stat6 deficiency on the responsiveness of C-moDCs and IL-4–moDCs to LPS stimulation. Production of the indicated cytokines by IL-4–moDCs from wild-type *(WT)* or Stat6-deficient *(STAT6$^{-/-}$)* mice 16 hours after LPS stimulation analyzed by means of ELISA. Significant differences, as determined by using the unpaired *t* test, are indicated as follows: ***$P < .001$. Data are expressed as means ± SDs of duplicates and are representative of 2 independent experiments with similar results.

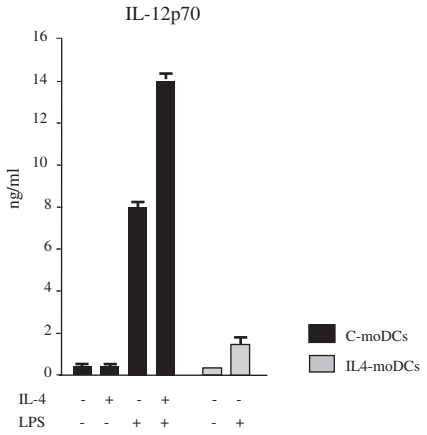

FIG E6. Synergistic effect of IL-4 and LPS on IL-12 production by moDCs. Production of IL-12p70 by C-moDCs unstimulated or stimulated with LPS, IL-4, or both *(black bars)* or IL-4–moDCs unstimulated or stimulated with LPS *(gray bars)*. Data are expressed as means ± SDs of duplicates and are representative of 2 independent experiments with similar results.

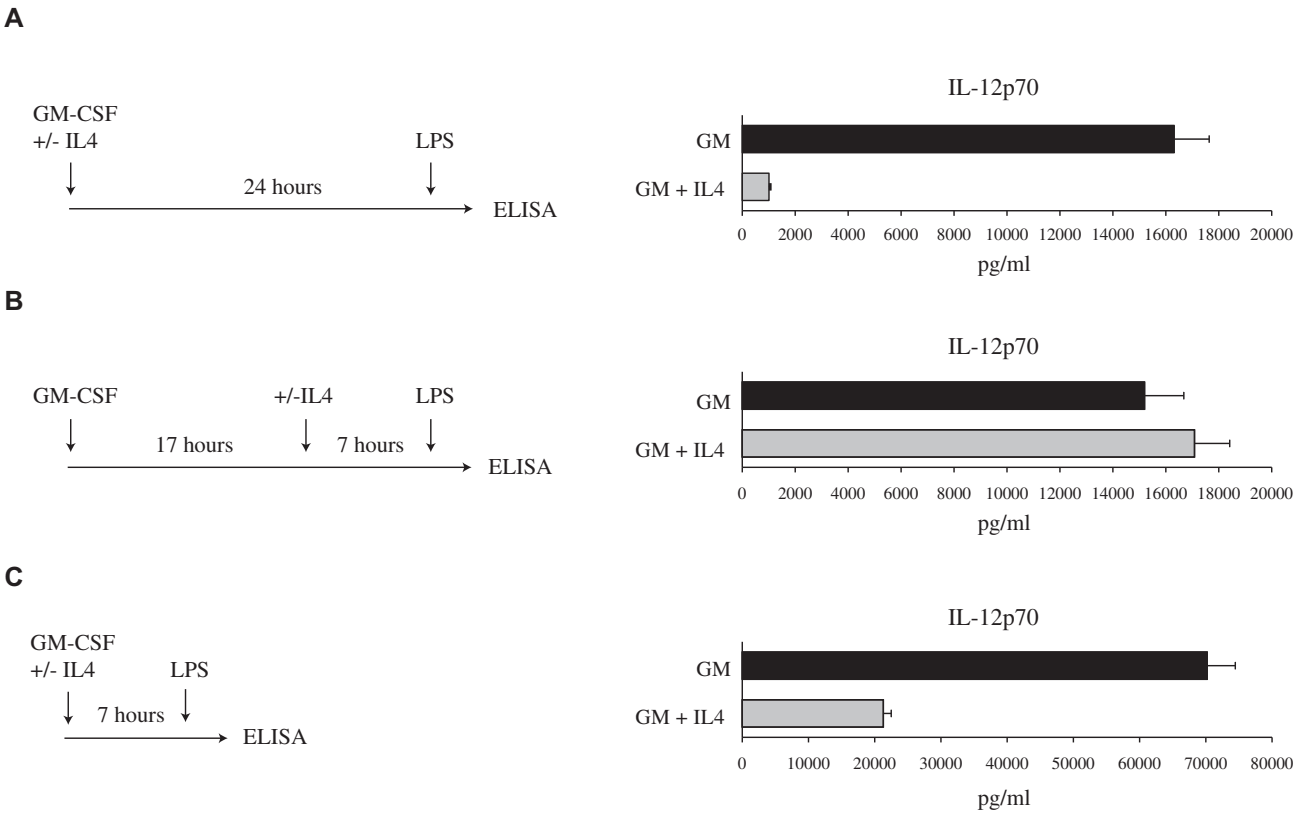

FIG E7. Differential effect of IL-4 during or after moDC differentiation. **A,** IL-12p70 production by C-moDCs *(black bars)* and IL-4–moDCs *(gray bars)* stimulated with LPS. **B,** IL-12p70 production by C-moDCs untreated *(black bars)* or treated with IL-4 *(gray bars)* for 7 hours before LPS stimulation. **C,** Production of IL-12p70 by monocytes cultured with GM-CSF for 7 hours in the absence *(black bars)* or presence *(gray bars)* of IL-4 for 7 hours before LPS stimulation is shown as a control of the ability of the 7-hour IL-4 treatment used in Fig E7, *B*, to block IL-12 production. IL-12p70 production was analyzed by means of ELISA 16 hours after LPS stimulation. Data are expressed as means ± SDs of duplicates and are representative of 2 independent experiments with similar results.

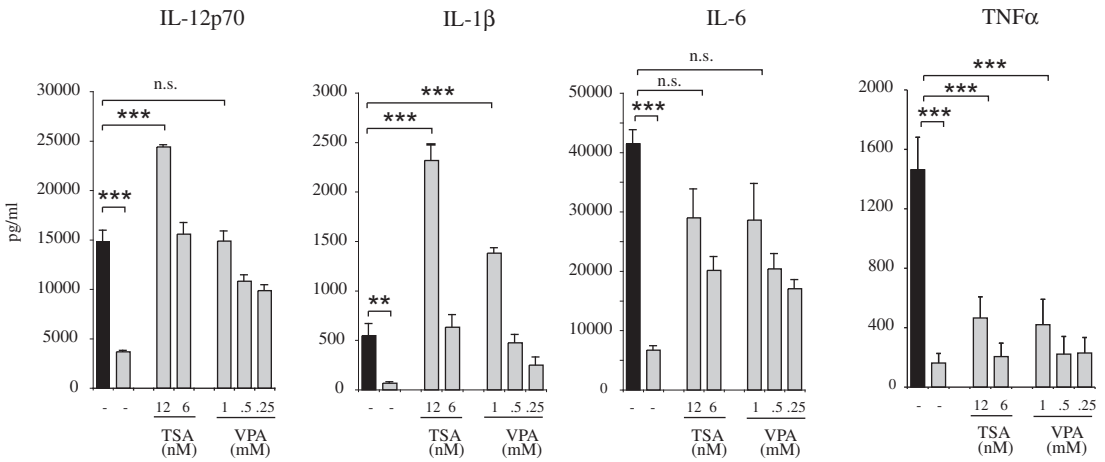

FIG E8. Reversal of T_H1/inflammatory cytokine production blockade on LPS-stimulated IL-4–moDCs by the HDAC inhibitor valproic acid *(VPA)*. Production of the indicated cytokines by untreated C-moDCs or IL-4–moDCs untreated or treated with TSA or VPA at the indicated concentrations analyzed 16 hours after LPS stimulation by means of ELISA. Significant differences, as determined by using the unpaired *t* test, are indicated as follows: *n.s.*, not significant; ***$P < .001$. Data are representative of 2 independent experiments with similar results.

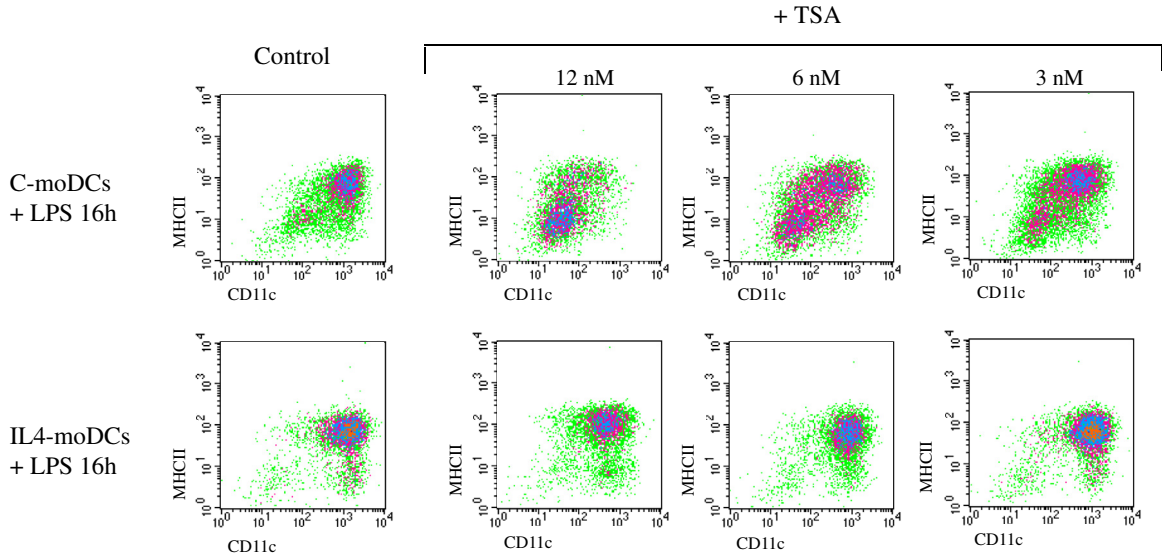

FIG E9. Effect of TSA on C-moDC differentiation and responsiveness to LPS stimulation. CD11c versus MHC class II expression by C-moDCs and IL-4–moDCs differentiated for 24 hours with GM-CSF in the absence or presence of TSA at the indicated concentrations and subsequently stimulated with LPS for 16 hours. Data are representative of at least 4 independent experiments with similar results.

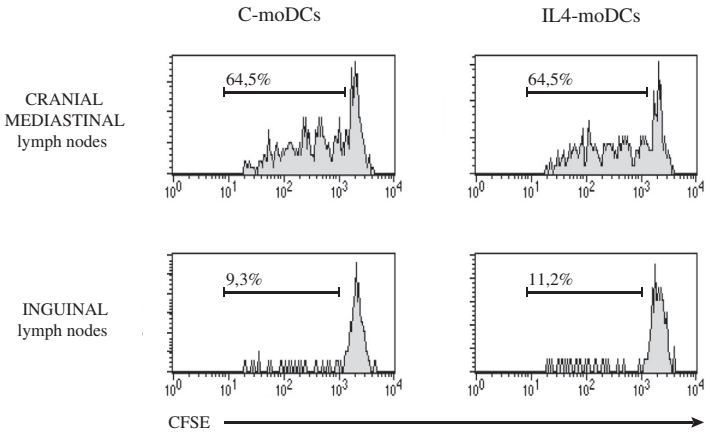

FIG E10. Migratory and *in vivo* T-cell stimulatory capacity of IL-4–moDCs and C-moDCs. Ovalbumin-pulsed C-moDCs or IL-4–moDCs (0.5×10^6) were injected intraperitoneally into C57BL/6 mice that had received an intravenous transfer of 5×10^6 carboxyfluorescein succinimidyl ester *(CFSE)*–labeled OVA-specific TCR transgenic OT-II $CD4^+$ T cells 24 hours before. OT-II cell proliferation was assessed in the draining cranial mediastinal lymph nodes and in the nondraining inguinal lymph nodes by means of CFSE dilution, 3 days after moDC transfer. The percentage of OT-II divided cells is indicated.

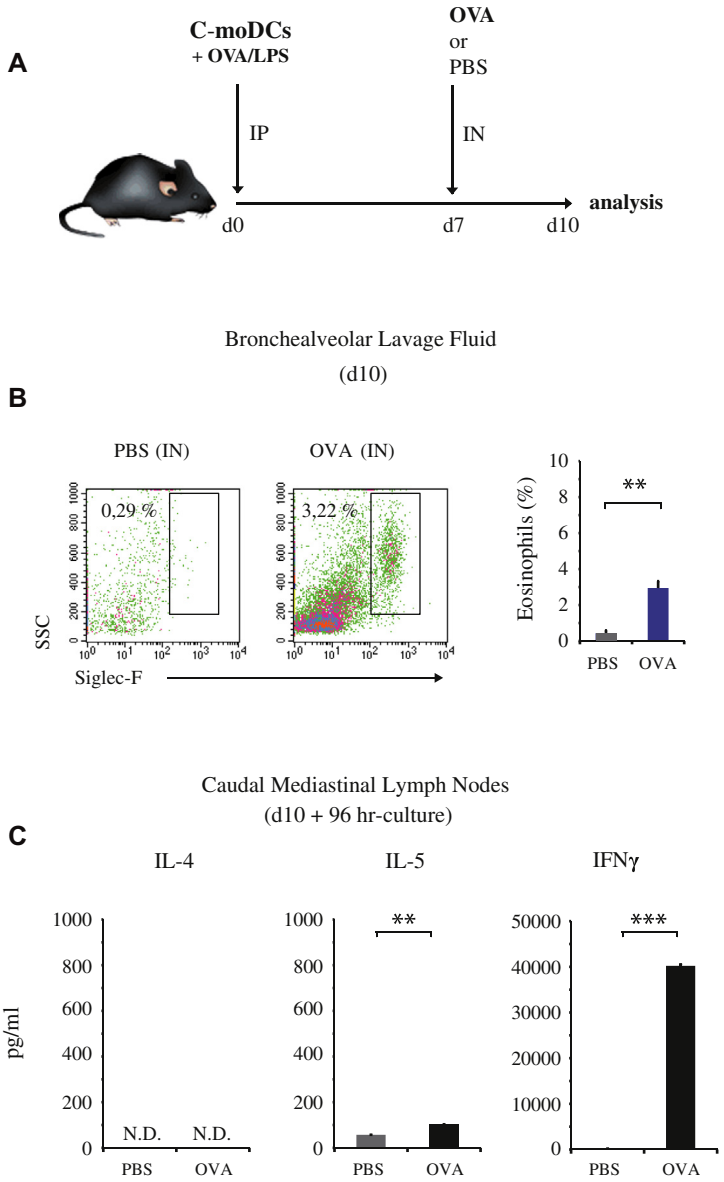

FIG E11. T_H1-polarized lung immune response induced by LPS-stimulated moDCs. **A,** Protocol for the induction of a T_H1-polarized lung immune response to ovalbumin *(OVA)* after intraperitoneal *(IP)* transfer of OVA-loaded, LPS-stimulated C-moDCs, followed by intranasal *(IN)* administration of OVA or PBS. **B,** Flow cytometric analysis and quantitation of eosinophil infiltration in the bronchoalveolar lavage fluid at day 10 after transfer of C-moDCs (means ± SDs of 4 mice per condition). **C,** Analysis by means of ELISA of the production of the indicated cytokines by cM-LN cells at day 10 after culture for 96 hours in the presence of OVA. Data are expressed as means ± SDs of duplicates. Significant differences, as determined by using the unpaired *t* test, are indicated as follows: $^{**}P < .01$; $^{***}P < .001$. *N.D.*, Not detectable. Data are representative of 2 independent experiments with similar results.

TABLE E1. Primers for qPCR used in this study

Gene	Sequence
IL-1β	Forward: 5′-TGGTGTGTGACGTTCCCATT-3′ Reverse: 5′-CAGCACGAGGCTTTTTTGTTG-3′
IL-6	Forward: 5′-GAGGATACCACTCCCAACAGACC-3′ Reverse: 5′-AAGTGCATCATCGTTGTTCATACA-3′
IL-12p35	Forward: 5′- CCACCCTTGCCCTCCTAAA-3′ Reverse: 5′-GGCAGCTCCCTCTTGTTGTG-3′
IL-12p40	Forward: 5′-GGAAGCACGGCAGCA GAATA-3′ Reverse: 5′- AACTTGAGGGAGAAGTAGGAATGG-3′
TNF-α	Forward: 5′-CCCCAAAGGGATGAGAAGTT-3′ Reverse: 5′-TGGGCTACAGGCTTGTCACT-3′
iNOS	Forward: 5′-CAGCTGGGCTGTACAAACCTT-3′ Reverse: 5′-CATTGGAAGTGAAGCGTTTCG-3′
β-Actin	Forward: 5′-GGCTCCTAGCACCATGAAGA-3′ Reverse: 5′-CCACCGATCCACACAGAGTA-3′
CD40	Forward: 5′-CAGACACTGTGAACCCAATCAAGG-3′ Reverse: 5′-TGGTGTCAGTGGCCATCTCCATAA-3′
CD86	Forward: 5′-TGTTTCCGTGGAGACGCAAG-3′ Reverse: 5′-CAGCTCACTCAGGCTTATGTTTT-3′
IL-12p35 promoter	Forward: 5′-GGGACGGTCCCGAATCTC-3′ Reverse: 5′-GGTGGCGCTTTCGAATTAAC-3′
IL-12p40 promoter	Forward: 5′-TTCCCCCAGAATGTTTTGACA-3′ Reverse: 5′-TGATGGAAACCCAAAGTAGAAACTG-3′
iNOS promoter	Forward: 5′-GGAGTGTCCATCATGAATGAG-3′ Reverse: 5′-CAACTCCCTGTAAAGTTGTGACC-3′
IL-1β promoter	Forward: 5′-GGACAATTGTGCAGATGGTG-3′ Reverse: 5′-CCTACCTTTGTTCCGCAGAT-3′
TNF-α promoter	Forward: 5′-CAACTTTCCAAACCCTCTGC-3′ Reverse: 5′-CTGGCTAGTCCCTTGCTGTC-3′