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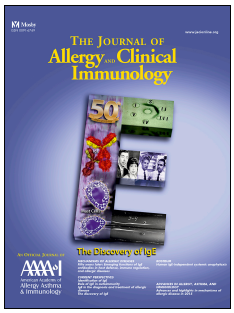

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Mast cells regulate CD4$^+$ T cell differentiation in absence of antigen presentation

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Abstract

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, while the precise mechanisms remained unknown.

Objective: The objective of this study is to further dissect the mechanism of actions of NAD^+, and to determine the impact of APCs on NAD^+-mediated T cell activation.

Methods: Isolated dendritic cells and bone marrow-derived mast cells 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* using WT C57BL/6, Mast $cell^{-/-}$, MHC class $II^{-/-}$, $WASP^{-/-}$, 5C.C7 $Rag2^{-/-}$ and CD11b-DTR transgenic mice. Finally, we tested the physiological impact of NAD^+ on the systemic immune response in the context of *Listeria monocytogenes* infection.

Results: Our *in vivo and in vitro* findings indicate that following NAD^+ administration MCs, exclusively, promote $CD4^+$ T cell differentiation, both in absence of antigen and independently of major APCs. Moreover, we found that MCs mediated $CD4^+$ T cell differentiation independently of MHC-II and TCR signaling machinery. More importantly, although treatment with NAD^+ resulted in a decreased MHC-II expression on $CD11c^+$ cells, MC-mediated $CD4^+$ T cell differentiation rendered mice resistant to the administration of lethal doses of *Listeria monocytogenes*.

Conclusions: Collectively, our study unravels a novel cellular and molecular pathway that regulates innate and adaptive immunity via MCs, exclusively, and underscores the therapeutic potential of NAD^+ in the context of primary immunodeficiencies and antimicrobial resistance.

Key Messages

- NAD^+ alone regulates $CD4^+$ T cell fate in 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 following NAD^+ administration

Capsule Summary

NAD^+ mediates an immune response via mast cells that is APC-MHC-TCR independent and protects from lethal doses of *Listeria monocytogenes.*

Keywords

nicotinamide adenine dinucleotide; mast cells; t cells; antigen presentation; MHC; TCR; $CD4^+$ T cell differentiation; dendritic cells; macrophages; Listeria monocytogenes; cytokine

Abbreviations (in order of appearance)

APCs	antigen presenting cells
MHC	major histocompatibility complex
TCR	T cell receptor
DCs	dendritic cells
CTL	cytotoxic T lymphocyte
PAMPs	pathogen-associated molecular patterns
DAMPs	damage-associated molecular patterns
PRRs	pattern recognition receptors
MCs	mast cells
Ig	immunoglobulin
EAE	experimental autoimmune encephalomyelitis
NAD^+	nicotinamide adenine dinucleotide
CD	cluster of differentiation
IL	interleukin
WT	wild type
CFU	colony-forming unit
PBS	phosphate-buffered saline
i.p.	intra-peritoneal
BMMCs	bone marrow-derived mast cells
SD	standard deviation
PID	primary immunodeficiency disease
MCMIR	mast-cell mediated immune regulator

Introduction

Antigen presenting cells (APCs) play a central role in the regulation of innate and adaptive immune responses[1]. APCs have the ability to capture, process and present antigens via their MHC cell surface molecules to the T cell-receptor (TCR) in order to mount a 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 as the major APCs bridging innate and adaptive immune responses[7, 8]. The mode of action of DCs is mediated at least through three signals: 1) TCR activation, 2) activation of co-stimulatory molecules, and 3) secretion of chemokines and pro-inflammatory cytokines[8]. Indeed, depletion of $CD11c^+$ DCs has been shown to alter cytotoxic T lymphocyte (CTL) 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 via intracellular or surface-expressed pattern recognition receptors (PRRs)[10-14].

Although considered as “atypical” APCs, Mast cells (MCs) have been mainly described 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].

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 co-stimulatory molecules and to secrete a myriad of chemokines and pro-inflammatory 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 so

far on the capacity of a direct antigen presentation to the TCR[8, 23]. Thus, the mechanisms by which MCs regulate T cell responses remain unclear and yet to be determined.

Recently, we have shown the role of nicotinamide adenine dinucleotide (NAD^+), a co-factor found in all living cells and in 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^+ via a 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 following NAD^+ administration MCs, exclusively, are able to induce $CD4^+$ 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 impact 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 following *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 diphteria toxin treatment

Eight- to ten-week old WT C57BL/6 (B6, $H2^b$) mice were purchased from Charles River Laboratories. Mast cell$^{-/-}$ (WBB6F1/J-*Kit*^{W}/*Kit*$^{W\text{-}v}$/*J* (*Kit*^{W}/*Kit*$^{W\text{-}v}$) and Kit$^{W\text{-}sh}$/HNihrJaeBsmJ (*kit*^{Wsh}/*Kit*^{Wsh}), MHCII$^{-/-}$ (B6.129S-H2dlAb1-Ea), WASP$^{-/-}$ (B6.129S6-*Was*^{tm1Sbs}/J) and CD11b-DTR (B6.FVB-Tg(ITGAM-DTR/EGFP)34Lan/J) mice were purchased from Jackson Laboratory. Rag2$^{-/-}$ γc$^{-/-}$ (B10; B6-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Wjl}), Rag2$^{-/-}$ and 5C.C7 Rag2$^{-/-}$ mice (both on B10.A background) were purchased from Taconic. For CD11b$^+$ cell depletion with diphtheria toxin treatment, CD11b-DTR transgenic mice weighing 25–30 g were injected with diphtheria toxin (25 ng/g body weight, Sigma-Aldrich) 24 hours before and 72 hours after beginning of NAD^+ or PBS administration.

Isolation of mouse naïve CD4$^+$CD44$^-$CD62L$^+$T cells and DCs

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

Listeria monocytogenes infection

Listeria monocytogenes bacteria (ATCC #35152) were cultured overnight at 37 ℃ in Brain Heart infusion (BHI, Teknova, CA) with gentle agitation. Eight- to ten-week old WT and MC$^{-/-}$ mice were infected i.p. with 0.1 ml of a solution containing 1 x 10^7 CFU (non-lethal dose) or 1 x 10^8 CFU (lethal dose) of viable *L. monocytogenes* cells in 0.01 M phosphate-buffered saline (PBS; pH 7.4). Weight loss and survival following infection was monitored. Prior to infection mice were pre-treated daily for a period of five days with NAD^+ (40mg, i.p.) or pre-treated 5 days

prior to infection and continuously treated daily post infection.

Cultivation of bone marrow-derived mast cells

BMMCs (bone marrow-derived mast cells) from eight- to ten-week-old C57BL/6J WT mice were obtained by culturing bone marrow cells from femurs and tibias. In short, mice were euthanized by cervical dislocation, intact femurs and tibias were removed, and bone marrow cells were harvested by 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 >95% c-kit^{high}FcεRIα^{high} by flow cytometry analysis using PEcy7 anti-mouse FcεRIα (clone MAR-1, eBioscience, San Diego, CA, USA) and ef450 anti-mouse c-kit/CD117 (Clone 2B8, BD eBioscience, San Diego, CA, USA).

Human mast cell line LAD-2 culture

The human mast cell line LAD-2 was a generous gift from Dr. A. Kirshenbaum (NIH/NIAID). LAD-2 mast cells were cultured in serum-free media (StemPro-34 SFM, Life Technologies) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 50 µg/ml streptomycin and 100 ng/ml recombinant SCF. The LAD2 cells were periodically tested for expression of Kit and FcεRI by flow cytometry.

Cell culture

Isolated naïve CD4$^+$ T cells or CD11c$^+$ DCs (1x10^6 cells per well) were cultured in 48-well flat bottom plates in 0.5ml of complete RPMI 1640 media supplemented with 10% FCS, 200mM L-glutamine, 100U/ml penicillin/streptomycin, 4.5 g/L glucose in presence of 10µg/ml plate-bound anti-mouse α-CD3 (17A2) and 2µg/ml soluble α-CD28 (37.51). NAD$^+$ (Sigma-Aldrich, Cat. # N3014) was diluted in PBS and added as indicated. LPS was added at a concentration of 1µg/ml. All recombinant cytokines and antibodies were purchased from eBioscience. After indicated day of culture, supernatants and cells were collected and analyzed by ELISA and flow

cytometry, respectively.

Co-culture of mouse naïve CD4+ T cells and bone marrow derived mast cells in transwell systems

The non-contacting co-cultured cells were prepared as follows: isolated naïve $CD4^+CD44^-CD62L^+$ T cells were plated on the bottom of the 24-well transwell cell culture system (Costar Corp., USA). Bone marrow-derived mast cells were co-cultured at a ratio of 1:100 in the upper transwell compartment. Cells were stimulated with NAD^+ (500μM) or PBS as control. Naïve $CD4^+$ T cells were cultured in complete media only or in presence of 10μg/ml plate-bound anti-mouse α-CD3 (clone 17A2) and 2μg/ml soluble α-CD28 (clone 37.51). For cell-cell contact experiments, BMMCs and naïve $CD4^+CD44^-CD62L^+$ T cells were co-cultured (at a ratio of 1:100) in complete media with NAD^+ (500μM) or PBS as control. Naïve $CD4^+$ T cells were cultured in presence of 10μg/ml plate-bound anti-mouse α-CD3 (clone 17A2) and 2μg/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 during 96 hours and $CD4^+IFN\gamma^+$, $CD4^+IL4^+$ and $CD4^+IL\text{-}17A^+$ T cell frequencies were assessed by flow cytometry.

Isolation and co-culture of human naïve $CD4^+$ T cells

Human naïve $CD4^+$T cells were isolated from peripheral blood mononuclear cells (PBMCs) by density gradient centrifugation using 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 the 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 naïve $CD4^+CD25^-CD45RA^+CD45RO^-CCR7^+CD62L^+$ T cells were then sorted by flow cytometry. Naive $CD4^+$ T cells were purified to >98% by cell sorting. Naïve human $CD4^+$ T cells were then plated

on the bottom of the 24-well transwell cell culture system (Costar Corp., USA) and LAD-2 cells were co-cultured at a ratio of 1:100 in the upper transwell compartment. Cells were stimulated with NAD^+ (500μM) or PBS as control. Naïve $CD4^+$ T cells were cultured in complete media only or in presence of 10μg/ml soluble anti-mouse α-CD3 (clone 17A2) and 5μg/ml soluble α-CD28 (clone 37.51). For cell-cell contact experiments, LAD-2 cells and naïve $CD4^+CD44^-CD62L^+$ T cells were co-cultured (at a ratio of 1:100) in complete media with NAD^+ (500μM) or PBS as control. Naïve $CD4^+$ T cells were cultured in presence of 10μg/ml soluble anti-mouse α-CD3 (clone 17A2) and 5μg/ml soluble α-CD28 (clone 37.51) or complete media only. After 96 hours, $CD4^+$ T cells IFNγ cytokine production was assessed by flow cytometry.

Dendritic cell and macrophage depletion

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

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), IFNγ (XMG 1.2), LAP (TW7-16B4), TNF-α (MP6-XT22) were obtained from eBioscience. All antibodies were used at a concentration of 2–5 μg per 1×10^6 cells. To set the gates, flow cytometry dot plots were based on comparison with isotype controls, fluorescence minus one, permeabilized and unpermeabilized unstained cells. Intracellular staining for IL-1β, IL-4, IL-6, IL-10, IL-12/IL-23p40, IL-17, IFNγ, LAP and TNF-α was performed according to manufacturer's protocols. Cells were fixed and permeabilized using Cytofix/Cytoperm solution (BD Biosciences). Flow cytometry

measurements were performed on a BD FACSCANTO II (BD Biosciences) using standard procedures and data were analyzed using FlowJo software (Tree Star, Inc.).

ELISA

Mouse IL-4, IL-17A and IFNγ were measured using commercial kits (eBioscience) as described previously[24, 25].

RNA extraction and quantitative PCR

BMMCs from C57BL/6 mice were cultured in presence of NAD^+ (500µM), LPS, or placebo (PBS). After 24 hours of culture, cells were collected and mRNA was extracted using the RNAqueous extraction kit according to the manufacturer's protocols (Applied Biosystems). 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-12alpha (Mm00434165_m1), IL-23 (Mm00518984_m1), TGF-β_1 (Mm01178820_m1), TNF-α (Mm00443260_g1), TLR2 (Mm00442346_m1), TLR4 (Mm00445273_m1), CD86 (Mm00444543_m1), CD80 (Mm00711660_m1), ICOS-L (Mm00497237_m1), OX40-L (Mm00437214_m1) and IL-33 (Mm00505403_m1) measurements were performed with Taqman primers and probes from Applied Biosystems. The housekeeping gene GAPDH (Mm99999915_g1) was used as control.

RNA-Sequencing Analysis

Bone marrow derived mast cells (BMMCs) from C57BL/6 mice were cultured in presence of NAD^+ (500µM), LPS (10µg/ml; *E. coli* O127:B8), or placebo (PBS). After 16 hours of culture, cells were collected and RNA was extracted using the RNAqueous extraction kit according to the manufacturer's protocols (Applied Biosystems) as described above. cDNA was obtained using New England Biolabs kits (NEBNext® Ultra™ Directional RNA Library Prep Kit for

Illumina® (New England Biolabs Inc., Ipswich, MA). Briefly, mRNA was extracted using polyT magnetic beads, then first and second strand syntheses were performed. Once double-strand cDNA was generated, DNA was cleaned up using 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 PCR. The final library was cleaned up by magnetic beads and ready for sequencing. FastQ-format files were aligned against the Ensembl GRCm38.75 genome using STAR aligner (v 2.3.1z4) using default parameters [28]. Alignment files (BAM format) were filtered to retain only primary alignments (Samtools view -F 0x0100) and inspected for duplication rate with PicardTools MarkDuplicates; for downstream analyses, duplicate reads were not removed due to the high-quality input RNA [29]. Reads were quantified at the gene level using featureCounts using annotated exon features in the Ensembl GRCm38.75 GTF file [30, 31]. The resulting count matrix was normalized and analyzed for differential expression using DESeq2 software. The Ingenuity Pathways Analysis (Ingenuity Systems) applications were used to generate canonical pathways.

Statistical analysis

Data are presented as mean ± SD. Statistical analysis was done using 2-tailed Student's t-test (between 2 groups) and 1-way ANOVA (among multiple groups) were appropriate. Survival was compared by Log-rank test. *P*-values <0.05 were considered statistically significant.

Study approval

Animal use and care 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 previously demonstrated 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 via an intermediate cell type. To characterize the direct effect of NAD^+ on $CD4^+$ T cells in absence of antigen challenge, C57BL/6 wild type (WT) naïve mice were treated daily with intraperitoneal injection of NAD^+ or a placebo solution (PBS) and $CD4^+$ T cell responses were assessed. After 7 days, mice were euthanized and systemic $CD4^+IFN\gamma^+$, $CD4^+IL\text{-}4^+$, $CD4^+IL\text{-}17A^+$ T cell frequencies were evaluated by flow cytometry. The data indicated that NAD^+ administration was sufficient to promote a significant increase in frequencies of $CD4^+IFN\gamma^+$, $CD4^+IL\text{-}4^+$, and $CD4^+IL\text{-}17^+$ T cells *in vivo* (**Figure 1A**). Of note, NAD^+ administration to $Rag2^{-/-}$ mice adoptively transferred with naïve $CD4^+$ T cells from Rag 5C.C7 transgenic mice promoted $CD4^+IFN\gamma^+$ T cell differentiation in absence of moth cytochrome c (MCC) peptide challenge (**Figure 1B**). Thus, we next assessed *in vitro* whether NAD^+ directly promotes $CD4^+$ T cell differentiation and cytokine production. Splenic naïve $CD4^+CD44^-CD62L^+$ T cells from C57BL/6 WT mice were cultured in 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 IFNγ, IL-4 and IL-17 production by flow cytometry and ELISA. Consistent with our previous reports, flow cytometry analysis revealed that NAD^+ promotes a robust increase of $CD4^+IFN\gamma^+$ T cell frequencies, following TCR activation, in particular in the presence of IL-2 (**Figure 1C**). These findings were confirmed by ELISA indicating an increased IFNγ, IL-4 and IL-17 production following CD3/CD28 activation (**Figure 1D**). Of note, the highest $CD4^+IL\text{-}17^+$ T cell frequencies and IL-17 secretion were observed following TCR activation and in absence of IL-2, which was consistent with previous reports indicating that IL-2 inhibits Th17 development. In contrast, no changes in $CD4^+IFN\gamma^+$ T cell frequencies or IFNγ, IL-4 and IL-17 cytokine production were observed with NAD^+ treatment in absence of TCR activation (**Figure 1C-D**). Furthermore, increasing NAD^+ concentrations in

absence of TCR activation did not result in a change of $CD4^+IFN\gamma^+$, $CD4^+IL\text{-}4^+$, $CD4^+IL\text{-}17^+$ T cell frequencies (**Supplemental Figure 1A**). Collectively, our *in vivo* data indicate that NAD^+ promotes $CD4^+$ T cell differentiation in absence of antigen challenge, while our *in vitro* data indicate that NAD^+ requires TCR activation suggesting that NAD^+-mediated $CD4^+$ T cell differentiation observed in C57BL/6 WT naïve mice requires an intermediary signal.

NAD^+ regulates $CD4^+$ T cell fate in 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 co-enzyme, can promote Th17 cells via IL-6, IL-23 and TGFβ cytokine production by $CD11c^+$ cells[32]. Since NAD^+ also acts as a co-enzyme, we thus 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 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 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, TNF-α but not IL-12 and a down-regulation of TLR4 [33, 34]. More importantly, in 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 (**Supplemental Figure 1B**), suggesting that NAD^+ alters $CD11b^+CD11c^+$ DC activation. Thus, we next assessed if 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 intraperitoneal injection. Consistent with our *in vitro* findings we found that NAD^+ treated WT mice showed an increased cytokine production, including IL-1β, IL-4, IL-6, IL-10, IL-12, TGF-β1 and TNFα by $CD11b^+CD11c^+$ cells when compared with PBS-treated WT mice (**Figure 2**). This is consistent with a previous study indicating that intracellular NAD^+ levels regulate TNFα cytokine production[35]. Taken together, our *in vitro* and *in vivo* data indicated that NAD^+

promotes $CD11b^+CD11c^+$ DCs activation and cytokine production and may play a central role in NAD^+-mediated $CD4^+$ T cell differentiation.

It is well established that DCs can promote $CD4^+$ T cell differentiation via the release of cytokines and chemokines. Moreover, ATP, a co-factor in energy metabolism like NAD^+, has been shown to enhance IL-6, IL-23 and TGFβ cytokine production by $CD11c^+$ cells and promote Th17 response[32]. Since 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], >99% of professional phagocytes, including both DCs and macrophages were depleted in WT mice by injections of clodronate liposomes (**Supplemental Figure 2A**). As shown in **Supplemental Figure 2B**, DC depletion did not abolish $CD4^+IFN\gamma^+$, $CD4^+IL\text{-}4^+$, $CD4^+IL\text{-}17^+$ 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 following macrophage and DC depletion, we could not rule out the compensation by other APCs such as B cells. To characterize the role of B cells in NAD^+-mediated $CD4^+$ T cell differentiation, we thus used transgenic *Rag2*$^{-/-}\gamma c^{-/-}$ mice that lack B, NK and γδ T cells. In addition, *Rag2*$^{-/-}\gamma c^{-/-}$ mice were subjected to depletion of DCs and macrophages. After depletion (**Supplemental Figure 2A**), *Rag2*$^{-/-}\gamma c^{-/-}$ received adoptive transfers of naïve $CD4^+CD44^-CD62L^+$ T cells and were subjected to treatment with NAD^+ or placebo solution. The results indicated that NAD^+ induced a significant increase in $CD4^+IFN\gamma^+$, $CD4^+IL\text{-}4^+$, $CD4^+IL\text{-}17^+$, $CD4^+IL\text{-}10^+$ when compared to the control group of mice treated with a placebo solution (**Supplemental Figure 3A**). Moreover, treatment with diphtheria toxin of CD11b-DTR transgenic mice did not abolish $CD4^+IFN\gamma^+$, $CD4^+IL\text{-}4^+$, $CD4^+IL\text{-}17^+$ differentiation (**Supplemental Figure 3B**). 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 via MCs exclusively

MCs have been mainly described for their role in allergic and autoimmune responses[8, 15, 16]. Although MCs express co-stimulatory molecules and secrete a myriad of chemokines and pro-inflammatory 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 following *Leishmania major* infection[36] and in EAE[37]. Furthermore, we have previously shown 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$^{W\text{-}v}$*) were treated daily with intraperitoneal injection of NAD$^+$. Treatment with NAD$^+$ in MC-deficient mice was not able to promote CD4$^+$IFNγ$^+$, CD4$^+$IL-4$^+$, CD4$^+$IL-17$^+$ T cells, indicating that MCs were required for NAD$^+$-mediated CD4$^+$ T cell differentiation (**Figure 3**).

Therefore, we next sought to dissect the role of MCs in NAD$^+$-mediated T cell differentiation *in vitro*. Bone marrow-derived mast cells (BMMCs) were generated as described in Materials and Methods. The average yield of MCs (FcεRI$^+$c-Kit$^+$ double-positive cells) increased with time and reached over 95% on day 90, as shown by flow cytometry (**Supplemental Figure 3C**). BMMCs were cultured over 6 weeks in order to express homogenous levels of KIT and FcεRI[8]. BMMCs were then directly co-cultured with naïve CD4$^+$CD44$^-$CD62L$^+$ T cells in presence of NAD$^+$ or PBS. Moreover, to determine whether MCs and T cells require cell-cell contact, MCs and T cells were co-cultured in separate compartments using a transwell system. As additional control, naïve CD4$^+$CD44$^-$CD62L$^+$ T cells were activated with α-CD3/α-CD28. The results indicated that in presence of NAD$^+$, MCs promoted CD4$^+$IFNγ$^+$, CD4$^+$IL-4$^+$ and CD4$^+$IL-17$^+$ T cell differentiation in absence of TCR activation (media + NAD$^+$) and independently of cell-cell contact (**Supplemental Figure 4**). Moreover, when MCs and naïve CD4$^+$CD44$^-$CD62L$^+$ T cells were co-cultured in presence of NAD$^+$ and T cells were activated with α-CD3/α-CD28, frequencies of CD4$^+$IFNγ$^+$ and CD4$^+$IL-17$^+$ T cells increased further (**Supplemental Figure 4**). These results were consistent with our initial findings indicating that cultured naïve CD4$^+$CD44$^-$CD62L$^+$ T cells

in presence of NAD^+ and α-CD3/α-CD28 promoted $CD4^+IFN\gamma^+$, $CD4^+IL\text{-}4^+$ and $CD4^+IL\text{-}17^+$ (**Figure 1C-D**). Like DCs, MCs express co-stimulatory molecules and secrete a myriad of cytokines that are known to regulate innate and adaptive immune responses. Thus, to determine if MCs mediate $CD4^+$ T cell differentiation via co-stimulatory molecules and/or cytokines, MCs were treated with NAD^+ *in vitro* and mRNA levels of OX40L, ICOSL, CD80, CD86, TNFα, IL-4, IL-6 and IL-33 were measured by real-time PCR. NAD^+ induced a modest increase of CD80, IL-33 and a decrease in IL-4 mRNA expression levels by mast cells (**Supplemental Figure 5A**). It is well established that CD80 can either promote or inhibit activation of naïve T cells by binding to CD28 or CTLA4, 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* using our cell-cell contact co-culture system. Our findings indicated that CD80 blockade did not reduce $CD4^+IFN\gamma^+$, $CD4^+IL\text{-}17^+$ T cell frequencies when compared to isotype control (**Supplemental Figure 5B-C**). Of note, CD80 blockade resulted in increased $CD4^+IL\text{-}4^+$ T cell frequencies (**Supplemental Figure 5D**), suggesting that CD80 may 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 co-stimulatory molecules such as CD80.

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

We next investigated whether this novel pathway is conserved in humans and if human MCs could regulate human $CD4^+$ T cell differentiation as well. Naïve $CD4^+$ T cells were isolated from healthy donors and co-cultured in direct contact or in our transwell system with LAD-2 cells, a well-established human mast cell line. Similarly to murine BMMCs, co-cultures were performed in presence of NAD^+ or PBS and with or without α-CD3/α-CD28. Flow cytometry analysis indicated that in presence of NAD^+, human MCs promoted $CD4^+IFN\gamma^+$ T cell differentiation (**Supplemental Figure 6A**). Consistent with our murine BMMCs data, human mast cells were able to promote Th1 polarization in absence of cell-cell contact. Taken together, these results

suggest that MCs-mediated $CD4^+$ T cell differentiation pathway via mast cells is conserved in human as well.

Unique gene expression profile by MCs following NAD^+ activation

To unravel a potential signaling pathway involved in MC-mediated $CD4^+$ T cell differentiation, we next performed an RNA-sequencing analysis on bone marrow derived mast cells (BMMCs) that were cultured for 16 hours in presence of NAD^+, LPS, or PBS. As shown in **Figure 4A** the results indicated that NAD^+ significantly up-regulated 603 genes and down-regulated 753 genes (with a p value < 0.05) when compared to MCs treated with PBS. Moreover, when compared to LPS conditions the changes in the number of genes marked as differentially expressed were dramatic. When comparing LPS versus NAD^+ conditions, 6053 genes were significantly up-regulated while 5845 were found down-regulated, suggesting that NAD^+-signaling machinery is distinct from LPS stimulation. As expected, dramatic changes in gene expression were observed when comparing PBS to LPS treatment (**Figure 4A**). To elucidate potential genes involved in MC- mediated $CD4^+$ T cell differentiation, we investigated the role of the most up-regulated genes in NAD^+ versus PBS conditions and compared them to gene expression characteristics in presence of LPS (**Figure 4B**). Among the first 10 genes up-regulated in PBS versus NAD^+ conditions, a robust up-regulation of Autotaxin (Enpp2), Dystrophia Myotonica Protein Kinase (DMPK), and FERM Domain Containing 5 (Frmd5) were specific to NAD^+ conditions when compared to PBS or LPS treatment. Both DMPK [38, 39] and FRMD5 [40] proteins have been implicated in stabilizing cell membranes and cytoskeletons. Autotaxin (ATX) 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 ATX [43]. Furthermore, Ingenuity Pathway Analysis predicted significant perturbations by NAD^+ of other signaling molecules involved in MC activation, such as *TNFAIP2* or IKBKG (NEMO) (**Figure 4C**). It is well established that the ectoenzyme ATX generates lysophosphatidic acid (LPA), a potent lipid mediator that acts on a series of specific G protein-coupled receptors, through the hydrolysis of lysophosphatidylcholine [41]. LPA can be produced

by a myriad of different cell types that include postmitotic neurons, adipocytes, mast cells, and other lymphoid cells. ATX has been recently described as regulating cytokine production and ATX-LPA pathways have been shown to play a critical role in asthma [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 $MHCII^{-/-}$ and $WASP^{-/-}$ mice following NAD^+ administration

Our findings indicate that NAD^+ promotes $CD4^+$ T cell differentiation via MCs and in 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 analysis showed that NAD^+ was able to promote a significant increase in $CD4^+IFN\gamma^+$, $CD4^+IL\text{-}4^+$, $CD4^+IL\text{-}17^+$ T cells in MHC-II deficient mice (**Supplemental Figure 6B**).

In addition, to assess the role of TCR in NAD^+-MC mediated $CD4^+$ T cell differentiation, $WASP^{-/-}$ mice, known to have an altered TCR activation and cytokine production, in particular IFNγ[48], were used. Thus, to assess if NAD^+-MC mediated $CD4^+$ T cell differentiation could promote IFNγ production also in the context of immunodeficiency, $WASP^{-/-}$ mice were treated daily with intraperitoneal injection of NAD^+ or PBS. The results indicated that NAD^+ was able to promote a robust increase of $CD4^+IFN\gamma^+$, $CD4^+IL\text{-}4^+$, $CD4^+IL\text{-}17^+$ T cell frequencies in $WASP^{-/-}$ mice when compared to the control group (**Supplemental Figure 6C**). Collectively, our results suggest that NAD^+-MCs mediated $CD4^+$ T cell differentiation is functional even in absence of MHC class II or with an altered TCR activation.

Daily NAD^+ treatment protects against lethal doses of Listeria monocytogenes via mast cells exclusively

To test whether the regulation of innate and adaptive immune cells via MCs following NAD^+ administration had a physiological impact, WT mice were pre-treated for five consecutive days with NAD^+ prior to infection with a *non*-lethal dose of *Listeria monocytogenes*. In addition, a control group of animals was pre-treated with PBS prior to infection. Mice treated with PBS had only a modest loss of weight, did not exhibit signs of lethargy, gained weight 7 days after infection and showed 100% survival (**Figure 5A**) when infected with a non-lethal dose of *L. monocytogenes*. In contrast, mice pre-treated with NAD^+ prior infection (non-lethal dose) had a more pronounced loss of weight, exhibited lethargy and were very susceptible to infection with 80% mortality rate (**Figure 5A**).

We thus tested whether MCs play a role in *L. monocytogenes* infection survival outcome following NAD^+ administration. Indeed, Kit^W/Kit^{Wv} MC deficient mice showed improved survival compared to NAD^+-treated WT mice. In contrast to WT mice pre-treated 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 pre-treated with NAD^+ showed an 80% survival rate (**Figure 5A**), confirming our *in vivo* and *in vitro* findings 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.* Similarly, to Kit^W/Kit^{Wv}, Kit^{Wsh}/Kit^{Wsh} mice exhibited 100% survival rate with no sign of lethargy. Collectively, NAD^+-mediated $CD4^+$ T cell differentiation dramatically altered the immune responses via MCs and significantly impacted physiological functions of protection against bacterial infection.

Our results indicated that pre-treatment with NAD^+ promoted Th1, Th2 and Th17 $CD4^+$ T cell subsets. Since Th1 has been shown to play a protective role in *L. monocytogenes* infection[35], we initially expected NAD^+ pre-treatment to protect mice following bacterial infection. However, we could not rule out that a continuous NAD^+ treatment may be required to sustain a robust Th1 response. Thus, we assessed whether a prolonged and continuous treatment with NAD^+ could

provide protection against *L. monocytogenes* infection. As shown in **Figure 5B**, WT mice continuously treated with NAD^+ exhibited resistance (> 60% survival rate) to even lethal doses of *L. monocytogenes* when compared to the control group of mice that exhibited 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 previously shown that NAD^+ regulates $CD4^+$ T cell differentiation via a pathway distinct of well-established transcription factors[25]. In addition, we showed that NAD^+ altered dramatically differentiated Th1 and Th2 $CD4^+$ T cells by repressing their cytokine production and transcription factors[25]. More importantly, second stimulation of differentiated Th1 and Th2 $CD4^+$ T cells was profoundly altered when they were cultured in presence of NAD^+ following first stimulation[25]. We thus next investigated whether pre-treatment with NAD^+ altered the classical $CD4^+$ T cell differentiation pathway[36] specifically in $CD4^+$ $IFN\gamma^+$ T cells that have been shown essential for host resistance to *L. monocytogenes* infection. Thus, we assessed whether NAD^+ altered frequencies of $CD4^+T\text{-}bet^+$ T cells. Mice pre-treated with NAD^+ had lower frequencies and total numbers of $CD4^+IFN\gamma^+T\text{-}bet^+$ when compared to mice treated with a placebo solution (**Supplemental Figure 7A**). Although treatment with NAD^+ resulted in a down-regulation of T-bet expression, $CD4^+$ T cells from mice treated with NAD^+ mounted a robust IFNγ production (**Supplemental Figure 7B**), which was consistent with our previous studies[24-25]. Although $CD4^+IFN\gamma^+$ 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^+ impacted the regulation and function of other important immune cells such as MCs and more importantly DCs. We thus investigated whether NAD^+ promotes IFNγ production that is not mediated by $CD4^+$ T cells. Our results indicated that mice treated with NAD^+ exhibited a significant increase of IFNγ production by non-$CD4^+$ T cells, suggesting that NAD^+ may promote IFNγ production by other non-$CD4^+$ lymphocytes (**Supplemental Figure 7C**). Taken together, our results indicate that continuous treatment with NAD^+ promotes a robust IFNγ production and protects against lethal doses of *L. monocytogenes* via mast cells exclusively.

Pre-treatment with NAD^+ renders mice susceptible to sub-lethal doses of Listeria monocytogenes by dampening the MCH-II-TCR pathway

Our data indicate that mice pre-treated with NAD^+ are susceptible to sub-lethal doses of *L. monocytogenes*, while continuous treatment protects against lethal doses. We thus next investigated how pre-treatment with NAD^+ rendered mice susceptible to a sub-lethal dose of bacteria.

Since antigen presentation via 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 absence of MHC class II molecules, we investigated the impact of NAD^+ pre-treatment on MHC class II expression of $CD11b^+CD11c^+$ DCs following *L. monocytogenes* infection. The results indicated that pre-treatment with NAD^+ resulted in a dramatic decrease of MHC class II expression when compared to the control group (**Supplemental Figure 7D**), indicating that NAD^+ dampens antigen presentation capacities of DCs following *L. monocytogenes* infection. WT mice pre-treated for five consecutive days with NAD^+ prior to infection with a non-lethal dose of *L. monocytogenes* had a more pronounced loss of weight, exhibited lethargy and were very susceptible to infection with 80% mortality rate (**Figure 5A**). In contrast, WT mice continuously treated with NAD^+ exhibited resistance (> 60% survival rate) to even lethal doses of *L. monocytogenes* when compared to the control group of mice that exhibited 100% mortality rate (**Figure 5B**).

Collectively, our results indicate that pre-treatment with NAD^+ will trigger the NAD^+-MC pathway while dampening the APC-MHC-II-TCR signaling machinery, suggesting that discontinuous administration of NAD^+ may subject the animals to an "*immunodeficient*" state that renders them susceptible to sub-lethal doses of *L. monocytogenes*. Collectively, our results indicate that NAD^+ profoundly alters, in absence of antigen, innate and adaptive immune responses via MCs exclusively and in independently from classical DC/MHC class II antigen presentation machinery that results in significant physiological changes (**Figure 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]. NAD^+ mechanisms of action in an antigen-independent manner and whether NAD^+ affects other immune cells remain however unknown. APCs, in particular DCs, have been considered as the most potent immune cells to prime naive $CD4^+$ T cells. In addition to their capacity of antigen processing and presentation to the TCR via MHC class II cell surface molecules, DCs can regulate $CD4^+$ T cell activation via co-stimulatory 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, released from injured cells, via intracellular or via 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 as 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 co-factor, induces $CD4^+$ differentiation in absence of antigen presentation, MHC class II expression and in absence of TCR activation. Furthermore, we demonstrate that effects of NAD^+ are not mediated by major APCs, including DCs, but via MCs exclusively. Moreover, our study indicated that major co-stimulatory 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 naïve $CD4^+$ T cells, most likely because of an altered co-stimulatory molecule expression[8]. Furthermore, previous studies have reported that MCs express MHC class II molecules; however, it has been found 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

598 molecules.

599 The co-factor ATP is well recognized for its role as a source of high energy and in cellular
600 metabolism; however, when released from cells following cellular damage, ATP acts as a DAMP
601 signal. DAMP signaling is mainly mediated through the pattern recognition receptors (PRRs)
602 that are expressed on APCs, in particular DCs and macrophages[52]. Although the primary
603 function of PRRs is to mediate innate immune responses to pathogen invasion and tumors,
604 increasing evidence has shown their role in the development of autoimmune and chronic
605 inflammation[52] by promoting Th17 responses. ATP, for instance, has been shown to convert
606 $CD4^+CD25^+Foxp3^+$ regulatory T cells into Th17 cells and to promote colitis by enhancing Th17
607 pro-inflammatory subset in the lamina propria via DCs[32, 53]. Similarly, to ATP, NAD^+, a co-factor
608 as well, has been described for its role in energy metabolism and more recently in aging[54-56]. In
609 contrast to ATP, NAD^+-mediated innate and adaptive immune regulation was intermediated by
610 MCs and was independent of major APCs including DCs and macrophages. Moreover, our
611 previous studies have underscored the robust immunosuppressive properties of NAD^+ by
612 promoting a systemic increase of IL-10 cytokine via regulatory type-1 cells[24, 25]. We have also
613 shown that NAD^+ regulates $CD4^+$ T cell fate in absence of major transcription factors. Indeed,
614 NAD^+ was able to promote a robust Th1 response in absence of T-bet, a transcription factor
615 considered indispensable for Th1 differentiation and IFNγ production[25]. In contrast to pro-
616 inflammatory responses induced by DAMPs, including ATP, NAD^+ protects against autoimmune
617 diseases and promotes allograft survival[24, 25]. Furthermore, our RNA-sequencing analysis
618 indicated that treatment with NAD^+ resulted in the upregulation of a distinct set of genes when
619 compared to LPS, a prototypical PAMP, suggesting that NAD^+ triggered a different signaling
620 pathway that remains yet to be determined in detail.

621 In line with our previous reports, NAD^+ treatment down regulated T-bet expression, but did not
622 alter $CD4^+IFN\gamma^+$ T cell responses when compared to the control group of mice. Although
623 $CD4^+IFN\gamma^+$ T cell responses remained similar in NAD^+ and placebo treated mice, continuous
624 treatment with NAD^+ conferred host protection against *Listeria monocytogenes*. Previous

studies have underscored the critical role of IFNγ in host protection against *Listeria monocytogenes*[9, 57]. Thus, NAD^+ may mediate host protection by promoting IFNγ production by other immune cells such as $CD8^+$ or innate lymphoid cells (ILCs). Although our study emphasizes the potent role of NAD^+ on many immune cells such as $CD4^+$ T cells, MCs and DCs, its impact on other important immune cells such as B, $CD8^+$ and innate lymphoid cells remain yet to be determined and its role in other inflammatory conditions or infections - including viral - 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 absence of antigen had a robust physiological impact following bacterial infection. Indeed, NAD^+ pre-treated mice were highly susceptible to even a non-lethal dose of bacterial infection that resulted in high mortality. In contrast, maintenance of NAD^+ signaling machinery by continuous treatment with NAD^+ conferred host protection following 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 may not only promote a robust host protection against bacterial infection but may be distinct from the classical DCs-MHC class II-TCR pathway. Indeed, with clodronate administration, DTR transgenic mice as well as the use of MC-deficient mice ($Kit^W/Kit^{W\text{-}v}$) and BMMCs we demonstrated that NAD^+-MC-mediated $CD4^+$ T cell differentiation is independent of the classical DCs-MHC class II-TCR pathway. More importantly, our data indicated that in presence of NAD^+, MCs mediated differentiation of both murine and human $CD4^+$ T cells. These findings suggest that NAD^+ may pave the way for novel therapies in the context of microbial infections or primary immunodeficiencies that are characterized by an altered DCs-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 a MC-mediated immune regulator (MCMIR) that is distinct from the classical APCs-MHC class II-TCR pathway.

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None.

Author contributions

H.R.C.B, T.H., H.U. V.C. and A.E. performed experiments with real time PCR, FACS and infection mouse model. S.K., T.H., H.U. and M.A.d.l.F. helped with Bone marrow derived mast cells experiment. K.M. and Y.N. helped with DTR transgenic mice and 5c.c7 Rag $2^{-/-}$ transgenic mice experiments. A.J.T., I.G., T.H, A.V., M.A.d.l.F, H.A. O.A and S.G.T. helped writing the manuscript. A.E. designed experiments, supervised and directed the work, interpreted data and wrote the manuscript. All authors discussed the results and contributed to the manuscript.

Conflicts of Interest

A.E. is inventor on the patent application WO WO/2014/169011 'METHODS FOR TREATING IMMUNE DISEASES'. All other authors declare no competing financial interests.

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Figure Legends

Figure 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 euthanized and CD4$^+$ T cells were isolated from spleens. Frequencies and total numbers of CD4$^+$IFNγ$^+$, CD4$^+$IL-4$^+$, CD4$^+$IL-17A$^+$ cells were analyzed by flow cytometry. **(B)** 3x10^6 CD4$^+$ T cells 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 sacrificed to analyze frequencies and total number of CD4$^+$IFNγ$^+$ cells by flow cytometry. **(C-D)** Sorted naïve CD4$^+$CD44$^-$CD62L$^+$ T cells were isolated from spleens of C57BL/6 mice and cultured in complete media alone, or with α-CD3/α-CD28, with or without IL-2, or in presence of 50μM NAD$^+$. After 96 hours, frequencies of CD4$^+$IFNγ$^+$ cells and IFNγ, IL-4 and IL-17A cytokine secretion were assessed by **(C)** flow cytometry and **(D)** ELISA, respectively. Statistics: n=15 **(A-B)** or 10 **(B-C)**; data derived from three independent sets of experiments. **P<0.01; ***P<0.001. Student's t-test and ANOVA tests were used accordingly to compare groups. Data given as mean±SD.

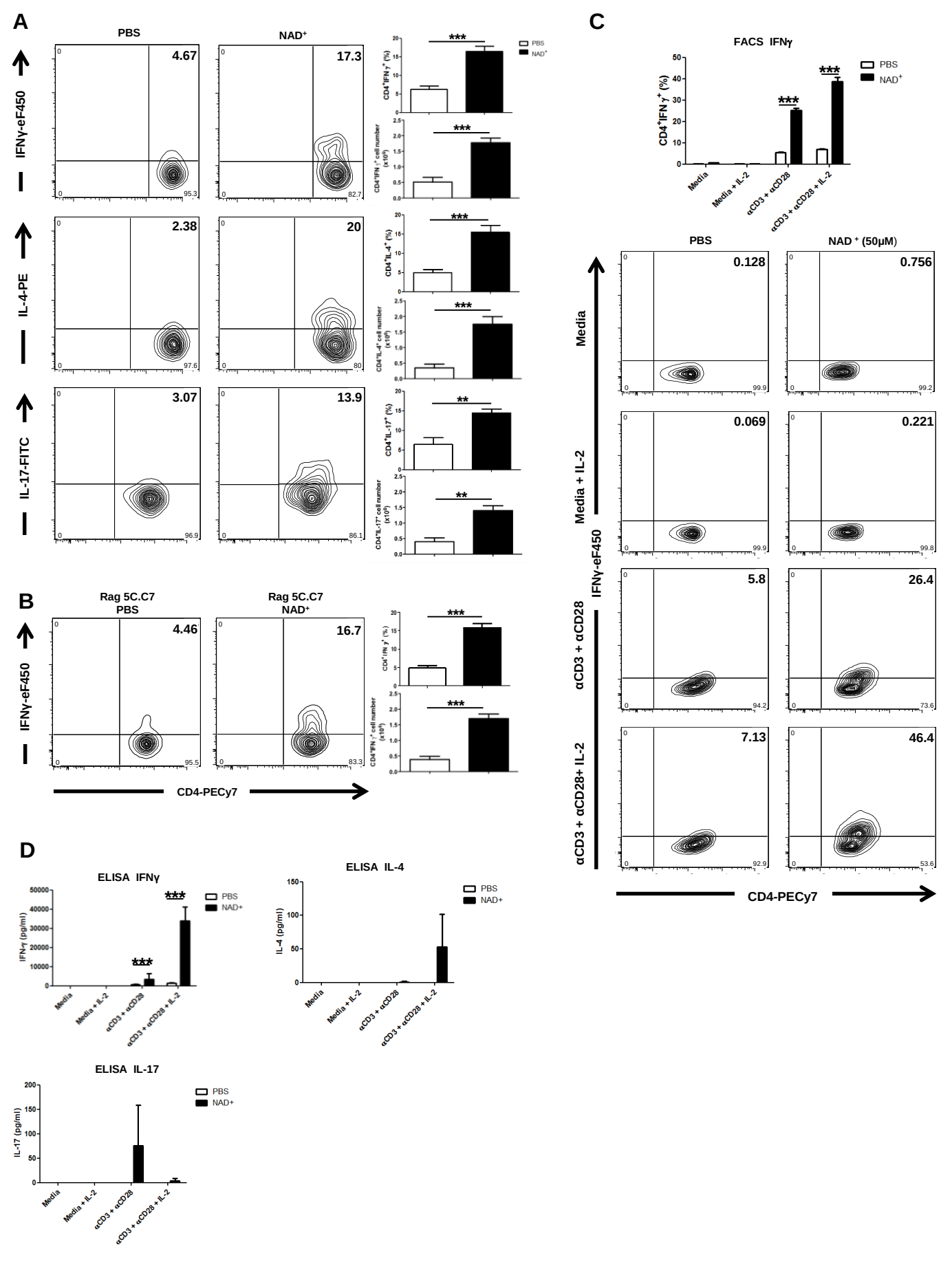
Figure 2: NAD$^+$ treatment induces increased cytokine production by CD11b$^+$CD11c$^+$ dendritic cells in vivo. C57BL/6 mice were treated daily with intraperitoneal injection of 40 mg of NAD$^+$ or a placebo solution (PBS). After 7 days, mice were euthanized and CD11b$^+$CD11c$^+$ dendritic cells were 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 flow cytometry. Statistics: n=15, data derived from three independent sets of experiments; data given as mean±SD. ***P<0.001. Student's t-test was used to compare groups.

Figure 3: Mast cells play a central role in NAD$^+$ mediated CD4$^+$ T cell differentiation. C57BL/6 wild type 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 euthanized and systemic levels of CD4$^+$IFNγ$^+$, CD4$^+$IL-4$^+$, CD4$^+$IL-17A$^+$ cells were assessed by flow cytometry. Data derived from two independent experiments (n=5-10); data represent mean±S.D. ***P<0.001 ANOVA tests were used to compare groups.

Figure 4: Unique MC gene expression profile following NAD$^+$ activation. Bone marrow derived mast cells (BMMCs) from C57BL/6 mice were cultured in presence of NAD$^+$ (500μM), LPS (10μg/ml; E. 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.

Figure 5: Mast cells mediate protection against lethal doses of L. monocytogenes during daily NAD$^+$ administration. C57BL/6 WT mice, Kit^W/Kit^{W-v} and kit^{Wsh}/Kit^{Wsh} MC-deficient mice were pre-treated for five consecutive days with daily intraperitoneal injections of NAD$^+$ (40mg) or placebo solution (PBS). Mice were then infected with an intraperitoneal **(A)** non-lethal dose (1x10^7 CFU) injection of viable Listeria monocytogenes. **(B)** C57BL/6 WT mice were pre-treated for five consecutive days with NAD$^+$ (i.p., 40mg) or placebo solution (PBS) prior infection. WT mice were then treated daily with NAD$^+$ (i.p., 40mg) or placebo solution (PBS) following a lethal dose (1x10^8 CFU) of viable Listeria monocytogenes. Weight loss and survival following infection was monitored. Data derived from three independent experiments (n=5-10 per group). Data represent mean±S.D. ns; not significant. ANOVA and Log-rank tests were used to compare groups, respectively.

Figure 6: NAD$^+$ regulates T cell differentiation through a novel mast cell-dependent signaling pathway. MC-mediated CD4$^+$ T cell differentiation following NAD$^+$ administration does not require antigen presentation through major APCs and MHC class II.


A
PBS
NAD+
4.67
17.3
95.3
82.7
IFNγ-eF450
2.38
20
97.6
80
IL-4-PE
3.07
13.9
96.9
86.1
IL-17-FITC
***
**
PBS
NAD+
B
Rag 5C.C7
PBS
Rag 5C.C7
NAD+
4.46
16.7
95.5
83.3
IFNγ-eF450
CD4-PECy7
C
FACS IFNγ
PBS
NAD+
Media
Media + IL-2
αCD3 + αCD28
αCD3 + αCD28 + IL-2
PBS
NAD+ (50μM)
0.128
0.756
99.9
99.2
0.069
0.221
99.9
99.8
5.8
26.4
94.2
73.6
7.13
46.4
92.9
53.6
Media
Media + IL-2
αCD3 + αCD28
αCD3 + αCD28+ IL-2
IFNγ-eF450
CD4-PECy7
D
ELISA IFNγ
IFNγ (pg/ml)
ELISA IL-4
IL-4 (pg/ml)
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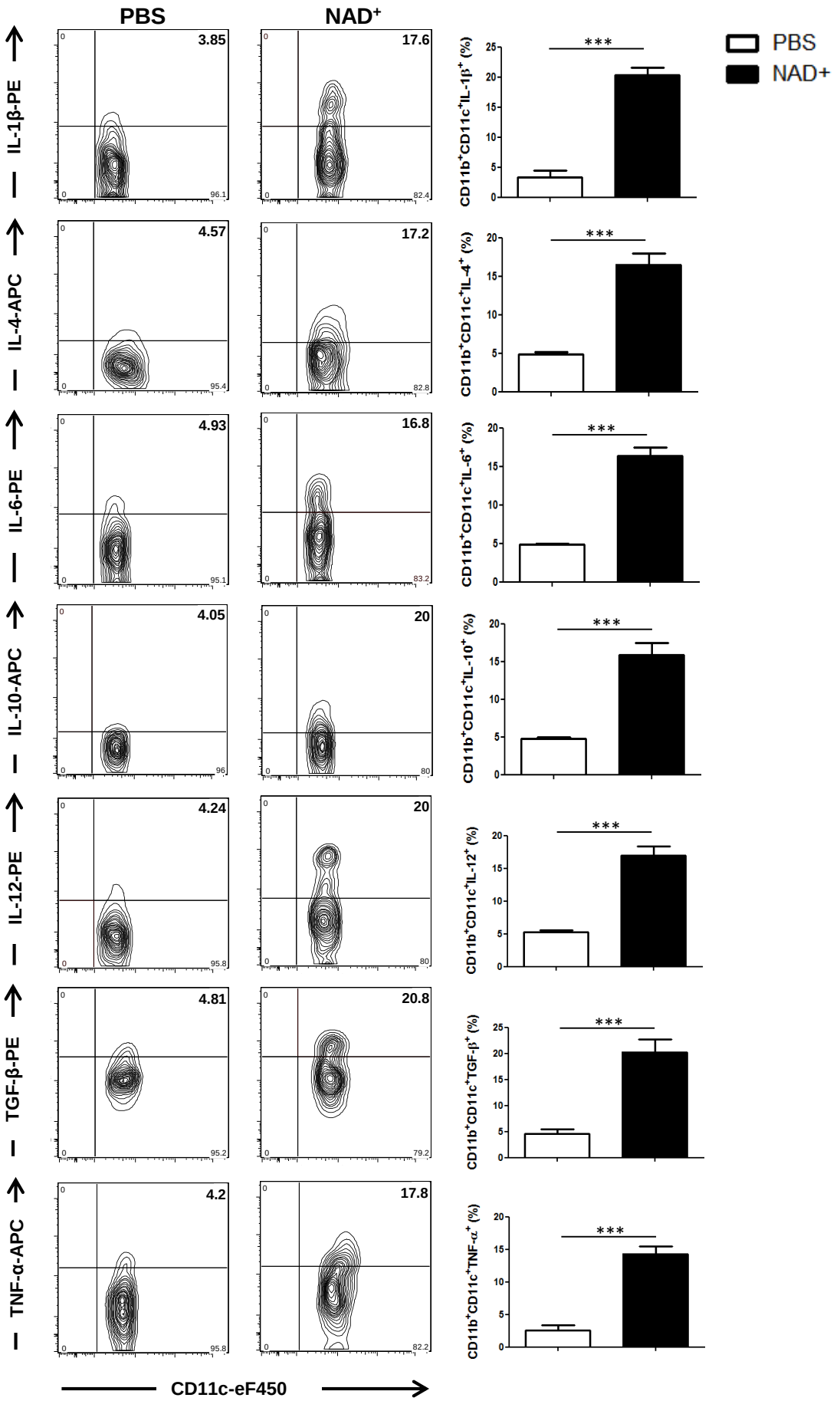
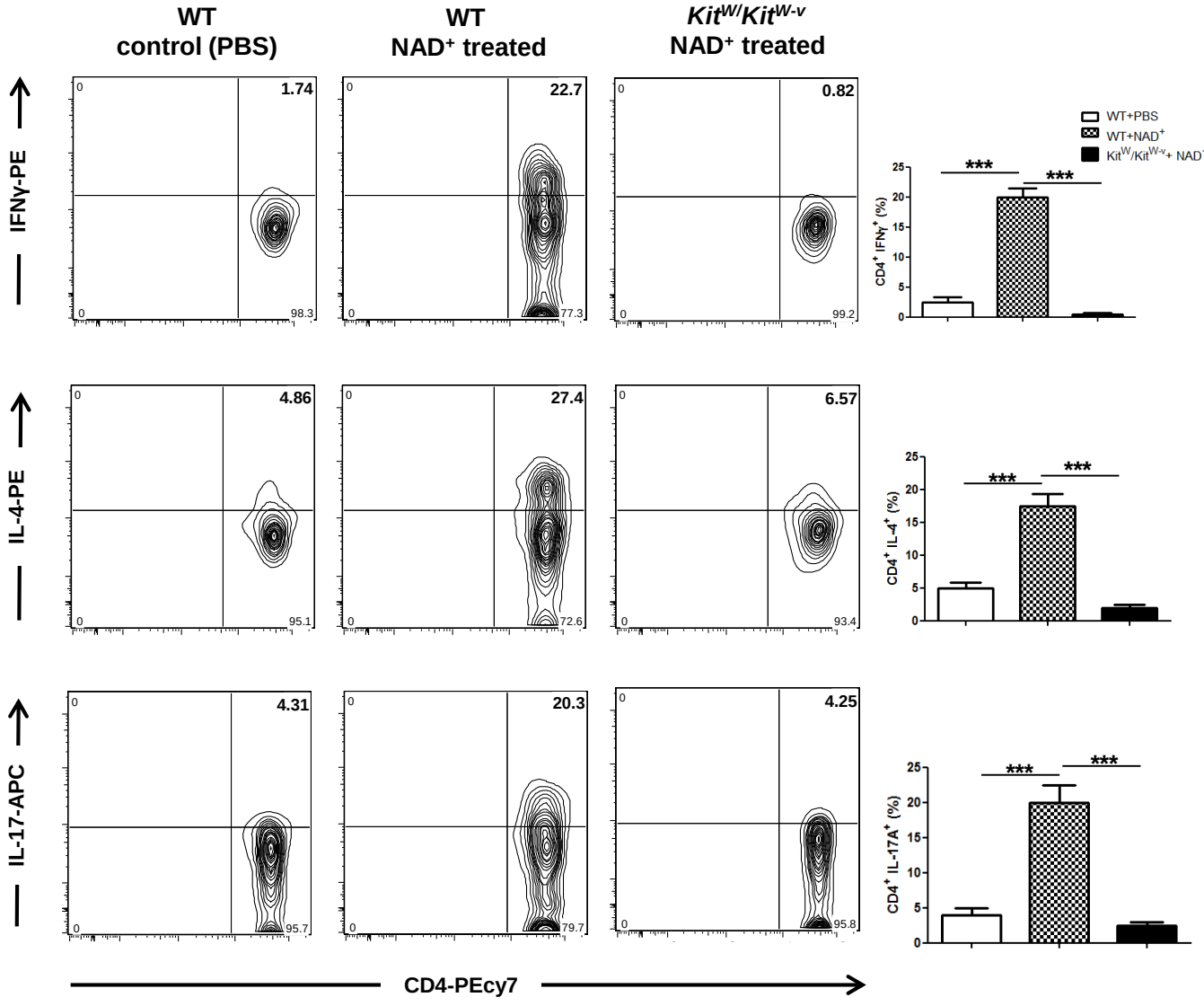
PBS
NAD+
Media
Media + IL-2
αCD3 + αCD28
αCD3 + αCD28 + IL-2

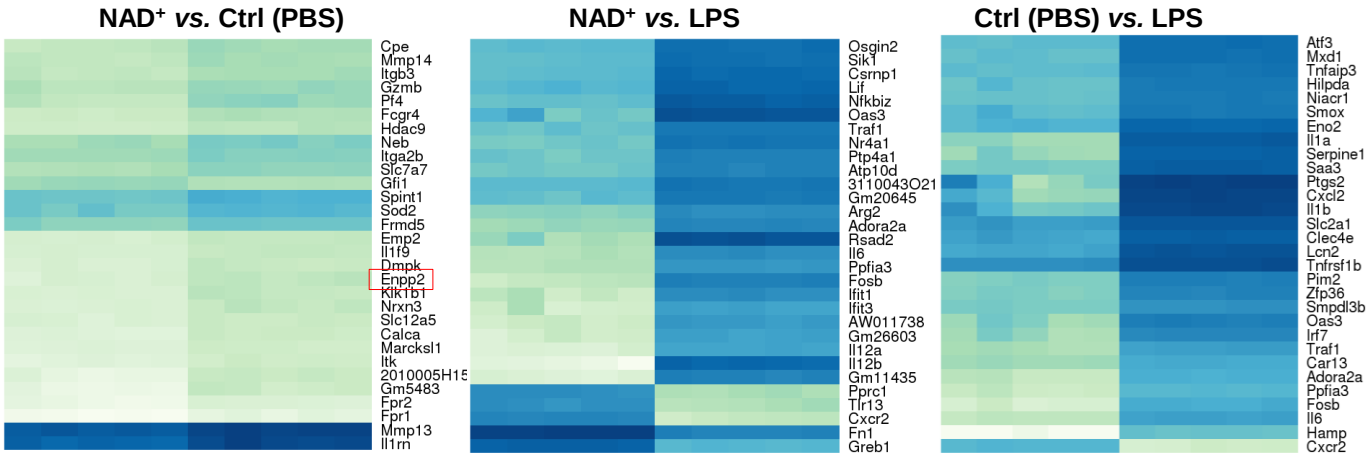

PBS
NAD+
IL-1β-PE
IL-4-APC
IL-6-PE
IL-10-APC
IL-12-PE
TGF-β-PE
TNF-α-APC
CD11c-eF450
3.85
96.1
17.6
82.4
4.57
95.4
17.2
82.8
4.93
95.1
16.8
83.2
4.05
96
20
80
4.24
95.8
20
80
4.81
95.2
20.8
79.2
4.2
95.8
17.8
82.2
CD11b+CD11c+IL-1β+ (%)
CD11b+CD11c+IL-4+ (%)
CD11b+CD11c+IL-6+ (%)
CD11b+CD11c+IL-10+ (%)
CD11b+CD11c+IL-12+ (%)
CD11b+CD11c+TGF-β+ (%)
CD11b+CD11c+TNF-α+ (%)
***
PBS
NAD+


WT
control (PBS)
WT
NAD+ treated
Kit^W/Kit^W-v
NAD+ treated
IFNγ-PE
IL-4-PE
IL-17-APC
CD4-PEcy7
1.74
98.3
22.7
77.3
0.82
99.2
4.86
95.1
27.4
72.6
6.57
93.4
4.31
95.7
20.3
79.7
4.25
95.8
WT+PBS
WT+NAD+
Kit^W/Kit^W-v+ NAD+
CD4+ IFNγ+ (%)
CD4+ IL-4+ (%)
CD4+ IL-17A+ (%)
***
***

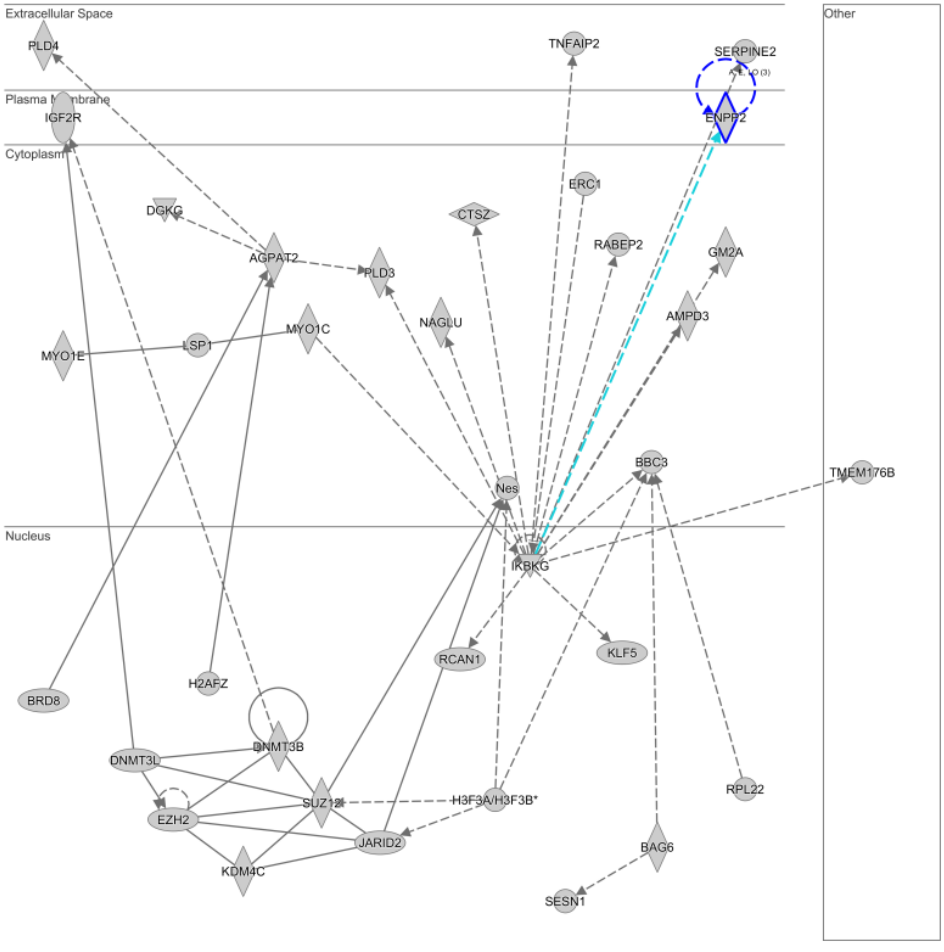
A

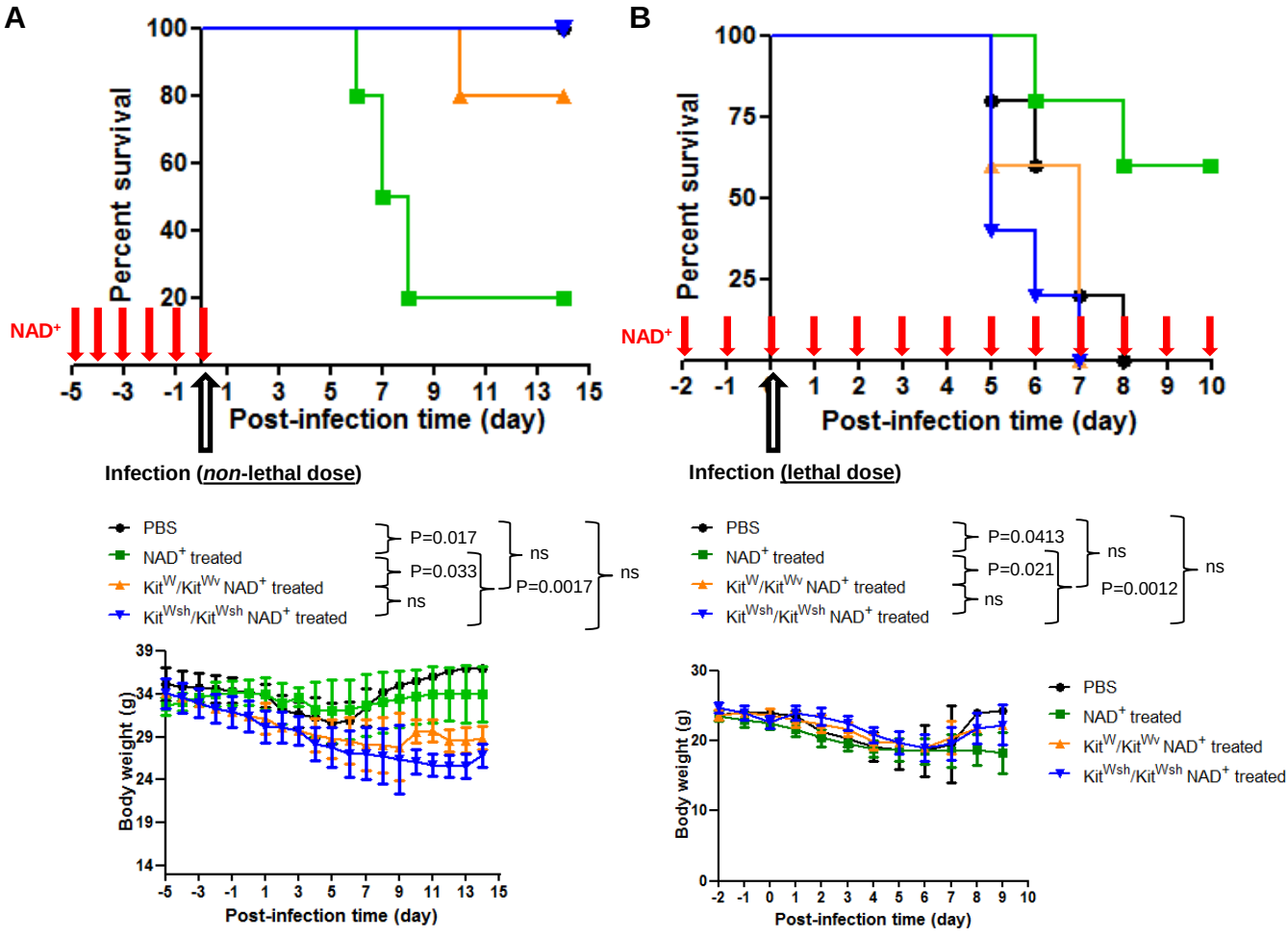
Contrast		Number of Genes	
Base/Control	Experimental	Upregulated	Downregulated
NAD^+	CTRL	603	753
LPS	CTRL	6092	5898
LPS	NAD^+	6053	5845

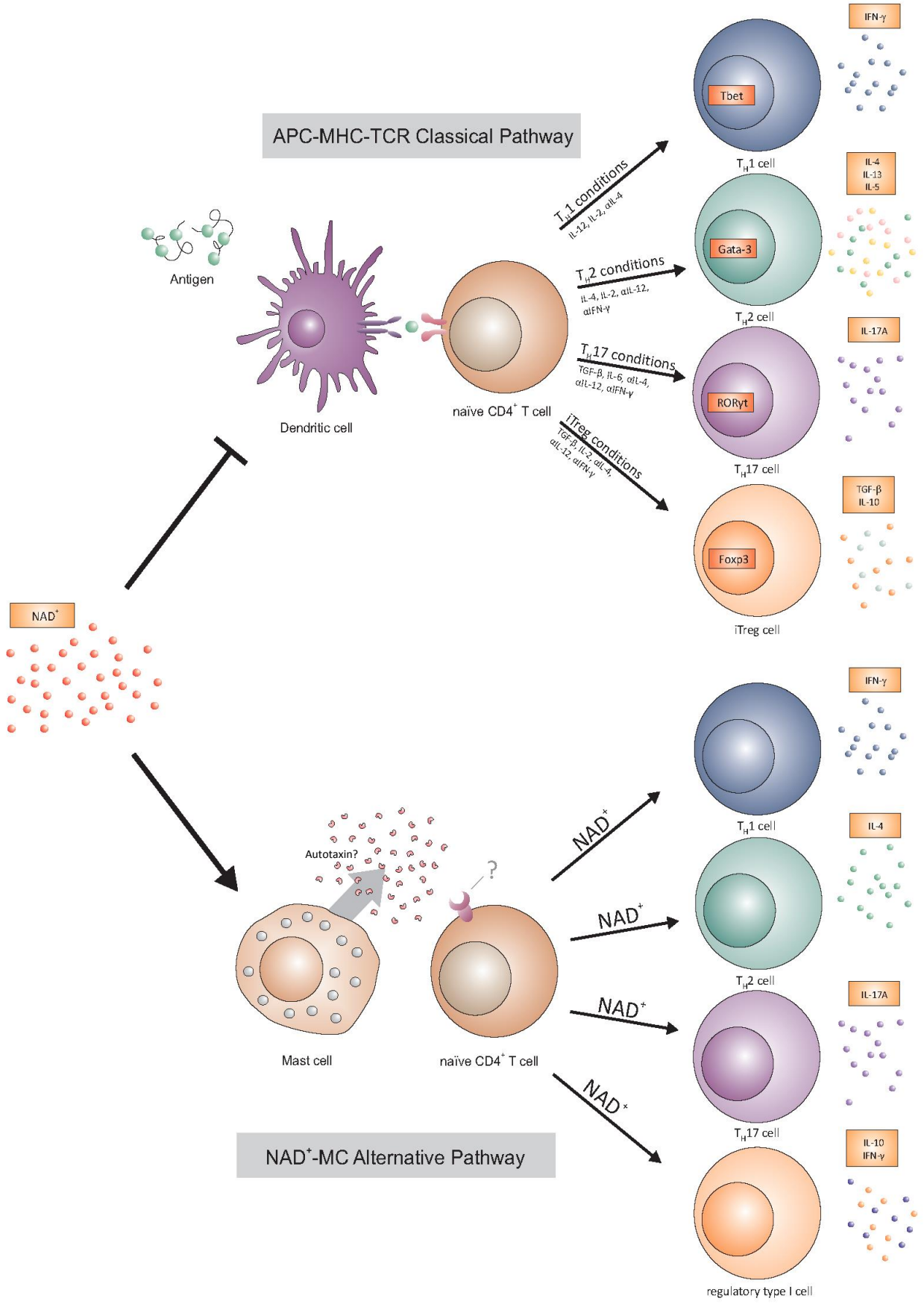
B



C




A
Percent survival
100
80
60
40
20
NAD+
-5 -3 -1 1 3 5 7 9 11 13 15
Post-infection time (day)
Infection (non-lethal dose)
PBS
NAD+ treated
KitW/KitWv NAD+ treated
KitWsh/KitWsh NAD+ treated
P=0.017
P=0.033
ns
ns
P=0.0017
ns
Body weight (g)
39
34
29
24
19
14
-5 -3 -1 1 3 5 7 9 11 13 15
Post-infection time (day)
B
Percent survival
100
75
50
25
NAD+
-2 -1 1 2 3 4 5 6 7 8 9 10
Post-infection time (day)
Infection (lethal dose)
PBS
NAD+ treated
KitW/KitWv NAD+ treated
KitWsh/KitWsh NAD+ treated
P=0.0413
P=0.021
ns
ns
P=0.0012
ns
Body weight (g)
30
20
10
0
-2 -1 0 1 2 3 4 5 6 7 8 9 10
Post-infection time (day)
PBS
NAD+ treated
KitW/KitWv NAD+ treated
KitWsh/KitWsh NAD+ treated


APC-MHC-TCR Classical Pathway
Antigen
Dendritic cell
naïve CD4+ T cell
T_H1 conditions
IL-12, IL-2, αIL-4
T_H2 conditions
IL-4, IL-2, αIL-12, αIFN-γ
T_H17 conditions
TGF-β, IL-6, αIL-4, αIL-12, αIFN-γ
iTreg conditions
TGF-β, IL-2, αIL-4, αIL-12, αIFN-γ
Tbet
T_H1 cell
IFN-γ
Gata-3
T_H2 cell
IL-4
IL-13
IL-5
RORγt
T_H17 cell
IL-17A
Foxp3
iTreg cell
TGF-β
IL-10
NAD+
Autotaxin?
?
Mast cell
naïve CD4+ T cell
NAD+
NAD+
NAD+
NAD+
T_H1 cell
IFN-γ
T_H2 cell
IL-4
T_H17 cell
IL-17A
regulatory type I cell
IL-10
IFN-γ
NAD+-MC Alternative Pathway

Supplemental EFigure 1: High doses of NAD^+ do not promote naïve $CD4^+$ T cell differentiation in vitro and NAD^+ regulates $CD11b^+CD11c^+$ dendritic cell cytokine production in vitro in a dose-dependent manner. **(A)** Sorted naïve $CD4^+CD44^-CD62L^+$ T cells were isolated from spleens of C57BL/6 mice and cultured in complete media with increasing concentrations of NAD^+ (500μM and 1mM) or PBS. After 96 hours, cell frequencies of $CD4^+IFN\gamma^+$, $CD4^+$IL- 4^+, $CD4^+$IL-$17A^+$ were assessed by flow cytometry (n=10; data derived from three independent experiments). **(B)** Sorted $CD11b^+CD11c^+$ dendritic cells were isolated from spleens of C57BL/6 mice and cultured (1x 10^6 cells per well) in complete media and in presence of increasing concentrations of NAD^+ (100μM and 500μM). As a positive control

$CD11b^+CD11c^+$ dendritic cells were cultured in the presence of LPS (1μg/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 real-time PCR. Values are expressed as fold expression relative to the house-keeping gene GAPDH. (n=5; data derived from two different experiments). *P<0.05; **P<0.01; ***P<0.001; ns, not significant, as determined by ANOVA, comparing the indicated groups. Data represent mean±S.D.

Supplemental EFigure 2: In vivo depletion of dendritic cells by liposomal clodronate administration does not alter NAD^+ mediated $CD4^+$ T cell differentiation. **(A)** C57BL/6 wild type, $MC^{-/-}$ and $Rag2^{-/-}\gamma c^{-/-}$ mice were treated

intravenously with liposomal clodronate at −8 days, −5 days, and −1 day before NAD^+ treatment. Data derived from two independent experiments (n=5 per group). **(B)** C57BL/6 wild type 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 euthanized and frequencies of $CD4^+IFN\gamma^+$, $CD4^+$IL-4^+ and $CD4^+$IL-$17A^+$ cells were analyzed by flow cytometry. Data derived from two independent experiments (n=10). Data represent mean±S.D. **P<0.01; ***P<0.001; ns, not significant. Student's t-test and ANOVA was used to compare between groups.

Supplemental EFigure 3: NAD$^+$ promotes T cell differentiation in Rag2$^{-/-}\gamma c^{-/-}$ mice and flow cytometry analysis of in vitro differentiation of bone marrow derived mast cells. **(A)** Rag2$^{-/-}\gamma$c$^{-/-}$ mice were treated intravenously with liposomal clodronate at −8 days, −5 days, and −1 day before NAD$^+$ treatment. Following depletion, FACS-sorted naïve CD4$^+$CD44$^-$CD62L$^+$ T cells from C57BL/6 WT mice were adoptively transferred (3x10^6 cells/adoptive transfer). Animals were then treated with daily intraperitoneal injections of 40 mg NAD$^+$ or a placebo solution (PBS). After 7 days, mice were euthanized and spleens were collected and frequencies of CD4$^+$IFNγ^+, CD4$^+$IL-4$^+$ and CD4$^+$IL-17A$^+$ cells were assessed by flow cytometry. Data derived from two independent experiments (n=10). **(B)** CD11b-DTR transgenic mice weighing 25–30 g were injected with diphtheria toxin (25 ng/g body weight) 24 hours before and 72 hours after beginning of NAD$^+$ or PBS administration for depletion of CD11b$^+$ cells. After 7 days of treatment with PBS or NAD$^+$, frequencies of CD4$^+$IFNγ^+, CD4$^+$IL-4$^+$ and CD4$^+$IL-17A$^+$ cells were assessed by flow cytometry. Data derived from two independent experiments (n=10). **(C)** Bone marrow derived mast cells (BMMCs) were obtained from femurs and tibias of 6-8 weeks old C57BL/6 WT mice. BMMCs were cultured in WEHI-3-conditioned medium over 90 days. Purities of c-kit$^+$Fc□R1$^+$ mast cells were then assessed by flow cytometry. Data represent mean±S.D. *P<0.05; **P<0.01. Student's t-test was used to compare between groups.

Supplemental EFigure 4: Murine mast cells promote CD4$^+$IFNγ$^+$, CD4$^+$IL-4$^+$ and CD4$^+$IL-17A$^+$ T cell differentiation in presence of NAD$^+$ both with and without cell-cell contact. Bone marrow derived mast cells (BMMCs) were co- cultured with isolated naïve CD4$^+$CD44$^-$CD62L$^+$ T cells from C57BL/6 mice (1:100 ratio) either in cell-cell contact or in separate compartments using a transwell system. Cells were then treated with NAD$^+$ (500µM) or PBS. After 96 hours, frequencies of **(A)** CD4$^+$IFNγ$^+$, **(B)** CD4$^+$IL-4$^+$ and **(c)** CD4$^+$IL-17A$^+$ cells were assessed by flow cytometry (n=6; the data derived from two independent experiments). *P<0.05; **P<0.01; ***P<0.001, as determined by ANOVA, comparing the indicated groups. Data represent mean±S.D.

Supplemental EFigure 5: MCs do not regulate CD4$^+$ T cell differentiation in presence of NAD$^+$ via CD80. **(A)** Bone marrow derived mast cells (BMMCs) from C57BL/6 mice were cultured in presence of NAD$^+$ (500µM) or placebo (PBS). After 24 hours of culture, cells were collected and mRNA was extracted. mRNA levels of CD86, CD80, TNF-α, IL-4, ICOS-L, OX40-L, IL-6 and IL-33 were determined

by real-time PCR. Values are expressed as fold expression relative to the house-keeping gene GAPDH. **(B-D)** Bone marrow derived mast cells (BMMCs) were co-cultured with isolated naïve $CD4^+CD44^-CD62L^+$ T cells from C57BL/6 mice (1:100 ratio) in cell-cell contact conditions in the presence of α- CD80, NAD^+ (500µM), or placebo (PBS), as indicated. After 96 hours, frequencies of **(B)** $CD4^+IFN\gamma^+$, **(C)** $CD4^+$IL-$17A^+$

and **(D)** $CD4^+$IL-4^+ cells were assessed by flow cytometry (n=6; data derived from two independent experiments).

*P<0.05; **P<0.01; ***P<0.001; ns, not significant, as determined by Student's t-test and ANOVA, comparing the indicated groups. Data represent mean±S.D.

Supplemental EFigure 6: Conserved MC-mediated $CD4^+$ T cell differentiation in human MC line LAD-2 in presence of NAD^+ and NAD^+ induces T cell differentiation in MHC class $II^{-/-}$ and $WASP^{-/-}$ mice. **(A)** Human mast cell line LAD-2 cells were co-cultured with isolated human naïve $CD4^+$ T cells from healthy donors (1:100 ratio) either in cell-cell contact or in separate compartments using a transwell system. Cells were then treated with NAD^+ (500µM) or PBS. After 96 hours, frequencies of $CD4^+IFN\gamma^+$ cells were assessed by flow cytometry (n=6; data derived from two independent experiments). **(B)** MHC class $II^{-/-}$ (B6.129S-$H2^{dlAb1\text{-}Ea}$) mice were treated daily with intraperitoneal injections of 40 mg of NAD^+ or a placebo solution (PBS). After 7 days, mice were euthanized and splenocytes were harvested. Systemic frequencies of $CD4^+IFN\gamma^+$, $CD4^+$IL-4^+, $CD4^+$IL-$17A^+$ cells were analyzed by flow cytometry (data derived from two 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 euthanized and frequencies of $CD4^+IFN\gamma^+$, $CD4^+$IL-4^+, $CD4^+$IL-$17A^+$ cells were assessed by flow cytometry (data derived from two independent

experiments; n=5). *P<0.05; **P<0.01; ***P<0.001; ns, not significant, as determined by Student's t-test and ANOVA, comparing the indicated groups. Data represent mean±S.D.

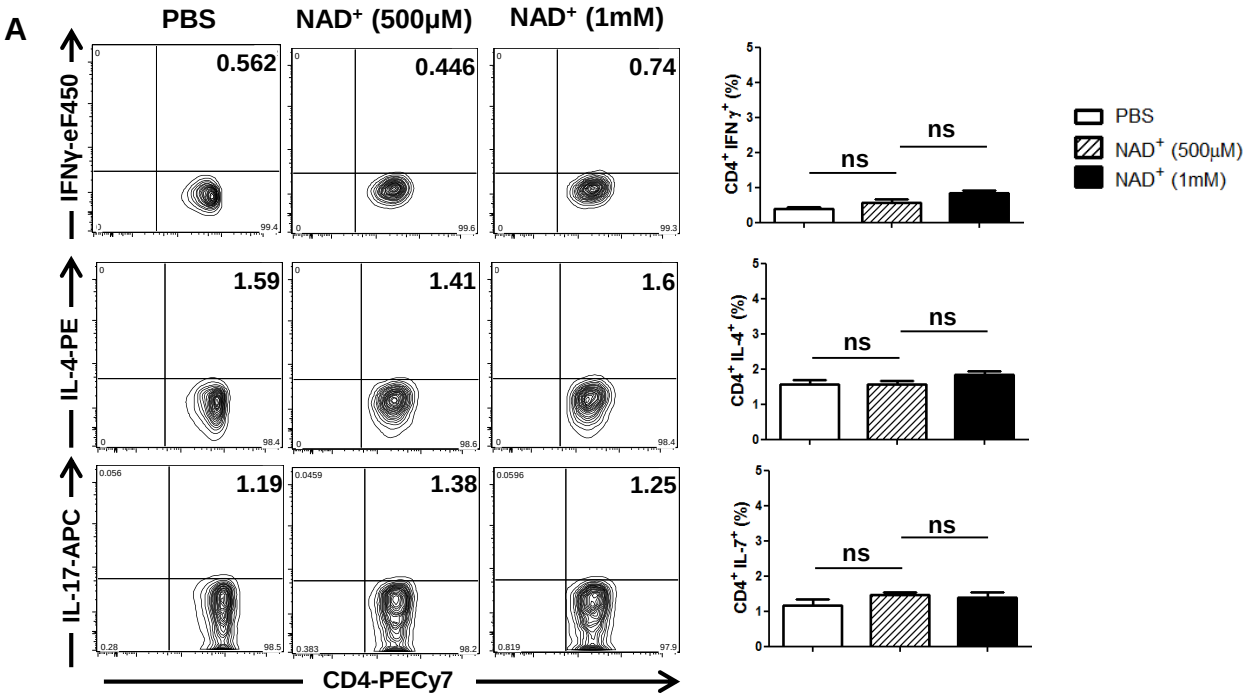
Supplemental EFigure 7: NAD^+ alters systemic frequencies of $CD4^+Tbet^+IFNg^+$ cells and $CD11c^+MHCII^+$ dendritic cells following L. monocytogenes infection. C57BL/6 mice were treated for five days with daily intraperitoneal injections of NAD^+ (40mg) or placebo solution (PBS). After 5 days, mice were infected with a non-lethal dose of *Listeria*

Monocytogenes ($1x10^7$ CFU) and euthanized three days later. Spleens were collected and

frequencies of **(A)**

$CD4^+Tbet^+IFN\gamma^+$ **(B)** $CD4^+$ $IFN\gamma^+$ **(C)** $CD4^-IFN\gamma^+$ T cells and **(D)** $CD11b^+CD11c^+MHCII^+$ dendritic cells were assessed by flow cytometry. Data derived from two independent experiments (n=5); data represent mean±s.d. *P<0.05;

***P<0.001: ns, not significant. Student's t-test was used to compare between groups.


A
PBS
NAD+ (500μM)
NAD+ (1mM)
IFNγ-eF450
IL-4-PE
IL-17-APC
CD4-PECy7
0.562
0.446
0.74
1.59
1.41
1.6
1.19
1.38
1.25
CD4+ IFN-γ+ (%)
CD4+ IL-4+ (%)
CD4+ IL-7+ (%)
ns
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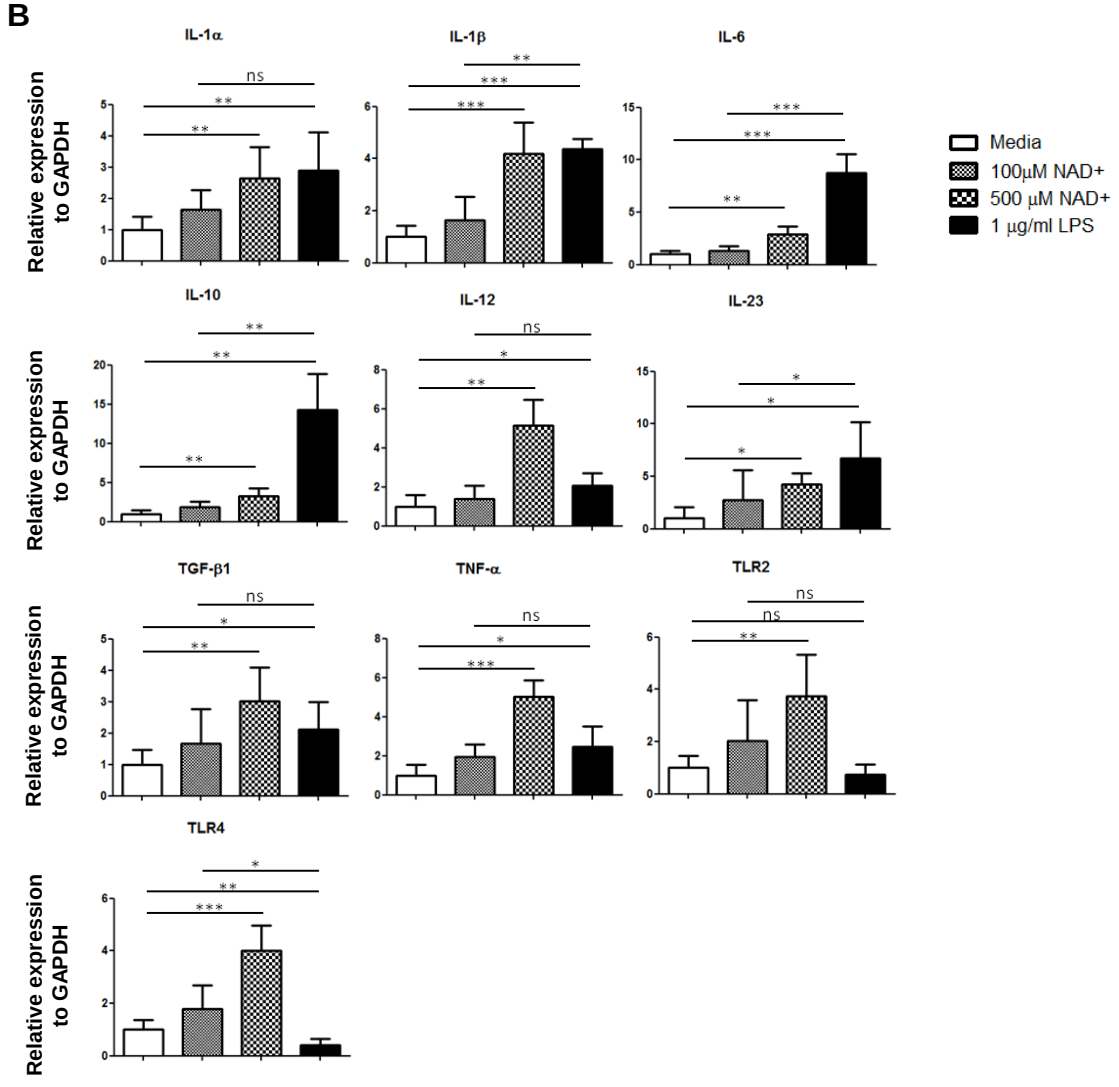
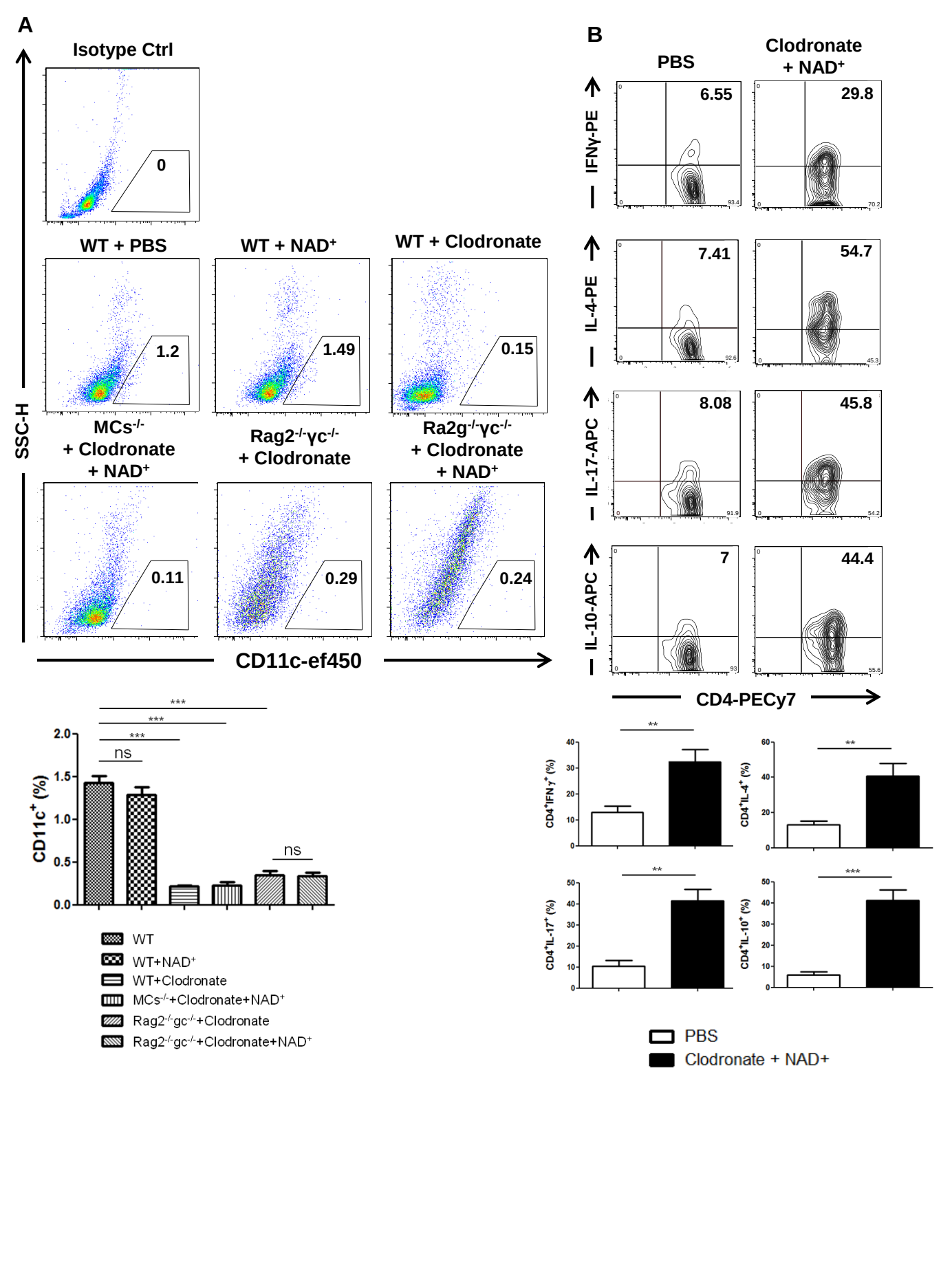
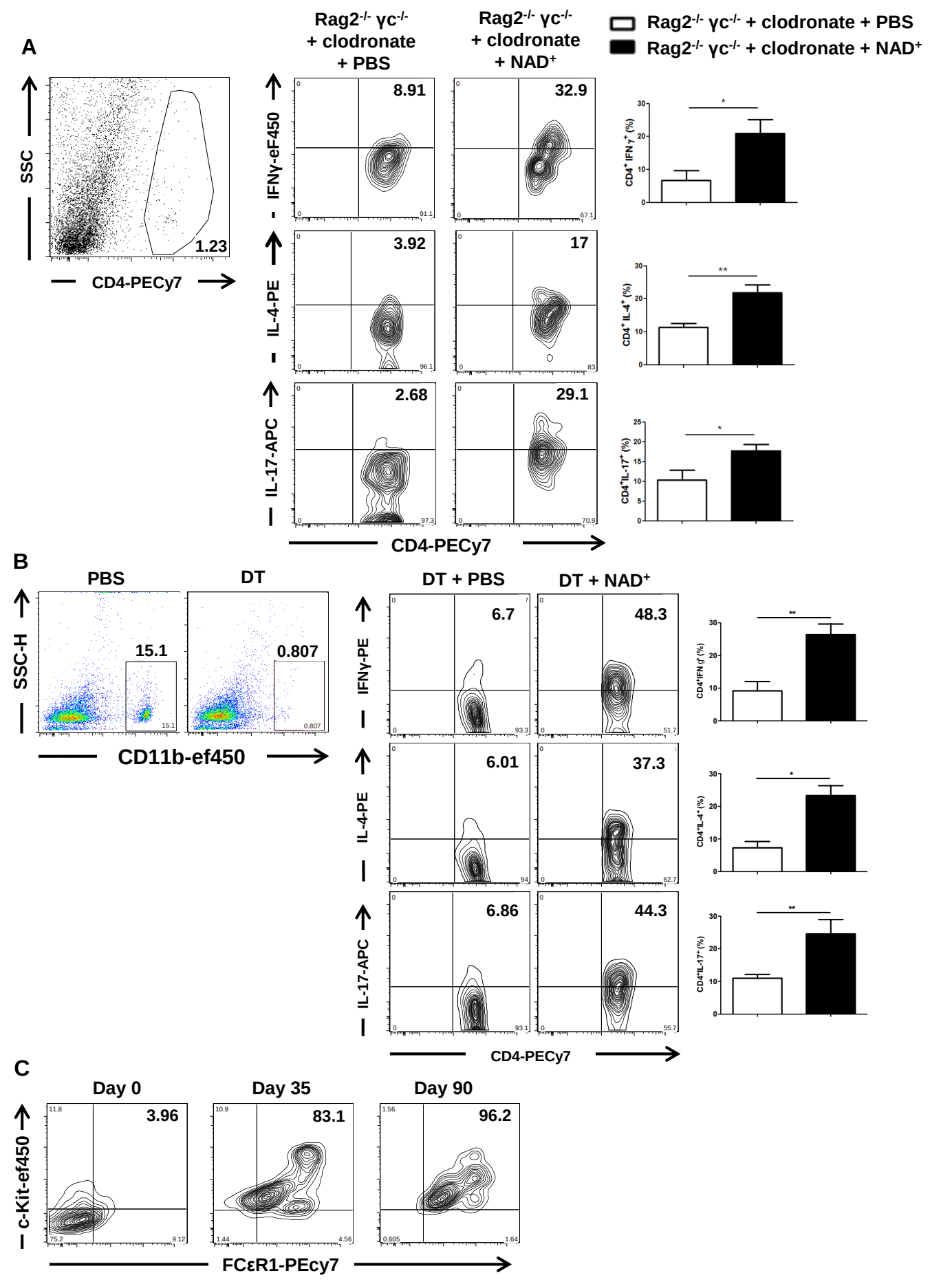
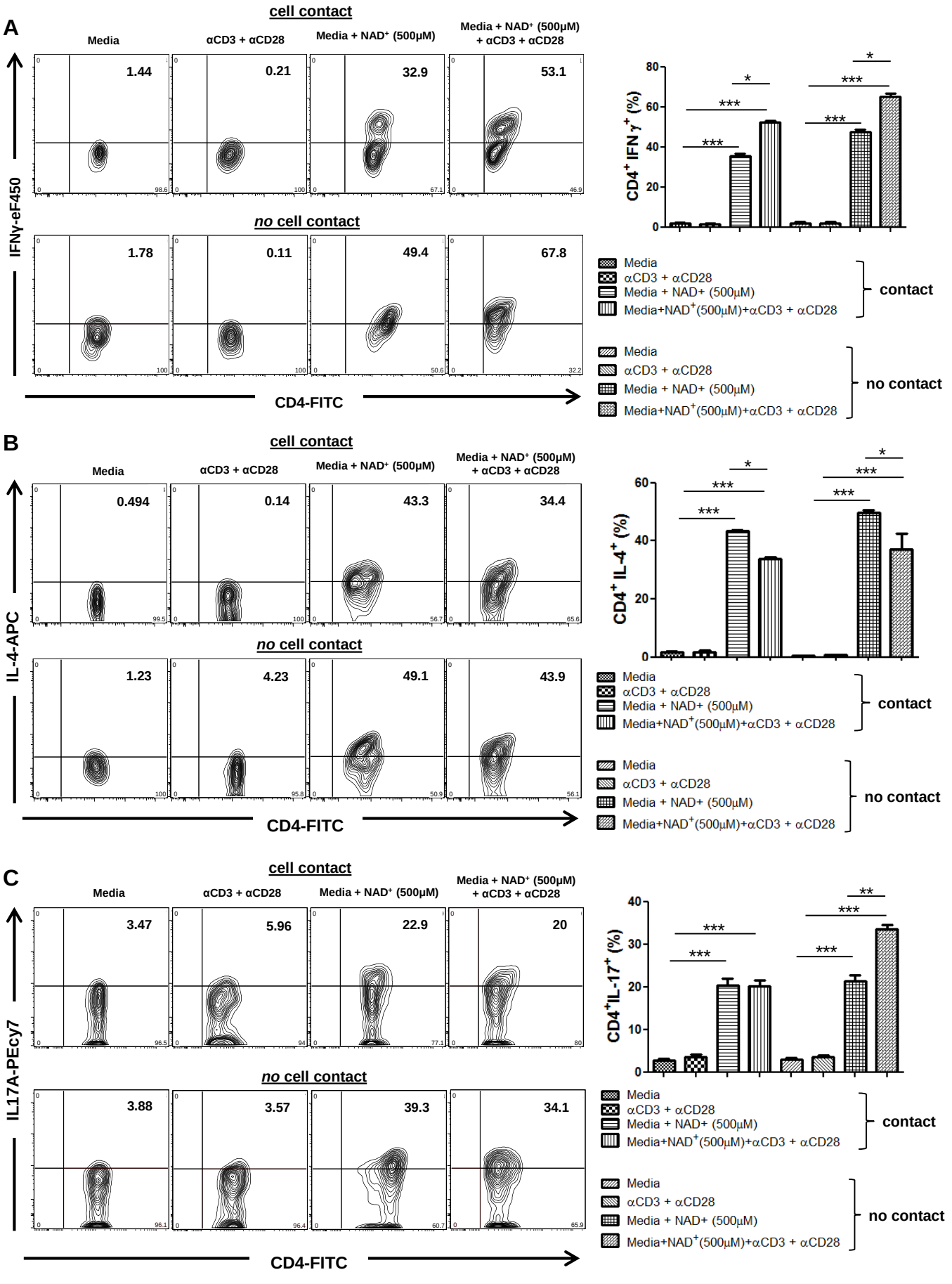
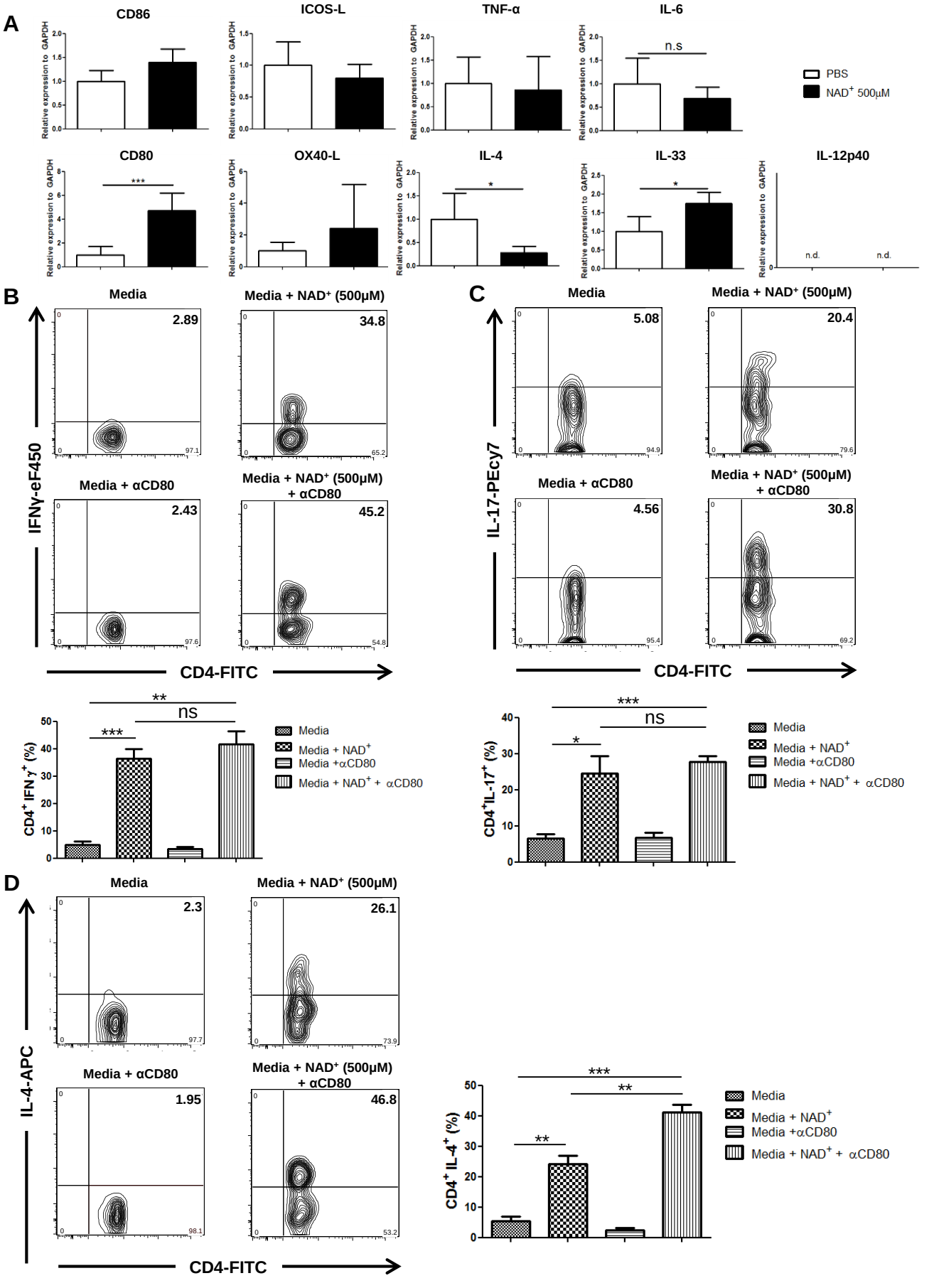
NAD+ (500μM)
NAD+ (1mM)

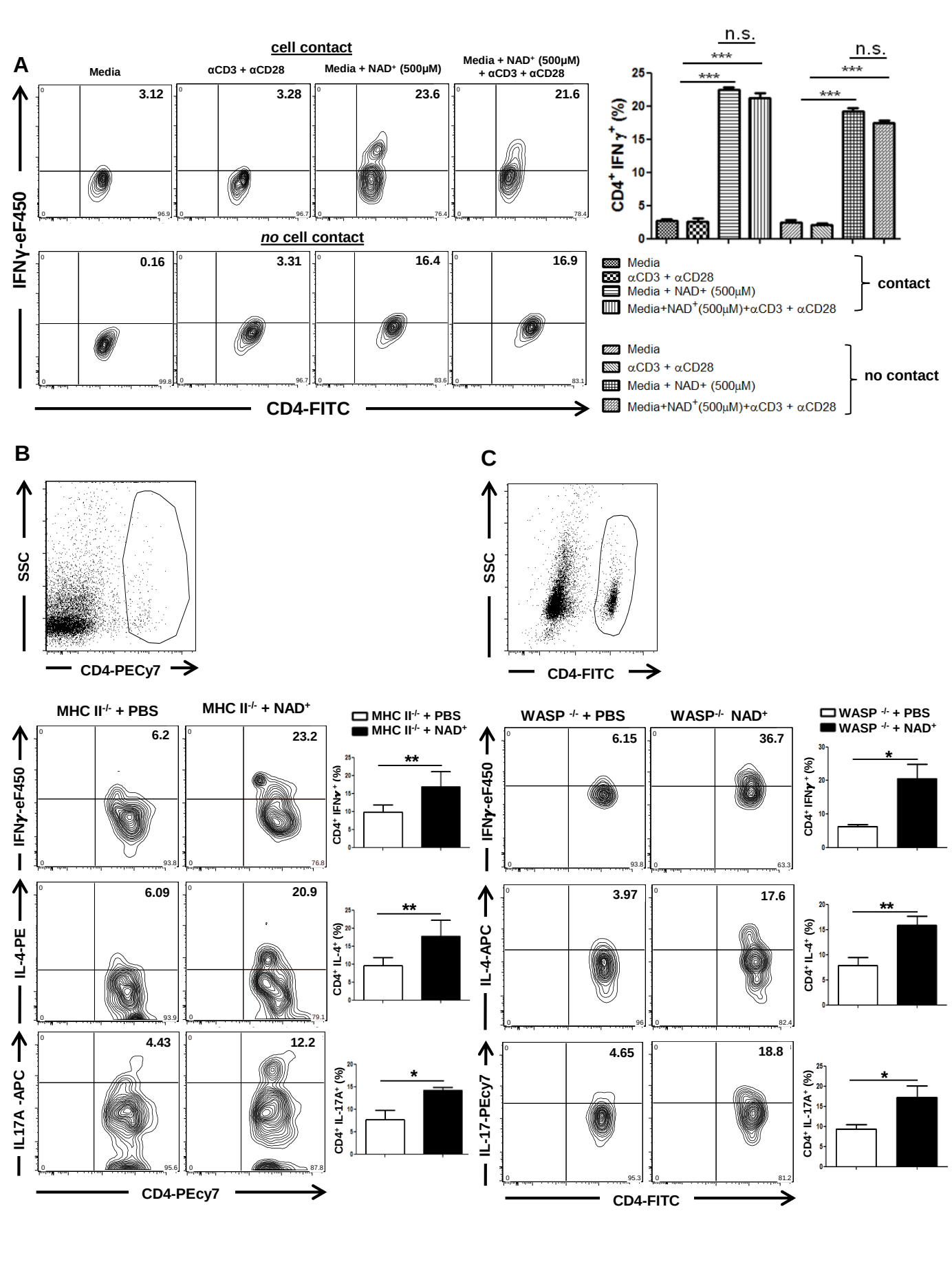

B
IL-1α
IL-1β
IL-6
IL-10
IL-12
IL-23
TGF-β1
TNF-α
TLR2
TLR4
Relative expression to GAPDH
Media
100μM NAD+
500 μM NAD+
1 μg/ml LPS

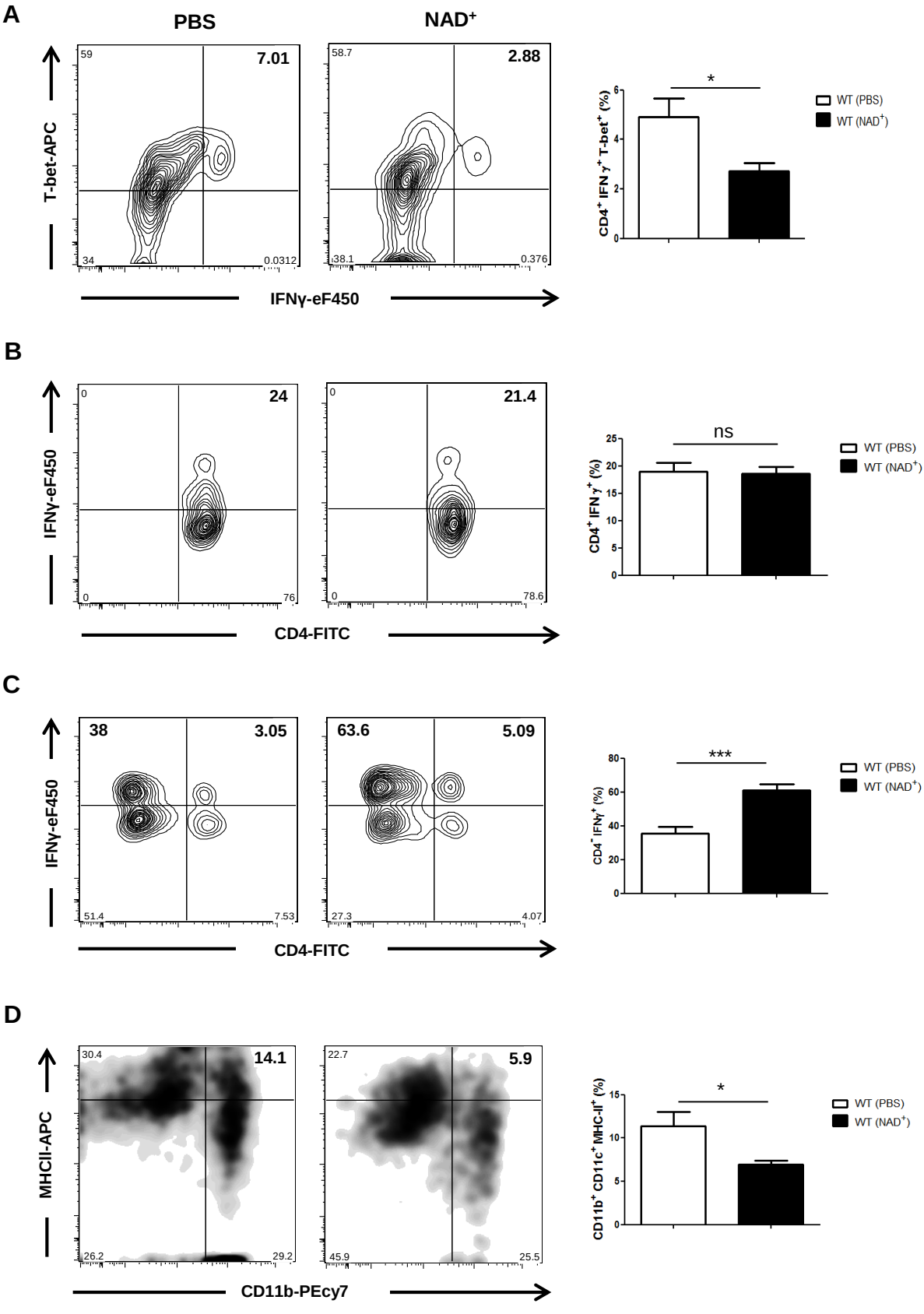

A
Isotype Ctrl
0
WT + PBS
1.2
WT + NAD+
1.49
WT + Clodronate
0.15
MCs-/-
+ Clodronate
+ NAD+
0.11
Rag2-/-γc-/-
+ Clodronate
0.29
Ra2g-/-γc-/-
+ Clodronate
+ NAD+
0.24
SSC-H
CD11c-ef450
B
PBS
Clodronate
+ NAD+
IFNγ-PE
6.55
29.8
IL-4-PE
7.41
54.7
IL-17-APC
8.08
45.8
IL-10-APC
7
44.4
CD4-PECy7
***
***
***
ns
ns
CD11c+ (%)
2.0
1.5
1.0
0.5
0.0
WT
WT+NAD+
WT+Clodronate
MCs-/-+Clodronate+NAD+
Rag2-/-gc-/-+Clodronate
Rag2-/-gc-/-+Clodronate+NAD+
**
**
**
**
***
CD4+IFN γ+ (%)
CD4+IL-4+ (%)
CD4+IL-17+ (%)
CD4+IL-10+ (%)
PBS
Clodronate + NAD+


A
Rag2-/- γc-/- + clodronate + PBS
Rag2-/- γc-/- + clodronate + NAD+
Rag2-/- γc-/- + clodronate + PBS
Rag2-/- γc-/- + clodronate + NAD+
SSC
CD4-PECy7
1.23
IFNγ-eF450
8.91
32.9
CD4+ IFN γ+ (%)
IL-4-PE
3.92
17
CD4+ IL-4+ (%)
IL-17-APC
2.68
29.1
CD4+IL-17+ (%)
CD4-PECy7
B
PBS
DT
DT + PBS
DT + NAD+
SSC-H
CD11b-ef450
15.1
0.807
IFNγ-PE
6.7
48.3
CD4+IFN g+ (%)
IL-4-PE
6.01
37.3
CD4+IL-4+ (%)
IL-17-APC
6.86
44.3
CD4+IL-17+ (%)
CD4-PECy7
C
Day 0
Day 35
Day 90
c-Kit-ef450
3.96
83.1
96.2
FCεR1-PEcy7


A
cell contact
Media
αCD3 + αCD28
Media + NAD+ (500μM)
Media + NAD+ (500μM) + αCD3 + αCD28
1.44
0.21
32.9
53.1
no cell contact
1.78
0.11
49.4
67.8
IFNγ-eF450
CD4-FITC
CD4+ IFNγ+ (%)
Media
αCD3 + αCD28
Media + NAD+ (500μM)
Media+NAD+(500μM)+αCD3 + αCD28
contact
no contact
B
cell contact
0.494
0.14
43.3
34.4
no cell contact
1.23
4.23
49.1
43.9
IL-4-APC
CD4+ IL-4+ (%)
C
cell contact
3.47
5.96
22.9
20
no cell contact
3.88
3.57
39.3
34.1
IL17A-PEcy7
CD4+IL-17+ (%)


A
CD86
ICOS-L
TNF-α
IL-6
n.s
PBS
NAD+ 500μM
CD80
OX40-L
IL-4
IL-33
IL-12p40
n.d.
Relative expression to GAPDH
B
Media
Media + NAD+ (500μM)
Media + αCD80
Media + NAD+ (500μM) + αCD80
IFNγ-eF450
CD4-FITC
CD4+ IFNγ+ (%)
Media + NAD+
Media +αCD80
Media + NAD+ + αCD80
C
IL-17-PEcy7
CD4+IL-17+ (%)
D
IL-4-APC
CD4+ IL-4+ (%)


A
cell contact
Media
αCD3 + αCD28
Media + NAD+ (500μM)
Media + NAD+ (500μM) + αCD3 + αCD28
3.12
3.28
23.6
21.6
no cell contact
0.16
3.31
16.4
16.9
IFNγ-eF450
CD4-FITC
n.s.
***
***
***
n.s.
***
CD4+ IFN γ+ (%)
Media
αCD3 + αCD28
Media + NAD+ (500μM)
Media+NAD+(500μM)+αCD3 + αCD28
contact
Media
αCD3 + αCD28
Media + NAD+ (500μM)
Media+NAD+(500μM)+αCD3 + αCD28
no contact
B
SSC
CD4-PECy7
C
SSC
CD4-FITC
MHC II-/- + PBS
MHC II-/- + NAD+
6.2
23.2
6.09
20.9
4.43
12.2
IFNγ-eF450
IL-4-PE
IL17A -APC
CD4-PEcy7
MHC II-/- + PBS
MHC II-/- + NAD+
CD4+ IFNγ+ (%)
CD4+ IL-4+ (%)
CD4+ IL-17A+ (%)
WASP -/- + PBS
WASP-/- NAD+
6.15
36.7
3.97
17.6
4.65
18.8
IFNγ-eF450
IL-4-APC
IL-17-PEcy7
CD4-FITC
WASP -/- + PBS
WASP -/- + NAD+
CD4+ IFNγ+ (%)
CD4+ IL-4+ (%)
CD4+ IL-17A+ (%)


A
PBS
NAD+
59
7.01
34
0.0312
58.7
2.88
38.1
0.376
T-bet-APC
IFNγ-eF450
CD4+ IFN γ+ T-bet+ (%)
*
WT (PBS)
WT (NAD+)
B
0
24
0
76
0
21.4
0
78.6
IFNγ-eF450
CD4-FITC
CD4+ IFN γ+ (%)
ns
WT (PBS)
WT (NAD+)
C
38
3.05
51.4
7.53
63.6
5.09
27.3
4.07
IFNγ-eF450
CD4-FITC
CD4- IFNγ+ (%)
***
WT (PBS)
WT (NAD+)
D
30.4
14.1
26.2
29.2
22.7
5.9
45.9
25.5
MHCII-APC
CD11b-PEcy7
CD11b+ CD11c+ MHC-II+ (%)
*
WT (PBS)
WT (NAD+)