Mast cells regulate CD4$^+$ T-cell differentiation in the absence of antigen presentation

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Background: Given their unique capacity for antigen uptake, processing, and presentation, antigen-presenting cells (APCs) are critical for initiating and regulating innate and adaptive immune responses. We have previously shown the role of nicotinamide adenine dinucleotide (NAD$^+$) in T-cell differentiation independently of the cytokine milieu, whereas the precise mechanisms remained unknown.
Objective: The objective of this study is to further dissect the mechanism of actions of NAD$^+$ and determine the effect of APCs on NAD$^+$-mediated T-cell activation.
Methods: Isolated dendritic cells and bone marrow–derived mast cells (MCs) were used to characterize the mechanisms of action of NAD$^+$ on CD4$^+$ T-cell fate *in vitro*. Furthermore, NAD$^+$-mediated CD4$^+$ T-cell differentiation was investigated *in vivo* by using wild-type C57BL/6, MC$^{-/-}$, MHC class II$^{-/-}$, Wiskott-Aldrich syndrome protein (WASP)$^{-/-}$, 5C.C7 recombination-activating gene 2 (Rag2)$^{-/-}$, and CD11b-DTR transgenic mice. Finally, we tested the physiologic effect of NAD$^+$ on the systemic immune response in the context of *Listeria monocytogenes* infection.

Results: Our *in vivo and in vitro* findings indicate that after NAD$^+$ administration, MCs exclusively promote CD4$^+$ T-cell differentiation, both in the absence of antigen and independently of major APCs. Moreover, we found that MCs mediated CD4$^+$ T-cell differentiation independently of MHC II and T-cell receptor signaling machinery. More importantly, although treatment with NAD$^+$ resulted in decreased MHC II expression on CD11c$^+$ cells, MC-mediated CD4$^+$ T-cell differentiation rendered mice resistant to administration of lethal doses of *L monocytogenes*.
Conclusions: Collectively, our study unravels a novel cellular and molecular pathway that regulates innate and adaptive immunity through MCs exclusively and underscores the therapeutic potential of NAD$^+$ in the context of primary immunodeficiencies and antimicrobial resistance. (J Allergy Clin Immunol 2018;142:1894-908.)

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Antigen-presenting cells (APCs) play a central role in regulation of the innate and adaptive immune responses.[1] APCs have the ability to capture, process, and present antigens through their MHC cell-surface molecules to the T-cell receptor (TCR) to mount an MHC-restricted immune response.[2-6] APCs include a myriad of immune cells, such as B cells, neutrophils, macrophages, eosinophils, basophils, and dendritic cells (DCs). Among these populations, DCs are considered the major APCs bridging innate and adaptive immune responses.[7,8] The mode of action of DCs is mediated through at least 3 signals: (1) TCR activation, (2) activation of costimulatory molecules, and (3) secretion of chemokines and proinflammatory cytokines.[8] Indeed, depletion of CD11c$^+$ DCs has been shown to alter cytotoxic T-lymphocyte responses to infection, as well as CD4$^+$ T-cell activation and antibody production.[9] In addition, DCs can also regulate innate and adaptive immune responses by recognizing pathogen-associated molecular patterns (PAMPs), such as microbial nucleic acids, lipoproteins, and carbohydrates, or damage-associated molecular patterns (DAMPs) released from injured cells through intracellular or surface-expressed pattern recognition receptors (PRRs).[10-14]

Although considered "atypical" APCs, Mast cells (MCs) have been described mainly for their role in allergic and autoimmune responses.[15,16] It is well established that MCs are important effector cells in IgE-mediated allergic inflammation, and MCs are also recognized to influence innate and adaptive immune responses.[8,16]

Abbreviations used
APC: Antigen-presenting cell
BMMC: Bone marrow–derived mast cell
DAMP: Damage-associated molecular pattern
DC: Dendritic cell
EAE: Experimental autoimmune encephalomyelitis
LPA: Lysophosphatidic acid
MC: Mast cell
NAD^+: Nicotinamide adenine dinucleotide
PAMP: Pathogen-associated molecular pattern
PRR: Pattern recognition receptor
Rag2: Recombination-activating gene 2
TCR: T-cell receptor
WASP: Wiskott-Aldrich syndrome protein
WT: Wild-type

MC-deficient mice have been shown to exhibit an altered $CD4^+$ T-cell response to infection and in experimental autoimmune encephalomyelitis (EAE), a mouse model for human multiple sclerosis, suggesting that MCs play a role in mediating T-cell responses.[17-20] Like DCs, MCs can directly present antigens to T cells *in vitro*, inducing an antigen-specific clonal expansion of T-cell populations, and are known to express costimulatory molecules and secrete a myriad of chemokines and proinflammatory cytokines.[8,15,16,21] However, MCs express MHC class II intracellularly rather than at the cell surface.[22] Moreover, MCs have been shown to promote T-cell activation in an antigen-independent manner, and no direct evidence has been provided thus far on the capacity of direct antigen presentation to the TCR.[8,23] Thus the mechanisms by which MCs regulate T-cell responses remain unclear and are yet to be determined.

Recently, we have shown the role of nicotinamide adenine dinucleotide (NAD^+), a cofactor found in all living cells and nutrients, in T-cell fate regulation.[24,25] We have demonstrated that NAD^+ was able to regulate $CD4^+$ T-cell differentiation through a novel pathway that is independent of the cytokine environment and well-established transcription factors.[25] More recently, we have reported the unique immunosuppressive properties mediated by NAD^+ through systemic IL-10 cytokine production.[24] Although we characterized the role of NAD^+ in regulating T-cell fate, the precise mechanisms of action remain largely unknown.

Here we show that after NAD^+ administration, MCs are able to induce exclusively $CD4^+$ T-cell differentiation *in vitro* and *in vivo* in the absence of antigen and major APCs. Furthermore, we demonstrate that MC-driven $CD4^+$ T-cell differentiation was independent of MHC class II or TCR activation. Furthermore, when assessing the functional effect of MC-mediated $CD4^+$ T-cell differentiation, we observed that treatment with NAD^+ resulted in profound alterations in innate and adaptive immunity and survival outcome after *Listeria monocytogenes* infection. Collectively, our study unravels a new cellular and molecular pathway regulating innate and adaptive immune responses that is mediated exclusively by MCs.

METHODS
Animals and diphtheria toxin treatment
Eight- to 10-week-old wild-type (WT) C57BL/6 (B6, $H2^b$) mice were purchased from Charles River Laboratories (Wilmington, Mass). $MC^{-/-}$ (WBB6F1/J-Kit^W/Kit^{W-v}/J [Kit^W/Kit^{W-v}] and Kit^{W-sh}/HNihrJaeBsmJ

[kit^{Wsh}/Kit^{Wsh}]), MHC class $II^{-/-}$ (B6.129S-H2dlAb1-Ea), Wiskott-Aldrich syndrome protein (WASP)$^{-/-}$ (B6.129S6-Was^{tm1Sbs}/J), and CD11b-DTR (B6.FVB-Tg[ITGAM-DTR/EGFP]34Lan/J) mice were purchased from Jackson Laboratory (Bar Harbor, Me). Recombination-activating gene 2 (Rag2)$^{-/-}$γc$^{-/-}$ (B10; B6-$Rag2^{tm1Fwa}$ $II2rg^{tm1Wjl}$), Rag2$^{-/-}$, and 5C.C7 Rag2$^{-/-}$ mice (both on B10.A background) were purchased from Taconic Biosciences (Albany, NY). For CD11b$^+$ cell depletion with diphtheria toxin treatment, CD11b-DTR transgenic mice weighing 25 to 30 g were injected with diphtheria toxin (25 ng/g body weight; Sigma-Aldrich, St Louis, Mo) 24 hours before and 72 hours after beginning NAD^+ or PBS administration.

Isolation of mouse naive $CD4^+CD44^-CD62L^+$ T cells and DCs
Single-cell leukocyte suspensions were obtained from spleens of 8- to 10-week-old C57BL/6 mice, and naive $CD4^+CD44^-CD62L^+$ T cells were isolated by means of flow cytometry, as described previously.[25] For isolation of CD11c$^+$ DCs, single-cell leukocyte suspensions were obtained from spleens of 8- to 10-week-old C57BL/6 WT mice. CD11c$^+$ DCs were then isolated with the EasySep Mouse CD11c Positive Selection Kit (STEMCELL Technologies, Vancouver, British Columbia, Canada), according to the manufacturer's protocol, followed by cell sorting (CD11c$^+$CD11b$^+$ cells).

L monocytogenes infection
L monocytogenes bacteria (ATCC #35152) were cultured overnight at 37°C in Brain Heart Infusion (Teknova, Hollister, CA) with gentle agitation. Eight- to 10-week-old WT and $MC^{-/-}$ mice were infected intraperitoneally with 0.1 mL of a solution containing 1×10^7 colony-forming units (nonlethal dose) or 1×10^8 colony-forming units (lethal dose) of viable *L monocytogenes* cells in 0.01 mol/L PBS (pH 7.4). Weight loss and survival after infection were monitored. Before infection, mice were pretreated daily for a period of 5 days with NAD^+ (40 mg administered intraperitoneally) or pretreated 5 days before infection and continuously treated daily after infection.

Cultivation of bone marrow–derived mast cells
Bone marrow–derived mast cells (BMMCs) from 8- to 10-week-old C57BL/6J WT mice were obtained by culturing bone marrow cells from femurs and tibias. In short, mice were killed by means of cervical dislocation, intact femurs and tibias were removed, and bone marrow cells were harvested by means of repeated flushing with sterile media. BM cells were cultured in WEHI-3–conditioned medium (containing IL-3) for 90 days, at which time the cells were greater than 95% c-Kit^{high}FcεRIα^{high}, as determined by using flow cytometric analysis with PE-Cy7 anti-mouse FcεRIα (clone MAR-1; eBioscience, San Diego, Calif) and ef450 anti-mouse c-Kit/CD117 (clone 2B8; eBioscience, San Diego, Calif).

Human MC line LAD-2 culture
The human MC line LAD-2 was a generous gift from Dr A. Kirshenbaum (National Institutes of Health/National Institute of Allergy and Infectious Diseases). LAD-2 MCs were cultured in serum-free media (StemPro-34 SFM; Life Technologies, Grand Island, NY) supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 50 μg/mL streptomycin, and 100 ng/mL recombinant stem cell factor. LAD-2 cells were tested periodically for expression of Kit and FcεRI by using flow cytometry.

Cell culture
Isolated naive $CD4^+$ T cells or CD11c$^+$ DCs (1×10^6 cells per well) were cultured in 48-well flat-bottom plates in 0.5 mL of complete RPMI 1640 medium supplemented with 10% FCS, 200 mmol/L L-glutamine, 100 U/mL penicillin/streptomycin, and 4.5 g/L glucose in the presence of 10 μg/mL plate-bound anti-mouse α-CD3 (17A2) and 2 μg/mL soluble α-CD28 (37.51). NAD^+ (catalog no. N3014; Sigma-Aldrich) was diluted in PBS and
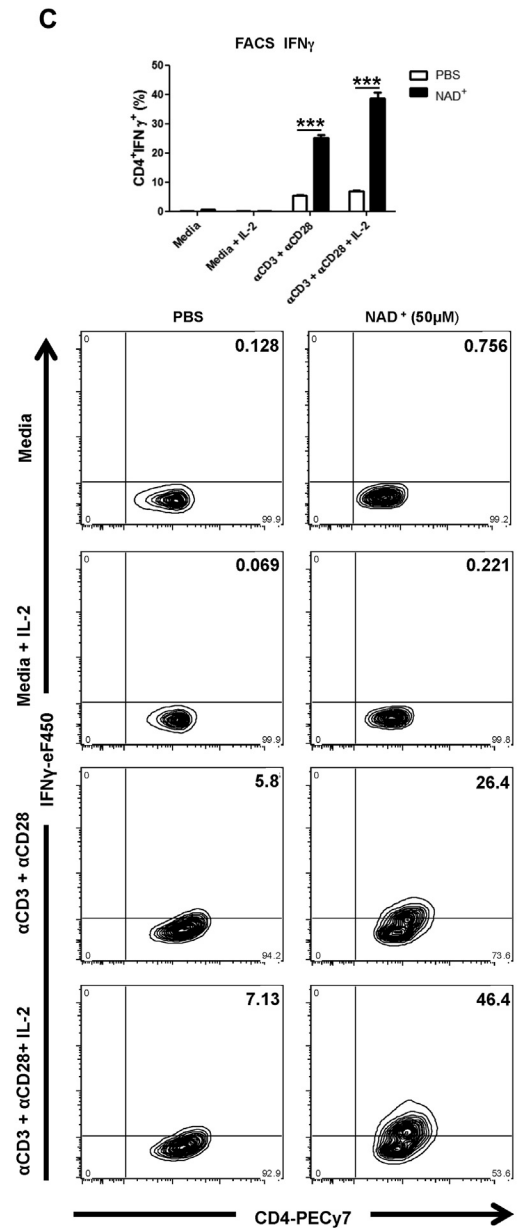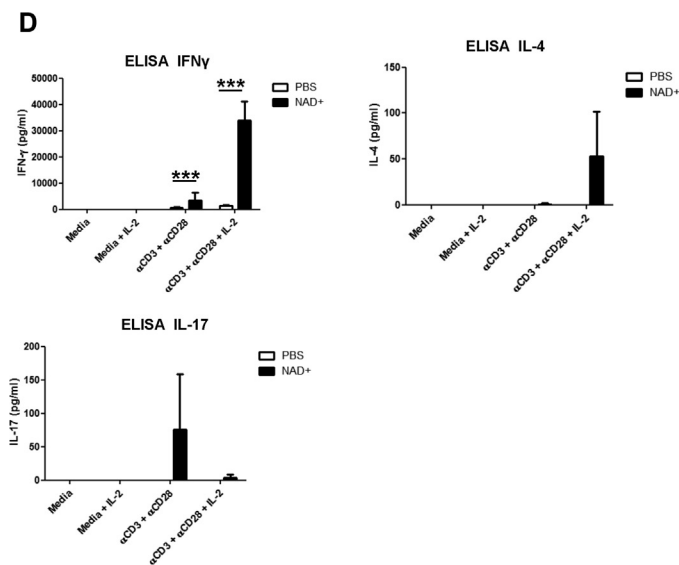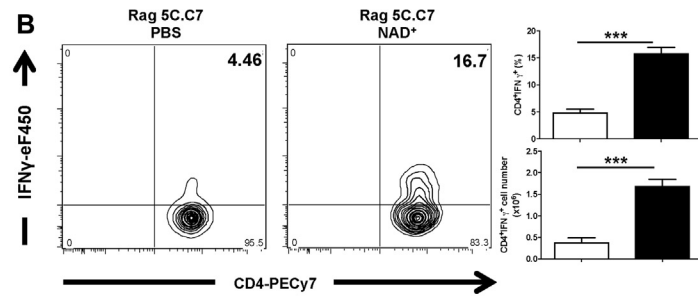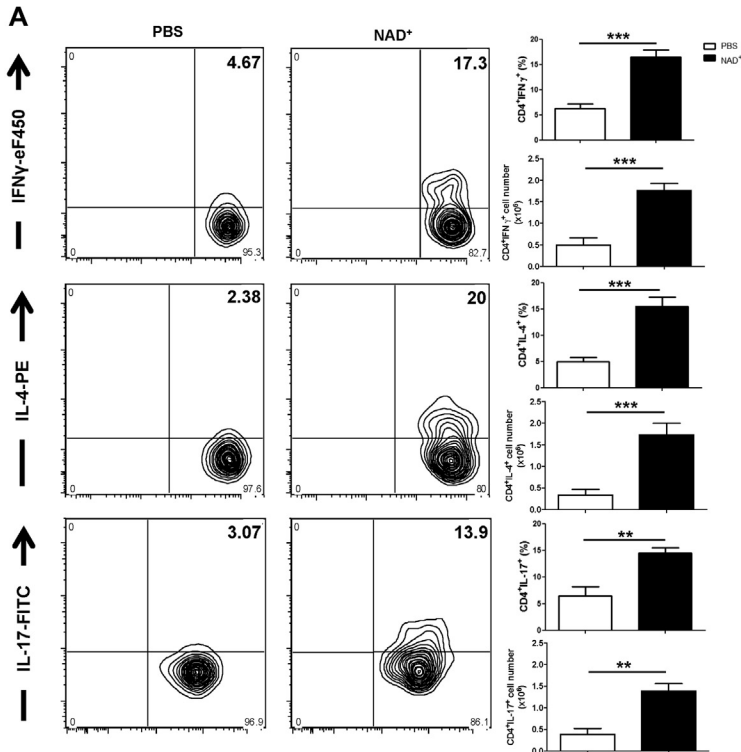
added as indicated. LPS was added at a concentration of 1 μg/mL. All recombinant cytokines and antibodies were purchased from eBioscience. After the indicated day of culture, supernatants and cells were collected and analyzed by means of ELISA and flow cytometry, respectively.

Coculture of mouse naive CD4$^+$ T cells and BMMCs in transwell systems

Noncontacting cocultured cells were prepared as follows: isolated naive CD4$^+$CD44$^-$CD62L$^+$ T cells were plated on the bottom of the 24-well transwell cell culture system (Costar, Cambridge, Mass). BMMCs were cocultured at a ratio of 1:100 in the upper transwell compartment. Cells were stimulated with NAD$^+$ (500 μmol/L) or PBS as a control. Naive CD4$^+$ T cells were cultured in complete media only or in the presence of 10 μg/mL plate-bound anti-mouse α-CD3 (clone 17A2) and 2 μg/mL soluble α-CD28 (clone 37.51). For cell-cell contact experiments, BMMCs and naive CD4$^+$CD44$^-$CD62L$^+$ T cells were cocultured (at a ratio of 1:100) in complete media with NAD$^+$ (500 μmol/L) or PBS as a control. Naive CD4$^+$ T cells were cultured in the presence of 10 μg/mL plate-bound anti-mouse α-CD3 (clone 17A2) and 2 μg/mL soluble α-CD28 (clone 37.51) or complete media only. For CD80 blockade, experiments were performed in cell-cell contact conditions, as described above, with α-CD80 neutralizing antibody (clone 16-10A1; eBioscience). Cells were cultured for 96 hours, and CD4$^+$IFN-γ$^+$, CD4$^+$IL-4$^+$, and CD4$^+$IL-17A$^+$ T-cell frequencies were assessed by using flow cytometry.

Isolation and coculture of human naive CD4$^+$ T cells

Human naive CD4$^+$ T cells were isolated from PBMCs by means of density gradient centrifugation with the SepMate kit (STEMCELL Technologies), followed by the EasySep Human Naïve CD4$^+$ T Cell Isolation Kit (STEMCELL Technologies). Blood was obtained from healthy adult volunteers in accordance with guidelines of and approved by the Institutional Review Board of Beth Israel Deaconess Medical Center. Informed consent was obtained from each volunteer in accordance with the Declaration of Helsinki. Human naive CD4$^+$CD25$^-$CD45RA$^+$CD45RO$^-$CCR7$^+$CD62L$^+$ T cells were then sorted by means of flow cytometry. Naive CD4$^+$ T cells were purified to greater than 98% by using cell sorting. Naive human CD4$^+$ T cells were then plated on the bottom of the 24-well transwell cell culture system (Costar), and LAD-2 cells were cocultured at a ratio of 1:100 in the upper transwell compartment. Cells were stimulated with NAD$^+$ (500 μmol/L) or PBS as a control. Naive CD4$^+$ T cells were cultured in complete media only or in the presence of 10 μg/mL soluble anti-mouse α-CD3 (clone 17A2) and 5 μg/mL soluble α-CD28 (clone 37.51). For cell-cell contact experiments, LAD-2 cells and naive CD4$^+$CD44$^-$CD62L$^+$ T cells were cocultured (at a ratio of 1:100) in complete media with NAD$^+$ (500 μmol/L) or PBS as a control. Naive CD4$^+$ T cells were cultured in the presence of 10 μg/mL soluble anti-mouse α-CD3 (clone 17A2) and 5 μg/mL soluble α-CD28 (clone 37.51) or complete media only. After 96 hours, CD4$^+$ T-cell IFN-γ cytokine production was assessed by using flow cytometry.

DC and macrophage depletion

For DC depletion, WT, Rag2$^{-/-}$γc$^{-/-}$, and MC$^{-/-}$ mice were treated intravenously with 0.5 mg of liposomal clodronate (Encapsula NanoSciences, Nashville, Tenn) at days 8, 5, and 1 before NAD$^+$ administration. This regimen ensured depletion of greater than 99% CD11c$^+$ DCs, as described previously.[26,27] As a control group, mice were injected with the same amount of isotype-matched rat IgG as control mice.

Flow cytometry

Fluorescence-labeled anti-mouse CD4 (clone GK1.5), CD11b (M1/70), CD11c (N418), CD41 (eBioMWReg30), CD61 (2C9. G3), IL-1β (NJTEN3), IL-4 (11B11), IL-6 (MP5-20F3), IL-10 (JES5-16E3), IL-12/IL-23p40 (C 17.8), IL-17 (eBio17B7), IFN-γ (XMG 1.2), latency-associated peptide (TW7-16B4), and TNF-α (MP6-XT22) were obtained from eBioscience. All antibodies were used at a concentration of 2 to 5 μg per 1 × 10^6 cells. To set the gates, flow cytometric dot plots were based on comparison with isotype controls, fluorescence minus one, and permeabilized and unpermeabilized unstained cells. Intracellular staining for IL-1β, IL-4, IL-6, IL-10, IL-12/IL-23p40, IL-17, IFN-γ, latency-associated peptide, and TNF-α was performed according to the manufacturer's protocols. Cells were fixed and permeabilized with Cytofix/Cytoperm solution (BD Biosciences, San Jose, Calif). Flow cytometry was performed on a BD FACSCanto II (BD Biosciences) by using standard procedures, and data were analyzed with FlowJo software (TreeStar, Ashland, Ore).

ELISA

Mouse IL-4, IL-17A, and IFN-γ levels were measured with commercial kits (eBioscience), as described previously.[24,25]

RNA extraction and quantitative PCR

BMMCs from C57BL/6 mice were cultured in the presence of NAD$^+$ (500 μmol/L), LPS, or placebo (PBS). After 24 hours of culture, cells were collected, and mRNA was extracted by using the RNAqueous extraction kit, according to the manufacturer's protocols (Applied Biosystems, Foster City, Calif). Briefly, cells were homogenized in lysis buffer (total volume of 0.5 mL) and passed through a column. After successive washes, RNA was eluted. For real-time PCR reactions, IL-1α (Mm00439620_m1), IL-β (Mm01336189_m1), IL-4 (Mm00445259_m1), IL-6 (Mm004446190_m1), IL-10 (Mm00439616_m1), IL-12α (Mm00434165_m1), IL-23 (Mm00 518984_m1), TGF-β$_1$ (Mm01178820_m1), TNF-α (Mm00443260_g1), TLR2 (Mm00442346_m1), TLR4 (Mm00445273_m1), CD86 (Mm00 444543_m1), CD80 (Mm00711660_m1), inducible costimulator ligand (Mm00497237_m1), OX40 ligand (Mm00437214_m1), and IL-33 (Mm00 505403_m1) measurements were performed with TaqMan primers and probes from Applied Biosystems. The housekeeping gene glyceraldehye-3-phosphate dehydrogenase (Mm99999915_g1) was used as a control.

FIG 1. NAD$^+$ induces T-cell activation *in vivo* and T-cell differentiation *in vitro* after TCR activation. **A,** C57BL/6 mice were treated daily with intraperitoneal injection of 40 mg of NAD$^+$ or a placebo solution (PBS). After 7 days, mice were killed, and CD4$^+$ T cells were isolated from spleens. Frequencies and total numbers of CD4$^+$IFN-γ$^+$, CD4$^+$IL-4$^+$, and CD4$^+$IL-17A$^+$ cells were analyzed by using flow cytometry. **B,** CD4$^+$ T cells (3 × 10^6) from 5C.C7 RAG-2–deficient mice were injected into Rag2$^{-/-}$ (B10.A background) mice as adoptive transfer recipients. Mice were then treated with NAD$^+$ or PBS for 7 days and subsequently killed to analyze frequencies and total numbers of CD4$^+$IFN-γ$^+$ cells by using flow cytometry. **C** and **D,** Sorted naive CD4$^+$CD44$^-$CD62L$^+$ T cells were isolated from spleens of C57BL/6 mice and cultured in complete media alone with α-CD3/α-CD28 with or without IL-2 or in the presence of 50 μmol/L NAD$^+$. After 96 hours, frequencies of CD4$^+$IFN-γ$^+$ cells and IFN-γ, IL-4, and IL-17A cytokine secretion were assessed by using flow cytometry (Fig 1, *C*) and ELISA (Fig 1, *D*), respectively. Statistics were as follows: n = 15 (Fig 1, *A* and *B*) or n = 10 (Fig 1, *C* and *D*). Data were derived from 3 independent sets of experiments. The Student *t* test and ANOVA were used accordingly to compare groups: **P < .01 and ***P < .001. Data are presented as means ± SDs. *FACS,* Fluorescein-activated cell sorting; *FITC,* fluorescein isothiocyanate; *PE,* phycoerythrin.

RNA sequencing analysis

BMMCs from C57BL/6 mice were cultured in the presence of NAD$^+$ (500 μmol/L), LPS (10 μg/mL; *Escherichia coli* O127:B8), or placebo (PBS). After 16 hours of culture, cells were collected and RNA was extracted with the RNAqueous extraction kit, according to the manufacturer's protocols (Applied Biosystems), as described above. cDNA was obtained by using New England Biolabs kits (NEBNext Ultra Directional RNA Library Prep Kit for Illumina; New England Biolabs, Ipswich, Mass). Briefly, mRNA was extracted with polyT magnetic beads, and then first- and second-strand syntheses were performed. Once double-strand cDNA was generated, DNA was cleaned up with magnetic beads and then went into library prep. Library preparation was performed by ligating on the P5 and P7 Illumina adaptors along with an index and amplifying the sequencing library by using PCR. The final library was cleaned up by using magnetic beads and made ready for sequencing. FastQ files were aligned against the Ensembl GRCm38.75 genome by using the STAR aligner (version 2.3.1z4) with default parameters.[28] Alignment files (BAM format) were filtered to retain only primary alignments (Samtools view-F 0x0100) and inspected for duplication rate with Picard tools MarkDuplicates; for downstream analyses, duplicate reads were not removed because of the high-quality input RNA.[29] Reads were quantified at the gene level by using featureCounts with annotated exon features in the Ensembl GRCm38.75 GTF file.[30,31] The resulting count matrix was normalized and analyzed for differential expression with DESeq2 software. Ingenuity Pathways Analysis (Ingenuity Systems, Redwood City, Calif) applications were used to generate canonical pathways.

Statistical analysis

Data are presented as means ± SDs. Statistical analysis was done with the 2-tailed Student *t* test (between 2 groups) and 1-way ANOVA (among multiple groups), where appropriate. Survival was compared by using the log-rank test. *P* values of less than .05 were considered statistically significant.

Study approval

Animal care and use were in accordance with the National Institutes of Health and Institutional Animal Care and Use Committee guidelines.

RESULTS
NAD$^+$ requires an intermediary signal to promote CD4$^+$ T-cell differentiation

We have demonstrated previously that NAD$^+$ regulated CD4$^+$ T-cell differentiation independently of the cytokine milieu and well-established transcription factors.[25] It remains unclear whether NAD$^+$ promotes CD4$^+$ T-cell differentiation by acting directly on CD4$^+$ T cells or through an intermediate cell type. C57BL/6 WT naive mice were treated daily with intraperitoneal injection of NAD$^+$ or a placebo solution (PBS) and CD4$^+$ T-cell responses were assessed to characterize the direct effect of NAD$^+$ on CD4$^+$ T cells in the absence of antigen challenge. After 7 days, mice were killed, and systemic CD4$^+$IFN-γ$^+$, CD4$^+$IL-4$^+$, and CD4$^+$IL-17A$^+$ T-cell frequencies were evaluated by using flow cytometry. Data indicated that NAD$^+$ administration was sufficient to promote a significant increase in frequencies of CD4$^+$IFN-γ$^+$, CD4$^+$IL-4$^+$, and CD4$^+$IL-17$^+$ T cells *in vivo* (Fig 1, *A*). Of note, NAD$^+$ administration to Rag2$^{-/-}$ mice adoptively transferred with naive CD4$^+$ T cells from Rag 5C.C7 transgenic mice promoted CD4$^+$IFN-γ$^+$ T-cell differentiation in the absence of moth cytochrome C peptide challenge (Fig 1, *B*).

Thus we next assessed *in vitro* whether NAD$^+$ directly promotes CD4$^+$ T-cell differentiation and cytokine production.

Splenic naive CD4$^+$CD44$^-$CD62L$^+$ T cells from C57BL/6 WT mice were cultured in the presence or absence of α-CD3/α-CD28 with or without IL-2 and in the presence of NAD$^+$ or PBS. After 96 hours of culture, CD4$^+$ T cells were assessed for IFN-γ, IL-4, and IL-17 production by using flow cytometry and ELISA. Consistent with our previous reports, flow cytometry revealed that NAD$^+$ promotes a robust increase of CD4$^+$IFN-γ$^+$ T-cell frequencies after TCR activation, particularly in the presence of IL-2 (Fig 1, *C*). These findings were confirmed by means of ELISA, indicating increased IFN-γ, IL-4, and IL-17 production after CD3/CD28 activation (Fig 1, *D*). Of note, the highest CD4$^+$IL-17$^+$ T-cell frequencies and IL-17 secretion were observed after TCR activation and in the absence of IL-2, which was consistent with previous reports indicating that IL-2 inhibits T$_H$17 development. In contrast, no changes in CD4$^+$IFN-γ$^+$ T-cell frequencies or IFN-γ, IL-4, and IL-17 cytokine production were observed with NAD$^+$ treatment in the absence of TCR activation (Fig 1, *C* and *D*). Furthermore, increasing NAD$^+$ concentrations in the absence of TCR activation did not result in a change in CD4$^+$IFN-γ$^+$, CD4$^+$IL-4$^+$, and CD4$^+$IL-17$^+$ T-cell frequencies (see Fig E1, *A*, in this article's Online Repository at www.jacionline.org). Collectively, our *in vivo* data indicate that NAD$^+$ promotes CD4$^+$ T-cell differentiation in the absence of antigen challenge, whereas our *in vitro* data indicate that NAD$^+$ requires TCR activation, suggesting that NAD$^+$-mediated CD4$^+$ T-cell differentiation observed in C57BL/6 WT naive mice requires an intermediary signal.

NAD$^+$ regulates CD4$^+$ T-cell fate in the absence of major APCs

We next investigated whether other immune cells that are known to activate CD4$^+$ T cells, in particular APCs, are involved in NAD$^+$-mediated CD4$^+$ T-cell differentiation. It has been shown that ATP, a coenzyme, can promote T$_H$17 cells through IL-6, IL-23, and TGF-β cytokine production by CD11c$^+$ cells.[32] Because NAD$^+$ also acts as a coenzyme, we first assessed *in vitro* the effects of NAD$^+$ on CD11c$^+$ DCs. CD11b$^+$CD11c$^+$ DCs were isolated from spleens of C57BL/6 mice and cultured in the presence of increasing NAD$^+$ concentrations or PBS. As a positive control, CD11b$^+$CD11c$^+$ DCs were cultured in the presence of 1 μg/mL LPS. After 16 hours, cells were collected and cytokine expression was quantified by using real-time PCR. Consistent with numerous reports, stimulation of CD11b$^+$CD11c$^+$ cells by LPS resulted in increased mRNA expression levels of IL-1α, IL-1β, IL-6, IL-23, and TNF-α but not IL-12 and a downregulation of TLR4.[33,34] More importantly, in the presence of NAD$^+$, CD11c$^+$CD11b$^+$ DCs exhibited increased mRNA expression levels of IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-23, TGF-β1, TNF-α, TLR2, and TLR4 in a dose-dependent manner (see Fig E1, *B*), suggesting that NAD$^+$ alters CD11b$^+$CD11c$^+$ DC activation.

Thus we next assessed whether NAD$^+$ administration can induce CD11c$^+$CD11b$^+$ DC activation *in vivo* as well. C57BL/6 WT mice were treated daily with 40 mg of NAD$^+$ or PBS by means of intraperitoneal injection. Consistent with our *in vitro* findings, we found that NAD$^+$-treated WT mice showed increased cytokine production, including IL-1β, IL-4, IL-6, IL-10, IL-12, TGF-β1, and TNF-α, by CD11b$^+$CD11c$^+$ cells when compared with levels in PBS-treated WT mice (Fig 2). This is consistent with a previous study indicating that
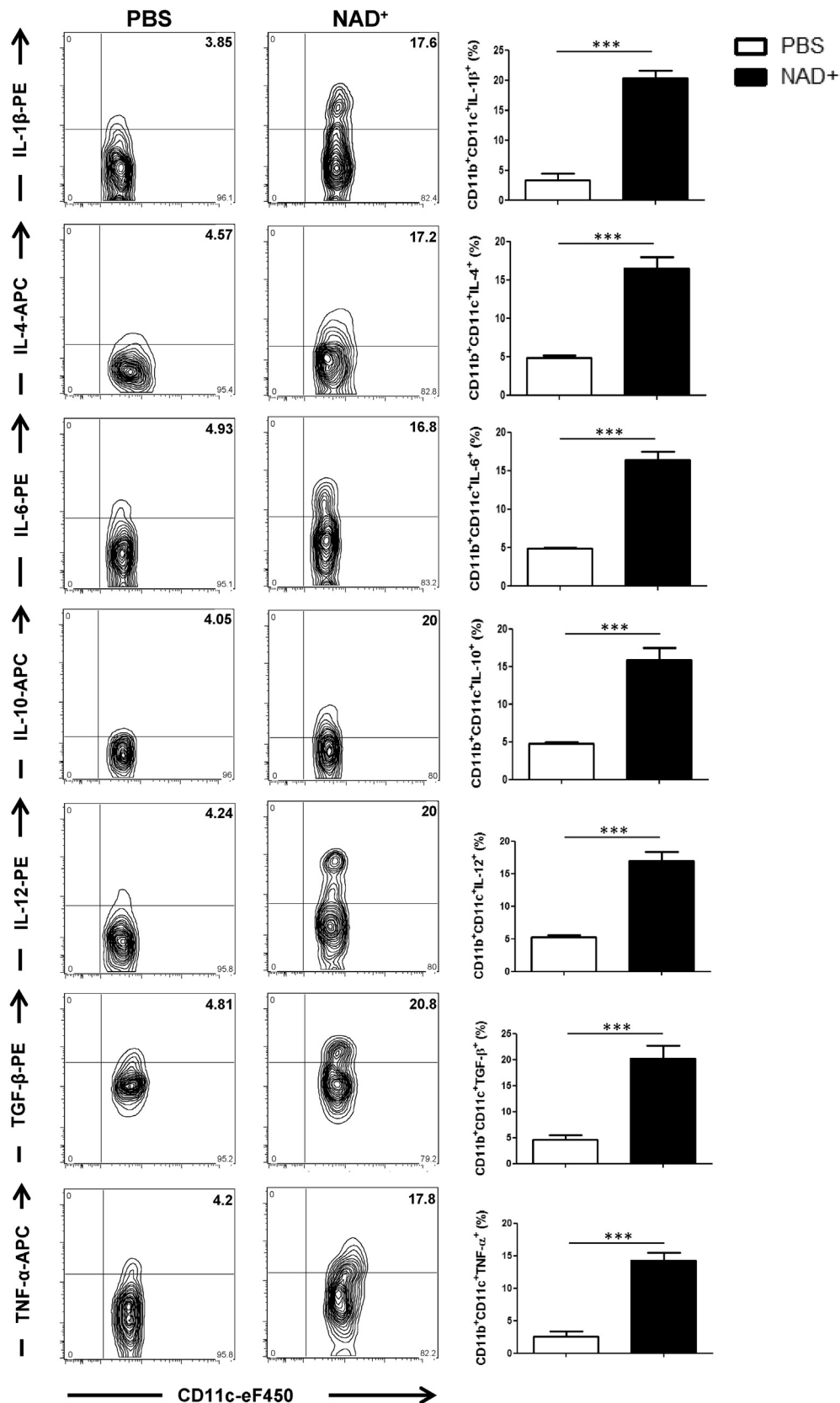
FIG 2. NAD$^+$ treatment induces increased cytokine production by CD11b$^+$CD11c$^+$ DCs *in vivo*. C57BL/6 mice were treated daily with intraperitoneal injection of 40 mg of NAD$^+$ or a placebo solution (PBS). After 7 days, mice were killed, and CD11b$^+$CD11c$^+$ DCs were isolated from spleens. Frequencies of CD11c$^+$IL-1β$^+$, CD11c$^+$IL-4$^+$, CD11c$^+$IL-6$^+$, CD11c$^+$IL-10$^+$, CD11c$^+$IL-12$^+$, CD11c$^+$TGF-β$^+$, and CD11c$^+$TNF-α$^+$ cells were analyzed by using flow cytometry. Statistics were as follows: n = 15. Data were derived from 3 independent sets of experiments. Data are presented as means ± SDs. The Student *t* test was used to compare groups: ***P < .001. *APC*, Allophycocyanin; *PE*, phycoerythrin.

intracellular NAD^+ levels regulate TNF-α cytokine production.[35] Taken together, our *in vitro* and *in vivo* data indicated that NAD^+ promotes $CD11b^+CD11c^+$ DC activation and cytokine production and might play a central role in NAD^+-mediated $CD4^+$ T-cell differentiation.

It is well established that DCs can promote $CD4^+$ T-cell differentiation through cytokine and chemokine release. Moreover, like NAD^+, ATP, a cofactor in energy metabolism, has been shown to enhance IL-6, IL-23, and TGF-β cytokine production by $CD11c^+$ cells and promote a T_H17 response.[32] Because our results indicated that NAD^+ promotes cytokine expression by $CD11b^+CD11c^+$ DCs, we tested whether $CD4^+$ T-cell differentiation resulted from NAD^+-mediated DC activation. As reported by us and others,[26,27] more than 99% of professional phagocytes, including both DCs and macrophages, were depleted in WT mice by means of injection of clodronate liposomes (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). As shown in Fig E2, *B*, DC depletion did not abolish $CD4^+IFN-γ^+$, $CD4^+IL-4^+$, and $CD4^+IL-17^+$ differentiation. These data suggest that NAD^+ regulates $CD4^+$ T-cell differentiation independently of DCs and macrophages.

Although NAD^+-mediated $CD4^+$ T-cell differentiation was not abolished after macrophage and DC depletion, we could not rule out compensation by other APCs, such as B cells. To characterize the role of B cells in NAD^+-mediated $CD4^+$ T-cell differentiation, we used transgenic $Rag2^{-/-}γc^{-/-}$ mice, which lack B, natural killer, and γδ T cells. In addition, $Rag2^{-/-}γc^{-/-}$ mice were subjected to depletion of DCs and macrophages. After depletion (see Fig E2, *A*), $Rag2^{-/-}γc^{-/-}$ mice received adoptive transfer of naive $CD4^+CD44^-CD62L^+$ T cells and were subjected to treatment with NAD^+ or placebo solution. The results indicated that NAD^+ induced a significant increase in numbers of $CD4^+IFN-γ^+$, $CD4^+IL-4^+$, $CD4^+IL-17^+$, and $CD4^+IL-10^+$ cells when compared with the control group of mice treated with a placebo solution (see Fig E3, *A*, in this article's Online Repository at www.jacionline.org). Moreover, treatment with diphtheria toxin of CD11b-DTR transgenic mice did not abolish $CD4^+IFN-γ^+$, $CD4^+IL-4^+$, and $CD4^+IL-17^+$ differentiation (see Fig E3, *B*). Taken together, our results indicate that NAD^+ promotes $CD4^+$ T-cell differentiation independently of B cells in addition to macrophages and DCs.

NAD^+ administration regulates $CD4^+$ T-cell fate through MCs exclusively

MCs have been mainly described for their role in allergic and autoimmune responses.[8,15,16] Although MCs express costimulatory molecules and secrete a myriad of chemokines and proinflammatory cytokines and have been shown to influence T-cell polarization, the mechanisms by which MCs regulate T-cell response remain unclear. Indeed, previous studies have reported that MC-deficient mice display defective $CD4^+$ but also $CD8^+$ T-cell responses after *Leishmania major* infection[36] and in the setting of EAE.[37] Furthermore, we have shown previously that NAD^+ protects against EAE.[25] Thus we next investigated the role of MCs in NAD^+-mediated $CD4^+$ T-cell differentiation. WT and MC-deficient mice (Kit^W/Kit^{W-v}) were treated daily with intraperitoneal injection of NAD^+. Treatment with NAD^+ in MC-deficient mice was not able to promote $CD4^+IFN-γ^+$, $CD4^+IL-4^+$, and $CD4^+IL-17^+$ T cells, indicating that MCs

were required for NAD^+-mediated $CD4^+$ T-cell differentiation (Fig 3).

Therefore we next sought to dissect the role of MCs in NAD^+-mediated T-cell differentiation *in vitro*. BMMCs were generated, as described in the Methods section. The average yield of MCs ($FcεRI^+c-Kit^+$ double-positive cells) increased with time and reached greater than 95% on day 90, as shown by using flow cytometry (see Fig E3, *C*). BMMCs were cultured over 6 weeks to express homogenous levels of KIT and FcεRI.[8] BMMCs were then directly cocultured with naive $CD4^+CD44^-CD62L^+$ T cells in the presence of NAD^+ or PBS. Moreover, MCs and T cells were cocultured in separate compartments by using a transwell system to determine whether these cells require cell-cell contact. As an additional control, naive $CD4^+CD44^-CD62L^+$ T cells were activated with α-CD3/α-CD28. The results indicated that in the presence of NAD^+, MCs promoted $CD4^+IFN-γ^+$, $CD4^+IL-4^+$, and $CD4^+IL-17^+$ T-cell differentiation in the absence of TCR activation (media plus NAD^+) and independently of cell-cell contact (see Fig E4 in this article's Online Repository at www.jacionline.org). Moreover, when MCs and naive $CD4^+CD44^-CD62L^+$ T cells were cocultured in the presence of NAD^+ and T cells were activated with α-CD3/α-CD28, frequencies of $CD4^+IFN-γ^+$ and $CD4^+IL-17^+$ T cells increased further (see Fig E4). These results were consistent with our initial findings indicating that cultured naive $CD4^+CD44^-CD62L^+$ T cells in the presence of NAD^+ and α-CD3/α-CD28 promoted $CD4^+IFN-γ^+$, $CD4^+IL-4^+$, and $CD4^+IL-17^+$ (Fig 1, *C* and *D*).

Like DCs, MCs express costimulatory molecules and secrete a myriad of cytokines that are known to regulate innate and adaptive immune responses. Thus to determine whether MCs mediate $CD4^+$ T-cell differentiation through costimulatory molecules, cytokines, or both, MCs were treated with NAD^+ *in vitro*, and mRNA levels of OX40 ligand, inducible costimulator ligand, CD80, CD86, TNF-α, IL-4, IL-6, and IL-33 were measured by using real-time PCR. NAD^+ induced a modest increase in CD80 and IL-33 and a decrease in IL-4 mRNA expression levels by MCs (see Fig E5, *A*, in this article's Online Repository at www.jacionline.org). It is well established that CD80 can either promote or inhibit activation of naive T cells by binding to CD28 or cytotoxic T lymphocyte–associated antigen 4, respectively. Therefore to assess the role of CD80 in NAD^+-MC–mediated $CD4^+$ T-cell differentiation, blockade of CD80 with a neutralizing antibody was performed *in vitro* by using our cell-cell contact coculture system. Our findings indicated that CD80 blockade did not reduce $CD4^+IFN-γ^+$ or $CD4^+IL-17^+$ T-cell frequencies when compared with an isotype control (see Fig E5, *B* and *C*). Of note, CD80 blockade resulted in increased $CD4^+IL-4^+$ T-cell frequencies (see Fig E5, *D*), suggesting that CD80 can play an inhibitory effect on MC-mediated IL-4 cytokine production. Collectively, our findings suggest that MC-mediated $CD4^+$ T-cell differentiation does not require cell-cell contact or involvement of conventional costimulatory molecules, such as CD80.

MC-mediated $CD4^+$ T-cell differentiation is conserved in the human MC line LAD-2

We next investigated whether this novel pathway is conserved in human subjects and whether human MCs could regulate human $CD4^+$ T-cell differentiation as well. Naive $CD4^+$ T cells were isolated from healthy donors and cocultured in direct contact or in
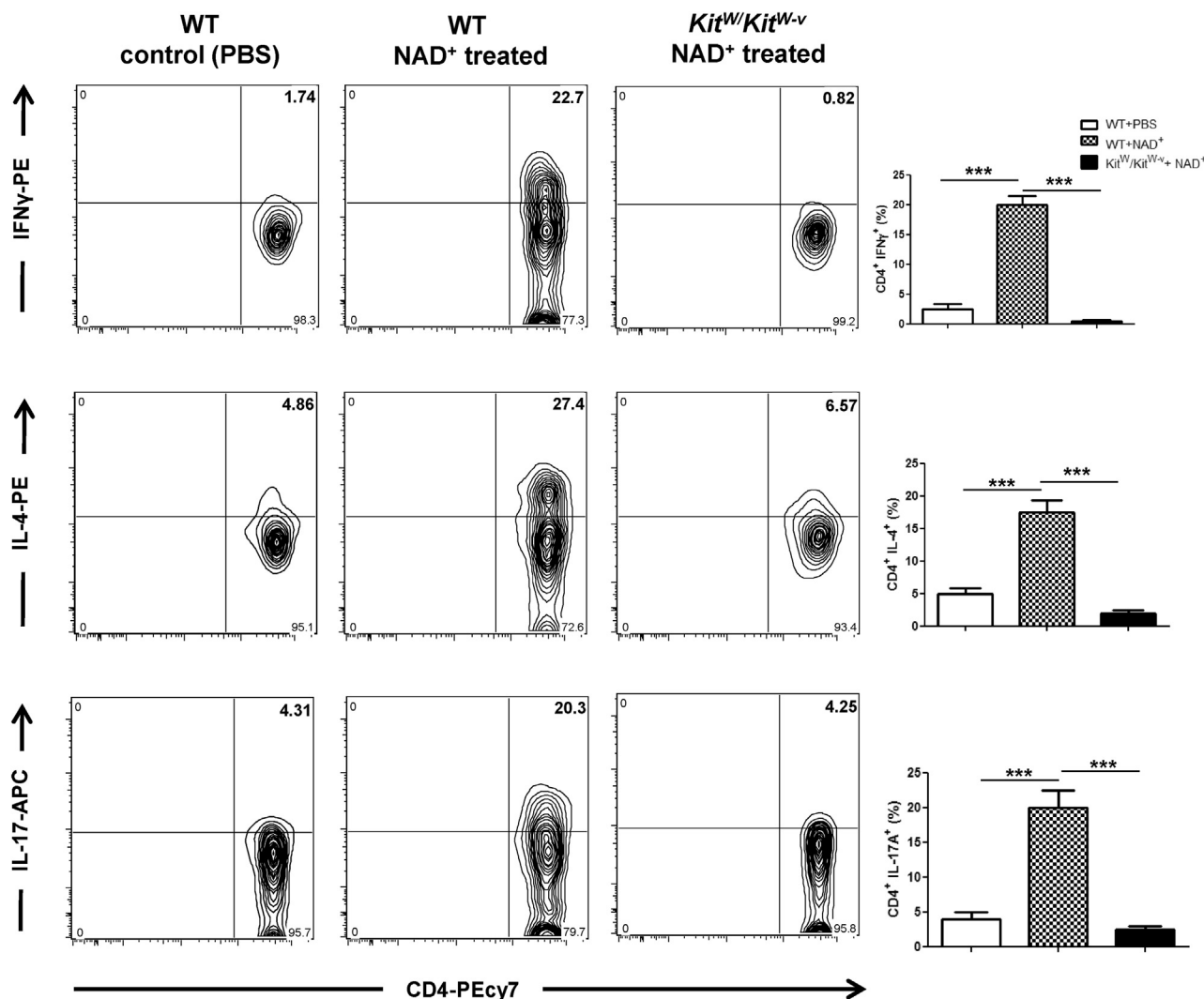
FIG 3. MCs play a central role in NAD$^+$-mediated CD4$^+$ T-cell differentiation. C57BL/6 WT and WBB6F1/ J*Kit*^{W}/*Kit*$^{W-v}$ (MC-deficient) mice were treated daily with intraperitoneal injection of 40 mg of NAD$^+$ or a placebo solution (PBS), as indicated. After 7 days, mice were killed, and systemic levels of CD4$^+$IFN-γ^+, CD4$^+$IL-4$^+$, and CD4$^+$IL-17A$^+$ cells were assessed by using flow cytometry. Data were derived from 2 independent experiments (n = 5-10). Data represent means $\pm$ SDs. ANOVA was used to compare groups: ***P < .001. *APC*, Allophycocyanin; *PE*, phycoerythrin.

our transwell system with LAD-2 cells, a well-established human MC line. Similar to murine BMMCs, cocultures were performed in the presence of NAD$^+$ or PBS and with or without α-CD3/α-CD28. Flow cytometry indicated that in the presence of NAD$^+$, human MCs promoted CD4$^+$IFN-γ^+ T-cell differentiation (see Fig E6, *A*, in this article's Online Repository at www.jacionline. org). Consistent with our murine BMMC data, human MCs were able to promote T$_H$1 polarization in the absence of cell-cell contact. Taken together, these results suggest that the MC-mediated CD4$^+$ T-cell differentiation pathway through MCs is conserved in human subjects as well.

Unique gene expression profile by MCs after NAD$^+$ activation

To unravel a potential signaling pathway involved in MC-mediated CD4$^+$ T-cell differentiation, we next performed an

RNA-sequencing analysis on BMMCs that were cultured for 16 hours in the presence of NAD$^+$, LPS, or PBS. As shown in Fig 4, *A*, the results indicated that NAD$^+$ significantly upregulated 603 genes and downregulated 753 genes (with a *P* value of less than .05) when compared with MCs treated with PBS. Moreover, when compared with LPS conditions, changes in the number of genes marked as differentially expressed were dramatic. When comparing LPS versus NAD$^+$ conditions, 6053 genes were significantly upregulated, and 5845 were found to be downregulated, suggesting that NAD$^+$ signaling machinery is distinct from LPS stimulation. As expected, dramatic changes in gene expression were observed when comparing PBS with LPS treatment (Fig 4, *A*).

To elucidate potential genes involved in MC-mediated CD4$^+$ T-cell differentiation, we investigated the role of the most upregulated genes in NAD$^+$ versus PBS conditions and compared them with gene expression characteristics in presence of LPS (Fig 4, *B*).
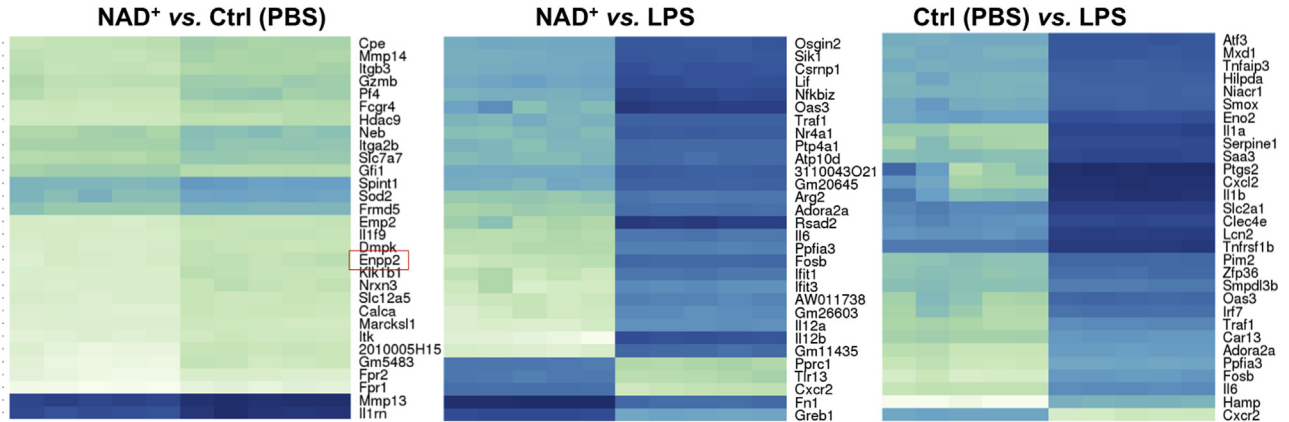
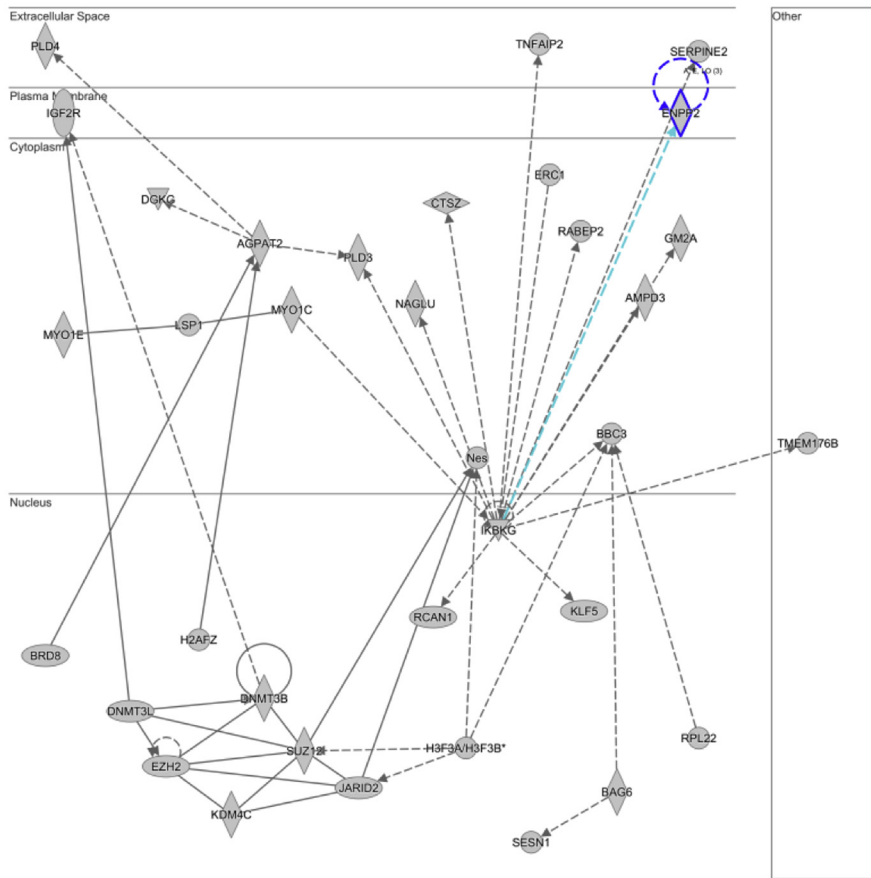**FIG 4.** Unique MC gene expression profile after NAD$^+$ activation. BMMCs from C57BL/6 mice were cultured in the presence of NAD$^+$ (500 μmol/L), LPS (10 μg/mL; *Escherichia coli* O127:B8), or placebo (PBS). After 16 hours of culture, cells were collected and RNA was extracted for RNA-sequencing analysis. **A,** Differential gene expression. **B,** Gene heat map expression profile. **C,** Ingenuity Pathway Analysis.

Among the first 10 genes upregulated in PBS versus NAD^{+} conditions, a robust upregulation of autotaxin (Enpp2), dystrophia myotonica protein kinase (DMPK), and FERM domain containing 5 (Frmd5) were specific to NAD^{+} conditions when compared with PBS or LPS treatment. Both DMPK[38,39] and FRMD5[40] proteins have been implicated in stabilizing cell membranes and cytoskeletons. Autotaxin has been shown to play a role in T-cell activation and chemotaxis,[41,42] and MCs have been shown to be able to produce autotaxin.[43] Furthermore, Ingenuity Pathway Analysis predicted significant perturbations by NAD^{+} of other signaling molecules involved in MC activation, such as *TNFAIP2* or *IKBKG* (NEMO; Fig 4, *C*). It is well established that the ectoenzyme autotaxin generates lysophosphatidic acid (LPA), a potent lipid mediator that acts on a series of specific G protein–coupled receptors through hydrolysis of lysophosphatidylcholine.[41] LPA can be produced by a myriad of different cell types that include postmitotic neurons, adipocytes, MCs, and other lymphoid cells. Autotaxin has been recently described as regulating cytokine production, and autotaxin-LPA pathways have been shown to play a critical role in asthmatic patients.[44] Both human and murine T cells express LPA receptors,[45,46] and LPA has been found to inhibit TCR-mediated calcium mobilization.[47]

Collectively, our results indicate that MC activation by NAD^{+} triggers a unique gene expression profile that is distinct from LPS stimulation and suggests the existence of an alternative pathway that remains to be determined.

MCs induce T-cell differentiation in MHC class II^{−/−} and WASP^{−/−} mice after NAD^{+} administration

Our findings indicate that NAD^{+} promotes CD4^{+} T-cell differentiation through MCs and in the absence of antigen and TCR activation. Thus we next sought to determine the role of MHC class II and TCR molecules in NAD^{+}-MC–mediated CD4^{+} T-cell differentiation. MHC class II^{−/−} mice were treated daily with NAD^{+} or a placebo solution (PBS). After 7 days, flow cytometry showed that NAD^{+} was able to promote a significant increase in CD4^{+}IFN-γ^{+}, CD4^{+}IL-4^{+}, and CD4^{+}IL-17^{+} T cells in MHC class II–deficient mice (see Fig E6, *B*).

In addition, WASP^{−/−} mice, which are known to have an altered TCR activation and cytokine production, in particular IFN-γ,[48] were used to assess the role of TCR in NAD^{+}-MC–mediated CD4^{+} T-cell differentiation. Thus WASP^{−/−} mice were treated daily with intraperitoneal injection of NAD^{+} or PBS to assess whether NAD^{+}-MC–mediated CD4^{+} T-cell differentiation could promote IFN-γ production also in the context of immunodeficiency. The results indicated that NAD^{+} was able to promote a robust increase in CD4^{+}IFN-γ^{+}, CD4^{+}IL-4^{+}, and CD4^{+}IL-17^{+} T-cell frequencies in WASP^{−/−} mice when compared with the control group (see Fig E6, *C*). Collectively, our results suggest that NAD^{+}-MC–mediated CD4^{+} T-cell differentiation is functional, even in the absence of MHC class II or with altered TCR activation.

Daily NAD^{+} treatment protects against lethal doses of *L monocytogenes* through MCs exclusively

WT mice were pretreated for 5 consecutive days with NAD^{+} before infection with a nonlethal dose of *L monocytogenes* to test whether regulation of innate and adaptive immune cells through MCs after NAD^{+} administration had a physiologic effect.

In addition, a control group of animals was pretreated with PBS before infection. Mice treated with PBS had only a modest loss of weight, did not exhibit signs of lethargy or gained weight 7 days after infection, and showed 100% survival (Fig 5, *A*) when infected with a nonlethal dose of *L monocytogenes*. In contrast, mice pretreated with NAD^{+} before infection (nonlethal dose) had a more pronounced loss of weight, exhibited lethargy, and were very susceptible to infection with an 80% mortality rate (Fig 5, *A*).

Thus we tested whether MCs play a role in *L monocytogenes* infection survival outcome after NAD^{+} administration. Indeed, *Kit^W/Kit^Wv* MC–deficient mice showed improved survival compared with NAD^{+}-treated WT mice. In contrast to WT mice pretreated with NAD^{+}, *Kit^W/Kit^Wv* transgenic mice had only a modest loss of weight and did not exhibit signs of lethargy. More importantly, *Kit^W/Kit^Wv* mice pretreated with NAD^{+} showed an 80% survival rate (Fig 5, *A*), confirming our *in vivo* and *in vitro* findings and indicating that MCs play a central role in the capacity of NAD^{+} to regulate innate and adaptive immunity. Furthermore, we tested another set of MC-deficient mice *(Kit^Wsh/Kit^Wsh)* in the context of *L monocytogenes* infection. Similar to *Kit^W/Kit^Wv* mice, *Kit^Wsh/Kit^Wsh* mice exhibited a 100% survival rate with no sign of lethargy. Collectively, NAD^{+}-mediated CD4^{+} T-cell differentiation dramatically altered the immune responses through MCs and significantly affected physiologic functions of protection against bacterial infection.

Our results indicated that pretreatment with NAD^{+} promoted T$_H$1, T$_H$2, and T$_H$17 CD4[+] T-cell subsets. Because T$_H$1 has been shown to play a protective role in *L monocytogenes* infection,[35] we initially expected NAD[+] pretreatment to protect mice after bacterial infection. However, we could not rule out that continuous NAD[+] treatment might be required to sustain a robust T$_H$1 response. Thus we assessed whether prolonged and continuous treatment with NAD[+] could provide protection against *L monocytogenes* infection. As shown in Fig 5, *B*, WT mice continuously treated with NAD[+] exhibited resistance (>60% survival rate) to even lethal doses of *L monocytogenes* when compared with the control group of mice that exhibited a 100% mortality rate. Consistent with our previous findings, NAD[+]-mediated protection against lethal doses of *L monocytogenes* was abolished in both *Kit^W/Kit^Wv* and *Kit^Wsh/Kit^Wsh* MC[−/−] mice. We have shown previously that NAD[+] regulates CD4[+] T-cell differentiation through a pathway distinct of well-established transcription factors.[25] In addition, we showed that NAD[+] altered dramatically differentiated T$_H$1 and T$_H$2 CD4[+] T cells by repressing their cytokine production and transcription factors.[25] More importantly, second stimulation of differentiated T$_H$1 and T$_H$2 CD4^{+} T cells was profoundly altered when they were cultured in the presence of NAD^{+} after first stimulation.[25]

Thus we next investigated whether pretreatment with NAD^{+} altered the classical CD4^{+} T-cell differentiation pathway,[36] specifically in CD4^{+}IFN-γ^{+} T cells that have been shown to be essential for host resistance to *L monocytogenes* infection. We assessed whether NAD^{+} altered frequencies of CD4^{+}T-bet^{+} T cells. Mice pretreated with NAD^{+} had lower frequencies and total numbers of CD4^{+}IFN-γ^{+}T-bet^{+} cells when compared with mice treated with a placebo solution (see Fig E7, *A*, in this article's Online Repository at www.jacionline.org). Although treatment with NAD^{+} resulted in downregulation of T-bet
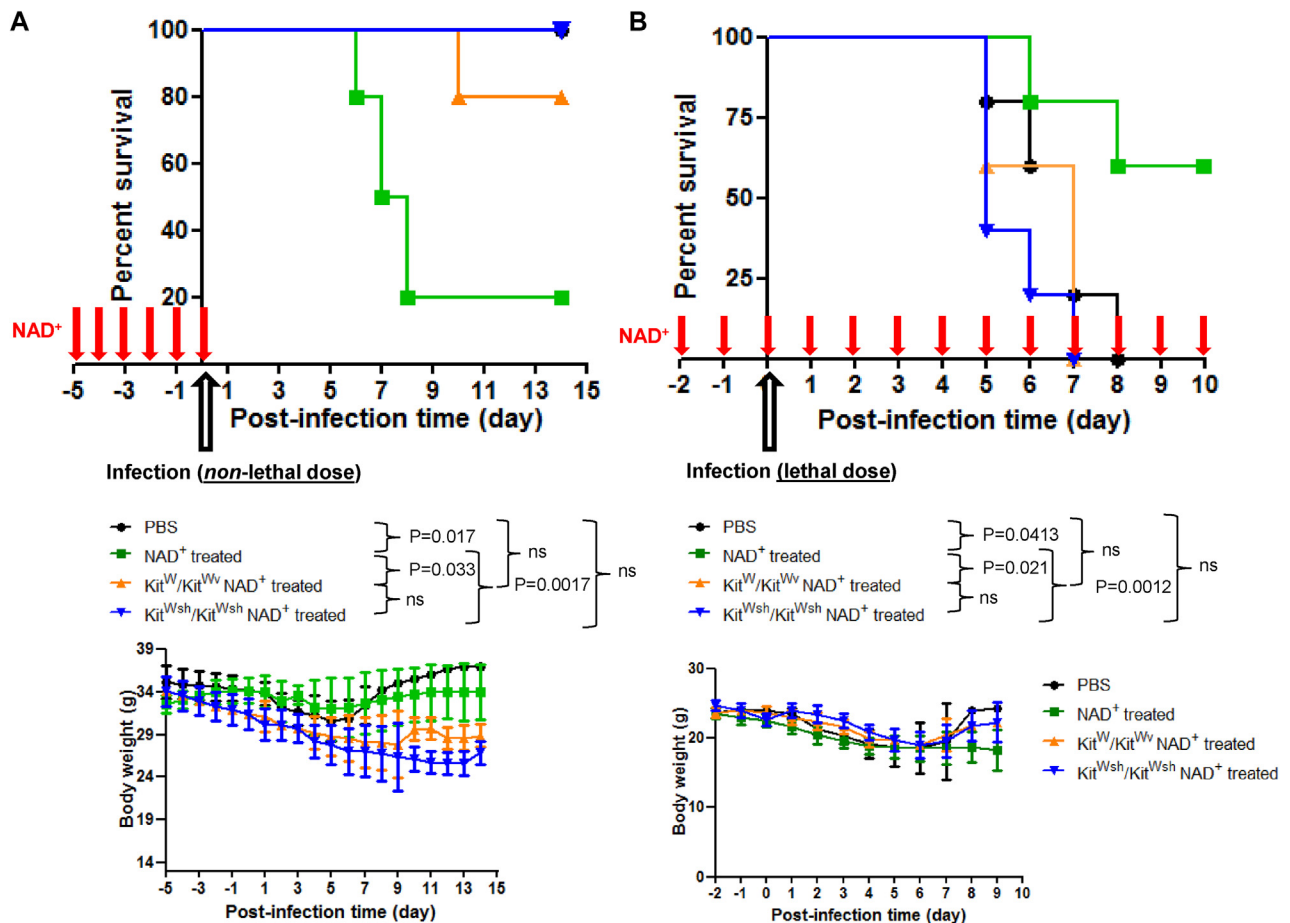
FIG 5. MCs mediate protection against lethal doses of *L monocytogenes* during daily NAD$^+$ administration. C57BL/6 WT, Kit^W/Kit^{W-v}, and kit^{Wsh}/Kit^{Wsh} MC-deficient mice were pretreated for 5 consecutive days with daily intraperitoneal injections of NAD$^+$ (40 mg) or placebo solution (PBS). **A,** Mice were then infected with an intraperitoneal nonlethal dose (1 × 10^7 colony-forming units) of viable *L monocytogenes*. **B,** C57BL/6 WT mice were pretreated for 5 consecutive days with NAD$^+$ (40 mg administered intraperitoneally) or placebo solution (PBS) before infection. WT mice were then treated daily with NAD$^+$ (40 mg administered intraperitoneally) or placebo solution (PBS) after a lethal dose (1 × 10^8 colony-forming units) of viable *L monocytogenes*. Weight loss and survival after infection were monitored. Data were derived from 3 independent experiments (n = 5-10 per group). Data represent means ± SDs. *ns*, Not significant. ANOVA and log-rank tests were used to compare groups, respectively.

expression, CD4$^+$ T cells from mice treated with NAD$^+$ mounted robust IFN-γ production (see Fig E7, *B*), which was consistent with our previous studies.[24,25] Although CD4$^+$IFN-γ$^+$ T-cell responses remained similar in NAD$^+$- and placebo-treated mice, continuous treatment with NAD$^+$ promoted host protection against a lethal dose of *L monocytogenes*. In addition, NAD$^+$ affected the regulation and function of other important immune cells, such as MCs and, more importantly, DCs. Thus we investigated whether NAD$^+$ promotes IFN-γ production that is not mediated by CD4$^+$ T cells. Our results indicated that mice treated with NAD$^+$ exhibited a significant increase in IFN-γ production by non-CD4$^+$ T cells, suggesting that NAD$^+$ can promote IFN-γ production by other non-CD4$^+$ lymphocytes (see Fig E7, *C*). Taken together, our results indicate that continuous treatment with NAD$^+$ promotes robust IFN-γ production and protects against lethal doses of *L monocytogenes* through MCs exclusively.

Pretreatment with NAD$^+$ renders mice susceptible to sublethal doses of *L monocytogenes* by dampening the MCH class II–TCR pathway

Our data indicate that mice pretreated with NAD$^+$ are susceptible to sublethal doses of *L monocytogenes*, whereas continuous treatment protects against lethal doses. Thus we next investigated how pretreatment with NAD$^+$ rendered mice susceptible to a sublethal dose of bacteria.

Because antigen presentation through the MHC class II cell-surface molecule is crucial in CD4$^+$ T-cell responses and our results indicated that NAD$^+$ promotes CD4$^+$ T-cell differentiation in the absence of MHC class II molecules, we investigated the effect of NAD$^+$ pretreatment on MHC class II expression of CD11b$^+$CD11c$^+$ DCs after *L monocytogenes* infection. The results indicated that pretreatment with NAD$^+$ resulted in a dramatic decrease in MHC class II expression when compared with the control group (see Fig E7, *D*), indicating that NAD$^+$
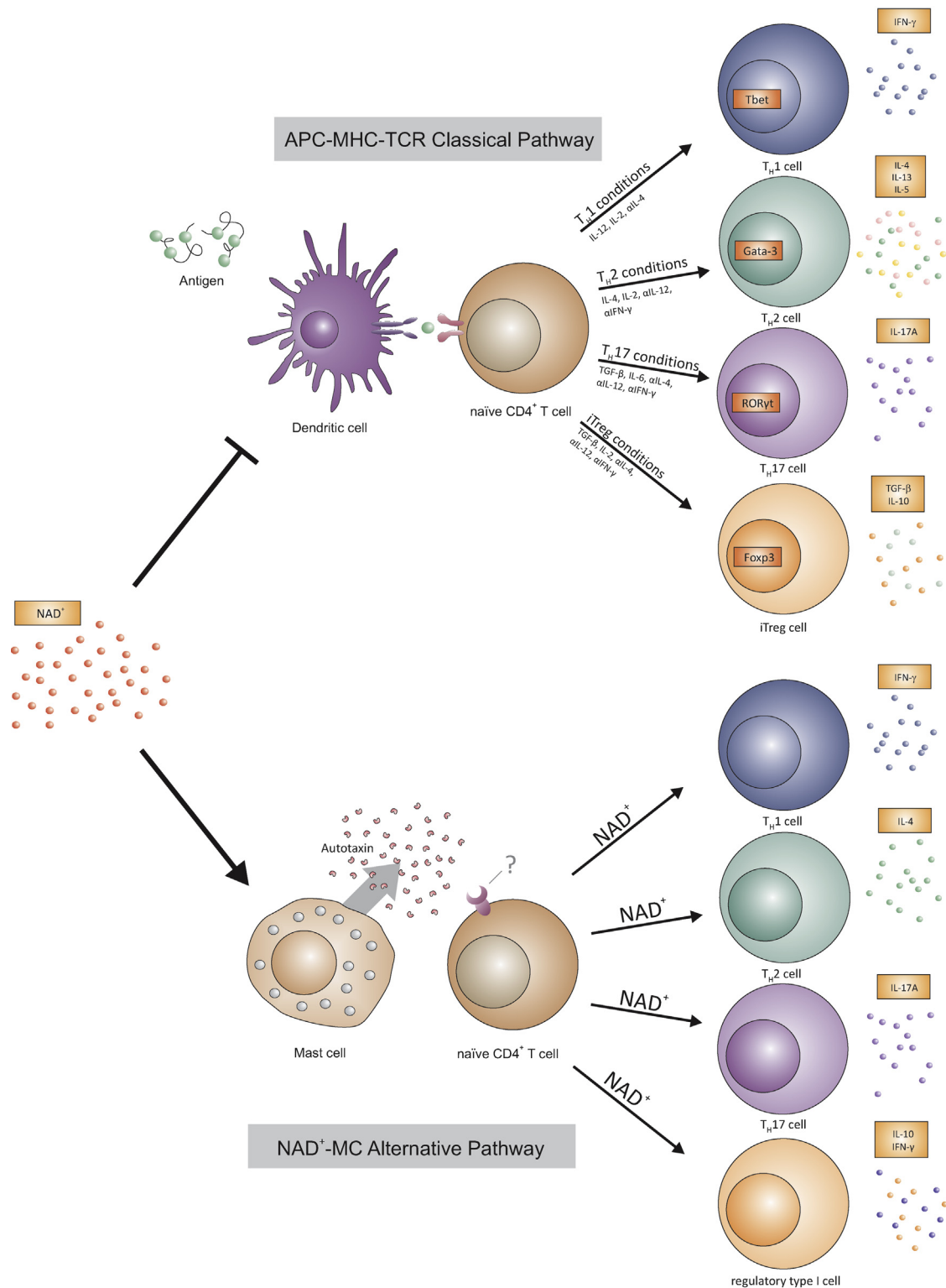
FIG 6. NAD$^+$ regulates T-cell differentiation through a novel MC-dependent signaling pathway. MC-mediated CD4$^+$ T-cell differentiation after NAD$^+$ administration does not require antigen presentation through major APCs and MHC class II.

dampens antigen presentation capacities of DCs after *L monocytogenes* infection. WT mice pretreated for 5 consecutive days with NAD$^+$ before infection with a nonlethal dose of *L monocytogenes* had a more pronounced loss of weight, exhibited lethargy, and were very susceptible to infection with an 80% mortality rate (Fig 5, *A*). In contrast, WT mice continuously treated with NAD$^+$ exhibited resistance (>60% survival rate) to even lethal doses of *L monocytogenes* when compared with the control group of mice that exhibited a 100% mortality rate (Fig 5, *B*).

Collectively, our results indicate that pretreatment with NAD$^+$ will trigger the NAD$^+$-MC pathway while dampening the APC–MHC class II–TCR signaling machinery, suggesting that discontinuous administration of NAD$^+$ can subject the animals to an immunodeficient state that renders them susceptible to sublethal doses of *L monocytogenes*. Collectively, our results indicate that NAD$^+$ profoundly alters, in the absence of antigen, innate and adaptive immune responses through MCs exclusively and independently from the classical DC/MHC class II antigen presentation machinery that results in significant physiologic changes (Fig 6).

DISCUSSION

Our previous studies have demonstrated a novel role for NAD$^+$ in regulating T-cell fate in the context of antigen-specific responses.[24,25] However, whether NAD$^+$ mechanisms of action operate in an antigen-independent manner and whether NAD$^+$ affects other immune cells remained unknown. APCs, in particular DCs, have been considered the most potent immune cells to prime naive CD4$^+$ T cells. In addition to their capacity for antigen processing and presentation to the TCR through MHC class II cell-surface molecules, DCs can regulate CD4$^+$ T-cell activation through costimulatory molecules and a myriad of secreted cytokines and chemokines.[8] Moreover, it has been reported that recognition of conserved PAMPs, such as microbial nucleic acids, lipoproteins, and carbohydrates, or DAMPs, which are released from injured, through intracellular or distinct PRRs expressed on DCs contribute to pathogen-specific CD4$^+$ T-cell responses.[12] Thus DCs and antigen presentation are regarded as fundamentally required and play a central role in CD4$^+$ T-cell activation and differentiation and are considered the bridge between innate and adaptive immune responses.[1,7,8,49] These observations are widely supported by studies based on transgenic animals with alteration in MHC class II, TCR, and DC depletion and confirmed in immunodeficient patients.[50,51]

Here we demonstrated that NAD$^+$, a natural cofactor, induces CD4$^+$ differentiation in the absence of antigen presentation, MHC class II expression, and absence of TCR activation. Furthermore, we demonstrate that effects of NAD$^+$ are not mediated by major APCs, including DCs, but through MCs exclusively. Moreover, our study indicated that major costimulatory molecules are not involved in NAD$^+$-MC–mediated CD4$^+$ T-cell differentiation, suggesting that MCs deliver T-cell stimulation through an alternative pathway. This is in line with previous studies indicating that MCs cannot prime naive CD4$^+$ T cells, most likely because of an altered costimulatory molecule expression.[8] Furthermore, previous studies have reported that MCs express MHC class II molecules; however, it has been found to be sequestered mainly in lysosomal intracellular compartments.[22] This is consistent with our findings indicating that NAD$^+$-MC–mediated CD4$^+$ T-cell differentiation does not require antigen presentation or MHC class II molecules.

The cofactor ATP is well recognized for its role as a source of high energy and in cellular metabolism; however, when released from cells after cellular damage, ATP acts as a DAMP signal. DAMP signaling is mainly mediated through the PRRs that are expressed on APCs, in particular DCs and macrophages.[52] Although the primary function of PRRs is to mediate innate immune responses to pathogen invasion and tumors, increasing evidence has shown their role in the development of autoimmune and chronic inflammation[52] by promoting T$_H$17 responses. ATP, for instance, has been shown to convert CD4$^+$CD25$^+$Foxp3$^+$ regulatory T cells into T$_H$17 cells and to promote colitis by enhancing the T$_H$17 proinflammatory subset in the lamina propria through DCs.[32,53] Similar to ATP, NAD$^+$, which is also a cofactor, has been described for its role in energy metabolism and more recently in aging.[54-56] In contrast to ATP, NAD$^+$-mediated innate and adaptive immune regulation was intermediated by MCs and was independent of major APCs, including DCs and macrophages. Moreover, our previous studies have underscored the robust immunosuppressive properties of NAD$^+$ by promoting a systemic increase in IL-10 cytokine levels through regulatory type 1 cells.[24,25] We have also shown that NAD$^+$ regulates CD4$^+$ T-cell fate in the absence of major transcription factors. Indeed, NAD$^+$ was able to promote a robust T$_H$1 response in the absence of T-bet, a transcription factor considered indispensable for T$_H$1 differentiation and IFN-γ production.[25] In contrast to proinflammatory responses induced by DAMPs, including ATP, NAD$^+$ protects against autoimmune diseases and promotes allograft survival.[24,25] Furthermore, our RNA-sequencing analysis indicated that treatment with NAD$^+$ resulted in upregulation of a distinct set of genes when compared with LPS, a prototypical PAMP, suggesting that NAD$^+$ triggered a different signaling pathway that remains yet to be determined in detail.

In line with our previous reports, NAD$^+$ treatment downregulated T-bet expression but did not alter CD4$^+$IFN-γ$^+$ T-cell responses when compared with those in the control group of mice. Although CD4$^+$IFN-γ$^+$ T-cell responses remained similar in NAD$^+$ and placebo-treated mice, continuous treatment with NAD$^+$ conferred host protection against *L monocytogenes*. Previous studies have underscored the critical role of IFN-γ in host protection against *L monocytogenes*.[9,57] Thus NAD$^+$ might mediate host protection by promoting IFN-γ production by other immune cells, such as CD8$^+$ cells or innate lymphoid cells. Although our study emphasizes the potent role of NAD$^+$ on many immune cells, such as CD4$^+$ T cells, MCs, and DCs, its effect on other important immune cells, such as B cells, CD8$^+$ cells, and innate lymphoid cells, remain yet to be determined, and its role in other inflammatory conditions or infections, including viral infections, requires further investigation.

More importantly, our study unravels a novel cellular mechanism that regulates CD4$^+$ T-cell differentiation, a process known to play a central role in many inflammatory conditions.[58] NAD$^+$-mediated immune changes in the absence of antigen had a robust physiologic effect after bacterial infection. Indeed, NAD$^+$-pretreated mice were highly susceptible to even a nonlethal dose of bacterial infection that resulted in high mortality. In contrast, maintenance of NAD$^+$ signaling machinery by continuous treatment with NAD$^+$ conferred host protection after infection. More importantly, NAD$^+$ treatment altered T-bet expression and MHC class II expression on CD11c$^+$ DCs, suggesting that the NAD$^+$-MC–mediated CD4$^+$ T-cell differentiation pathway might not only promote robust host protection against bacterial infection

but also might be distinct from the classical DC–MHC class II–TCR pathway. Indeed, with clodronate administration, DTR transgenic and MC-deficient mice (Kit^W/Kit^{W-v}), and BMMCs, we demonstrated that NAD^+-MC–mediated $CD4^+$ T-cell differentiation is independent of the classical DC–MHC class II–TCR pathway. More importantly, our data indicated that in the presence of NAD^+, MCs mediated differentiation of both murine and human $CD4^+$ T cells. These findings suggest that NAD^+ can pave the way for novel therapies in the context of microbial infections or primary immunodeficiencies that are characterized by altered DC–MHC class II–TCR signaling machinery.

In summary, our study demonstrates the emerging role of NAD^+ as a new paradigm in innate and adaptive immune responses with its unique property as an MC-mediated immune regulator that is distinct from the classical APC–MHC class II–TCR pathway.

Key messages

- NAD^+ alone regulates $CD4^+$ T-cell fate in the absence of antigen.
- NAD^+ regulates $CD4^+$ T-cell fate independently of major APCs and MHC-TCR signaling machinery.
- Mast cells exclusively regulate $CD4^+$ T-cell differentiation after NAD^+ administration.

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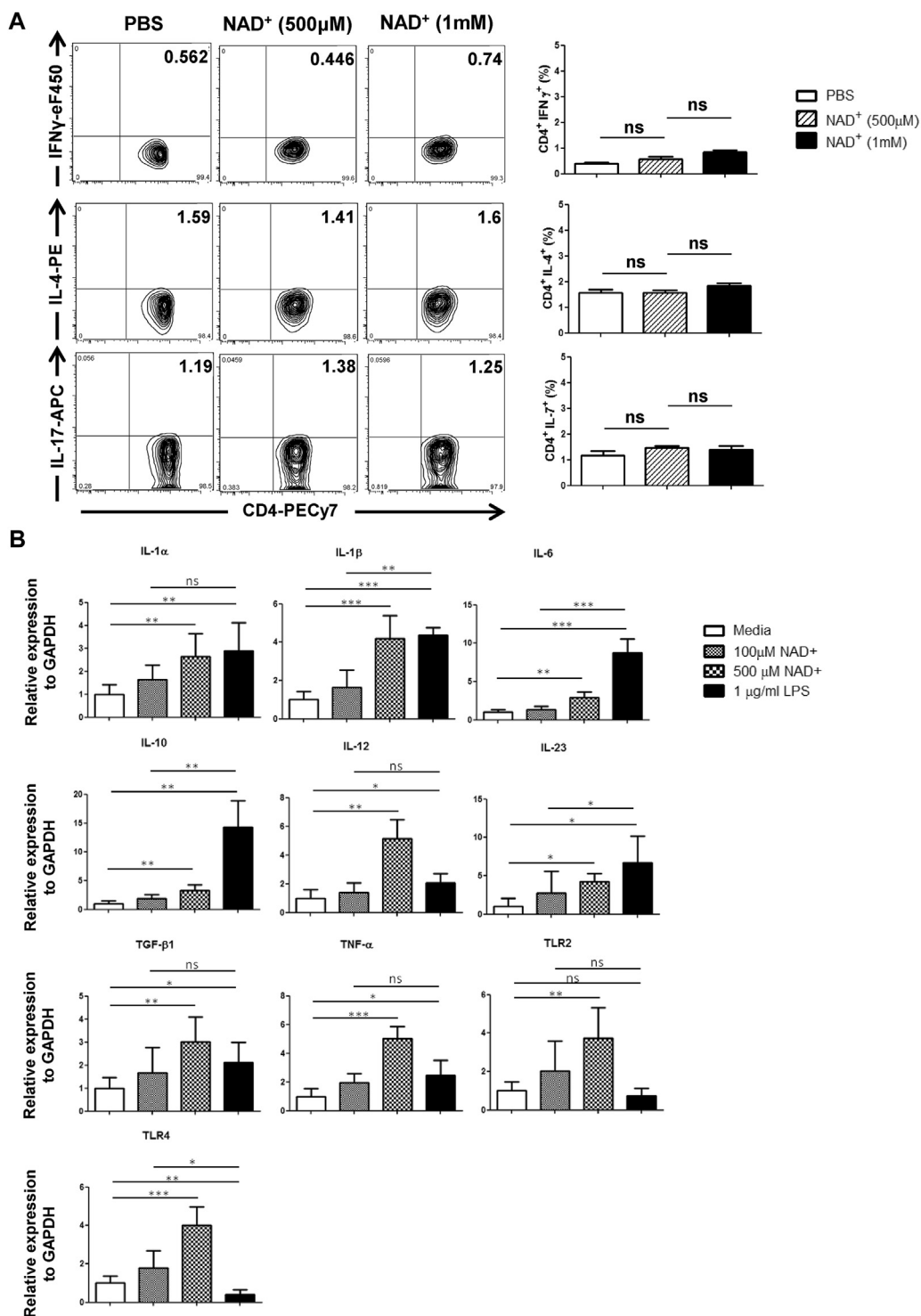**FIG E1.** High doses of NAD$^+$ do not promote naive CD4$^+$ T-cell differentiation *in vitro*, and NAD$^+$ regulates CD11b$^+$CD11c$^+$ DC cytokine production *in vitro* in a dose-dependent manner. **A,** Sorted naive CD4$^+$CD44$^-$CD62L$^+$ T cells were isolated from spleens of C57BL/6 mice and cultured in complete media with increasing concentrations of NAD$^+$ (500 μmol/L and 1 mmol/L) or PBS. After 96 hours, frequencies of CD4$^+$IFN-γ$^+$, CD4$^+$IL-4$^+$, and CD4$^+$IL-17A$^+$ cells were assessed by using flow cytometry (n = 10). Data were derived from 3 independent experiments. **B,** Sorted CD11b$^+$CD11c$^+$ DCs were isolated from spleens of C57BL/6 mice and cultured (1 × 10^6 cells/well) in complete media and in the presence of increasing concentrations of NAD$^+$ (100 μmol/L and 500 μmol/L). As a positive control, CD11b$^+$CD11c$^+$ DCs were cultured in the presence of LPS (1 μg/mL). After 16 hours of culture, cells were collected, and mRNA expression levels of IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-23, TGF-β, TNF-α, TLR2, and TLR4 were determined by using real-time PCR. Values are expressed as fold expression relative to the housekeeping gene glyceraldehye-3-phosphate dehydrogenase (*GAPDH*; n = 5). Data were derived from 2 different experiments. *ns,* Not significant. *$P < .05$, **$P < .01$, and ***$P < .001$, as determined by means of ANOVA, comparing the indicated groups. Data represent means ± SDs. *APC,* Allophycocyanin; *PE,* phycoerythrin.
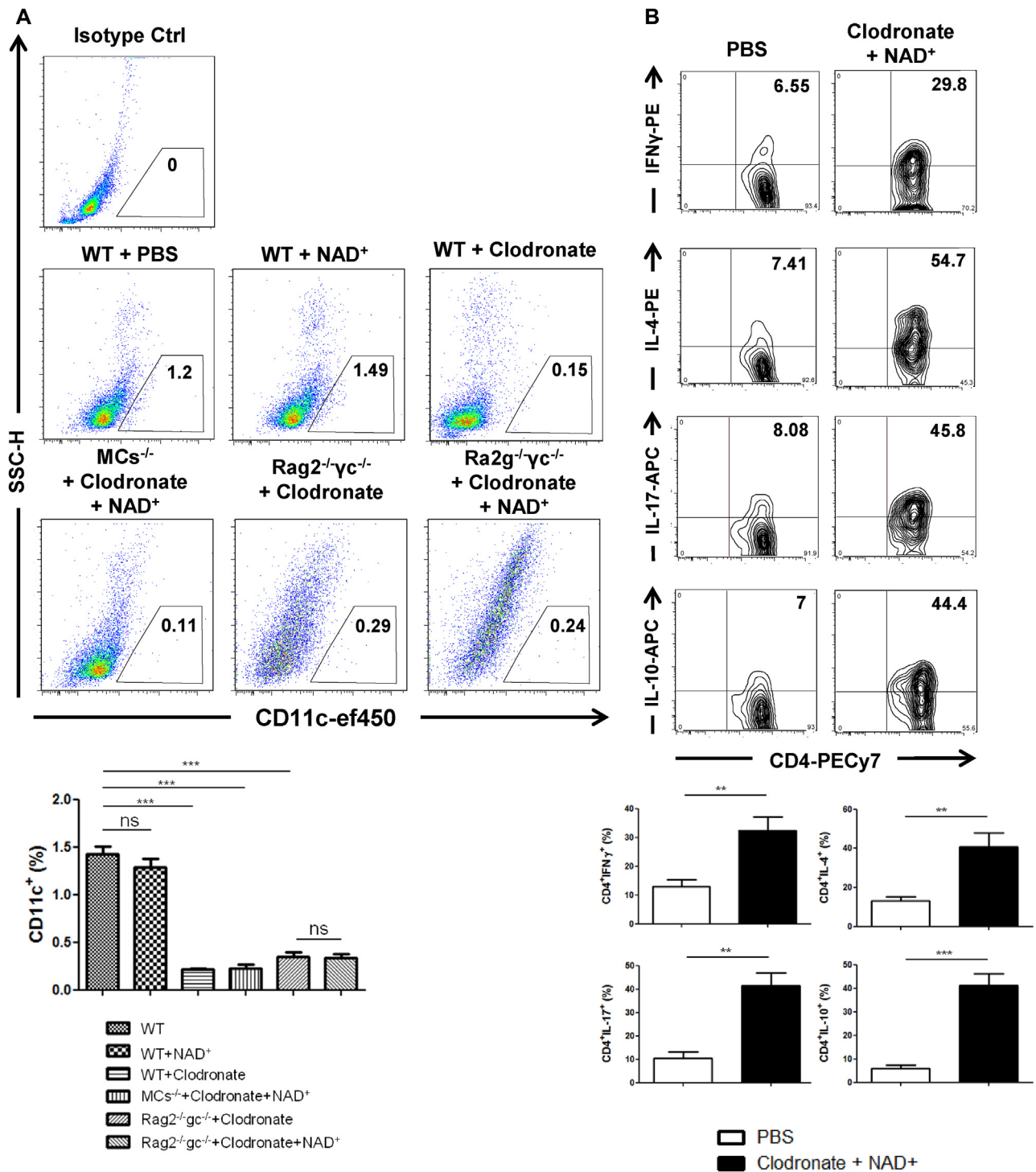
FIG E2. *In vivo* depletion of DCs by means of liposomal clodronate administration does not alter NAD⁺-mediated CD4⁺ T-cell differentiation. **A,** C57BL/6 WT, MC$^{-/-}$, and Rag2$^{-/-}$γc$^{-/-}$ mice were treated intravenously with liposomal clodronate at −8 days, −5 days, and −1 day before NAD⁺ treatment. Data derived from 2 independent experiments (n = 5 per group). **B,** C57BL/6 WT mice were treated intravenously with liposomal clodronate at −8 days, −5 days, and −1 day before NAD⁺ treatment. Mice were then treated with daily intraperitoneal injections of 40 mg of NAD⁺ or a placebo solution (PBS). After 7 days, mice were killed, and frequencies of CD4⁺IFN-γ⁺, CD4⁺IL-4⁺, and CD4⁺IL-17A⁺ cells were analyzed by using flow cytometry. Data were derived from 2 independent experiments (n = 10). Data represent means ± SDs. *ns,* Not significant. The Student *t* test and ANOVA were used to compare between groups: **P < .01 and ***P < .001. *APC,* Allophycocyanin; *PE,* phycoerythrin; *SSC,* side scatter.
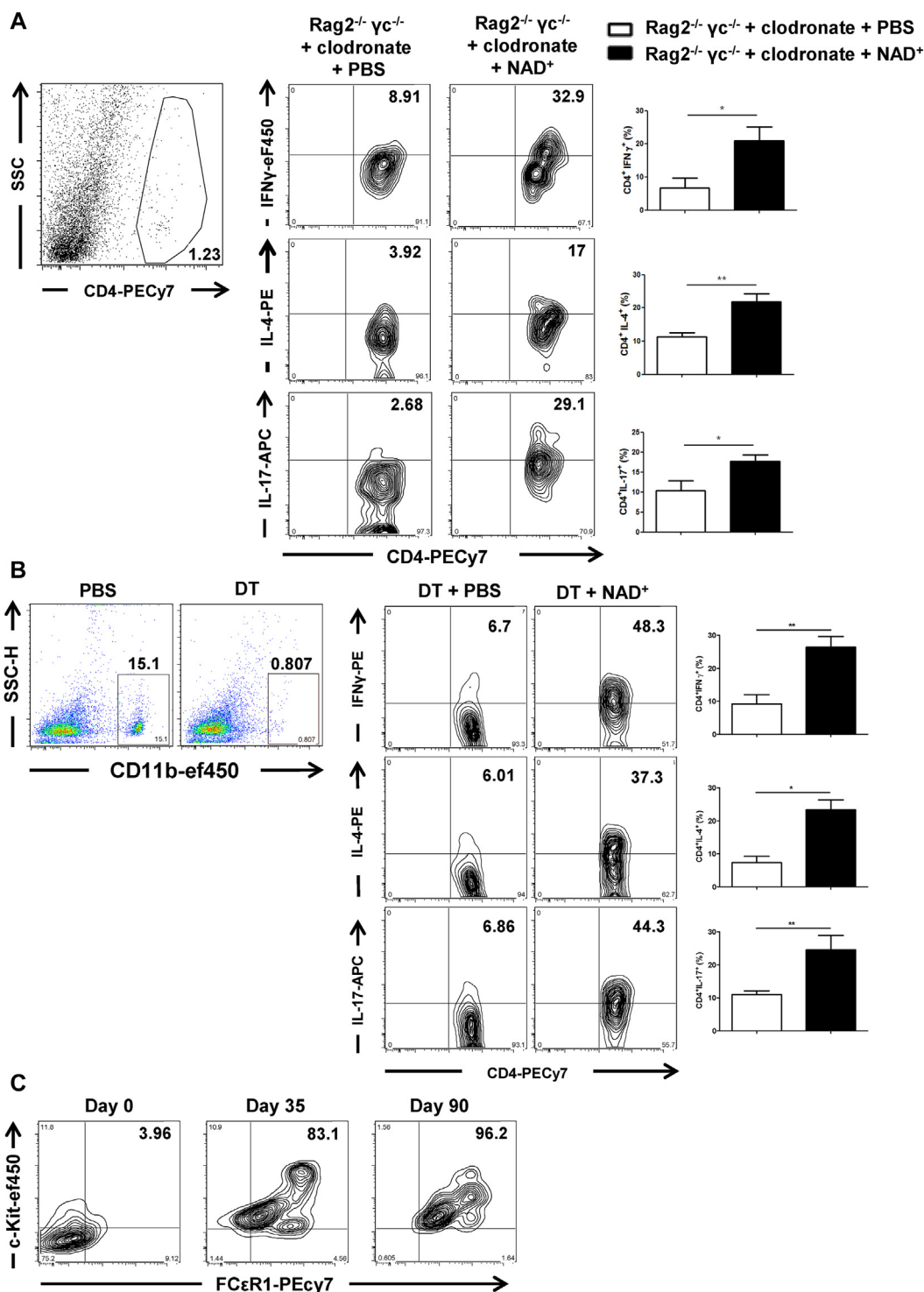
FIG E3. NAD$^+$ promotes T-cell differentiation in Rag2$^{-/-}$γc$^{-/-}$ mice and flow cytometry of *in vitro* differentiation of BMMCs. **A,** Rag2$^{-/-}$γc$^{-/-}$ mice were treated intravenously with liposomal clodronate at −8 days, −5 days, and −1 day before NAD+ treatment. After depletion, sorted naive CD4$^+$CD44$^-$CD62L$^+$ T cells from C57BL/6 WT mice sorted by means of fluorescence-activated cell sorting were adoptively transferred (3 × 10^6 cells per adoptive transfer). Animals were then treated with daily intraperitoneal injections of 40 mg of NAD$^+$ or a placebo solution (PBS). After 7 days, mice were killed, spleens were collected, and frequencies of CD4$^+$IFN-γ$^+$, CD4$^+$IL-4$^+$, and CD4$^+$IL-17A$^+$ cells were assessed by using flow cytometry. Data were derived from 2 independent experiments (n = 10). **B,** CD11b-DTR transgenic mice weighing 25 to 30 g were injected with diphtheria toxin (*DT*; 25 ng/g body weight) 24 hours before and 72 hours after beginning NAD$^+$ or PBS administration for depletion of CD11b$^+$ cells. After 7 days of treatment with PBS or NAD$^+$, frequencies of CD4$^+$IFN-γ$^+$, CD4$^+$IL-4$^+$, and CD4$^+$IL-17A$^+$ cells were assessed by using flow cytometry. Data were derived from 2 independent experiments (n = 10). **C,** BMMCs were obtained from femurs and tibias of 6- to 8-week-old C57BL/6 WT mice. BMMCs were cultured in WEHI-3–conditioned medium over 90 days. Purities of c-Kit$^+$FcεRI$^+$ MCs were then assessed by using flow cytometry. Data represent means ± SDs. The Student *t* test was used to compare between groups: *P < .05 and **P < .01. *APC*, Allophycocyanin; *PE*, phycoerythrin; *SSC*, side scatter.
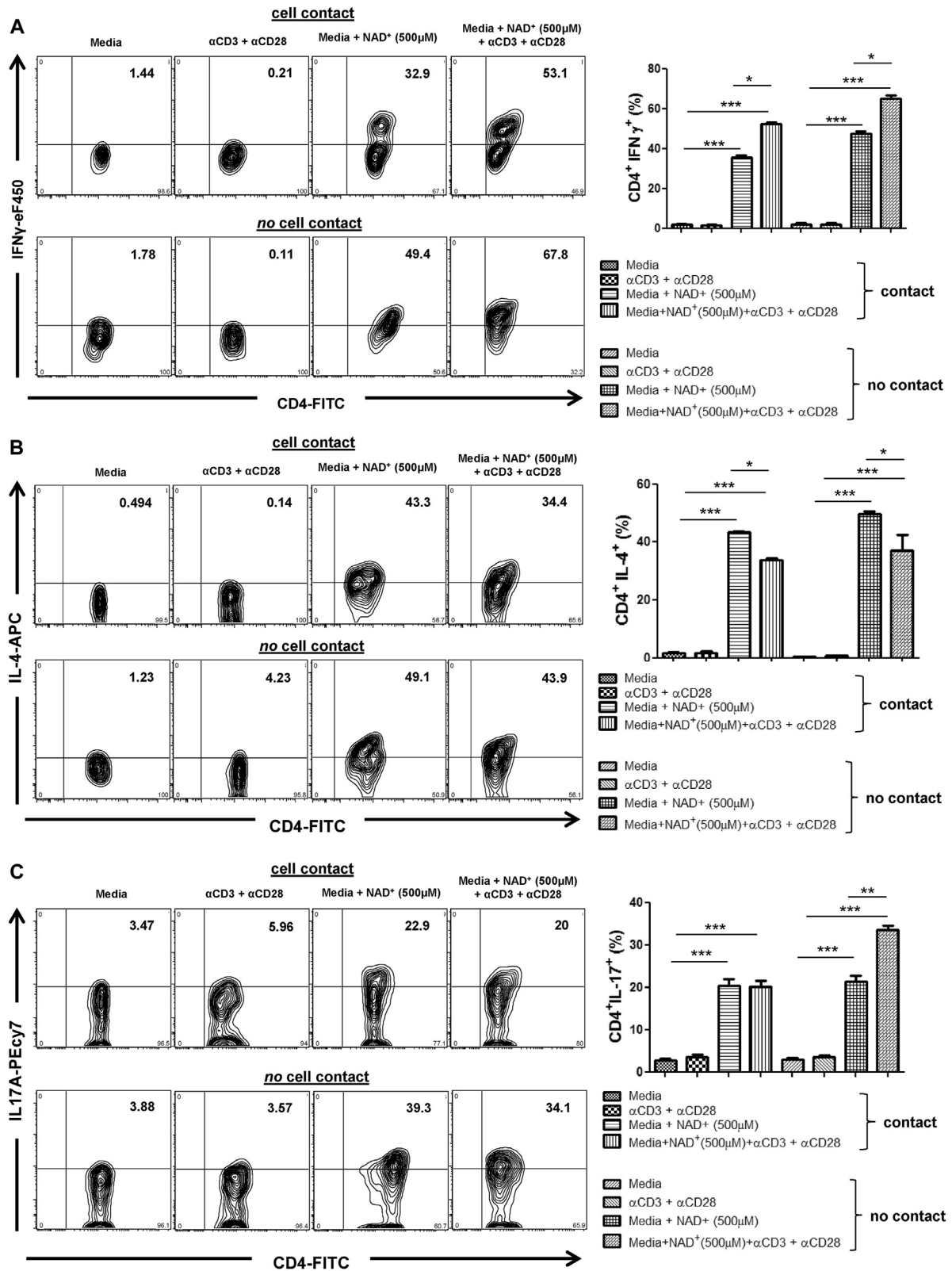
FIG E4. Murine MCs promote CD4$^+$IFN-γ^+, CD4$^+$IL-4$^+$, and CD4$^+$IL-17A$^+$ T-cell differentiation in the presence of NAD$^+$ both with and without cell-cell contact. BMMCs were cocultured with isolated naive CD4$^+$CD44$^-$CD62L$^+$ T cells from C57BL/6 mice (1:100 ratio) either in cell-cell contact or in separate compartments by using a transwell system. Cells were then treated with NAD$^+$ (500 μmol/L) or PBS. After 96 hours, frequencies of CD4$^+$IFN-γ^+ **(A)**, CD4$^+$IL-4$^+$ **(B)**, and CD4$^+$IL-17A$^+$ **(C)** cells were assessed by using flow cytometry (n = 6). Data were derived from 2 independent experiments. *P < .05, **P < .01, and ***P < .001, as determined by means of ANOVA, comparing the indicated groups. Data represent means ± SDs. *APC,* Allophycocyanin; *FITC,* fluorescein isothiocyanate; *PE,* phycoerythrin.
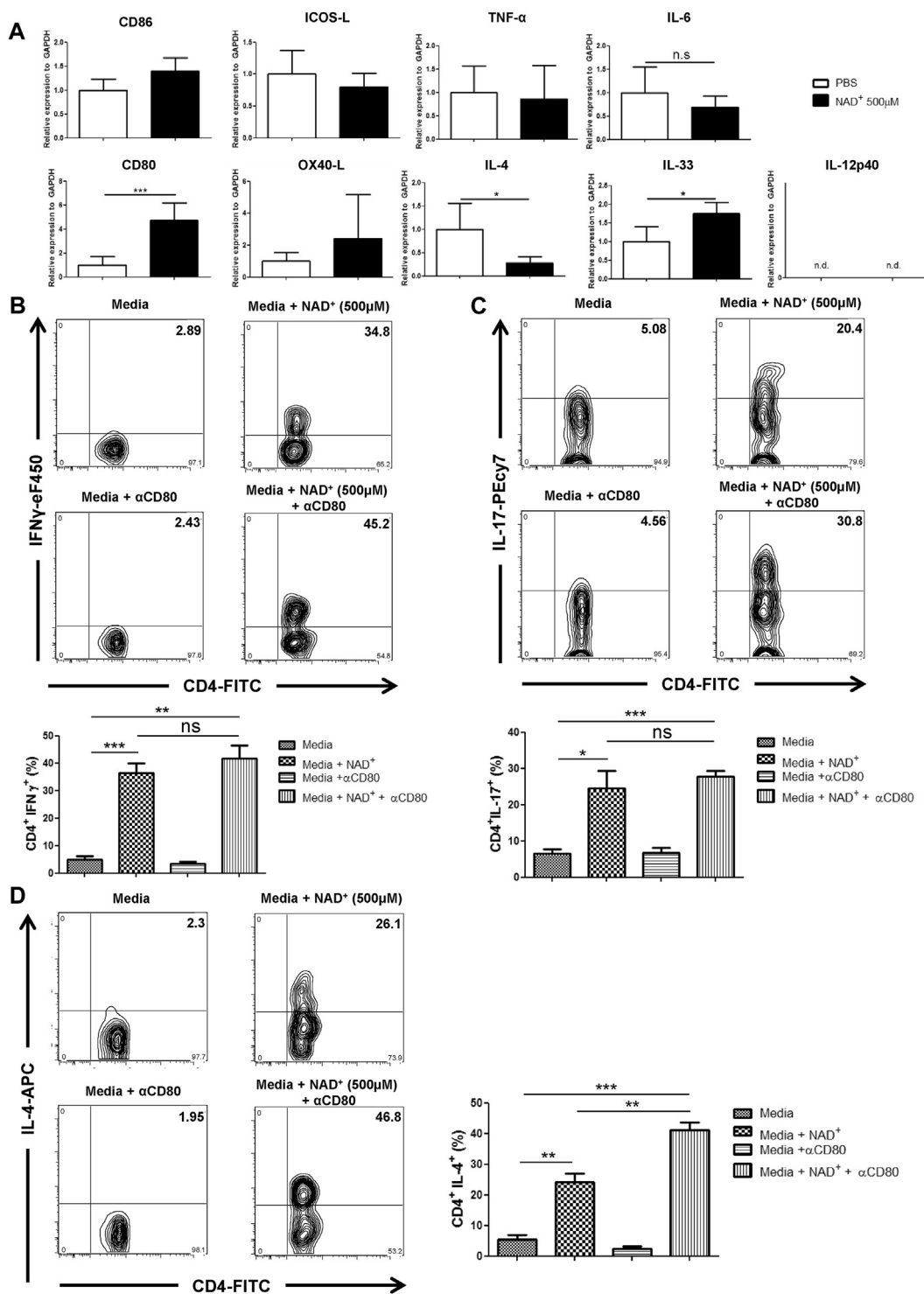
FIG E5. MCs do not regulate CD4$^+$ T-cell differentiation in the presence of NAD$^+$ through CD80. **A,** BMMCs from C57BL/6 mice were cultured in the presence of NAD$^+$ (500 μmol/L) or placebo (PBS). After 24 hours of culture, cells were collected, and mRNA was extracted. mRNA levels of CD86, CD80, TNF-α, IL-4, inducible costimulator ligand *(ICOS-L)*, OX40 ligand *(OX40-L)*, IL-6, and IL-33 were determined by using real-time PCR. Values are expressed as fold expression relative to the housekeeping gene glyceraldehye-3-phosphate de-hydrogenase *(GAPDH)*. **B** and **C,** BMMCs were cocultured with isolated naive CD4$^+$CD44$^-$CD62L$^+$ T cells from C57BL/6 mice (1:100 ratio) in cell-cell contact conditions in the presence of α-CD80, NAD$^+$ (500 μmol/L), or placebo (PBS), as indicated. **B-D,** After 96 hours, frequencies of CD4$^+$IFN-γ$^+$ (Fig E5, *B*), CD4$^+$IL-17A$^+$ (Fig E5, *C*), and CD4$^+$IL-4$^+$ (Fig E5, *D*) cells were assessed by using flow cytometry (n = 6). Data were derived from 2 independent experiments. *ns,* Not significant. *$P < .05$, **$P < .01$, and ***$P < .001$, as determined by using the Student *t* test and ANOVA, comparing the indicated groups. Data represent means ± SDs. *APC,* Allophycocyanin; *FITC,* fluorescein isothiocyanate; *PE,* phycoerythrin.
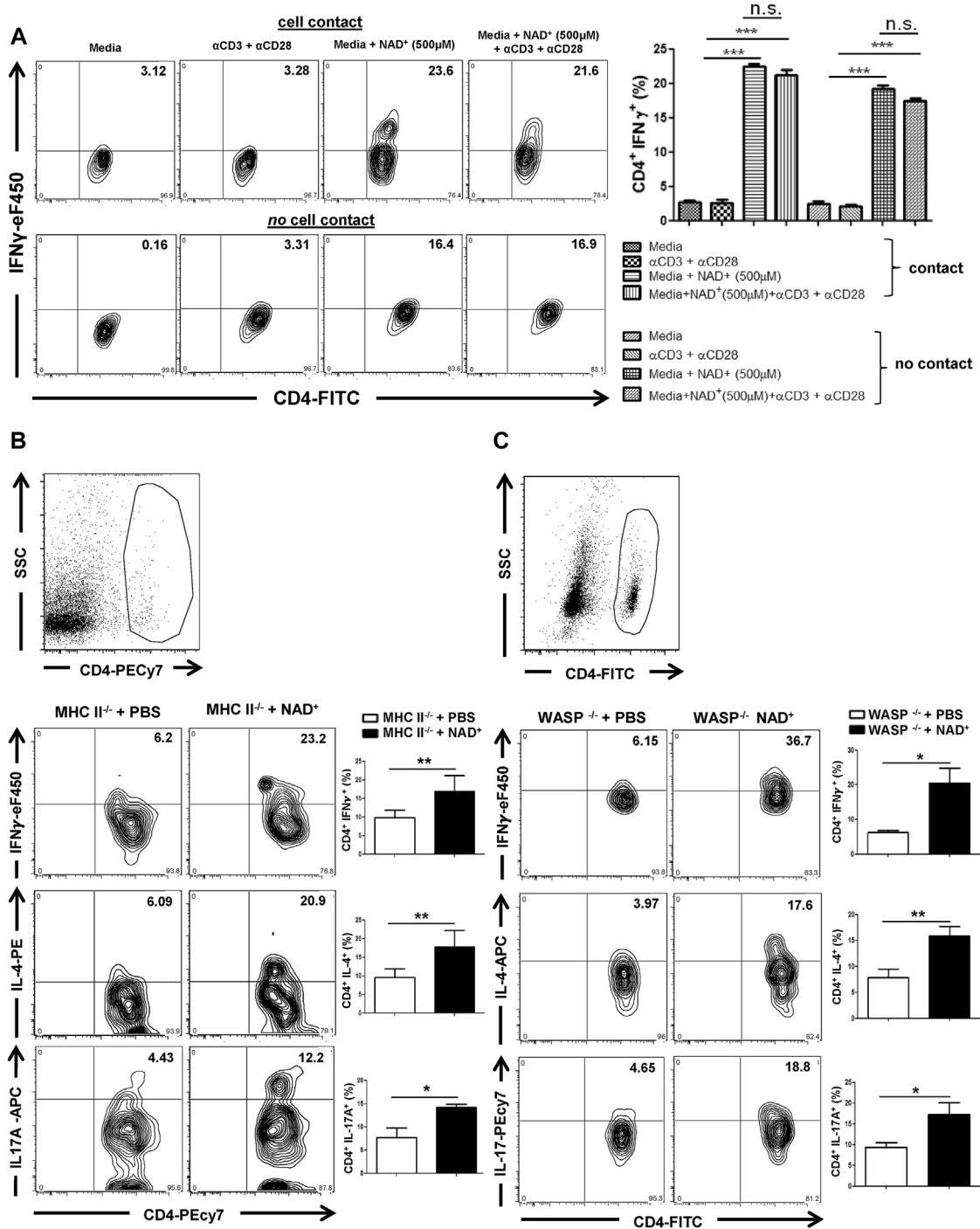
FIG E6. Conserved MC-mediated CD4$^+$ T-cell differentiation in the human MC line LAD-2 in the presence of NAD$^+$. NAD$^+$ induces T-cell differentiation in MHC class II$^{-/-}$ and WASP$^{-/-}$ mice. **A,** Human MC line LAD-2 cells were cocultured with isolated human naive CD4$^+$ T cells from healthy donors (1:100 ratio) either in cell-cell contact or in separate compartments by using a transwell system. Cells were then treated with NAD$^+$ (500 μmol/L) or PBS. After 96 hours, frequencies of CD4$^+$IFN-γ$^+$ cells were assessed by using flow cytometry (n = 6). Data were derived from 2 independent experiments. **B,** MHC class II$^{-/-}$ (B6.129S-H2$^{dlAb1-Ea}$) mice were treated daily with intraperitoneal injections of 40 mg of NAD$^+$ or a placebo solution (PBS). After 7 days, mice were killed, and splenocytes were harvested. Systemic frequencies of CD4$^+$IFN-γ$^+$, CD4$^+$IL-4$^+$, and CD4$^+$IL-17A$^+$ cells were analyzed by means of flow cytometry. Data were derived from 2 independent experiments (n = 5). **C,** WASP$^{-/-}$ (B6.129S6-Was^{tm1Sbs}/J) mice were treated daily with intraperitoneal injections of 40 mg of NAD$^+$ or a placebo solution (PBS). After 7 days, mice were killed, and frequencies of CD4$^+$IFN-γ$^+$, CD4$^+$IL-4$^+$, and CD4$^+$IL-17A$^+$ cells were assessed by means of flow cytometry. Data were derived from 2 independent experiments (n = 5). *ns,* Not significant. *P < .05, **P < .01, and ***P < .001, as determined by using the Student *t* test and ANOVA, comparing the indicated groups. Data represent means ± SDs. *APC,* Allophycocyanin; *FITC,* fluorescein isothiocyanate; *PE,* phycoerythrin; *SSC,* side scatter.
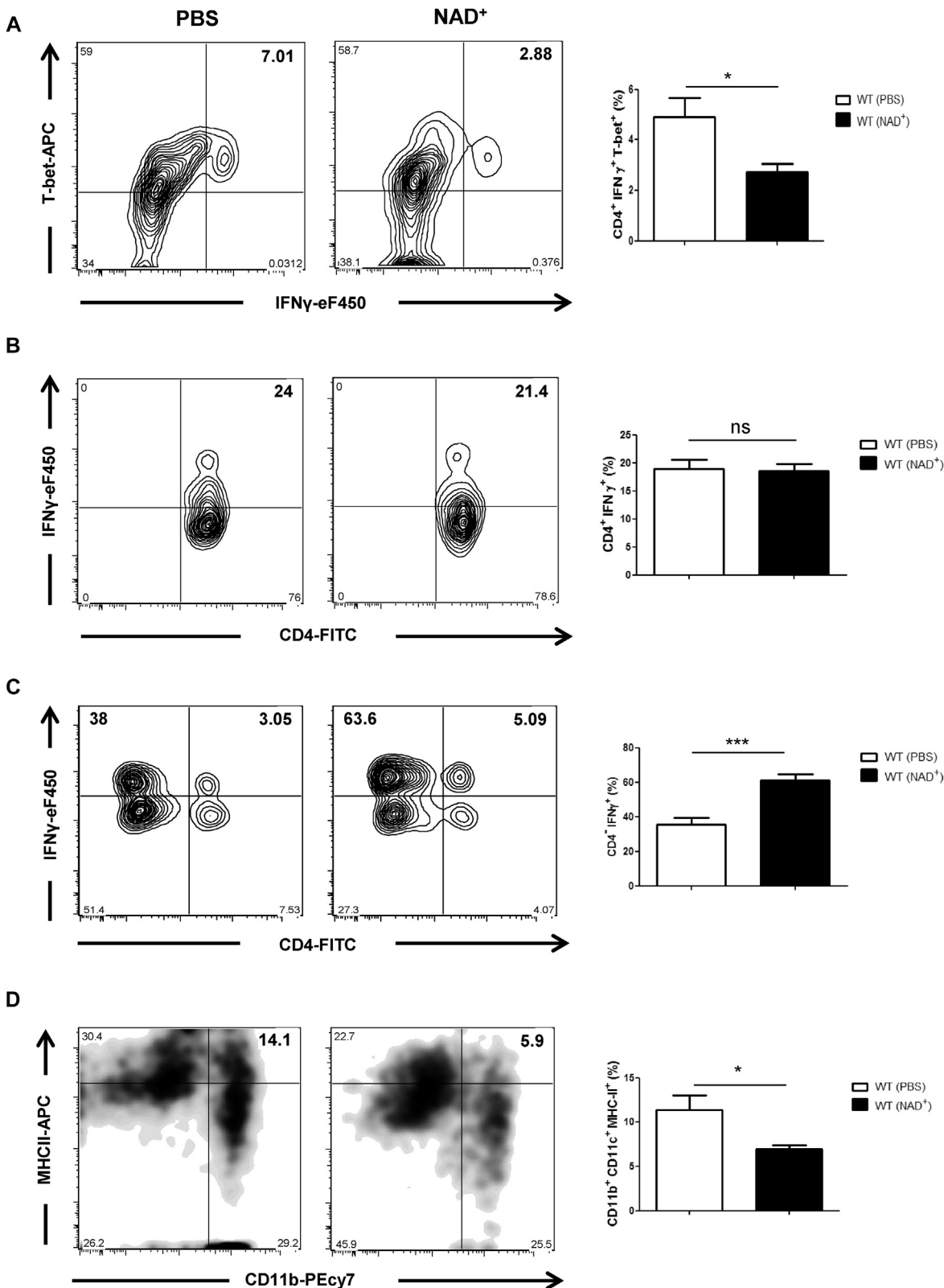
FIG E7. NAD$^+$ alters systemic frequencies of CD4$^+$T-bet$^+$IFN-γ^+ cells and CD11c$^+$MHC class II$^+$ DCs after *L monocytogenes* infection. C57BL/6 mice were treated for 5 days with daily intraperitoneal injections of NAD$^+$ (40 mg) or placebo solution (PBS). After 5 days, mice were infected with a nonlethal dose of *L monocytogenes* (1 × 10^7 colony-forming units) and killed 3 days later. Spleens were collected, and frequencies of CD4$^+$T-bet$^+$IFN-γ+ **(A)**, CD4$^+$IFN-γ^+ **(B)**, and CD4$^-$IFN-γ^+ **(C)** T cells and CD11b$^+$CD11c$^+$MHC class II$^+$ DCs **(D)** were assessed by using flow cytometry. Data were derived from 2 independent experiments (n = 5). Data represent means ± SDs. *ns*, Not significant. The Student *t* test was used to compare between groups: *P < .05 and ***P < .001.