Implication of the Nuclear Hormone Receptors in Immunity and Anti-pathogen Response of Dendritic Cells

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ABSTRACT

Nuclear hormone receptors (NRs) are a family of intracellular receptors, which mediate biological actions of lipophilic hormones and metabolites of various lipid compounds. They play essential roles in numerous physiological functions, such as intermediary metabolism, cell fate decision, homeostasis and development of the central nervous system and immune organs. Humans express 48 members of the family proteins and their expression is tissue- and cell type-specific, so that timely and appropriate response can be induced according to changes from internal and external environment.

Dendritic cells (DCs) are a sparsely distributed, migratory group of bone-marrow-derived leukocytes that are specialized for the uptake, transport, processing and presentation of antigens to T cells. They play pivotal roles in the initiation and regulation of pathogen-directed immune response promoted by innate and acquired immunity. Because DCs have such indispensible activities in immunity and NRs plays numerous important roles in every aspect of organism activity, I examined the mRNA expression profile of all NRs in mouse bone marrow-derived DCs. I found that these cells expressed most of the lipophilic hormone- and metabolite-binding NRs, while they expressed only half of the orphan receptors. Upon viral infection, mRNA expression of half of the expressed NRs was significantly modulated in DCs. The differences in kinetics and duration of the changes in NR mRNA expression may indicate the specific and distinctive roles of NRs in DC-mediated anti-viral immune response.

Since the expression of NOR1 was greatly increased in virus-infected DCs, we measured serum cytokine concentrations of NOR1$^{-/-}$ mice that were stimulated by Toll-like receptor 3 (TLR3) or infected by protozoa *Toxoplasma Gondii*. The serum concentration of Th1 cytokine

interferon (IFN) γ and the proinflammatory cytokines tumor necrosis factor α and interleukin (IL) 12 were significantly lowered and delayed in NOR1$^{-/-}$ mice when compared to wild type mice. Since IL-12 is mainly secreted from DCs and is essential for IFNγ induction, it is likely that NOR1 is required for anti-pathogen response organized by DCs.

Glucocorticoid receptor (GR) is one of the NR family proteins that mediate the biological action of glucocorticoids. Physical or mental stress stimulates the hypothalamic-pituitary-adrenal axis and induces adrenal glands to secrete the end-effector hormones glucocorticoids to cope with stressed conditions. It is well known that stress increases infectivity and course of infectious diseases, however, little is known about actions of glucocorticoids and GR on DC-mediated anti-pathogen immunity and cytokines expression profile. Hence, the mRNA expression of cytokines and TLR-related molecules was examined while DCs were infected with the Newcastle disease virus under the influence of synthetic glucocorticoid dexamethasone. Synergistically increased mRNA expression and secretion of anti-inflammatory cytokine IL-10 was found. Multiple signaling pathways were evaluated on this synergism and activation of the extracellular signal-regulated kinase 1/2 was found to be involved. Thus, these findings may partly explain the underlying mechanism of increased susceptibility of stressed individuals to pathogen infection.

In conclusion, this study has showed that expression of NRs was modulated upon viral infection and they may function as important regulators in controlling cytokine expression organized by DCs. Pathogen such as viruses may hence target NR signaling in promoting their infection and survival.

摘要

核受體家族在各項生物過程中扮演重要角色，例如細胞代謝，生理平衡調控，免疫系統及神經系統的發展。人類擁有四十八種核受體，他們於生物體面對各種內在及外在的改變中所需的調節尤其重要。

樹狀細胞是一種特殊的白血球，其專門對病原體偵查，分解及向 T 細胞表達抗原的功能，能有效地促進激化免疫系統。因此，我在這個研究中探討了核受體對樹狀細胞在免疫系統中的影響。

首先，我先查測四十九個核受體在樹狀細胞的表演模式，我發現樹狀細胞在轉錄調節中表現大部份的賀爾蒙類受體及代謝物類受體，但他們只表現約一半的無配體類受體。當樹狀細胞被紐卡素病毒感染後，當中一半的核受體 mRNA 轉錄表現被顯著地改變。

NOR1 是其中一個被發現於被病毒感染的樹狀細胞中大大地提高的無配體類受體。因此，我們分別用弓型蟲感染 NOR1 基因剔除小鼠，及注射 Toll 樣受體 3 的配體，然後收取其血液樣本，並量度血清中部份細胞因子的含量。結果顯示，對比野生型小鼠，NOR1 基因剔除小鼠含有較低濃度的干擾素 γ ，腫瘤壞死因子，及細胞因子 12(IL-12)。由於 IL-12 主要由樹狀細胞產生，並於刺激干擾素 γ 的產生具有決定性作用。所以，這反映 NOR1 對樹狀細胞的抗原體反應，特別是細胞因子的規控有重要功用。

當生物體受到內在或外在的壓力刺激，腎上腺會大量分泌糖皮質激素，而糖皮質激素受體(GR)是調控靶基因的份子。文獻顯示壓力提高生物體被病原體感染的機會，但當中描述樹狀細胞所涉及的機制卻十分有限。故此，我檢測了地塞米松 (DEX: 一種人工合成的腎上腺皮質激素) 對被病毒感染的樹狀細胞的影響，我發現 toll 樣受體的五個靶基因，其轉錄調控受 DEX 影響。當中我仔細地檢測了細胞因子 10 (IL-10) 在不同時段及使用不同 DEX 劑量的影響。我除了發現 DEX 能增加被病毒感染的樹狀細胞分泌更多的 IL-10。這結果能一定程度上解釋壓力對病毒感染的影響。

由此可見，這個研究顯示核受體家族的表現在感染後的樹狀細胞中被顯著地改變，表明核受體在免疫系統中，特別在細胞因子的調控上扮演重要功能，而病毒也會採用干擾核受體的靶基因而助長其感染過程。

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Ng SS, Li A, Elenkov I, Ozato K, Kino T. Dexamethasone Pre-treatment Enhances Viral Infection-induced Interleukin-10 Production in the Murine Dendritic Cells. Under review

Publication that are related to this work:

Nader N, Ng SS, Lambrou GI, Pervanidou P, Wang Y, Chrousos GP, Kino T. 2010. AMPK Regulates Metabolic Actions of Glucocorticoids by Phosphorylating the Glucocorticoid Receptor through p38 MAPK. Mol Endocrinol. 24: 1748-1764

Nader N, Ng SS, Abel BS, Chrousos GP, Kino T. Gene-specific Regulation of Glucocorticoids Receptor Action by Liver X Receptors. Under review

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ABBREVIATIONS

AP-1	Activator protein 1
AR	Androgen receptor
CCL2	Monocyte chemoattractant protein 1
cDC	Conventional DC
Clec4e	C-type lectin domain family 4 member e
CORT	Corticosterone
CpG	Unmethylated CpG oligodeoxynucleotide
C_t	Threshold cycle
DC	Dendritic cell
DEX	Dexamethasone
dsDNA	Double-stranded DNA
ELISA	Enzyme-linked immunosorbent assay
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinases
FCS	Fetal calf serum
FLT3L	Fms-related tyrosine kinase 3 ligand
Gapdh	Glyceraldehyde 3-phosphate dehydrogenase
GC	Glucocorticoid hormones
GR	Glucocorticoid receptor
HDAC	Histone deacetylase
HPA	Hypothalamic-pituitary-adrenal
i.p.	Intra-peritoneally
IFN	Interferon
IFNAR	Type I IFN receptor
IL	Interleukin
IRF	Interferon regulatory factors
ISG	Interferon stimulated genes
JNK	c-Jun N-terminal kinases
LPS	Lipopolysaccharides
LXR	Liver X receptor
MAPK	Mitogen-activated protein kinase
MCMV	Murine cytomegalovirus
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NCOA2	Nuclear coactivator 2
NCOR2	Nuclear coreppresssor 2
NDV	Newcastle disease virus

NFAT	Nuclear factor of activated T-cells
NF-κB	Nuclear factor of κB
NK	Natural killer
NOR1	Neuron-derived orphan receptor 1
NR	Nuclear hormone receptor
Nur77	Nerve growth factor-induced factor B
NURR1	Nuclear receptor related 1 protein
pDC	Plasmacytoid DC
PKA/Syk	Protein kinase A/spleen tyrosine kinase
Poly IC	Polyinosine-polycytidylic acid
PPAR	Peroxisome proliferator-activated receptor
PR	Progesterone receptor
PRR	Pattern recognition receptors
Ptgs2	Prostaglandin-endoperoxide synthase 2
qPCR	Real-time quantitative PCR
R848	Resiquimod
RAR	Retinoic acid receptor
ROR	Retinol acid receptor related orphan receptor
SEM	Standard error
STAT	Signal transducer and activator of transcription
T. gondii	*Toxoplasma gondii*
Th	T-helper
TLR	Toll-like receptors
TNFα	Tumor necrosis factor α
VDR	Vitamin D receptor

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Chapter 1

General Introduction

1.1 The nuclear hormone receptor (NR) superfamily

Nuclear hormone receptors (NRs) are a group of DNA-binding transcription factors that consists of over 130 members from nematodes to humans (Flamant, Baxter et al. 2006). They regulate a broad spectrum of physiological processes from cell cycle turnover, cellular energy metabolism and architectural components to embryonic development and homeostasis of virtually all organs and tissues (Glass and Ogawa 2006). Since the glucocorticoid receptor was first cloned in 1985, other NRs have subsequently been identified and their functional and structural analysis has been emerged rapidly (Bourguet, Germain et al. 2000). So far, 48 NR members are discovered in humans and 49 in rodents, and are classified into 7 groups according to their structural similarity (NR1 to NR7, Table 1.1).

Among all NRs, 24 bind to their specific ligands, such as steroid hormones (e.g. cortisol, estrogen & progesterone) and metabolites of various lipophilic molecules (e.g. cholesterol and fatty acids) (Table 1.1). The rest of the NRs do not have known ligands, therefore they are called orphan receptors (Gronemeyer, Gustafsson et al. 2004). With the progressive improvement of biochemical engineering technology, synthetic agonists and antagonists for particular NRs have been developed, and they are now considered as essential therapies for numerous disorders in the clinic (Table 1.1 and 1.2).

Table 1.1 The mouse NR protein family.

Name	Abbreviation	Nomenclature	Ligand
Thyroid hormone receptor	TRα	NR1A1	Thyroid hormone
	TRβ	NR1A2	Thyroid hormone
Retinoic acid receptor	RARα	NR1B1	Retinoic acid
	RARβ	NR1B2	Retinoic acid
	RARγ	NR1B3	Retinoic acid
Peroxisome proliferator activated receptor	PPARα	NR1C1	Fatty acids, leukotriene B4, fibrates
	PPARβ	NR1C2	Fatty acids
	PPARγ	NR1C3	Fatty acids, prostaglandin J2
Reverse erbA	Rev-erbα	NR1D1	Orphan
	Rev-erbβ	NR1D1	Orphan
RAR-related orphan receptor	RORα	NR1F1	Cholesterol, cholesterol sulphate
	RORβ	NR1F2	Retinoic acid
	RORγ	NR1F3	Retinoic acid
Liver X receptor	LXRα	NR1H3	Oxysterols, T0901317, GW3965
	LXRβ	NR1H2	Oxysterols, T0901317, GW3965
Farnesoid X receptor	FXRα	NR1H4	Bile acids, Fexaramine
	FXRβ	NR1H5	Inactivated
Vitamin D receptor	VDR	NR1I1	1,25-dihydroxy vitamin D, lithocholic acid
Pregnane X receptor	PXR	NR1I2	Xenobiotics, PCN
Constitutive androstane receptor	CAR	NR1I3	Xenobiotics, phenobarbital
Human nuclear factor 1	HNF4α	NR2A1	Orphan
	HNF4γ	NR2A2	Orphan
Retinoid X receptor	RXRα	NR2B1	Retinoic acid
	RXRβ	NR2B2	Retinoic acid
	RXRγ	NR2B3	Retinoic acid
Testis receptor	TR2	NR2C1	Orphan
	TR4	NR2C2	Orphan
Tailless	TLL	NR2E2	Orphan
Photoreceptor specific nuclear receptor	PNR	NR2E3	Orphan
Chicken ovalbumin upstream promoter-transcription factor	COUP-TFI	NR2F1	Orphan
	COUP-TFII	NR2F2	Orphan
ErbA2-related gene-6	EAR6	NR2F6	Orphan
Oestrogen receptor	ERα	NR3A1	Oestradiol 17β, tamoxifen, raloxifene
	ERβ	NR3A2	Oestradiol 17β, various synthetic compounds
Oestrogen receptor related receptor	ERRα	NR3B1	Orphan
	ERRβ	NR3B2	DES, 4-OH tamoxifen
	ERR	NR3B3	DES, 4-OH tamoxifen
Glucocorticoid receptor	GR	NR3C1	Cortisol, dexamethasone, RU486
Mineralocorticoid receptor	MR	NR3C2	Aldosterone, spironolactone
Progesterone receptor	PR	NR3C3	Progesterone, medroxyprogesterone acetate, RU486
Androgen receptor	AR	NR3C4	Testosterone, flutamide
NGF-induced factor B	NGFIB	NR4A1	Orphan
Nur-related factor 1	NURR1	NR4A2	Orphan
Neuron-derived orphan receptor 1	NOR1	NR4A3	Orphan
Steroidogenic factor 1	SF1	NR5A1	Orphan
Liver receptor homologous protein 1	LRH1	NR5A2	Orphan
Germ cell nuclear factor	GCNF	NR6A1	Orphan
DSS-AHC critical region on the chromosome, gene 1	DAX1	NR0B1	Orphan
Short heterodimeric partner	SHP	NR0B2	Orphan

*The human *FXRβ* is the pseudogene, thus it does not express a functional receptor. DES, diethylstilbestrol; DSS-AHC, dosage-sensitive sex reversal-adrenal hypoplasia congenital; NGF, nerve growth factor; PCN, pregnenolone 16α-carbonitrile. The table is adapted from (Gronemeyer, Gustafsson et al. 2004).

Table 1.2 Clinical use of agonists or antagonists for NRs and their coregulators.

NRs	Disease	Disease Pathogenesis	Agonists	Antagonists/Inhibitors	Mechanism	Reference
RARα	Acute promeylocytic leukemia	Translocation of RARα to the promyelocytic leukemia (PML) gene leading to production of the PML-RXRα fusion protein	All trans retinoic acid		Induces differentiation of the transformed promyelocytes into functional leukocytes	(Degos and Wang 2001)
ERα	Breast cancer	Estrogen-dependent cancer cells		Tamoxifen and other mixed ligands	Inhibition of tumor growth by suppressing ERα activity	(Sommer and Fuqua 2001)
GR	Allergic, autoimmune and inflammatory diseases, leukemia and lymphoma	Production of inflammatory cytokines and bioactive compounds, carcinogenic transformation of myeloid and lymphoid lineages	Predonisolone, methypredonisolone, dexamethasone and other synthetic agonists		Transrepression of inflammatory genes by ligand-activated GR, induction of apoptosis	(Barnes 2006)
PPARγ	Type II diabetes mellitus	High blood glucose level due to resistance to insulin in skeletal muscles and adipose tissues	Thiazolidinediones		Increase tissue insulin sensitivity by stimulating PPARγ activity	(Khanderia, Pop-Busui et al. 2008)
AR	Prostate cancer	Androgen stimulates the growth of AR-expressing cancer cells		Cyproterone	Inhibits binding of dihydrotestosterone or testosterone to the androgen receptor	(Denmeade and Isaacs 2002)
PR	Abortion, emergency contraceptive			RU486 (mifepristone)	Antiprogesterone in the luteal phase of the cycle and for early pregnancy interruption	(Cadepond, Ulmann et al. 1997)

VDR, ER	Osteoporosis	Loss of calcium in bones due to menopause or cortisol therapy	1, 25 dihydroxy-vitamin D and Raloxifene		Increase calcium deposition in bones	(Rosen 2005; Jackson, LaCroix et al. 2006)
Class I HDAC	Lymphoma, leukemia and sarcoma	Multiple mechanisms, such as induction of cancer-related genes or suppression/inactivation of tumor suppressor genes		Suberoylanilide hydroxamic acid, FK228, LBH-589, MGCD0103, PXD101, Valproic acid	Induce activity of tumor suppressor genes (e.g., p21) , promote ER silencing effect	(Vigushin and Coombes 2002)
HDAC1	Psychiatric and neurological disease (e.g. epileptics, Alzehmier and Huntington's disease)	Unknown		Valproic acid	Inhibit HDAC1 activity	(McElroy, Keck et al. 1989)

1.2 Molecular mechanisms for NR transcriptional activity

NRs modulate expression of their target genes through both stimulation and suppression of transcription. Once ligands bind NRs at their ligand-binding domain (LBD), the activated NRs bind to their specific DNA sequences called "response elements" located in the promoter or enhancer region of their target genes either as a homodimer (e.g. glucocorticoid receptor) or as a heterodimer (e.g. liver X receptors (LXR) and retinoid X receptors (RXRs)) (Figure 1.1a & b). Some orphan receptors also bind to their response elements as a monomer or as a heterodimer; in the latter case, they employ RXRs as partner molecules (e.g. NURR1).

Coregulators are essential accessory molecules to facilitate or inhibit the transcriptional activity induced by NRs (Li, Carey et al. 2007; Thakur and Paramanik 2009). They are attracted to the promoter region through DNA-bound NRs, and participate both in the initiation of transcription and in the elongation of transcripts (Li, Carey et al. 2007; Thakur and Paramanik 2009). Coregulators consist of two groups of molecules with opposite functions; coactivators and corepressors. Coactivators are required for NRs to stimulate the transcription by facilitating the accessibility of chromatins to other transcription factors and components of general transcription factors through chemical modification of chromatin-associated histones and other molecules (Lonard and O'Malley 2005). Corepressors, on the other hand, repress the transcription of NR-responsive genes by attracting numerous inhibitory molecules and protein complexes to DNA-bound NRs (Glass and Rosenfeld 2000). At present, approximately 285 coregulators are reported, in connection with numerous physiological functions and pathological roles (Lonard, Lanz et al. 2007).

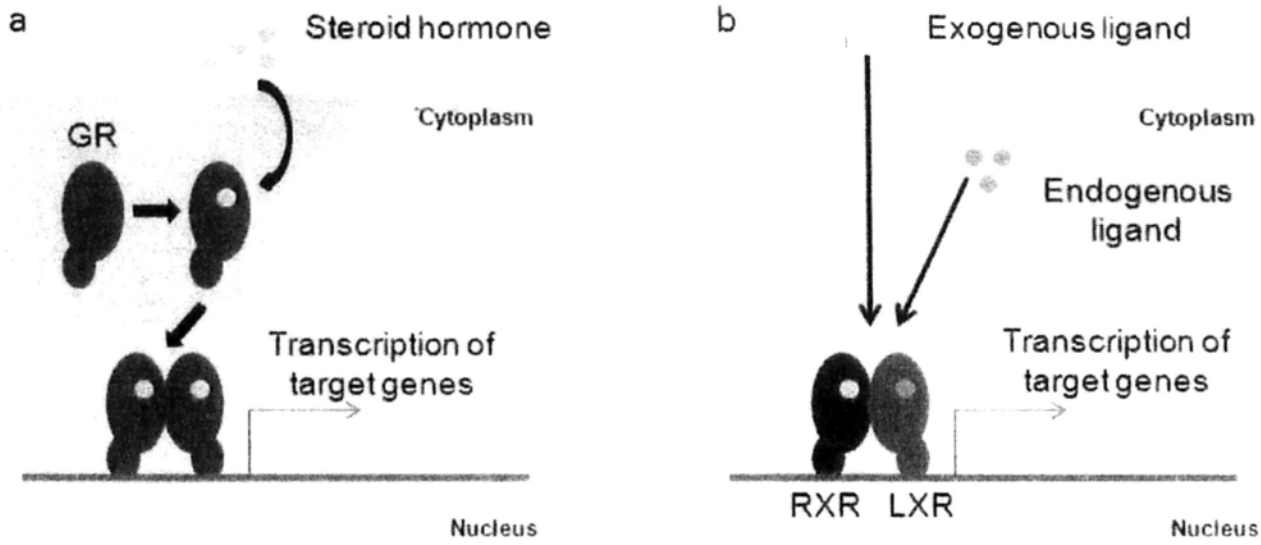


Figure 1.1 Mechanisms of transcriptional regulation by NRs.

(a) Steroid hormone receptors are located in cytoplasm in their inactive forms. Once these receptors bind to ligand, they stimulate the transcriptional activity of their responsive genes by binding to their responsive elements as homodimers. (b) Heterodimeric NRs bind constitutively to DNA with RXRs as obligate partners. In the absence of liagnd, they attract corepressors to suppress basal transcriptional activity of target genes. Upon ligand-binding, NRs dissociate corepressors, while recruit coactivators to stimulate the transcription. Figure is modified from (Glass and Ogawa 2006).

1.3 Action of NRs on immunity

1.3.1 Direct transcriptional regulation on the immune-related genes by NRs

The importance of NRs in the action of the immune system has recently been recognized in the scientific community, as several NRs emerge to possess important regulatory functions in the immune system and inflammation through various mechanisms. For instance, ligand-activated glucocorticoid receptor (GR) and peroxisome proliferator-activated receptor (PPARs) α inhibit expression of the inflammatory genes by inducing proteins with inhibitory activity (e.g. GR stimulates expression of inhibitor of nuclear factor of κB (IκB), which suppresses the transcriptional activity of the nuclear factor of κB (NF-κB) by segregating NF-κB in the cytoplasm (Auphan, DiDonato et al. 1995; Delerive, Martin-Nizard et al. 1999)) (Figure 1.2A).

Other mechanisms include the recruitment of corepressors to the promoter region of the inflammatory genes in a ligand-dependent fashion to suppress the transcriptional activity, as observed in the PPARγ-mediated inhibition of the expression of the inducible nitric-oxide synthase and the prostaglandin synthase (Mendez and LaPointe 2003). However, the transrepression action on other transcription factors that important for the expression of inflammatory genes (e.g. NF-κB, interferon regulatory factors (IRFs), activator protein 1 (AP-1), nuclear factor of activated T-cells (NFAT) and Signal Transducer and Activator of Transcription (STATs)), appears to be the major mechanism for NRs to suppress the inflammatory reaction (Pascual and Glass 2006) (Figure 1.2B). In addition, NRs also recruit molecules with various biological activities (e.g. histone deacetylase (HDAC) or SUMO transferase) to alter the formation and activity of the transcriptional initiation complex on the promoter region of the inflammatory genes, and suppress their transcriptional activity (Figure 1.2C).

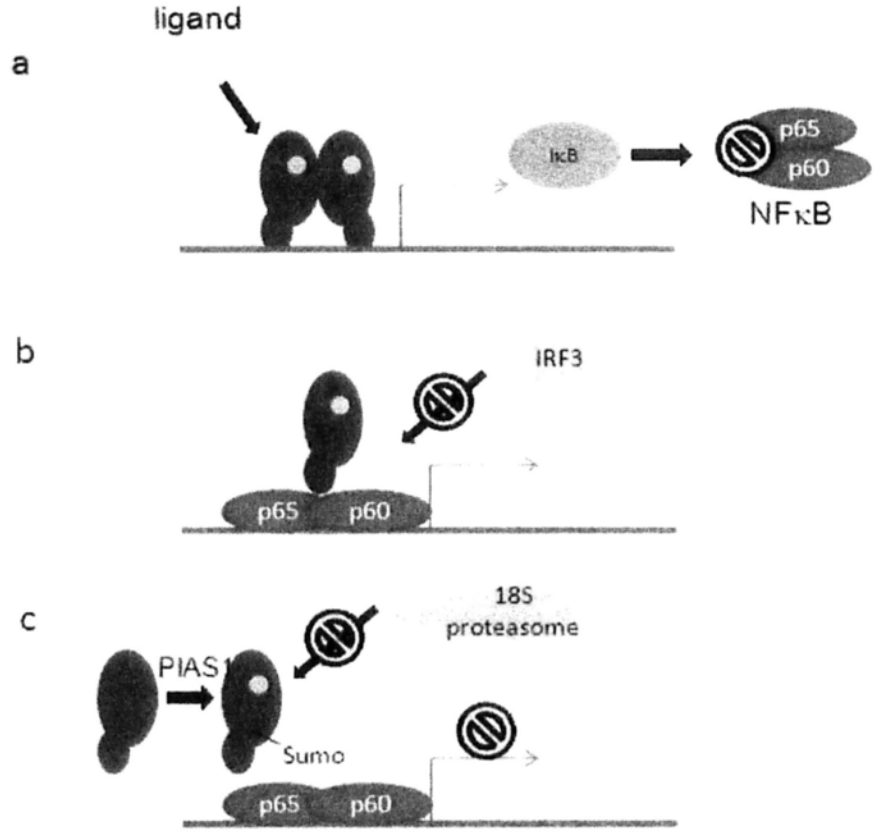


Figure 1.2. Mechanisms of NR-mediated anti-inflammatory response.

(a) Induction of IκBα expression. **(b)** GR-p65 interaction prevents p65 from being tethered to the IRF3, which functions as a coactivator required for the transcriptional activation; **(c)** inhibition of signal-dependent clearance of the NCoR corepressor complexes containing SUMO ligases that blocks the recruitment of the Ubc5/19S proteosome machinery that is necessary for the clearance of the corepressor machinery, a prerequisite for gene activation (e.g. PPARγ). Abbreviations: PIAS1, protein inhibitor of activated signal transducer and activation of transcription 1; Su, SUMO. Figure is modified from (Pascual and Glass 2006).

1.3.2 Lipid- and cholesterol metabolite-responsive NRs and their actions on inflammation

Metabolic disorders, such as atherosclerosis and type II diabetes mellitus, are associated with chronic low-level inflammation, while such smoldering inflammation acts as a stimulator of their disease progression (Ross 1999; Wellen and Hotamisligil 2005). Increasing evidence suggests that PPARs and LXRs, which employ lipid and cholesterol metabolites as their ligands, play regulatory roles in the pathogenesis and pathophysiology of these metabolic diseases (Zelcer and Tontonoz 2006; Varga and Nagy 2008). For example, PPARγ is an important regulator in the development and differentiation of macrophages: macrophage-specific deletion of PPARγ results in increased insulin resistance in the liver and skeletal muscles, a condition frequently observed in patients with type II diabetes mellitus (Odegaard, Ricardo-Gonzalez et al. 2007). Besides, whole body knockout of PPARα increases susceptibility to atherosclerosis by inhibiting cholesterol efflux from macrophages and subsequent induction of their transformation into foam cells in vascular walls: the initial step of atherosclerosis (Tordjman, Bernal-Mizrachi et al. 2001; Babaev, Ishiguro et al. 2007).

On the other hand, LXRs control inflammation and demyelination in the central nervous system, which plays important pathological roles in the development of Alzheimer disease and experimental allergic encephalitis (mouse model of brain inflammation) (Patel and Forman 2004; Xu, Wagoner et al. 2009). For example, ligand-activated LXRs upregulate the cholesterol-efflux transporter ATP-binding cassette transporter 1, resulting in reduction of cellular cholesterol content, which is important for formation of myelin sheath and cell membrane in neuronal cells (Bensinger and Tontonoz 2008). LXRs also decrease amyloid-β secretion in a mouse model of Alzheimer's disease, suggesting the therapeutic application of LXR ligands to this degenerative disease (Koldamova, Lefterov et al. 2005; Bensinger and Tontonoz 2008).

1.3.3 NRs control embryonic development, cellular differentiation, apoptosis and immunity

Orphan receptors are essential players in early embryonic development and adult cell growth and differentiation (Benoit, Cooney et al. 2006). They have also gained attention in immunity recently, especially on the regulatory effects on T cells. Recent discovery has revealed that two orphan receptors RORγ and RORα are indispensible for the development of T-helper (Th) 17 lineage, thus controlling the balance of Th subtypes in T cell population (Yang, Pappu et al. 2008). Besides, NR4A family members NUR77 and NOR1 control T cell apoptosis and mediate negative clonal selection in the thymus (Cheng, Chan et al. 1997; He 2002).

1.4 Dendritic cells (DCs): the versatile regulator for the immune system

1.4.1 Functional components of the immune system

The two arms of immune systems: innate and adaptive immunity, operate through more than a dozen of cell types and protein components to orchestrate a complex but well-organized immune response against invading pathogens. The innate immunity includes anti-microbial peptides, macrophages and granulocytes for phagocytosis, the complement system, chemokines, cytokines and natural killer lymphocytes. Its activation often occurs rapidly (in minutes or hours) by recognizing common characteristics of pathogens, such as lipopolysaccharides (LPS) of the bacterial cell wall and genomic materials (DNA and RNA) of viruses (Janeway and Medzhitov 2002; Takeda and Akira 2004). The adaptive immunity includes antibody-producing B cells, various subtypes of helper T cells, killer T cells and regulatory T cells (Banchereau and Steinman 1998; Janeway and Medzhitov 2002). Activation of the adaptive immunity is slower than that of innate immunity, but is more long lasting, with high specificity towards antigens and is associated with memory of antigens. Importantly, dendritic cells (DCs) play a central and indispensible role in both innate and acquired immunity, and is able to bridge these two components of the immune system (Banchereau and Steinman 1998) (Figure 1.3).

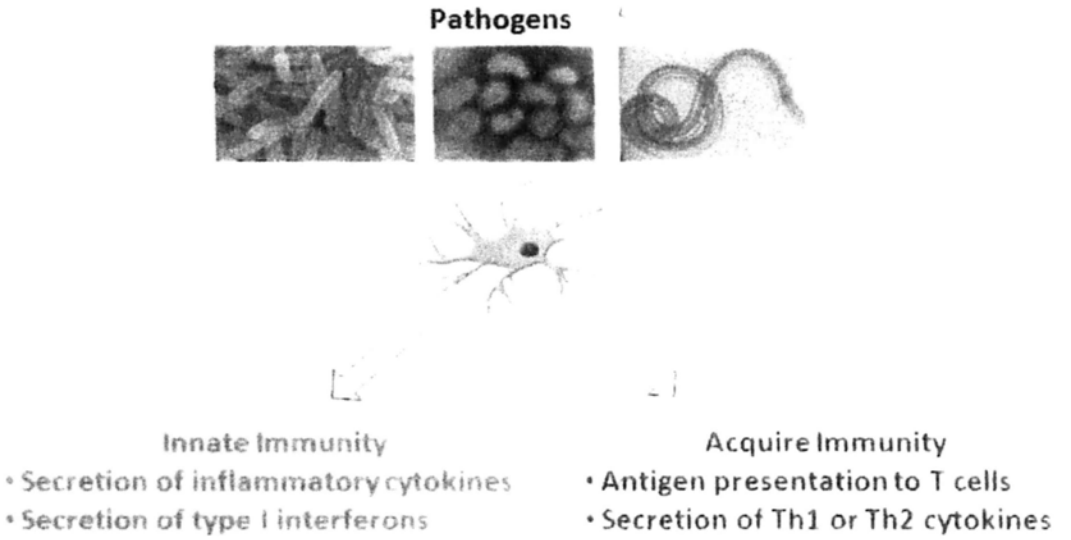


Figure 1.3 DCs in innate and adaptive immunity.

Upon infection, DCs sense pathogen-derived antigens and act as effector cells to initiate and sustain immune response by secreting interferons and various cytokines to stimulate T cells.

1.4.2 DCs and their actions in the human body

The name of "DCs" came from its cellular shape of dendrite-like protrusions (Steinman 2007). They are abundant in "surfaces" of the human body, such as the skin, pharynx, upper oesophagus and vagina, and mucosa of ectodermal organs, such as respiratory and gastrointestinal systems, as this characteristic distribution helps DCs to detect invading pathogens and exogenous antigens (Niess 2005) (Figure 1.4). Once DCs are activated by such antigens, they leave residential tissues and migrate into draining lymph nodes under the guidance of chemokines (Cyster 1999; Randolph, Angeli et al. 2005). The most notable property of DCs is their extremely high antigen presentation ability to T cells (Banchereau and Steinman 1998). Although many immune cells, such as macrophages and B cells, can also present antigens and activate T cells, DCs are at least 300 times more potent in stimulating T cells and in initiating their clonal expansion than other antigen-presenting cells (Steinman and Hemmi 2006).

DCs are also densely localized in intact lymphoid tissues, such as the spleen and lymph nodes (Steinman and Hemmi 2006). Isolation of DCs from these mouse organs are frequently used in *ex vivo* experiments or for assessing their response to treatment with antigens or pathogens *in vivo* (Inaba, Swiggard et al. 2001). Because of the extremely low DC cell number in humans and animals (only ~1% of cellular components in blood and spleen), DCs derived from bone marrow hematopoietic stem cells under the presence of soluble differentiation and clonal expansion factors are widely used in *in vitro* experiments (Inaba, Swiggard et al. 2001): The Fms-related tyrosine kinase 3 ligand (FLT3L) or combination of the granulocyte-macrophage colony-stimulating factor and interleukin (IL)-4 are commonly used to generate bone marrow-derived DCs (Inaba, Swiggard et al. 2001).

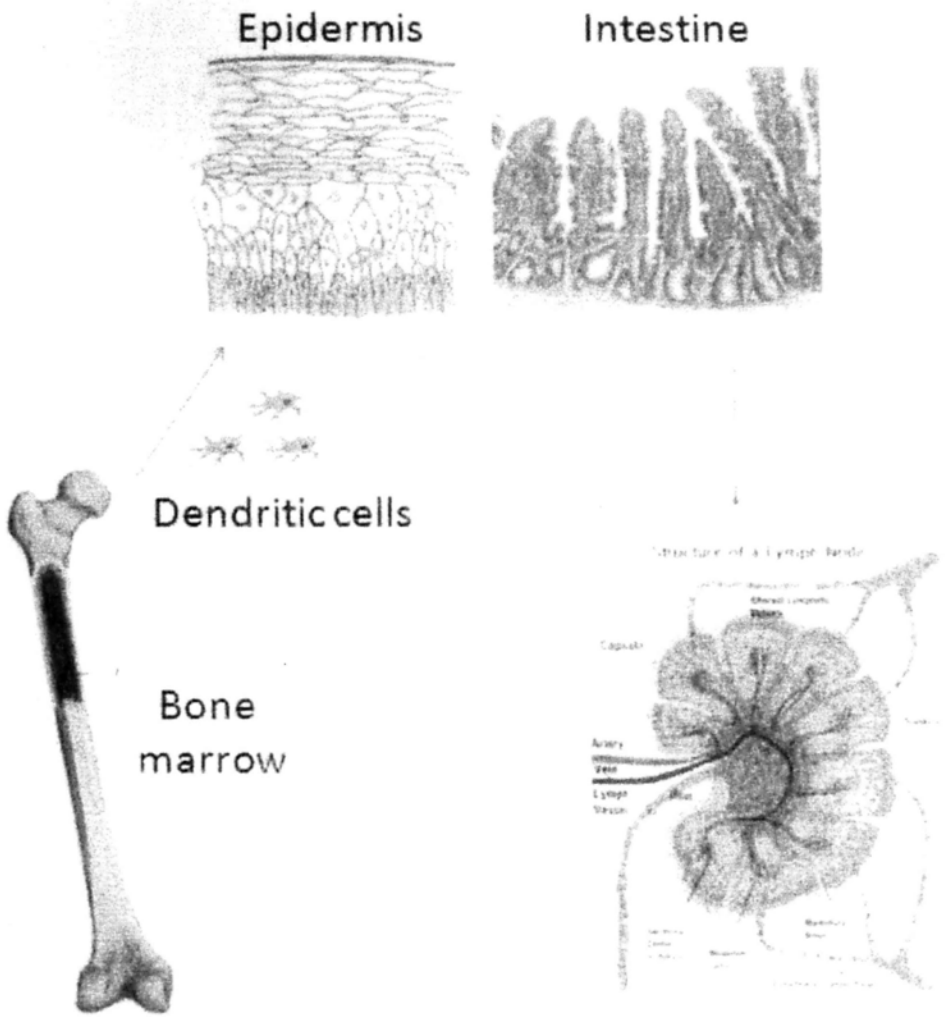


Figure 1.4 Generation, migration and maturation of DCs.

DC precursors (bone marrow-derived cells) leave the bone marrow and migrate into various peripheral tissues (e.g. epidermis of the skin, intestinal mucosa or vascular walls) as immature DCs. These cells sense various signals from surrounding tissues. Antigen-uptake associated with 'danger signals' leads to maturation of DCs and their migration into draining lymph nodes. In lymph nodes, matured DCs present captured antigens to naive T cells and activate them.

1.4.3 Actions of DCs and their subtypes

DCs play essential roles in immune response against viral infection: They produce type I interferons (IFNα and β), and subsequently stimulate the expression of hundreds of interferon-stimulated genes (ISGs) at various local effector cells to orchestrate anti-viral immune response (Steinman and Banchereau 2007; Sadler and Williams 2008). DCs contain a specialized endocytotic system to deliver captured antigens to intra-cellular compartments for digestion, then the processed peptides are presented with the major histocompatibility complex (MHC) class I and class II to downstream effector cells, such as CD4+ and CD8+ T cells, NKT cells and B cells, for activation (Trombetta and Mellman 2005; Dudziak 2007). With their high expression of costimulatory molecules (CD80, CD86 and CD40), DCs stimulate naive T-cells in lymphoid organs very efficiently (Honda, Sakaguchi et al. 2003; Steinman and Hemmi 2006).

Among the DC subtypes, two major components, the conventional (cDCs) and the plasmacytoid (pDCs) DCs have been identified (Shortman and Liu 2002). In the mouse model, cDCs express surface molecules CD11c and CD4 and/or CD8, and are more potent than pDCs in antigen presentation and secretion of the Th1 cytokine IL-12 (Shortman and Liu 2002). The pDCs, on the other hand, express B220 on their cell surface, and are specialized for producing tremendous amounts of type I IFNs upon pathogen infection (Shortman and Liu 2002; Liu 2005).

1.5 Toll-like receptors (TLRs) and anti-viral response

In 2002, Charles Janeway proposed a hypothesis on innate immunity, that sentinel cells such as DCs, express pattern recognition receptors (PRRs) for sensing microbial infection (Janeway and Medzhitov 2002). PRRs recognize the molecular signatures expressed on pathogens but not on host cells to distinguish self and non-self (Janeway and Medzhitov 2002). Among PPR family, the Toll-like receptor (TLR) family proteins are the most studied. While mice have 11 TLRs, humans have only 10 functional TLRs (Gabriele and Ozato 2007). Among the TLR family members, TLR3, TLR7, TLR8 and TLR9 specifically detect genetic materials of infecting agents (Bowie and Unterholzner 2008) (Figure 1.5).

Binding of antigens (ligands) to their respective TLRs activates intracellular components of these receptors and downstream intracellular signaling pathways to finally stimulate the transcription factors, such as the IRF3, IRF7 and NF-κB, which in turn initiate expression and secretion of type I IFNs (IFNα and IFNβ) and numerous inflammatory cytokines by binding to specific DNA sequences located in the promoter region of these genes (Gabriele and Ozato 2007). The secreted type I IFNs then bind to the type I IFN receptor (IFNAR) expressed on the surface of their target cells, and induce the expression of more than 300 Interferon stimulated genes in these cells through activation of signaling cascades that include STAT1 and IRF9 (Bowie and Unterholzner 2008) (Figure 1.5). Particularly in DCs, expression and production of type I IFN is further amplified through activation of IRF8 (in positive feedback mechanism), resulting in the formation of the second phase of IFN secretion upsurge (Gabriele and Ozato 2007).

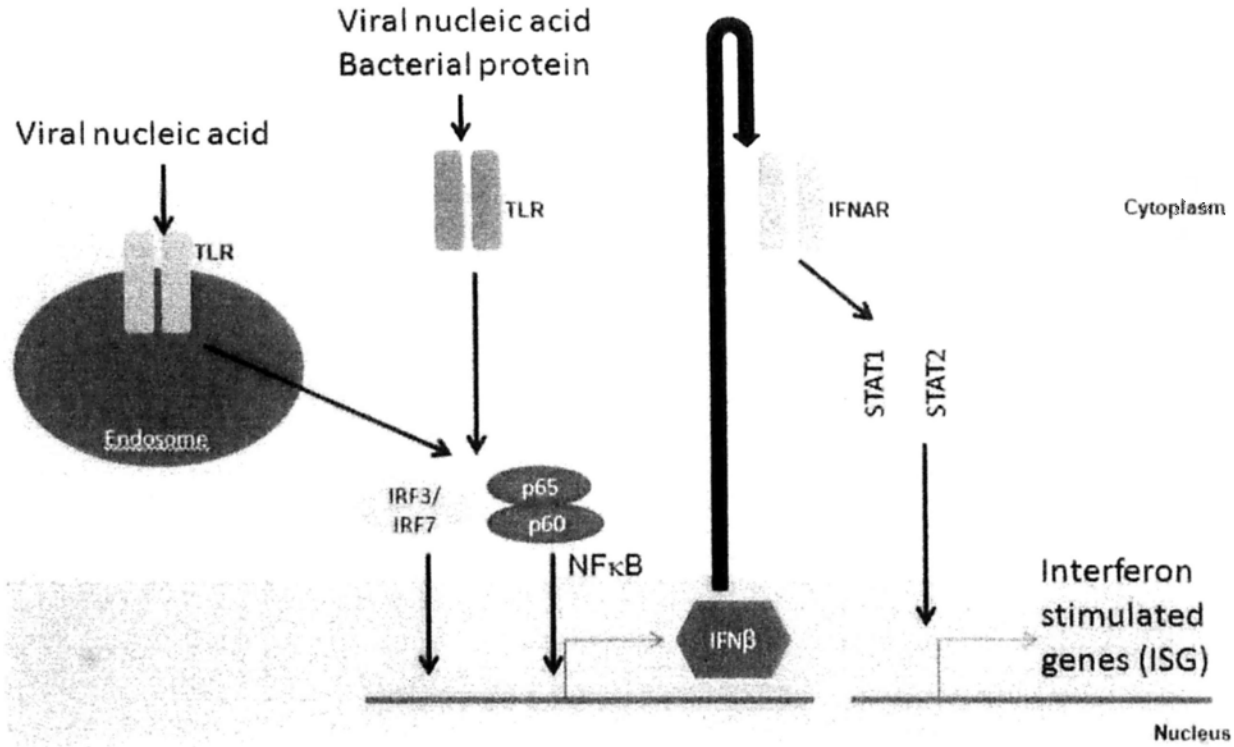


Figure 1.5 Pattern-recognition receptors (PRRs) and their signaling pathways.

The activation of PRRs initiates the signaling pathways and converges to the activation of common transcription factors, such as the interferon-regulatory factor 3 (IRF3), IRF7 and/or nuclear factor-κB (NF-κB); Activation of these transcription factors then leads to the expression of type I IFNs including IFNβ. Secreted IFNβ in turn initiates the antiviral program in the infected cells and neighboring cells by inducing expression of numerous IFN-stimulated genes (ISGs). Figure is modified from (Bowie and Unterholzner 2008).

1.6 NRs in the regulation of DC biology and their immune activities

As discussed in 1.3, NR regulates the function of various immune cells, including DCs. The versatile roles of NRs in the regulation of immune activities are mediated through modulation of the development, differentiation and functions of DCs. Steroid hormone-binding NRs show potent immunosuppressive effects on DCs (Szatmari and Nagy 2008): One of such NRs, GR, inhibits differentiation of immature DCs and their ability to stimulate T cells *in vitro* and *in vivo* (Rozkova, Horvath et al. 2006; Szatmari and Nagy 2008). GR does this in part by up-regulating the expression of the glucocorticoid-induced TNFα-related protein in pDCs that results in the induction of the indolamine 2,3-dioxygenase, a key enzyme to suppress lymphocyte proliferation (Grohmann, Volpi et al. 2007). The estrogen receptor (ER) and the progesterone receptor (PR) inhibit maturation and differentiation of some DC subtypes (Paharkova-Vatchkova, Maldonado et al. 2004; Liang, Sun et al. 2006; Escribese, Kraus et al. 2008), which may explain gender-specific as well as pregnancy-associated alteration of immune activity.

Besides, some lipid metabolite-binding NRs, such as LXRα and the vitamin D receptor (VDR), reduce the ability of DCs to present antigens to T cells (Penna and Adorini 2000; Geyeregger, Zeyda et al. 2007) (Figure 1.5). VDR regulates development and expansion of some DC populations, as VDR deficient mice develop hypertrophy of the lymph nodes and show elevated numbers of mature DCs in these organs (Griffin, Lutz et al. 2001). PPARγ inhibits maturation of DCs, and suppresses their cytokine expression and potency to activate T-cells (Straus and Glass 2007). Roles of retinoid (vitamin A and its derivatives)-binding RXRs and RARs in the differentiation of DCs are still under debate, as both the repressive and the enhancing effect are reported (Szatmari and Nagy 2008). Interestingly, DCs can produce some

NR ligands themselves, such as vitamin A and vitamin D, which may regulate the homing of lymphocytes and antibody production in B cells (Szatmari and Nagy 2008).

In contrast, very limited information is available on the regulation and expression of orphan receptors in DCs. The NR4A family member NOR1 is the only member that was recently found to control apoptosis in activated DCs and to regulate the anti-tumor effect of these cells (Wang, Jiang et al. 2009).

1.7 Experimental design and aim of study

Growing importance of NRs in the field of immunity and inflammation has attracted my interest in studying the relationship between the two systems. Therefore, this study has focused on how DCs regulate their NR expression and activity in response to viral infection. First, the expression profile of all NRs and selected coregulators was examined at a basal (un-stimulated) condition and under viral infection in DCs. As some TLRs play primary roles in the detection of infecting viruses, the effects of specific TLR ligands on the NR expression were compared to the viral-induced alteration in NR expression. Potential biological importance of the viral-mediated modification of some NR expression was further investigated in DCs, particularly focusing on their regulation on type I IFN expression.

Second, the significance of orphan receptor NOR1, which I identified to be highly expressed in DCs upon viral infection, in the regulation on immunity was addressed by employing an animal model. $NOR1^{+/+}$ and $NOR1^{-/-}$ mice were challenged with a TLR3 ligand or were infected with the intracellular protozoa, *Toxoplasma gondii*. Mice survival and serum cytokine levels were monitored to address the importance of NOR1 in DC-organizing immune response against the TLR ligand and the pathogen *in vivo*.

Third, the effects of dexamethasone (DEX), a synthetic ligand for the steroid hormone receptor GR, on the cytokine response and regulation of the TLR-related gene expression in DCs upon viral infection were examined. Among the significantly regulated genes, the synergistic effect between DEX and viral infection on IL-10 expression was studied in detail. IL-10 is an anti-inflammatory cytokine secreted from DCs and other immune cells. Detailed time points, dose dependent effects and the converging signaling molecule for the observed synergism were examined. Stress, which strongly induces glucocorticoid secretion from the adrenal glands,

increase host's susceptibility to viral infection. Thus, the synergistic induction of IL-10 by viral infection and glucocorticoids could partly explain the increased susceptibility to viral infection in stressed people.

In summary, this study aims to investigate the biological significance and molecular mechanisms of NRs on the immune function, specifically focusing on the key cellular components DCs. I believe that this study helps understanding the inter-relationship between the endocrine system and the immune system and further provides important information on the development of anti-viral therapy that targets DCs and NRs.

Chapter 2

Regulation of NRs and Coregulators in DCs upon Viral Infection

2.1 Introduction

Viruses are the most abundant, diverse, ancient but rapidly evolving pathogens, which challenge the host immune system, and thus, are serious threat to human health. Infection of viruses to host cells initiates anti-viral innate immune response organized by DCs, which is characterized by massive induction of the type I IFNs and proinflammatory cytokines in these cells (Steinman and Banchereau 2007; Sadler and Williams 2008). Besides, virus-infected host cells adjust or reset their cellular activities, ranging from induction of apoptosis to regulate glucose, lipid and energy metabolism (Bukrinsky and Sviridov 2006; Galluzzi, Brenner et al. 2008; Negro and Sanyal 2009). Since NR family members play potent and diverse roles in every aspect of cellular functions, their expression may be modulated as a result of infection and participate in regulating anti-viral immune response throughout the infection.

Modulation of gene expression is the fundamental mechanism to regulate stimulus-induced cellular functions. Since NRs are ligand-dependent transcription factors that exhibit diverse activities to modulate transcription of important target genes, expression profiles of all members of NRs (49 in mice) have been performed in several important immune cells, including B cells and macrophages stimulated by virus infection or inflammatory cytokines. Epstein Barr virus-immortalized B cells demonstrated elevation or repression of more than 20 of NRs at their expression levels, which may be responsible for their transformation into immortalized cells, instead of fighting against the infecting viruses (Yenamandra, Lundin et al. 2009). In

macrophages, classic stimulation with LPS or IFNγ altered mRNA expression of half of the NR family members whose expression profiles correlated with the induction of inflammatory cytokines produced from macrophages, suggesting a possible role of NRs in the regulation of cytokine production (Barish, Downes et al. 2005).

Although previous studies showed the expression of lipid metabolite-binding NRs in DCs and their effects on DC-mediated-immunity, the profound expression profiling of all NR family members in DCs infected with live viruses has not been reported as yet (Szatmari and Nagy 2008). In this chapter, I sought to identify thorough and comprehensive expression profiles of all NR family members and some of their coregulators in mouse bone marrow-derived DCs. Protein expression of selected NRs and their coregulators were confirmed with their consistency to the patterns observed at the transcriptional level. Since TLRs are the most important PRRs for DCs to detect invading pathogens, the transcriptional regulation of NRs in DCs after stimulation with TLR ligands was also studied. Finally, biological significance of the expression changes observed in some NRs was addressed by examining their effects on the IRF-mediated induction of type I IFNs.

2.2 Material and methods

2.2.1 Mice

Male and female C57BL/6 mice at 6–8 week old were used as sources of hematopoietic cells to generate bone marrow-derived DCs in culture. Animal studies were conducted in accordance with guidelines of the NIH animal care and use committee under the approved animal study proposal 09-008.

2.2.2 DC culture and stimulation

Bone marrow-derived DCs were generated by culturing bone marrow mononuclear cells in the presence of the FLT3L for 8 days, as described previously (Tailor, Tamura et al. 2007). Briefly, bone marrow cells were collected by flushing femurs and tibiae with RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (FCS). They were then layered on Histoplaque 1083 (Sigma-Aldrich, St. Louis, MO), and were centrifuged at 400 Xg for 25 minutes (min) to remove red blood cells. Isolated mononuclear cells were cultured in RPMI 1640 medium supplemented with 10 % FCS, 1 mmol/l sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin and 55 μM 2-mercaptoethanol (Invitrogen, Carlsbad, CA), supplemented with 100 ng/ml FLT3L (Peprotech, Rocky Hill, NJ). After 4 days of culture, half of the medium was replaced with fresh medium supplemented with FLT3L (100 ng/ml), and the cells were incubated for an additional 4 days to allow differentiation into bone marrow-derived DCs. For purification of pDCs and cDCs, total DCs were incubated with the B220 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) at 4°C for 20 min to label pDCs. The pDCs bound to B220 microbeads were retained in LS columns (Miltenyi Biotech) under strong magnetic field. Unlabeled cDCs ran through the columns and were harvested immediately. After removal of the

columns from the magnetic field, retained pDCs were eluted by washing the columns with PBS supplemented with 2% FCS. Purity of pDCs was assessed by the flow cytometry by using B220-APC antibody and was > 95%. Total DCs, pDCs or cDCs (1 x 10^6 cells) were infected with the Newcastle disease virus (NDV) (Hertz strain, at multiplicity of infection (MOI)=10) or the murine cytomegalovirus (MCMV) (Smith strain, at MOI=10) at 37°C for indicated time periods. TLR ligands, including the polyinosine-polycytidylic acid (TLR 3 ligand: Poly IC, 20 μg/ml; Invitrogen, San Diego, CA), the resiquimod (TLR7 ligand: R848, 100 nM; Alexis, San Diego, CA) and the unmethylated CpG oligodeoxynucleotides (TLR9 ligand: CpG, 1 μg/ml; Operon, Huntsville, AL) were used to stimulate DCs.

2.2.3 Real-time quantitative PCR (qPCR) and PCR array

Total RNA was extracted and treated with DNase I by using the RNeasy mini kit (Qiagen, Valencia, CA). Total RNA (0.5 μg) was converted to cDNA using the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). qPCR was performed using 20 ng of cDNA, 5 μM of primers and the SYBR green PCR Master Mix (Applied Biosystems) in the ABI Prism 7500 Real-time PCR System (Applied Biosystems). Sequences of the primers used in qPCR were listed in Supplementary Table 1. RT^2 Profile Custom PCR Arrays (SA Bioscience, Frederick, MD) were used to examine simultaneously the mRNA levels of 89 genes and five housekeeping genes (β-glucuronidase (*Gusb*), hypoxanthine guanine phosphoribosyl transferase 1 (*Hprt1*), heat shock protein 1 (*Hspcb*), glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) and β-actin (*Actb*) in 96-well plates. Each reaction included 380 ng of cDNA. Obtained Ct (threshold cycle) values were normalized by those of five housekeeping genes (for PCR array) or *Gapdh* (for qPCR), and fold changes were calculated by using the comparative threshold cycle

(C_t) method ($2^{-\Delta\Delta Ct}$ where $\Delta\Delta C_t = \Delta C_{t,sample} - \Delta C_{t,reference}$). The data obtained in the absence of infection were employed as baseline. All experiments were conducted in triplicate.

2.2.4 Preparation of cell extracts and immunoblot

DCs (1×10^7 cells) were infected/stimulated with NDV (MOI=10) or CpG (1 μg/ml) for indicated time periods. Whole cell extracts or nuclear extracts were then prepared by using the nuclear protein extraction kit (Active Motif, Carlsbad, CA), according to manufacturer's instruction. Whole cell extracts (5 μg) were run on 8-12% NuPAGE Bis-Tris gels (Invitrogen), transferred to nitrocellulose membranes and immunoblotted with the rabbit anti-NOR1, anti-RXRα, anti-LXRα or anti-β-actin antibody (Santa Cruz Biotechnologies Inc., Santa Cruz, CA), followed by the anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnologies Inc.).

2.2.5 Evaluation of the HDAC1 activity

The HDAC1 activities of DCs infected/stimulated with NDV or CpG were examined using the HDAC activity kit (Active Motif). Briefly, 1 μg of the nuclear extract was incubated with the short peptide substrate containing an acetylated lysine residue at 37°C for 10 min. Deacetylation was monitored by measuring the emitted fluorescence ($\lambda_{excitation}$ = 340-360 nm; $\lambda_{emission}$ = 440-465 nm), which was produced through reaction of the deacetylated lysine on the substrate with the supplied developing solution using the spectrophotometer Victor 3 (Perkin Elmer, Waltham, MA). The HDAC1 enzymatic activities were then calculated by comparing their fluorescence intensities to standards. The value obtained in the absence of infection/stimulation at each time point was defined as 100 % of the HDAC1 activity.

2.2.6 Luciferase reporter assay

Human colon cancer HCT116 cells were maintained and transfected as previously described (Nader, Ng et al. 2010). Specifically, the cells were transfected with 0.2 μg/ml of the NOR1-expressing plasmid (gift from Dr. Naganari Ohkura, Osaka University, Osaka) or the LXRα- and RXRα-expressing plasmids (gifts from Dr. David Mangelsdorf, Southwestern Medical Center, Dallus), 0.2 μg/ml of the IRF-expressing plasmids, 0.5 μg/ml of the IFNα4 promoter-driven firefly luciferase reporter plasmid and 0.5 μg/ml of the pGL4.73[*hRluc*/SV40] renilla control plasmid (Promega, Madison, MI) for 24 h. The cells transfected with LXRα/RXRα were treated with 10^{-6} M of the LXR synthetic agonist GW3965 (Sigma Aldrich) for an additional 24 h. The firefly and renilla luciferase activities were measured using the dual-luciferase assay kit (Promega).

2.2.7 Statistical analysis

GraphPad Prism 5 was used for unpaired Student's t test with a two-tailed p value (GraphPad Software, La Jolla, CA). The calculated p values were regarded as statistically significant if $p < 0.05$.

2.3 Results

2.3.1 Basal expression of NRs and coregulators in DCs

The mRNA expression of NRs and coregulators in DCs was surveyed by using custom PCR arrays, which enabled measurement of mRNA levels of 49 NRs and 35 selected coregulators *en masse*. In the absence of infection or stimulation, DCs expressed most of the steroid and metabolic ligand receptors, which respectively bind to steroid hormones and metabolites of cholesterol, fatty acids and other bioactive lipids (Table 2.1A & B). However, DCs expressed only half of the orphan receptors. Upon the 35 coregulators examined, DCs expressed 34 of them except PPARγ coactivator 1α (Table 2.1C & D).

Table 2.1A NRs expressed in DCs.

Systemic Nomenclature	Name	Abbreviation	Ligand	GenBank Accession Number
Nr1a1	Thyroid hormone receptor α	TRα	Thyroid hormones	NM_178060
Nr1a2	Thyroid hormone receptor β	TRβ	Thyroid hormones	NM_009380
Nr1b3	Retinoic acid receptor γ	RARγ	Retinoic acids (Vitamin A)	NM_011244
Nr1c2	Peroxisome proliferator activator receptor δ	PPARδ	Fatty acids	NM_011145
Nr1c3	Peroxisome proliferator activated receptor γ	PPARγ	Fatty acids, prostaglandin J2, thiazolidinediones	NM_011146
Nr1f1	RAR-related orphan receptor α	RORα	Cholesterol, cholesteryl sulfate	NM_013646
Nr1h2	Liver X receptor β	LXRβ	Oxysterols, T0901317, GW3965	NM_009473
Nr1h3	Liver X receptor α	LXRα	Oxysterols, T0901317, GW3965	NM_013839
Nr1i1	Vitamin D receptor	VDR	Vitamin D, 1,25-dihydroxyvitamin D3	NM_009504
Nr1i3	Constitutive androstane receptor	CAR	Xenobiotics, phenobarbital	NM_009803
Nr2b1	Retinoid X receptor α	RXRα	Retinoic acids (Vitamin A)	NM_011305
Nr2b2	Retinoid X receptor β	RXRβ	Retinoic acids (Vitamin A)	NM_011306
Nr2c1	Testicular receptor 2	TR2	None	NM_011629
Nr2c2	Testicular receptor 4	TR4	None	NM_011630
Nr2f6	ErbA2-related gene-2	EAR2	None	NM_010150
Nr3a1	Estrogen receptor α	ERα	Estradiol-17β, tamoxifen, raloxifene	NM_007956
Nr3b1	Estrogen related receptor α	ERRα	Orphan	NM_007953
Nr3c1	Glucocorticoid receptor	GR	Cortisol, dexamethasone, RU486	NM_008173
Nr3c3	Progesterone receptor	PR	Progesterone, medroxyprogesterone acetate, RU486	NM_008829
Nr3c4	Androgen receptor	AR	Testosterone, flutamide	NM_013476
Nr4a1	Nerve growth factor IB	NUR77	None	NM_010444
Nr4a2	Nuclear receptor-related 1	NURR1	None	NM_013613
Nr4a3	Neuron-derived orphan receptor 1	NOR1	None	NM_015743
	G protein-coupled receptor 30	GPR30	Estrogen (membrane receptor)	NM_029771
	Progesterone receptor membrane component 1	PGRM1	Progesterone (membrane receptor)	NM_016783

Highly expressed gene (Ct>25)
Mildly expressed gene (25>Ct>27)
Lowly expressed gene (Ct<27)

Table 2.1B NRs not expressed in DCs.

Systemic Nomenclature	Name	Abbreviation	Ligand	GenBank Accession Number
Nr1b1	Retinoic acid receptor α	RARα	Retinoic acids (Vitamin A)	NM_009024
Nr1b2	Retinoic acid receptor β	RARβ	Retinoic acids (Vitamin A)	NM_011243
Nr1c1	Peroxisome proliferator activated receptor α	PPARα	Fatty acids, leukotriene B4, fibrates	NM_011144
Nr1f2	RAR-related orphan receptor β	RORβ	Retinoic acids (Vitamin A)	NM_146095
Nr1f3	RAR-related orphan receptor γ	RORγ	Unknown	NM_011281
Nr1h4	Farnesoid X receptor α	FXRα	Bile acids, fexaramine	NM_009108
Nr1h5	Farnesoid X receptor β	FXRβ	Lanosterol	NM_198658
Nr1i2	Pregnane X receptor	PXR	Xenobiotics, 16-cyanopregnenolone	NM_010936
Nr2a1	Hepatic nuclear factor 4 α	HNF4α	None	NM_008261
Nr2a2	Hepatic nuclear factor 4 γ	HNF4γ	None	NM_013920
Nr2b3	Retinoid X receptor γ	RXRγ	Retinoic acids (Vitamin A)	NM_009107
Nr2e2	Tailless	TLL	None	NM_152229
Nr2e3	Photoreceptor-specific nuclear receptor	PNR	None	NM_013708
Nr2f1	Chicken ovalbumin upstream promoter-transcription factor I	COUP-TFI	None	NM_010151
Nr2f2	Chicken ovalbumin upstream promoter-transcription factor II	COUP-TFII	None	NM_009697
Nr3a2	Estrogen receptor β	ERβ	Estradiol-17, various synthetic compounds	NM_009803
Nr3b2	Estrogen-related receptor β	ERRβ	None	NM_011934
Nr3b3	Estrogen-related receptor γ	ERRγ	None	NM_011935
Nr3c2	Mineralcorticoid receptor	MR	Aldosterone, spirolactone	XM_356093
Nr5a1	Steroidogenic factor 1	SF1	None	NM_139051
Nr5a2	Liver receptor homologous protein 1	LRH1	None	NM_030676
Nr6a1	Germ line nuclear factor	GCNF	None	NM_010264
Nr0b1	DSS-AHC critical region on the chromosome, gene 1	DAX1	None	NM_007430
Nr0b2	Short heterodimeric partner	SHP	None	NM_011850

Table 2.1C Coregulators expressed in DCs.

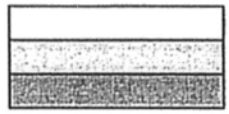
i. NR Corepressor	Abbreviation	GenBank Accession Number
Nuclear receptor co-repressor 1	NCOR1	NM_011308
Nuclear receptor co-repressor 2	NCOR2	NM_011424

ii. Histone Modifiers	Abbreviation	GenBank Accession Number
a. Histone Acetyltransferase		
Nuclear receptor coactivator 1	NCOA1	NM_010881
Nuclear receptor coactivator 2	NCOA2	NM_008678
Nuclear receptor coactivator 3	NCOA3	NM_008679
Nuclear receptor coactivator 6	NCOA6	NM_019825
p300	p300	NM_177821
CREB binding protein	CBP	NM_001025432
P300/CBP-associated factor	P/CAF	NM_020005
b. Histone Deacetylases		
Histone deacetylase 1	HDAC1	NM_008228
Histone deacetylase 2	HDAC2	NM_008229
Histone deacetylase 3	HDAC3	NM_010411
Histone deacetylase 4	HDAC4	NM_207225
Histone deacetylase 6	HDAC6	NM_010413
Histone deacetylase 7A	HDAC7a	NM_019572
Histone deacetylase 10	HDAC10	NM_199198
Histone deacetylase 11	HDAC11	NM_144919
Transcriptional regulator, SIN3A	SIN3a	NM_011378
c. Histone Methyltransferases		
Heterogeneous nuclear ribonucleoproteins methyltransferase-like 2	HRMT1l2	NM_019830
Suppressor of variegation 3-9 homolog 1	SUV39h1	NM_011514

iii. Specific NR Coregulators	Abbreviation	GenBank Accession Number
A kinase (PRKA) anchor protein 13	AKAP13	XM_133543
Coactivator-associated arginine methyltransferase 1	CARM1	NM_021531
Cofactor required for Sp1 transcriptional activation, subunit 2	CRSP2	NM_012005
Nuclear receptor interacting protein 1	NRIP1	NM_173440
Peroxisome proliferator activated receptor binding protein	PPARBP	NM_013634
Steroid receptor RNA activator 1	SRA1	NM_025291

iv. Cell Cycle/Growth-realted Coregulators	Abbreviation	GenBank Accession Number
Growth arrest specific 5	GAS5	NR_002840
Set template-activating factor-1 β	Set-TAF-Iβ	NM_023871
SNF2 histone linker PHD RING helicase	SHPRH	NM_172937
Structural maintenance of chromosomes 1-like 1	SMC1l1	NM_019710
Structural maintenance of chromosomes 4	SMC4	NM_133786

v. Others	Abbreviation	GenBank Accession Number
Mediator of RNA polymerase II transcription, subunit 6 homolog	MED6	NM_027213
SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	SMARCA4	NM_011417
C-terminal binding protein 1	CTBP1	NM_013502



Highly expressed gene (Ct>25)
Mildly expressed gene (25>Ct>27)
Lowly expressed gene (Ct<27)

Table 2.1D Coregulators not expressed in DCs.

General Coregulators	Abbreviation	GenBank Accession Number
Peroxisome proliferative activated receptor γ coactivator 1α	PGC1α	NM_008904

2.3.2 Virus-mediated modulation of NR and coregulator mRNA expression in DCs

The mRNA expression of NRs and coregulators in DCs after viral infection were examined next. Two different types of viruses, NDV and MCMV, were used in this study to compare their effects on the mRNA expression of the indicated molecules in DCs. NDV represents the single-stranded RNA viruses, which is widely used in murine models as it potently induces type I IFNs (Akira, Uematsu et al. 2006). MCMV is the double-stranded DNA virus of the herpesvirus family. It is often used as a mouse model to study the life-long persistent viral infection (Brune, Hengel et al. 2001). After infecting DCs with NDV or MCMV, the mRNA expression of these molecules at three time points (1, 7 and 13 h post-infection) was monitored (Table 2.2). Activation of DCs by these viruses was confirmed by pronounced up-regulation of type I IFNs, IL-12 and costimulatory molecules (Figure 2.1). Although the two viruses induced unique patterns of NR and coregulator mRNA expression, they commonly regulated six NRs (Figure 2.2A & B) and six coregulators (Figure 2.2C & D).

Regarding to the time course of mRNA expression after infection with NDV or MCMV, some NRs/coregulators (e.g. NOR1 and HDAC10) demonstrated the highest expression at 7 h time-point after the infection, while others (e.g. the neuron growth factor-induced factor-IB (NUR77) and HDAC4) developed the peaks at 13 h (Table 2.2). Interestingly, two groups of NRs showed significant and characteristic changes after viral infection: The NR4A family (NOR1, NURR1 and NUR77) and three metabolite receptors (LXRα, PPARγ and RXRα). While NOR1 was significantly upregulated (over 20-fold), NUR77 and NURR1 were strongly downregulated (by almost 80%). LXRα and PPARγ, which are master regulators for the cholesterol homeostasis and the carbohydrate metabolism respectively, were significantly modulated after viral infection (LXRα: ~3-6 fold up-regulation; PPARγ and RXRα ~60-80%

down-regulation). RXRα is essential for LXRα and PPARγ to form functional heterodimers (Germain, Chambon et al. 2006).

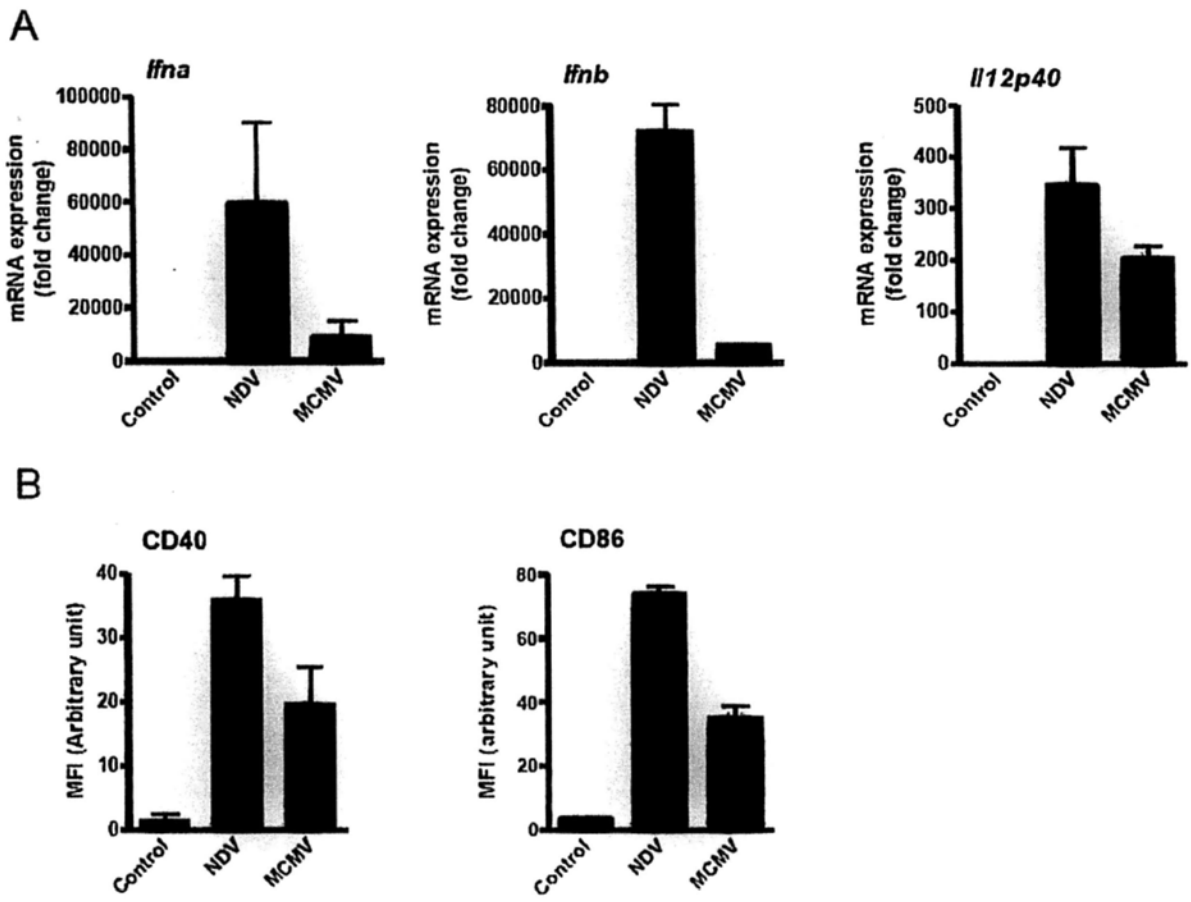


Figure 2.1 Virus infection activates DCs.

DCs were infected with NDV (MOI=10) or MCMV (MOI=10) for (A) 7 h and (B) 16 h. (A) The mRNA expression of *Ifna, Ifnb* and *Il12p40* was measured with qPCR. Data were normalized with *Gapdh* mRNA abundance and the mean values +/- SEM of three independent experiments were shown. (B) Expression of costimulatory molecules on the DC surface was detected by flow cytometry using anti-CD40-PE or -CD86-FITC antibodies. Values were subtracted by isotypic control and mean values +/- SEM of three independent experiments are shown.

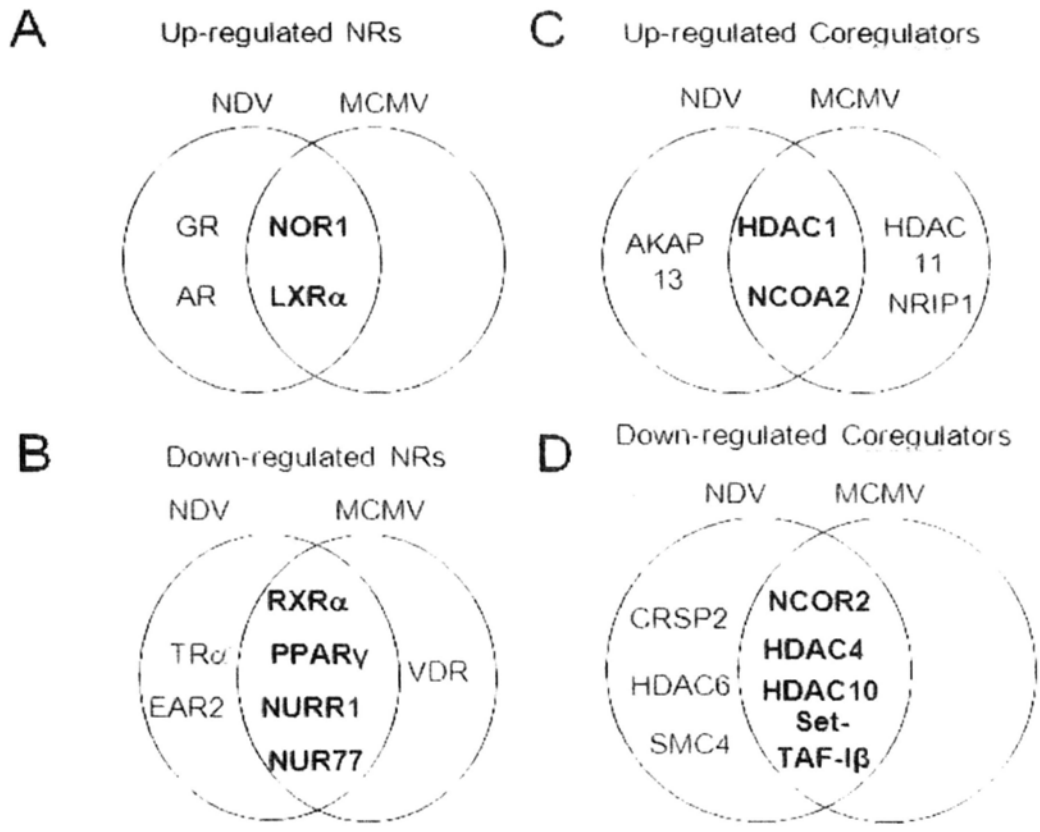


Figure 2.2 Venn diagrams demonstrating the NRs or coregulators that were significantly upregulated or downregulated by NDV or MCMV infection in DCs.

Table 2.2 Viral infection modulates NR and coregulator mRNA expression in DCs.

A. NDV infection

Nuclear Receptors

Name	1h		7h		13h	
	fold change	p-value	fold change	p-value	fold change	p-value
NOR1	-1.04	0.513	23.09	0.0002	3.24	0.118
AR	-1.34	0.458	4.64	0.030	3.06	0.181
LXRα	-1.31	0.039	4.18	0.016	7.77	0.034
GR	-1.13	0.239	3.23	0.017	2.19	0.162
TRα	-1.08	0.210	-2.41	0.0003	-5.63	0.036
NURR1	1.70	0.326	-3.16	0.014	-2.83	0.047
EAR2	-1.15	0.056	-3.18	0.001	-2.37	0.327
RXRα	-1.01	0.691	-4.60	0.001	-5.20	0.016
NUR77	1.69	0.232	-6.19	0.003	-16.30	0.009
PPARγ	-1.25	0.192	-6.28	0.002	-19.36	0.010

Coregulators

Name	1h		7h		13h	
	fold change	p-value	fold change	p-value	fold change	p-value
i. General Coregulators						
NCOA2	1.06	0.650	2.08	0.013	4.93	0.010
NCOR2	-1.11	0.169	-3.89	0.003	-2.87	0.034
ii. Histone Modifiers						
HDAC1	-1.16	0.091	3.70	0.001	2.41	0.226
HDAC4	-1.37	0.072	-4.88	0.0003	-5.60	0.015
HDAC9	1.07	0.735	-3.57	0.0001	-2.93	0.023
HDAC10	1.03	0.809	-7.99	0.0001	-8.20	0.005
iii. Specific NR Coregulators						
AKAP13	-1.03	0.350	3.02	0.002	1.31	1.729
CRSP2	1.05	0.795	-1.93	0.005	-3.04	0.044
iv. Cell Cycle/Growth Coregulators						
Set-TAF-Iβ	-1.02	0.363	-3.30	0.016	-5.54	0.296
SMC4	-1.22	0.147	-1.91	0.001	-3.77	0.078

Strongly induced genes (fold change>5)
Weakly induced genes (3>fold change>5)
Weakly suppressed genes (-5<fold change<-3)
Strongly suppressed genes (-5<fold change)

B. MCMV infection

Nuclear Receptors

Name	1h		7h		13h	
	fold change	p-value	fold change	p-value	fold change	p-value
NOR1	1.25	0.314	20.72	0.0003	5.95	0.003
LXRα	-1.56	0.059	2.07	0.242	3.35	0.035
NURR1	1.61	0.972	-3.66	0.013	-2.46	0.031
VDR	1.07	0.150	-3.69	0.001	-1.15	0.403
RXRα	-1.28	0.055	-4.17	0.0005	-3.06	0.002
PPARγ	-1.16	0.188	-4.33	0.003	-5.87	0.001
NUR77	1.88	0.625	-6.37	0.002	-11.71	0.001

Coregulators

Name	1h		7h		13h	
	fold change	p-value	fold change	p-value	fold change	p-value
i. General Coregulators						
NCOA2	-1.06	0.659	2.04	0.011	5.79	0.011
NCOR2	-1.07	0.150	-3.79	0.002	-2.32	0.009
ii. Histone Modifiers						
HDAC1	-1.45	0.034	3.33	0.003	3.07	0.022
HDAC4	-1.36	0.078	-4.51	0.003	-5.19	0.001
HDAC10	1.04	0.676	-3.67	0.004	-2.39	0.030
HDAC11	-2.07	0.013	-1.25	0.071	3.10	0.003
iii. Specific NR Coregulators						
NRIP1	-1.42	0.092	2.26	0.057	3.30	0.030
iv. Cell Cycle/Growth Coregulators						
Set-TAF-Iβ	-1.36	0.247	-3.80	0.002	-3.56	0.0002

2.3.3 Virus-mediated modulation of NR and coregulator mRNA expression in DCs subtypes

To investigate whether the above-mentioned transcriptional regulation occurs differently in DC subtypes, cDCs and pDCs were purified from total DCs and were infected with NDV or MCMV separately. The mRNA expression of selected NRs and coregulators (the 12 overlapping genes shown in Figure 2.2) were further monitored by qPCR. cDCs and pDCs exhibited similar induction/reduction profiles on most of the genes examined, while some genes showed stronger response in particular DC subtypes (Figure 2.3 & Supplementary Figure 2). For example, cDCs showed stronger induction of NOR1 and HDAC1 mRNA than pDCs upon viral infection. pDC, on the other hand, exhibited stronger repression on NURR1 and HDAC4 transcription than cDCs (Figure 2.3).

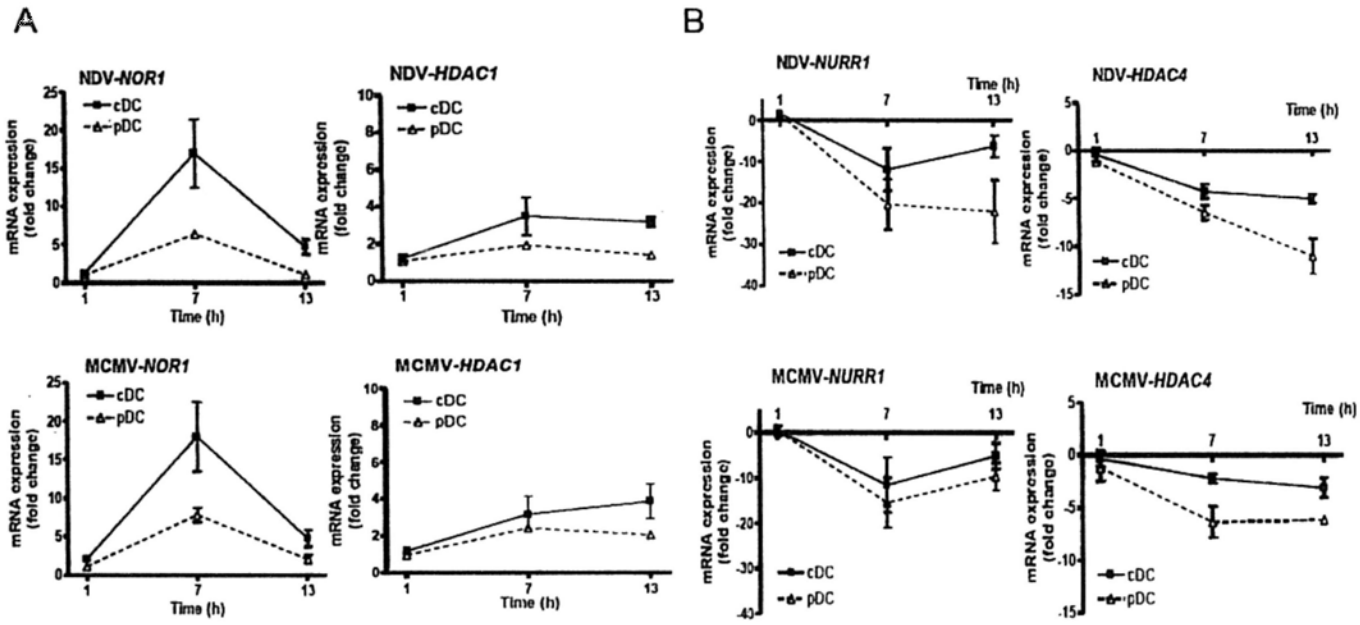


Figure 2.3 Viral infection alters NR and coregulator mRNA expression in pDCs and cDCs.

pDCs and cDCs were purified from total DCs, and were infected with NDV (MOI=10) or MCMV (MOI=10). Total RNA was isolated at 1 h, 7 h or 13 h of post-infection. The mRNA levels of indicated NRs and coregulators were measured with qPCR. Data were normalized with *Gapdh* mRNA abundance and the mean values +/- SEM of three independent experiments are shown.

2.3.4 TLR ligands-mediated modulation of NR and coregulator mRNA expression in DCs

The above results indicate that NDV and MCMV infection modulated mRNA expression of a similar set of NRs and coregulators in DCs. DCs express numbers of PRRs including TLRs, which sense specific viral genetic materials including viral DNA and RNA (Akira, Uematsu et al. 2006). The effects of selected TLR ligands were therefore examined. Synthetic ligands for TLR3 (Poly IC), TLR7 (R848) and TLR9 (CpG) were used to stimulate DCs and the mRNA expression of selected NRs and coregulators were quantified to verify if TLRs participate in the transcriptional regulation of the NR and coregulator genes upon NDV (single strand RNA virus) or MCMV (double strand DNA virus) infection. All of the tested TLR ligands strongly activated DCs, based on the robust mRNA expression of IFNα and induction of the costimulatory molecules (Figure 2.4). We found that all of the tested TLR ligands induced similar effects on the mRNA expression of selected NRs and coregulators (Upregulated gene: NOR1; Downregulated genes: RXRα, PPARγ, HDAC4, HDAC10 and NCOR2) as observed after viral infection, although they demonstrated differential potencies (Figure 2.5 and Table 2.2). These results suggested that activation of all or some of the TLRs may participate in the modulation of NR and coregulator mRNA expression caused by viral infection.

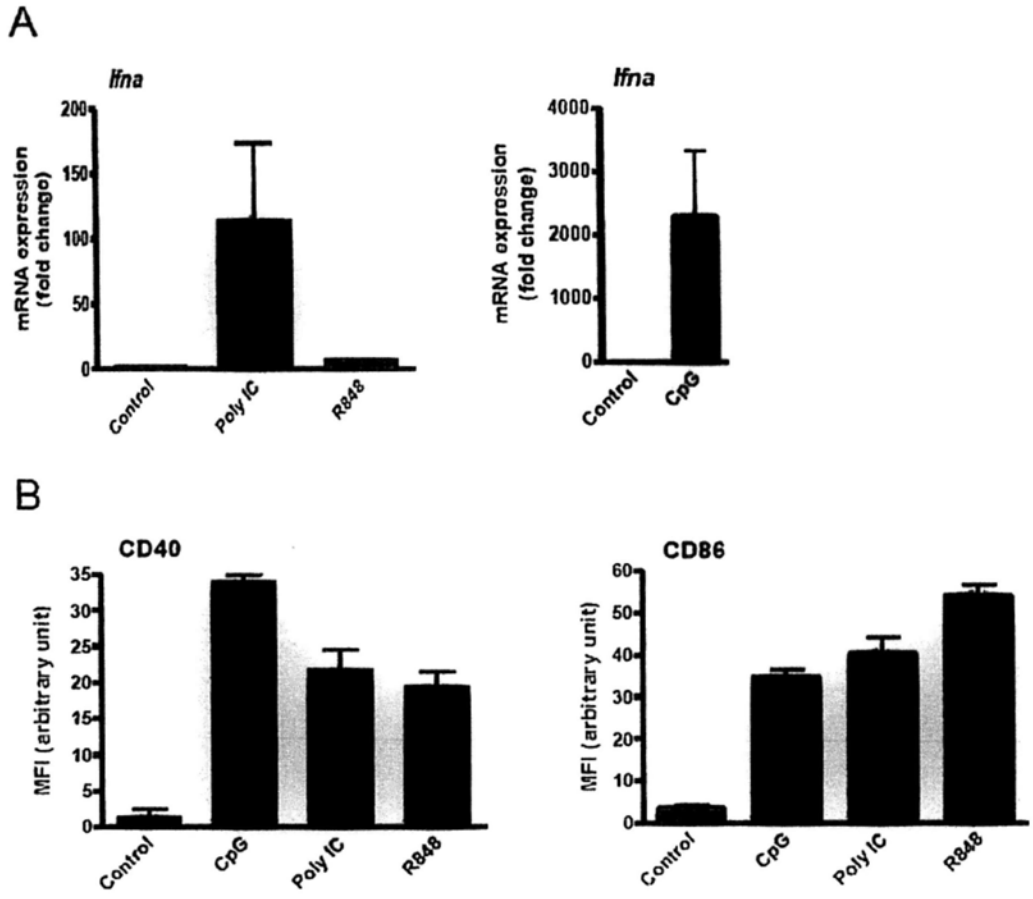


Figure 2.4 TLR ligands activate DCs.

DCs were treated with Poly IC (TLR3 ligand; 20 μg/ml), resiquimod (R848, TLR7 ligand; 100nM) or CpG (TLR9 ligand; 1 μg/ml). (A) The mRNA expression of *Ifna* was measured by qPCR after 7 h of activation. Data were normalized with *Gapdh* mRNA abundance and the mean values +/- SEM of three independent experiments are shown. (B) Costimulatory molecules expressed on the DC surface after 16 h stimulation were detected with the flow cytometry using anti-CD40-PE or -CD86-FITC antibodies. Values were corrected with those of isotypic control and mean values +/- SEM of three independent experiments are shown.

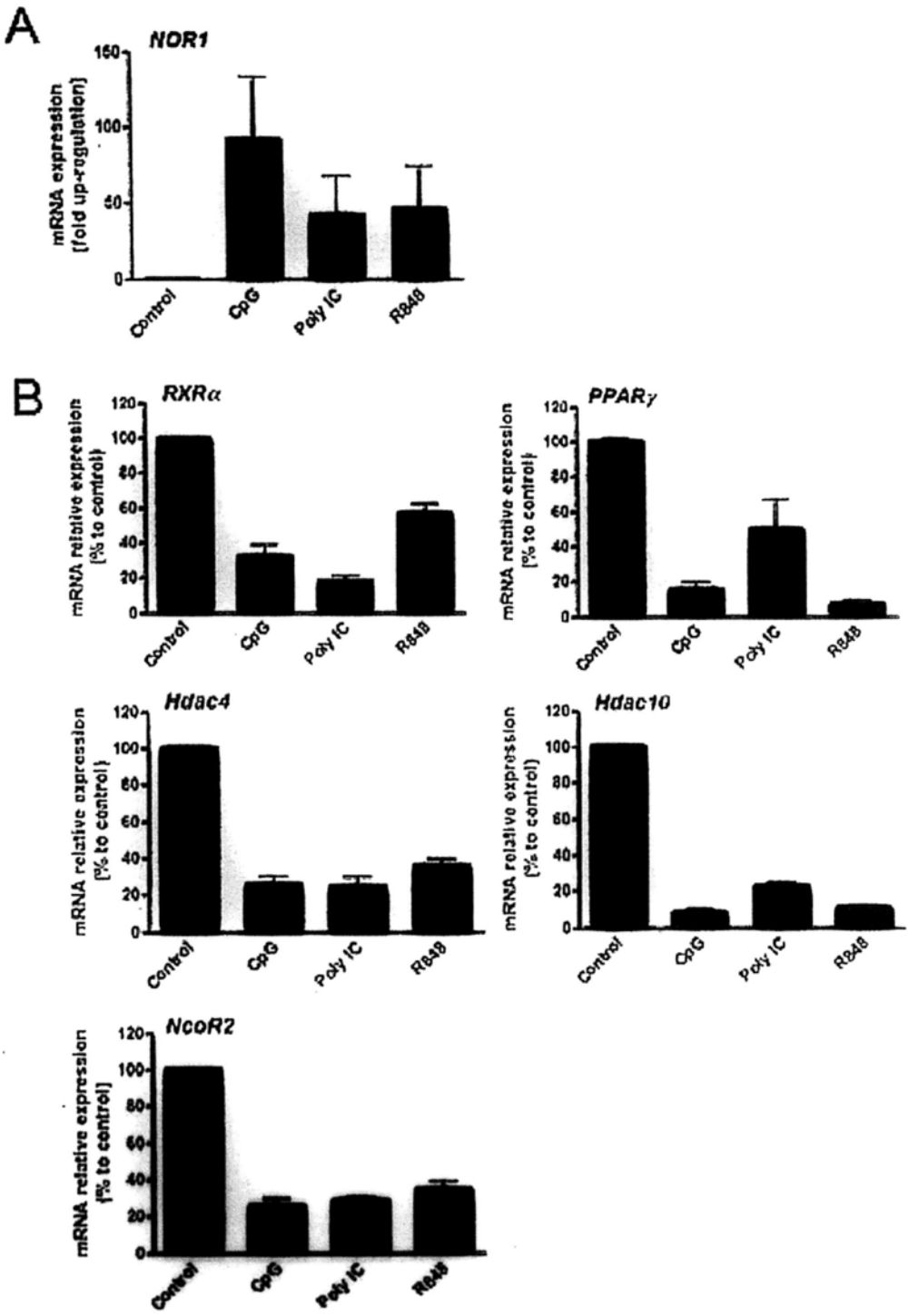


Figure 2.5 TLR ligands alter NR and coregulator mRNA expression in DCs.

DCs were treated with polyinos -polycytidylic acid (Poly IC, TLR3 ligand; 20 μg/ml), resiquimod (R848, TLR7 ligand; 100nM) or unmethylated CpG oligodeoxynucleotides (CpG, TLR9 ligand; 1 μg/ml) for 7 h. The mRNA levels of NRs and coregulators upregulated (A) or downregulated (B) by the treatment were shown. Expression levels of control in (B) were regarded as 100%. Data were normalized with *Gapdh* mRNA abundance, and the mean values +/- SEM of three independent experiments are shown.

2.3.5 NDV and CpG changed NR protein expression in DCs

To verify whether the changes found at the mRNA level were also observed at a protein level, the protein expression of the selected NRs in DCs after NDV infection or CpG stimulation (TLR9 ligand) were examined. NOR1 protein was not detected in DCs at baseline, whereas both NDV and CpG strongly upregulated its expression at 16 h time-point after the infection/stimulation and sustained to 24 h (Figure 2.6, first panel). The infection/stimulation also upregulated LXRα protein expression at 24 h, although the change was not as obvious as NOR1 (Figure 2.6, second panel). RXRα was highly expressed before infection/stimulation, while it was significantly downregulated at time-points 16 h and 24 h after the treatments (Figure 2.6, third panel).

2.3.6 NDV and CpG increased the HDAC1 activity in DCs

Among the HDACs found to be modulated upon viral infection (Figure 2.1C & D), HDAC1 belongs to class I HDACs, which constitutively localizes in the nucleus and mainly deacetylates chromatin-associated histones (Minucci and Pelicci 2006). In contrast, HDAC4 and HDAC10 are class II HDACs, which are localized in the cytoplasm and deacetylate various cytosolic proteins (Minucci and Pelicci 2006). Nuclear extracts obtained from DCs infected/stimulated with NDV or CpG showed increased HDAC activity (upregulated by 50-75 %) after 13 h of treatment (Figure 2.7), which was consistent with the up-regulation of HDAC1 mRNA expression after NDV infection (Table 2.2). The HDAC activity was returned to baseline after 19 h of the treatments, indicating that the effect of infection/treatment was transient.

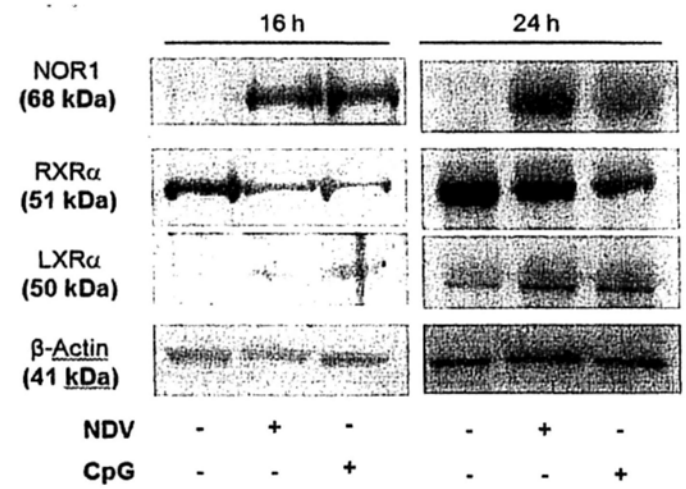


Figure 2.6 Viral infection modulates NRs protein expression in DCs.

Nuclear extracts were prepared from DCs infected with NDV (MOI=10) or stimulated with TLR9 ligand (CpG; 1 μg/ml) for 16 h or 24 h. Equal amounts of protein (5 μg) were loaded in each lane, and Western blots evaluating expression of NOR1, RXRα, LXRα and control β-actin were performed. Representative images of three independent experiments are shown.

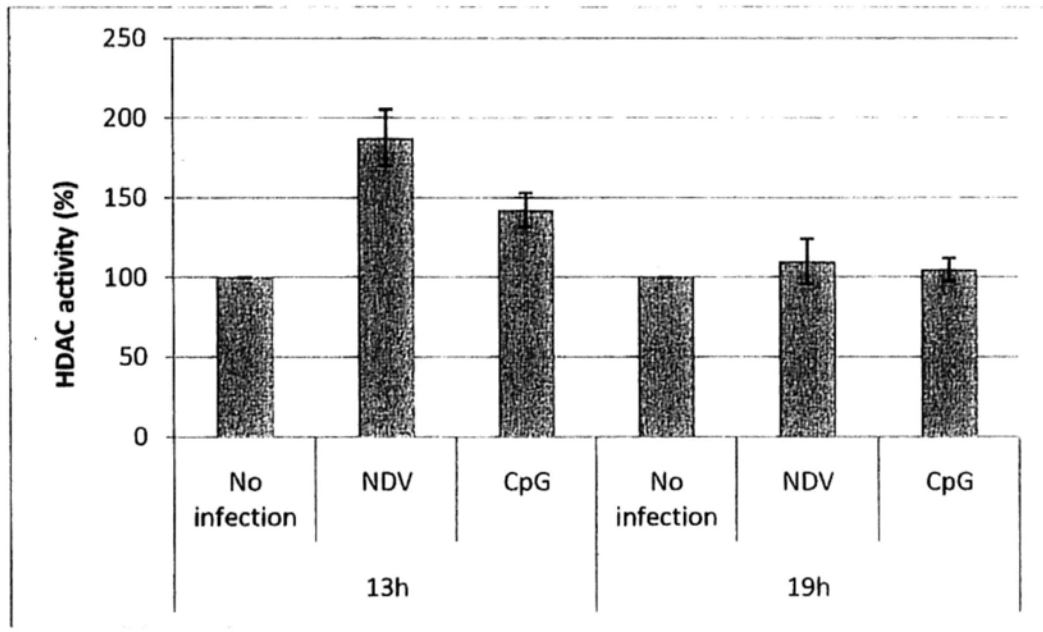


Figure 2.7 Viral infection modulates HDAC1 activity in DCs.

Nuclear extracts were prepared from DCs 13 h and 19 h after infection with NDV (MOI=10) or stimulation with TLR9 ligand (CpG; 1 μg/ml). The HDAC1 activity of each time-point is shown as % to the control obtained in the absence of viral infection or CpG at the same time-point. The mean values +/- SEM of two independent experiments are shown.

2.3.7 NOR1 and LXRα repressed IRF3- and IRF7-induced transcriptional activity on the IFNβ promoter

Pathogen-stimulated TLRs activate several transcription factors including IRF3 and IRF7 for induction of the type I IFNs and inflammatory cytokines in DCs (Kawai and Akira 2009). Specifically, TLR3 activates IRF3, while TLR7 and TLR9 activate IRF7 (Kawai and Akira 2009). Among the upregulated NRs after viral infection, NOR1 and LXRα were upregulated most significantly (Table 2.2). To address the biological consequences of viral infection-induced alteration of NR expression in DCs, the influence of NOR1 or LXRα to the transcriptional activity of IRF3 or IRF7 on the IFNβ promoter were addressed. Overexpression of NOR1 strongly repressed IRF3- or IRF7-induced transcriptional activity of the IFNβ promoter (80% and 50% respectively, Figure 2.8, left and middle panel). Since RXRα is required for LXRα to form a functional heterodimer, it was coexpressed with LXRα. LXRα/RXRα also repressed IRF3- and IRF7-induced transcriptional activity of the IFNβ promoter (30% and 50% respectively, Figure 2.8). These results suggest that NOR1 and LXRα may act as counter regulatory factors for viral-mediated induction of type I IFNs in DCs through repression of IRF-mediated transactivation of their promoters. We did not observe the negative effect of NOR1 and LXRα on IRF8-induced transcriptional activity on the IFNβ promoter (Figure 2.8, right panels), indicating the selectivity and functional specificity of these NRs to IRF subtypes.

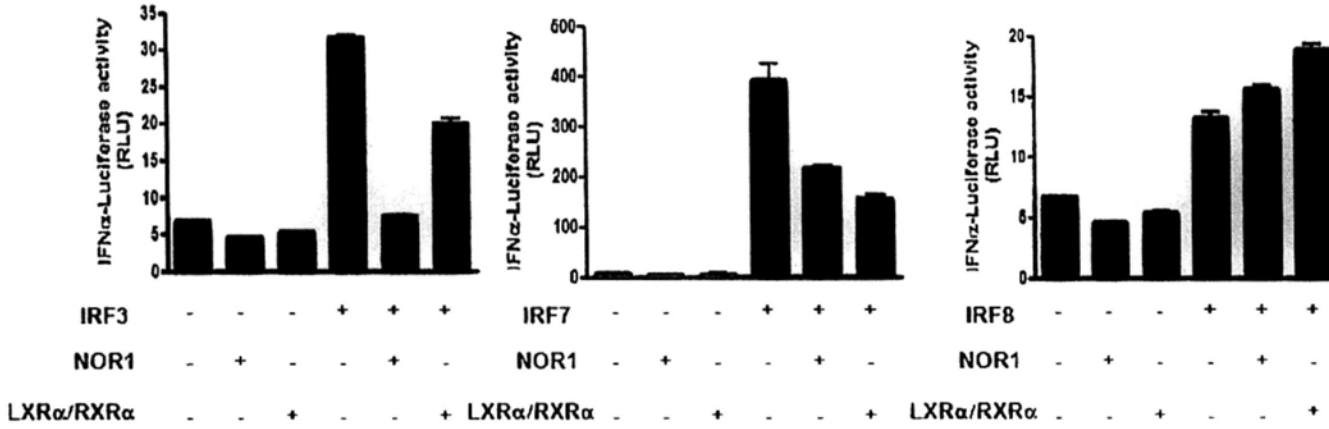


Figure 2.8 NOR1 or LXRα represses IRF3- and IRF7-induced transcriptional activity on the IFNβ promoter.

HCT116 cells were transfected with the indicated protein-expressing plasmids together with the pRL-TK reporter carrying the IFNβ promoter and the pGL4.73[*hRluc*/SV40] control plasmid. Firefly luciferase activities were normalized by renilla luciferase activities to account for transfection efficiency. The mean values +/- SEM of three independent experiments are shown.

2.4 Discussion

NRs play diverse roles in virtually all aspects of mammalian homeostasis (Gronemeyer, Gustafsson et al. 2004). Essentially, many of them demonstrate important regulatory activities on immunity and pathogen-related inflammation by modulating transcriptional rates of various cytokines, bioactive peptides and their receptors (Glass and Ogawa 2006; Pascual and Glass 2006). Further, NRs alter TLR-activated signaling pathways by interacting with their downstream signaling molecules or transcription factors (Barnes 1998; Glass and Ogawa 2006; Pascual and Glass 2006; Mora, Iwata et al. 2008).

Among all the NRs expressed in mice, the NR4A family members showed distinct expression profiles in response to viral infection: NOR1 was significantly upregulated, while NUR77 and NURR1 were strongly downregulated. It was also found that expression of LXRα and PPARγ were modulated significantly by viral infection: mRNA expression of LXRα was upregulated by ~3-6 fold, while PPARγ was downregulated by ~60-80%. In reporter assays employing the IFNα promoter construct, the NOR1 and LXRα/RXRα strongly repressed IRF3- and IRF7-induced transcriptional activities. The suppressive effect of NOR1 and LXRα may be mediated by direct interaction with IRF3 and IRF7, as LXRα is suggested to associate physically with IRF3 (Castrillo, Joseph et al. 2003). These NRs may act as counter regulatory factors to prevent prolonged production of type I IFNs, which may exert detrimental effects on local inflammatory tissues. Importantly, these NRs did not influence the transcriptional activity of IRF8 on the IFNα promoter. IRF8 promotes the second phase of the type I IFN production in DCs upon pathogen infection but not responsible for its first phase, which is specifically mediated by IRF3 and IRF7 (Tailor, Tamura et al. 2007). Thus, these results indicate the functional specificity of these NRs to the regulation of the first phase production of type I IFNs.

All three members of the NR4A family are expressed in macrophages (Barish, Downes et al. 2005; Pei, Castrillo et al. 2005), and LPS and IFNγ strongly induce their expression in these cells possibly through activation of the NF-κB pathway (Barish, Downes et al. 2005; Pei, Castrillo et al. 2005). Since both DCs and macrophages are professional antigen presenting cells, it is possible that regulation on the expression of NR4A family members in DCs upon viral infection might be similar to that seen in macrophages, and probably involves the NF-κB pathway. It is also known that NOR1 participates in the induction of apoptosis in DCs, T-cells and the bone marrow myeloid cells, while simultaneous inactivation of NOR1 and NUR77 develops acute leukemia in mice (Cheng, Chan et al. 1997; Mullican, Zhang et al. 2007; Wang, Jiang et al. 2009). Thus, NOR1 and other NR4A family members might play essential roles in the regulation of various cellular activities in DCs, such as differentiation, cytokine production and apoptosis, in addition to the regulation of the IRF activity in the production of the type I IFNs.

In regard to LXRα and PPARγ, these NRs have strong and diverse modulatory effects on immunity and inflammation *in vitro* and *in vivo* (Chinetti, Fruchart et al. 2000; Zelcer and Tontonoz 2006). For example, stimulation of LXRs in macrophages alleviates inflammation and relieves plaque formation in atherosclerotic vasculatures (Zelcer and Tontonoz 2006), while LXR-null macrophages are defective in response to intracellular pathogens (Joseph, Bradley et al. 2004). Further, PPARγ redirects DCs toward a less stimulatory condition, and modulates migration of Langerhan cells (the epidermal resident forms of DCs) from the site of infection to draining lymph nodes to activate T-cells (Faveeuw, Fougeray et al. 2000; Nencioni, Grunebach et al. 2002). Moreover, leukotriene, a prostanoid inflammatory mediator, acts as a ligand for PPARγ and modulates expression of IL-10 and IL-12 through activation of this NR in DCs

(Jozefowski, Biedron et al. 2005). These previous studies indicate the importance of LXRα and PPARγ in many aspects of the DC biology, and further suggest the possibility that viral infection could influence them by modulating expression of these NRs.

Many of the viral infection-responsive NRs found in this study, such as NOR1, LXRα, PPARγ, GR and TR, exhibit potent effects on energy metabolism (Penna and Adorini 2000; Chiang 2005; Flamant, Baxter et al. 2006; Pearen, Myers et al. 2008; Pyper, Viswakarma et al. 2010). Since immune response and subsequent inflammation consume tremendous amounts of cellular energy resources (Ritz and Gardner 2006), virus-infected DCs may regulate their cellular energy balance in addition to modulating immune reaction through these NRs.

Most of the coregulators examined were expressed at baseline and their mRNA levels after viral infection were relatively stable compared to those of NRs, suggesting that viral infection mainly alters DC activity by regulating the expression of DNA-binding factors including NRs. Nevertheless, DCs significantly altered mRNA levels of Set-TAF-Iβ, NCOR2, NCOA2, and HDACs upon viral infection. They are either chromatin modifying enzymes or essential cofactors for assembling basal transcriptional machineries. Specifically, mRNA levels of four members of the HDAC family were significantly altered in DCs by viral infection: HDAC1 was upregulated, while HDAC4, HDAC6 and HDAC10 were downregulated. HDACs modulate DC-mediated immune activity in part by inducing differentiation of precursor DCs into appropriate subtypes and by stimulating the expression of DC surface costimulatory molecules for antigen presentation (Adcock 2006; Brogdon, Xu et al. 2007). Among the HDACs significantly regulated by viral infection, HDAC1 is essential for induction of the interferon-responsive genes by deacetylating the histone H4 in DCs (Nusinzon and Horvath 2003). Given that viral infection strongly stimulated HDAC1 mRNA expression/activity in DCs, it is likely

that DCs alter IFN-mediated anti-viral response in part through regulation of HDAC1 expression/activity.

In conclusion, this part of my study demonstrated that viral infection dramatically altered expression of some NRs and their coregulators in DCs. Here I propose that these NRs and coregulators are critical for DCs to regulate production of type I IFNs, and possibly, other cytokines in response to viral infection. Further study to elucidate the detailed molecular mechanisms and biological consequences of such NR-mediated anti-viral response warrants our understanding on the overall DC-mediated immune response directed against viruses and other pathogens.

Chapter 3

In vivo Study Examining the Role of NOR1 in DC-mediated Immune Activity

3.1 Introduction

According to previous results on the expression of NRs in virus-infected DCs shown in Chapter 2, the expression of NOR1 was highly up-regulated in these cells both at the mRNA and protein levels. Because DCs are pivotal in the anti-pathogen immune response, NOR1 may play important roles in the regulation of such immune response organized by these cells.

The neuron-derived orphan receptor (NOR) 1 is a member of the NR4A subfamily and is regarded as an immediate response protein after exposure to various cellular stressors, such as inflammation, membrane depolarization, changes in the magnetic field and mechanical agitation (Maxwell and Muscat 2006). A wide range of extracellular compounds and molecules, such as fatty acids, prostaglandins, growth factors, ionic calcium, inflammatory cytokines, peptide hormones and neurotransmitters are also reported to induce NOR1 (Maxwell and Muscat 2006). In certain cell types, NOR1 plays specific physiological roles: It is essential for neuron development, as it was named based on this activity. In addition, NOR1 plays a regulatory role in glucose metabolism, energy expenditure and lipid homeostasis in adipocytes and skeletal muscle cells (Mahoney, Parise et al. 2005; Pei, Waki et al. 2006). Moreover, NOR1 participates in the development of atherosclerosis by mediating thrombin-stimulated proliferation of endothelial cells in the vasculature (Martorell, Rodriguez et al. 2008).

To explore the importance of NOR1 in the anti-pathogen immune response, $NOR1^{-/-}$ mice were employed. The *NOR1* genomic locus was destroyed by insertion of the *lacZ* gene into the exon 2 area with a homologous recombination technique (Ponnio, Burton et al. 2002). Knockout

animals are deaf due to defective semi-circular canal development, and are prone to seizures (Ponnio, Burton et al. 2002). In another study, mice with double knockout of *NOR1* and another NR4A member *NUR77* develop acute myeloid leukemia due to abnormal expansion of hematopoietic stem cells (Mullican, Zhang et al. 2007). Although $NOR1^{-/-}$ mice develop above-described phenotypes, their survival rates and life expectancy are the same as those of the wild type mice. By using these mice, roles of NOR1 in DC-mediated anti-pathogen immunity were examined by challenging them with TLR 3 ligand or *Toxoplasma Gondii (T. Gondii)*.

The TLR family members function as PRR in DCs. Some of them (TLR3, TLR7, TLR8 and TLR9) are responsible for recognizing nucleic acids of invading microorganisms and for activating downstream signaling cascades to induce immune and inflammatory response (Kawai and Akira 2009). dsRNA, the genetic component or by-product during the replication of some viruses, is ligand of TLR3. Activation of TLR3 *in vivo* leads to induction of type I IFNs (IFNα and IFNβ), proinflammatory cytokines and chemokines, and facilitates DC maturation through activation of NF-κB and IRF3 (Oshiumi, Matsumoto et al. 2003; Yamamoto, Sato et al. 2003). The secreted types I IFNs then induce ISG to exert anti-viral response. TLR3-mediated DC maturation is potent in priming both innate and adaptive immunity, hence TLR3 ligand is employed as adjuvant for vaccination, and is used as a potent cytokine inducer in some experimental inflammatory models (Matsumoto and Seya 2008; Longhi, Trumpfheller et al. 2009).

T. gondii is an intracellular protozoan that commonly infects mammals (particularly cats) and birds throughout the world (Dubey 1998). Although *T. gondii* infection is usually asymptomatic or self-limiting with just causing flu-like symptoms in healthy individuals, it develops lethal encephalitis in fetus of the infected mother and in immunocompromised hosts,

such as those with HIV-1 infection or drug-induced immune suppression (Dubey 1998). Pathogenesis of toxoplasmosis is complicated, but cell-mediated immunity and related cytokines play a central role in elimination of *T. gondii* (Yap and Sher 1999). *T. gondii* bradyzoites (a slowly replicating form of *T. gondii* generated from its oocytes)-infected cells secrete CCL2 (*monocyte chemoattractant protein 1, also named MC*P-1) to attract immune cells, such as DCs, macrophages, monocytes and T cells, to the site of infection (Bhopale 2003). Attracted DCs and macrophages, recognize *T. gondii* through several TLRs, such as TLR2, TLR4 and TLR11, and secrete IL-12 to activate CD4+ T cells, CD8+ T cells and NK cells, which in turn release IFNγ into circulation (Bhopale 2003; Aliberti, Jankovic et al. 2004; Denkers 2010). DCs and macrophages also secrete TNFα, which stimulates downstream immune cells to secrete IL-12 and serves as an autocrine cytokine for fully activating themselves (Bhopale 2003). TNFα and IFNγ cooperatively stimulate the phagocytic activity of DCs and macrophages against this protozoan (Bhopale 2003). During the acute phase of *T. gondii* infection that lasts about 7 days, tachyzoites (a motile, asexually reproducing form of *T. gondii* originated from bradyzoites) are released into circulation from infected cells, and eventually form cysts particularly in the brain of infected animals, although circulating levels of tachyzoites is lowered by cell-mediated immunity organized by DCs and their downstream effector cells (Bhopale 2003). Since DCs are essential for detection of infected *T. gondii* at the acute phase of its infection as well as for their clearance in a later phase, *T. gondii* infection in $NOR1^{-/-}$ mice is a useful model system to examine roles of NOR1 in DC-mediated anti-pathogen response (Aliberti, Jankovic et al. 2004).

In this study, roles of NOR-1 in DC-mediated anti-pathogen activity were examined by employing $NOR1^{-/-}$ mice. Sera were collected from wild type and $NOR1^{-/-}$ mice that were

injected with Poly IC or infected with *T. gondii*. This part of study provides preliminary information on the regulatory roles of NOR1 in anti-pathogen immunity promoted by DCs.

3.2 Materials and Methods

3.2.1 Animals

$NOR1^{+/+}$ (wild type) and $NOR1^{-/-}$ mice were kindly provided by Dr. Orla M. Conneely (Baylor College of Medicine, Houston, TX). Male and female mice at 6-8 week old were used for *in vivo* experiments. Mice were maintained at the animal facility of the *Eunice Kennedy Shriver* National Institute of Child Health & Human Development, and animal studies were conducted under the approved animal study proposal 09-008 in accordance with guidelines of the NIH animal care and use committee.

3.2.2 Histology staining

Thymuses, spleens and lymph nodes were excised from $NOR1^{+/+}$ and $NOR1^{-/-}$ mice and were fixed with 10% formaldehyde in PBS. These organs were then embedded in paraffin block. Slides of cross section were prepared and stained with hematoxylin and eosin.

3.2.3 Cell population analysis

Thymuses, spleens and lymph nodes of $NOR1^{+/+}$ and $NOR1^{-/-}$ animals were obtained and weighed. Lymph nodes and Thymuses were cut into small pieces, grained, and washed with RPMI medium once. Tissue debris was removed by passing through the cell strainer (BD Bioscience). Spleens were digested with liberase TL (100 U/sample, Roche, Indianapolis, IN) for 30 min at 37 ^{o}C. Undigested debris was removed by passing through the cell strainer. Cells were then washed with and resuspended in RPMI medium for cell counting. Cells in single cell suspension were stained with the fluorescence-labeled antibodies for surface markers for 20 min on ice in dark. The antibodies used are listed in Supplementary Table 3. Labeled cells were then

washed twice with FACS medium and were resuspended in the same medium. Data acquisition was performed by analyzing 30,000 cells/sample using the FACSCalibur (BD Bioscience). Mean fluorescence intensity values were calculated by subtracting the mean values of IgG isotypic control from the mean values of the antibody for a respective surface marker. Cell Quest software (BD Bioscience) was used for data analysis.

3.2.4 Synthetic TLR 3 ligand (Poly IC) stimulation

$NOR1^{+/+}$ and $NOR1^{-/-}$ mice were injected with vehicle PBS or Poly IC (100 μg/mice) intra-peritoneally (i.p.) (Hou, Reizis et al. 2008). Three mice were used at each time point. After 3 h or 6 h post-injection, mice were euthanized by carbon dioxide. Blood was drawn from vena cava and hearts, and was allowed to clot at room temperature for 30 min. The blood clot was removed by centrifugation at 3000 rpm for 15 min. Hemolysis was not observed after centrifugation. The sera obtained from the blood samples were frozen immediately at -80 °C until future use. $NOR1^{+/+}$ and $NOR1^{-/-}$ mice with no injection were used as controls.

3.2.5 *T. gondii* infection

Eight $NOR1^{+/+}$ and seven $NOR1^{-/-}$ mice (6-8 week old, include male and female mice) were injected i.p. with a sub-lethal dose of the virulent HF strain of *T. Gondii* in a volume of 0.5 ml PBS (20 cysts/animal, provided by Dr. Carl Feng, NIAID, NIH) at day 0 as suggested by a previous publication (Goldszmid, Bafica et al. 2007). To monitor the serum cytokine changes in infected mice, tail bleeding (~80 μl) of all infected mice were conducted on Day 4 and Day 7. After collecting blood samples, bleeding was stopped immediately by applying silver nitrate on the cut sites. Collected blood samples were allowed to clot at room temperature for 30 min. Sera

were collected after centrifugation at 3,000 rpm for 15 min. They were kept in an -80°C freezer for future use. $NOR1^{+/+}$ and $NOR1^{-/-}$ mice with no infection were used as controls.

3.2.6 Cytokine quantification

Serum concentrations of IFNβ were measured with ELISA (PBL Inc, Piscataway, NJ) and were quantified by comparing to the standard curve of the recombinant IFNβ provided by the kit. The concentrations of IFNγ, IL-6, IL-10, IL-12, CCL2 and TNFα were quantified by mouse cytometric bead analysis inflammation kit (BD Bioscience). Sera for analysis were diluted by 4-times by using the diluent provided by the kits. Samples and standards were run in FACSCalibur flow cytometer (BD Bioscience).

3.2.7 Statistical analysis

The Student's t test and Mann-Whitney test were performed with the GraphPad Prism 5 software (GraphPad Inc, La Jolla, CA) for the evaluation of statistical significance. *P*-values less than 0.05 were considered as statistically significant.

3.3 Results

3.3.1 NOR1$^{-/-}$ mice have abnormal spleens

Before testing their immune response towards pathogens, organ histology and cell population of the thymus, spleen and lymph nodes were evaluated in NOR1$^{+/+}$ and NOR1$^{-/-}$ mice to verify presence of developmental and/or pre-existing alterations. These immune organs have distinctive functions in the immune system: Thymus is specialized in generating mature T cells (Szatmari and Nagy 2008), while spleen is the site for filtering foreign antigen and degraded endogenous particles circulating in blood, which are further recognized and processed by DCs and macrophages reside in this organ (Cesta 2006). Draining lymph nodes are the place for activated DCs and macrophages to present captured antigens to lymphocytes (Szatmari and Nagy 2008)

Spleens of NOR1$^{-/-}$ mice were larger by twice than those of the NOR1$^{+/+}$ mice (Figure 3.1A & 3.3A), and the total splenocyte number of NOR1$^{-/-}$ mice was also significantly higher than that of NOR1$^{+/+}$ mice (Figure 3.3D). Microscopically, the spleen of NOR1$^{-/-}$ mice had enlarged red pulp area but diminished white pulp area (Figure 3.2A). The red pulp is responsible for filtration of wearing red blood cells, and is also a site of hematopoiesis in rodents, while the white pulp is the site for accumulating lymphocytes. Whereas in thymus and lymph nodes, size, weight, total cell number and organ histology were similar between these mice (Figure 3.1B & C, Figure 3.2B & C, Figure 3.3B, C, E & F).

Next, composition of cell-type population was characterized by staining the cells isolated from these organs with the fluorescence-labeled antibodies against indicated surface markers (Supplementary Table 3), percentages of each cell types were measured by using the flow cytometer. Spleens of NOR1$^{-/-}$ mice showed significantly higher numbers of total cells, while

percentages of most of the examined cell types except NK cells were similar between $NOR1^{+/+}$ and $NOR1^{-/-}$ mice (Figure 3.4A). In lymph nodes, percentages of the CD8+ T cells were elevated in $NOR1^{-/-}$ mice compared to $NOR1^{+/+}$ mice (Figure 3.4B). The T cell subpopulation of thymus showed huge variation, hence no statistical difference was found (Figure 3.3C).

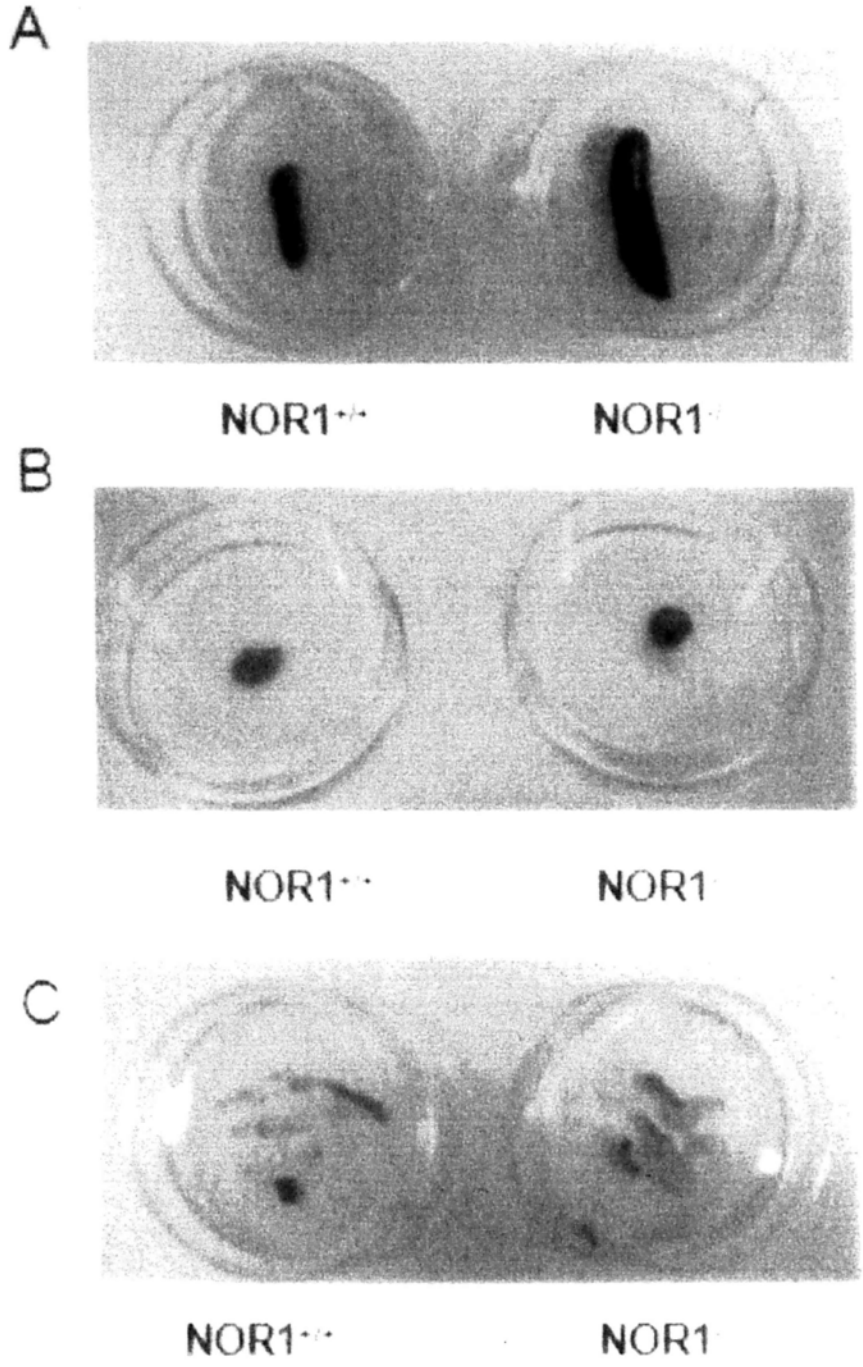
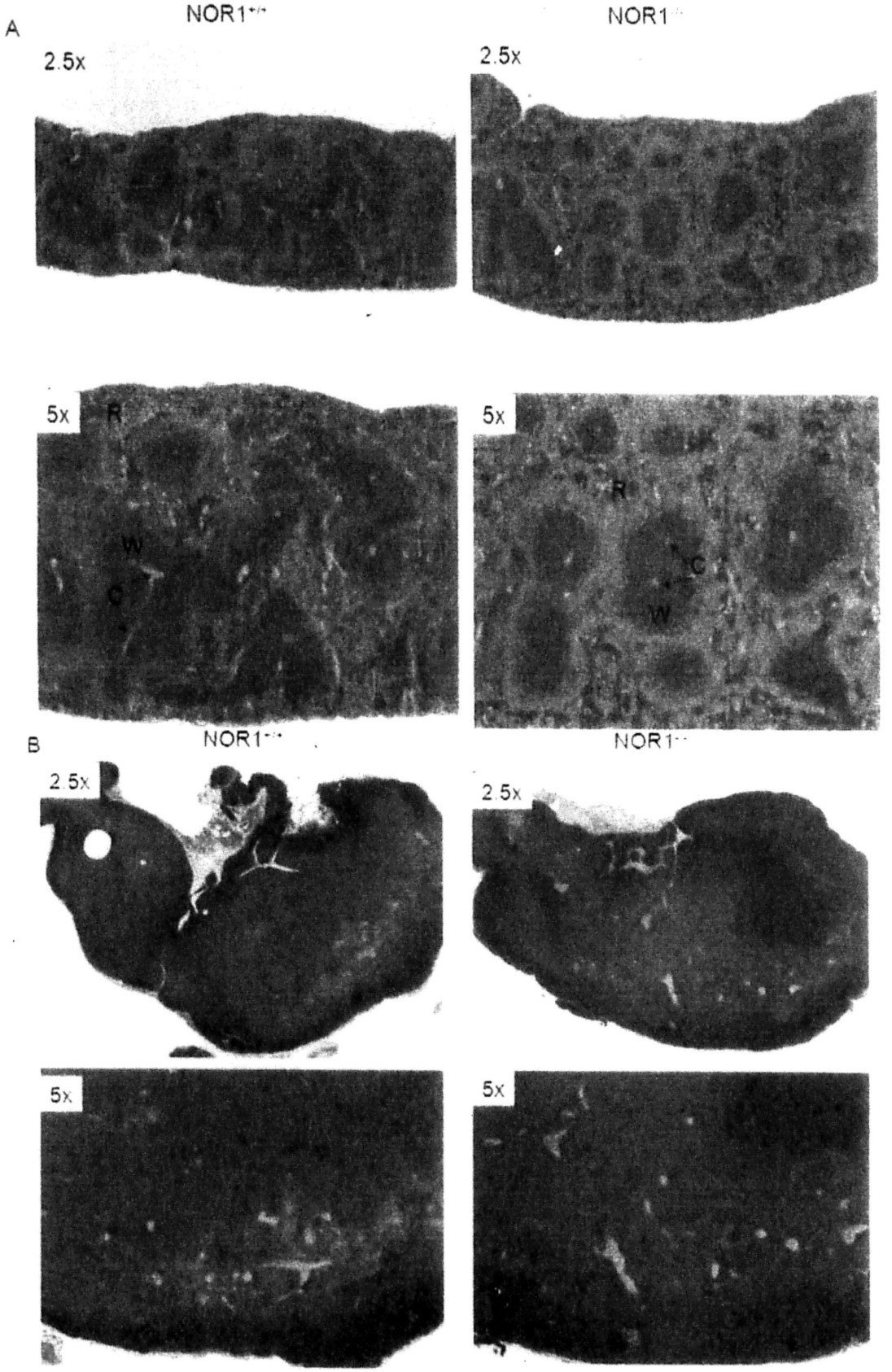


Figure 3.1 Gross appearances of the spleens, Thymuses and lymph nodes of NOR1$^{+/+}$ and NOR1$^{-/-}$ mice.

Spleens (A), Thymuses (B) and lymph nodes (C) were excised from NOR1$^{+/+}$ and NOR1$^{-/-}$ mice. Representative images of these organs from three animals of 6-8 week old are shown.


A
NOR1+/+
NOR1-/-
2.5x
2.5x
5x
R
W
C
5x
R
C
W
B
NOR1+/+
NOR1-/-
2.5x
2.5x
5x
5x

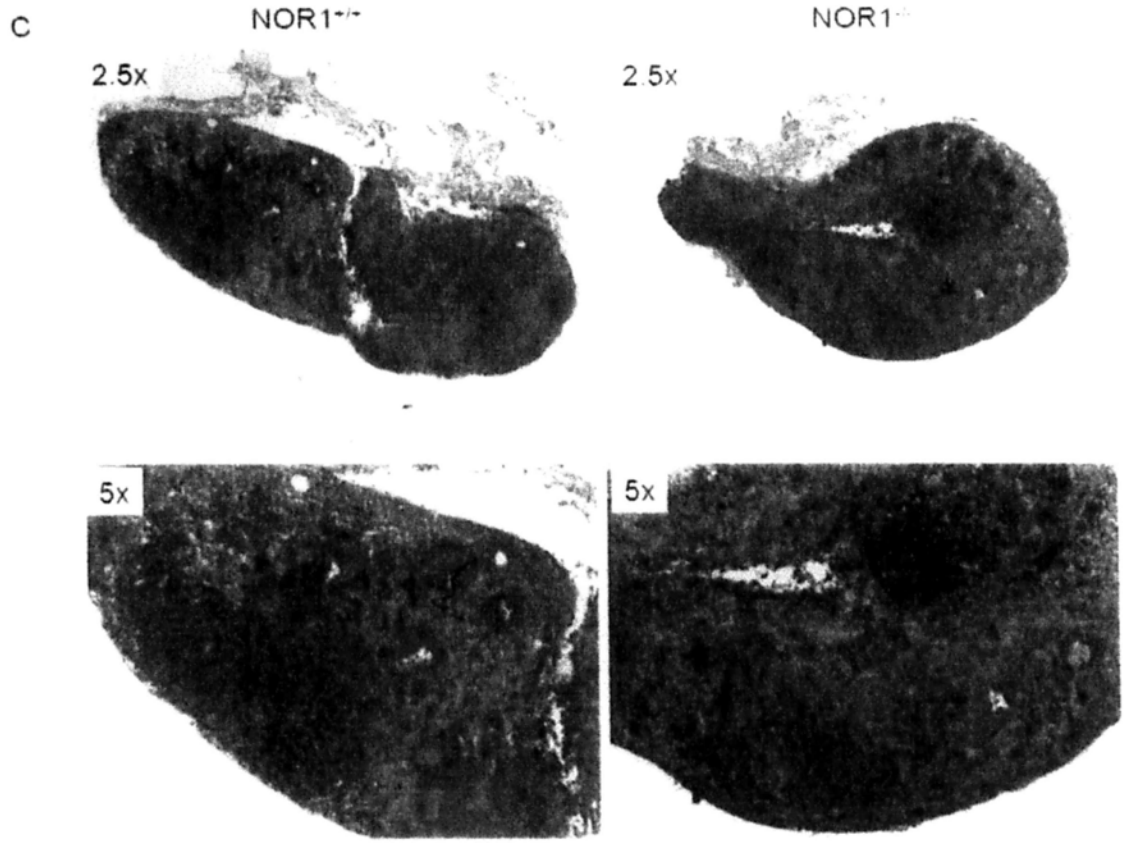


Figure 3.2 Histology of the spleen, thymus and lymph nodes of NOR1$^{+/+}$ and NOR1$^{-/-}$ mice.

Spleens (A), Thymuses (B) and inguinal lymph nodes (C) of NOR1$^{+/+}$ and NOR1$^{-/-}$ mice were excised and fixed. The cross sections of these organs were stained with hematoxylin and eosin. Images in 2.5x (upper panels) and 5x (lower panels) magnification are shown. Representative images of two animals of 6-8 week old are demonstrated. R, red pulp; W, white pulp; C, central artery.

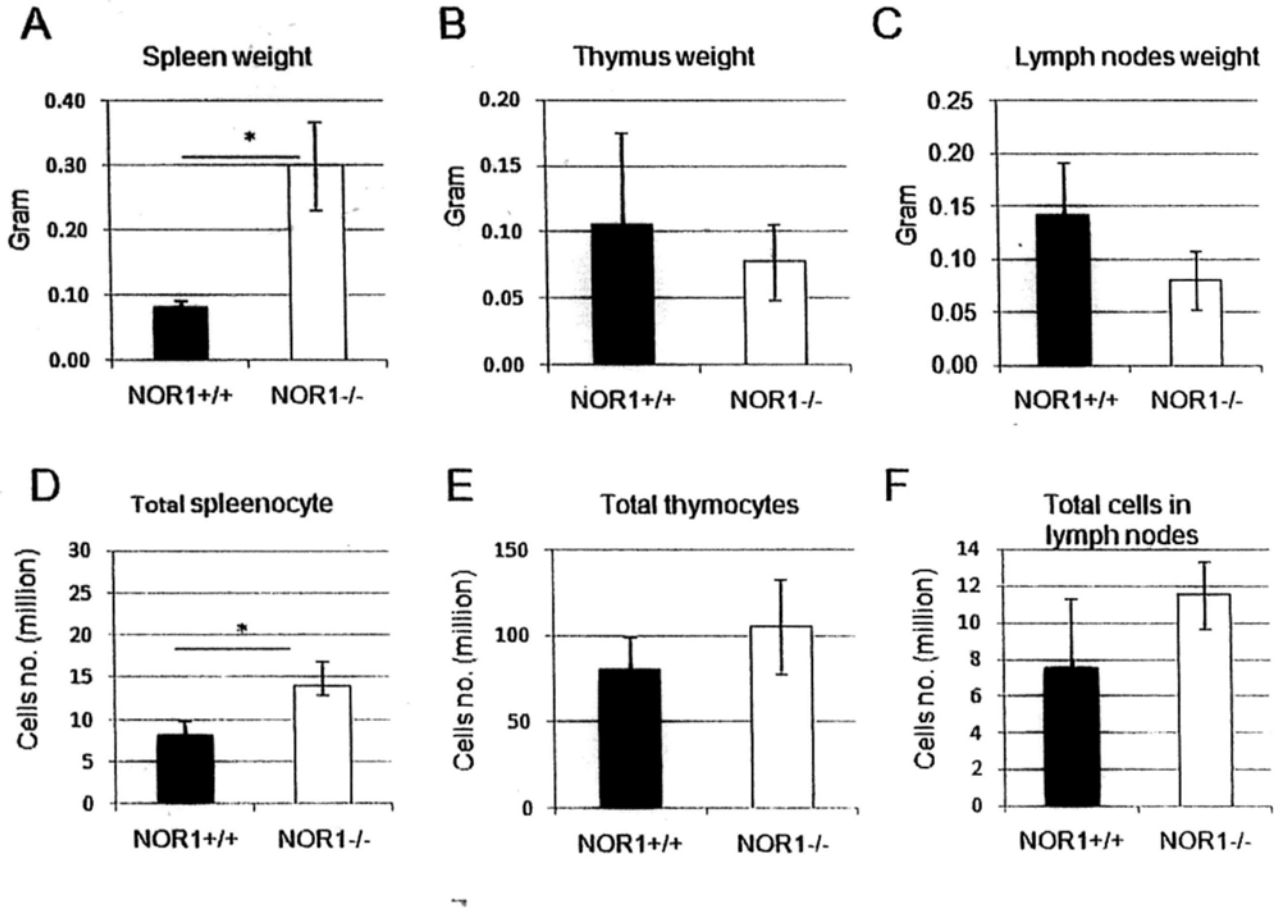
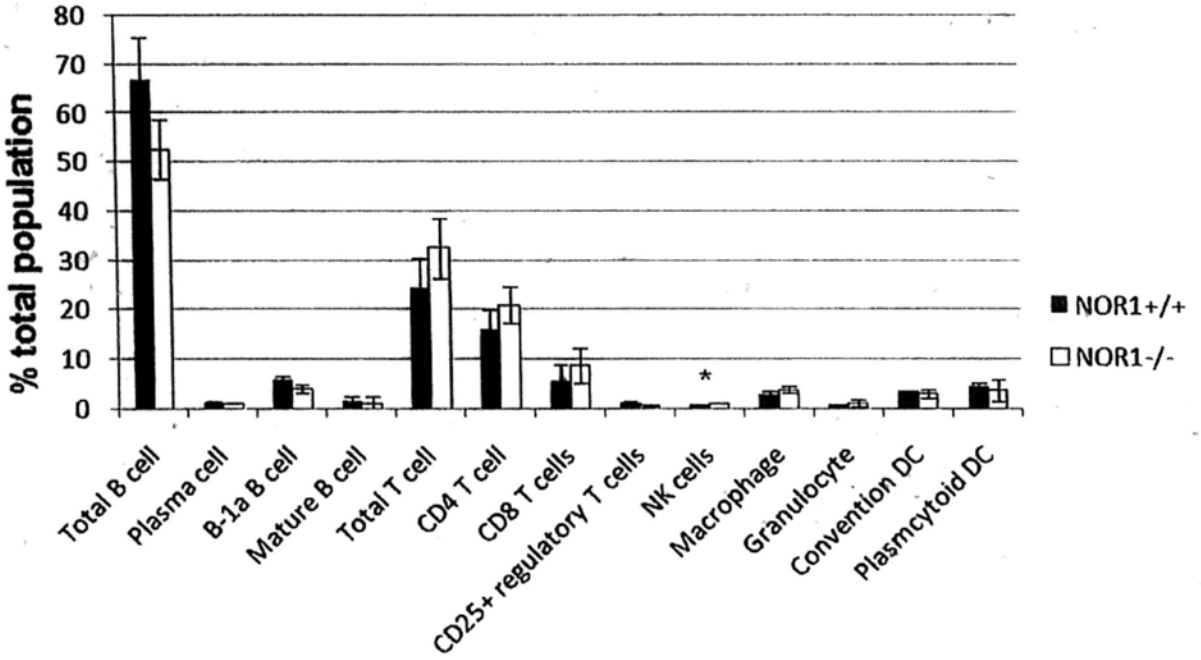


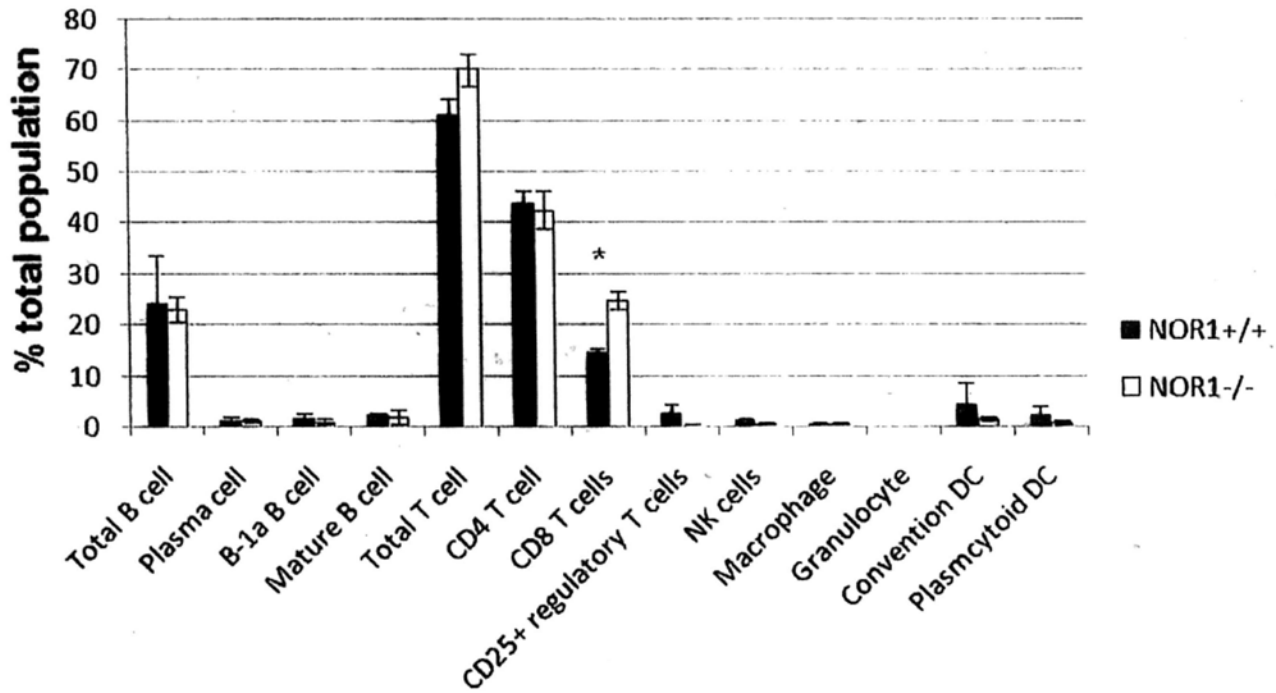
Figure 3.3 Weight and total cell numbers of the spleen, thymus and lymph nodes of NOR1$^{+/+}$ and NOR1$^{-/-}$ mice.

NOR1$^{+/+}$ and NOR1$^{-/-}$ mice at 6-8 week old were euthanized and their spleens, Thymuses and lymph nodes were obtained. Bars show mean values ± SEM of weight (A, B and C) and cell numbers (D, E, and F) of the indicated organs from three independent experiments. *: $p<0.05$.

A


% total population
80
70
60
50
40
30
20
10
0
*
NOR1+/+
NOR1-/-
Total B cell
Plasma cell
B-1a B cell
Mature B cell
Total T cell
CD4 T cell
CD8 T cells
CD25+ regulatory T cells
NK cells
Macrophage
Granulocyte
Convention DC
Plasmcytoid DC

B


% total population
80
70
60
50
40
30
20
10
0
*
NOR1+/+
NOR1-/-
Total B cell
Plasma cell
B-1a B cell
Mature B cell
Total T cell
CD4 T cell
CD8 T cells
CD25+ regulatory T cells
NK cells
Macrophage
Granulocyte
Convention DC
Plasmcytoid DC

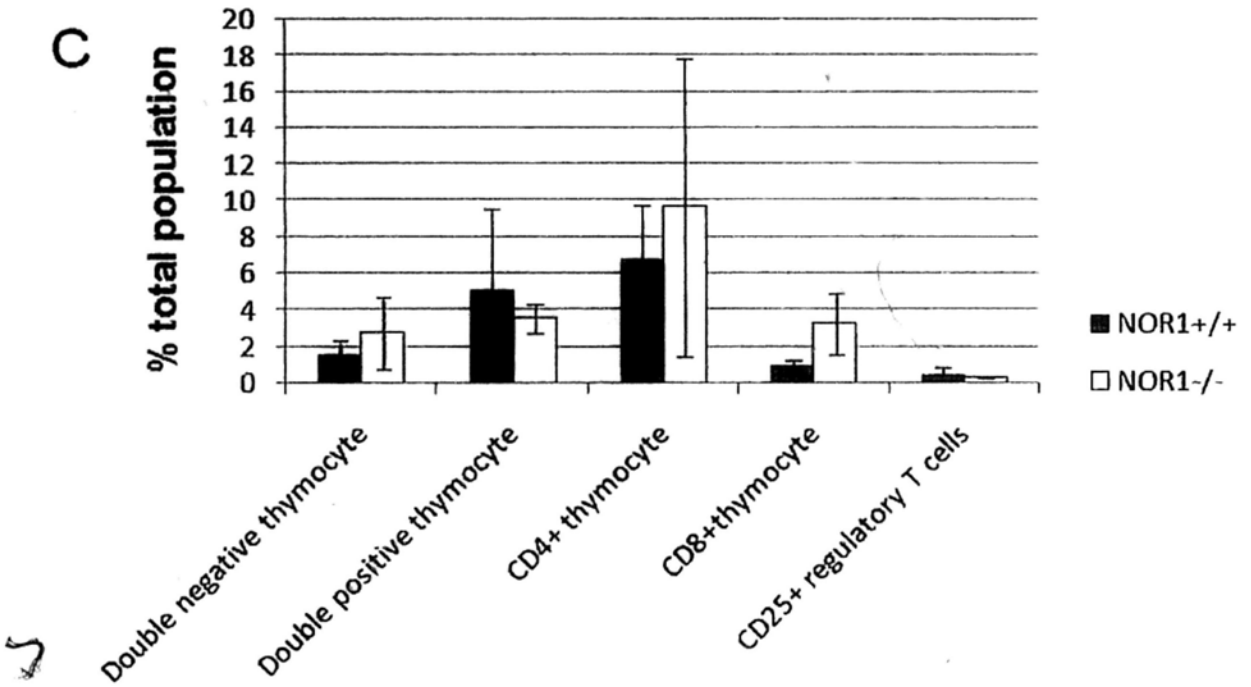


Figure 3.4 Percentages of immune cell types in the spleens, lymph nodes and Thymuses of NOR1$^{+/+}$ and NOR1$^{-/-}$ mice.

Spleens (A), lymph nodes (B) and Thymuses (C) were obtained from NOR1$^{+/+}$ and NOR1$^{-/-}$ mice at 6-8 week old and total organ cells were purified. Their cell surface markers were stained with fluorescence-conjugated antibodies and percentages of each cell type were analyzed with the flow cytometer. Bars indicate mean percentages ± SEM of three independent experiments. *: $p<0.05$.

3.3.2 NOR1$^{-/-}$ mice demonstrated altered cytokine response against Poly IC injection

To compare the inflammatory response induced by TLR3 activation, Poly IC was injected into the peritoneal cavity of NOR1$^{+/+}$ and NOR1$^{-/-}$ mice. Early time points were employed in this experiment to monitor the primary response of TLR3 activation, which could avoid noise from the secondary/positive feedback effects of primarily secreted cytokines (Longhi, Trumpfheller et al. 2009). Sera at 3 h and 6 h post-stimulation were collected and concentration of the representative Th1 cytokines (IFNγ and IL-12), Th2 cytokines (IL-10), inflammatory cytokines (TNFα) and type I IFNs (IFNβ) were quantified (Figure 3.5). IFNγ is mainly secreted from T cells under the influence of DCs and macrophages, while majority of IL-12, IL-10, TNFα and IFNβ are produced by DCs and macrophages (Schroder, Hertzog et al. 2004; Matsumoto and Seya 2008).

Intraperitoneal injection of Poly IC increased serum levels of IFNγ in both animals, but its peak level was >1000-times lower in NOR1$^{-/-}$ mice than in NOR1$^{+/+}$ mice at both time points (Figure 3.5A). Serum levels of TNFα demonstrated 40% and 80% lower increases in NOR$^{-/-}$ mice than in NOR1$^{+/+}$ mice at 3 h and 6 h time-point, respectively (Figure 3.5C). Secretion of IL-10 was detected in NOR1$^{-/-}$ mice but not in NOR1$^{+/+}$ mice at 3 h time-point (around 30 pg/ml), while reduced secretion of this cytokine was observed in NOR1$^{-/-}$ mice at 6 h time-point (Figure 3.5B). NOR1$^{-/-}$ and NOR$^{+/+}$ mice demonstrated similar serum levels of IL-12 p70 at the two time-points examined (Figure 3.5A). IFNβ showed no difference between these two mouse groups (Figure 3.5D).

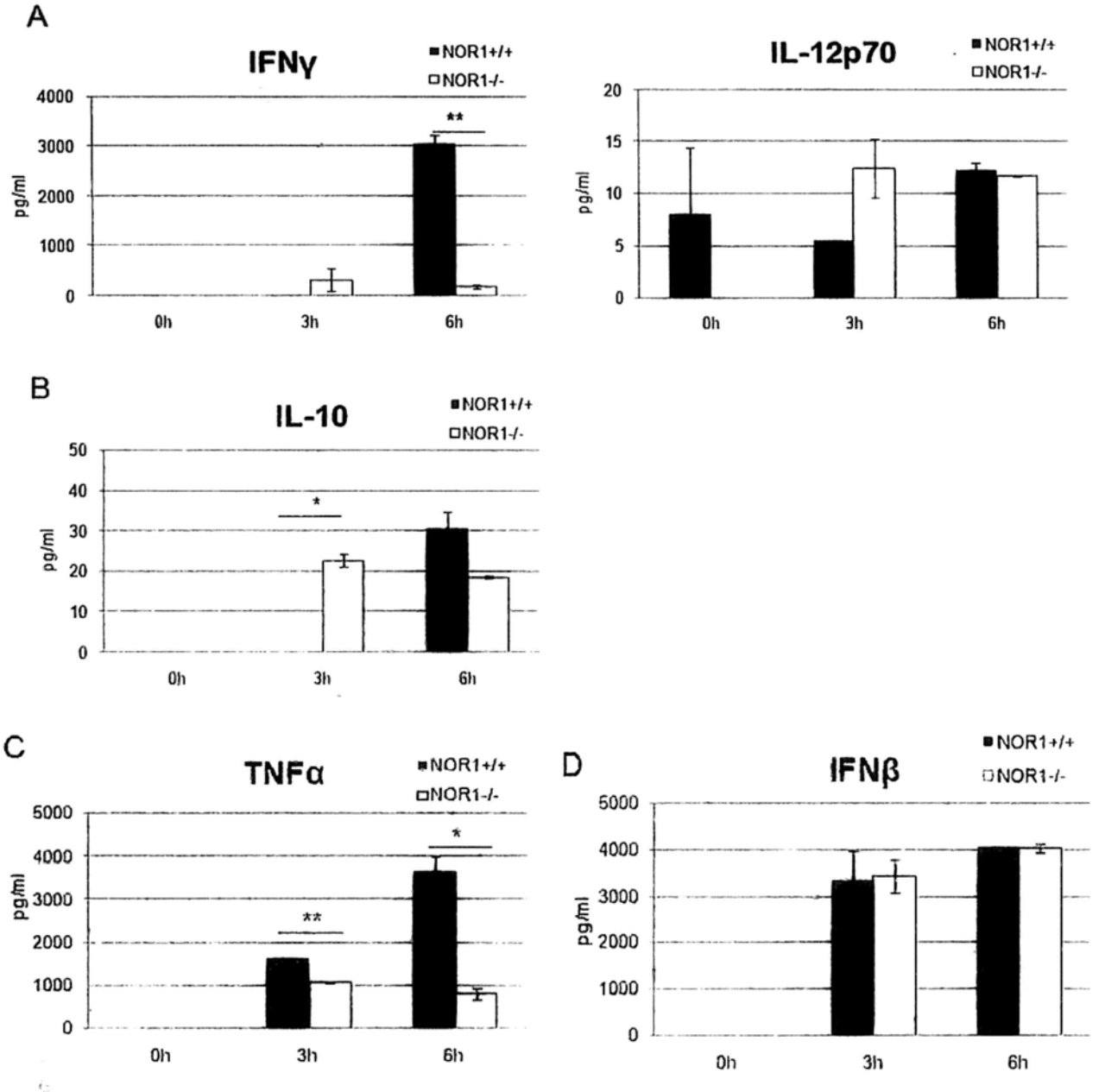


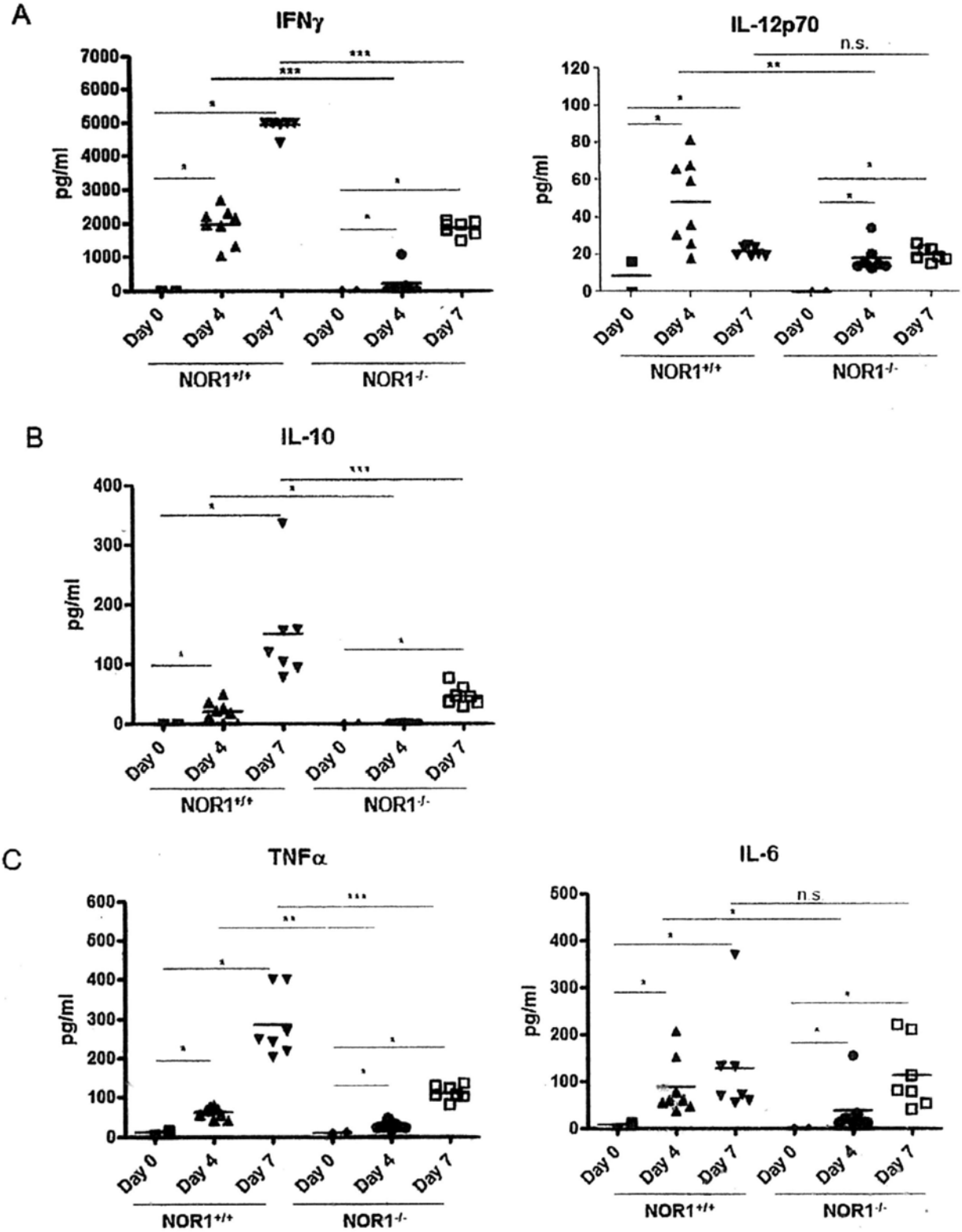
Figure 3.5 Serum cytokine levels in NOR1$^{+/+}$ and NOR1$^{-/-}$ mice injected with Poly IC.

NOR1$^{+/+}$ and NOR1$^{-/-}$ mice were injected with the TLR3 ligand Poly IC (100 μg/mice) and serum concentrations of Th1 cytokines: IFNγ and IL-12 (A), Th2 cytokine: IL-10 (B), inflammatory cytokines: TNFα (C), type I interferon: IFNβ (D) were measured at 0, 3 and 6 h after the injection. Bars represent mean values ± SEM obtained from three independent experiments. *: $p<0.05$, **: $p<0.01$.

3.3.3 NOR1$^{-/-}$ mice demonstrated attenuated cytokine production against *T. Gondii* infection

Because NOR1$^{-/-}$ mice had reduced production on several cytokines in response to the synthetic TLR3 ligand Poly IC, their response against live infectious agents were investigated. For this purpose, an infection model, the intracellular protozoa *T. Gondii,* was employed and the time frame for analysis was extended to 7 days. Unlike synthetic ligand that can directly bind to TLRs and activate downstream signaling pathways, live pathogens need to be detected and processed by antigen-presenting cells before presenting to downstream effector cells. During the 7-day infection course, all NOR1$^{+/+}$ and NOR1$^{-/-}$ mice survived and appeared active. In two time-points (day 4 and 7), blood samples from infected mice were collected and representative cytokines were quantified (Figure 3.6).

In all NOR1$^{+/+}$ and NOR1$^{-/-}$ mice examined, *T. Gondii* significantly upregulated serum levels of measured cytokines (Figure 3.6). Levels of IFNγ, TNFα and CCL2 after infection with *T. Gondii* were significantly lower in NOR1$^{-/-}$ mice than in NOR1$^{+/+}$ mice at day 4 and day 7 (Figure 3.6A, C & D). Levels of IL-12 and IL-6 were significantly lower in NOR1$^{-/-}$ mice than in NOR1$^{+/+}$ mice at day 4 but not at day 7, suggesting that NOR1 is effective at the earlier time period for these cytokines (Figure 3.6A & C). Peak levels of IFNβ after infection with *T. Gondii* (~300 pg/ml) were much lower than that induced by Poly IC (~5000 pg/ml), consistent with a previous report indicating that IFNβ is not the major IFN against *T. Gondii.* (Bhopale 2003). In contrast, serum levels of IFNγ (2000-5000 pg/ml; Figure 3.5D & 3.6E) was much higher than that of IFNβ, confirming its central role in the immune response against this protozoan (Bhopale 2003). Levels of IFNβ after infection with this pathogen did not show obvious difference between the two animal groups.


A
IFNγ
pg/ml
7000
6000
6000
4000
3000
2000
1000
0
***
***
*
*
*
*
Day 0
Day 4
Day 7
Day 0
Day 4
Day 7
NOR1+/+
NOR1-/-
IL-12p70
n.s.
**
120
100
80
60
40
20
0
pg/ml
B
IL-10
400
300
200
100
0
pg/ml
C
TNFα
600
600
400
300
200
100
0
pg/ml
IL-6
n.s
500
400
300
200
100
0
pg/ml

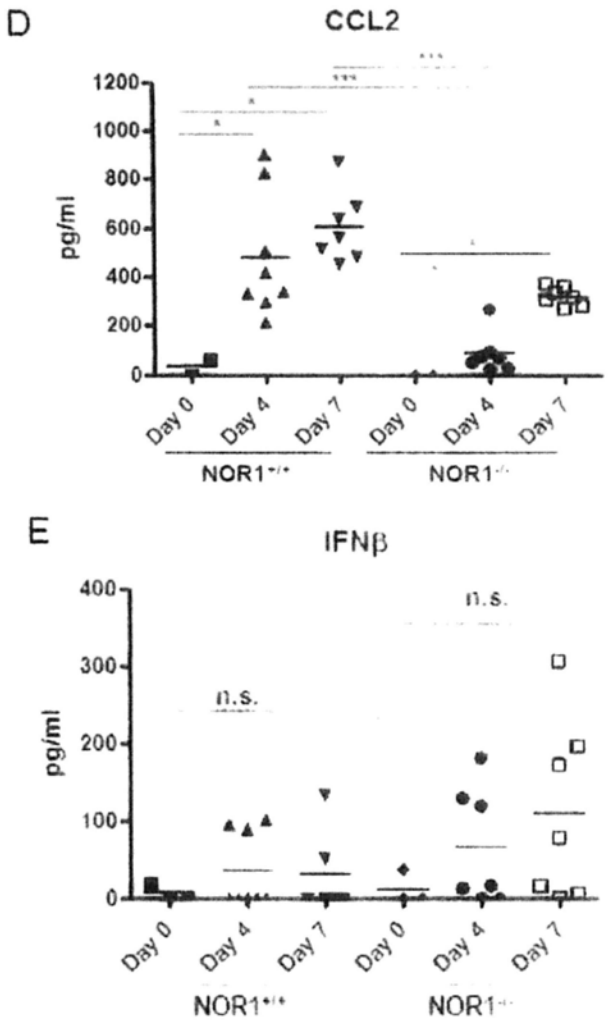


Figure 3.6 Serum levels of several cytokines in NOR1$^{+/+}$ and NOR1$^{-/-}$ mice infected with *T. Gondii*.

NOR1$^{+/+}$ and NOR1$^{-/-}$ mice were infected with *T. Gondii* (20 cysts/mice) and were monitored for 7 days. Serum concentrations of Th1 cytokines: IFNγ and IL-12 (A), Th2 cytokine: IL-10 (B), inflammatory cytokines: IL-6 and TNFα (C), chemokine: CCL2 (D) and type 1 interferon: IFNβ (E) were measured at Day 4 and Day 7. Each symbol represents the value from one animal out of 8 NOR1$^{+/+}$ and 7 NOR1$^{-/-}$ mice used in this experiment. Lines indicate mean values. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, n.s.: No statistical significance.

3.4 Discussion

This study aimed to compare the *in vivo* immune response, particularly cytokine production, between NOR1$^{-/-}$ mice and wild type mice. Among the immune organs examined, spleens exhibited significant alteration in morphology and cell composition between these animal groups. Spleen is the largest secondary lymphoid organ in mice, which contains one-fourth of the total body lymphocytes, and acts as a surveillance organ to detect circulating pathogens and to initiate immune response (Cesta 2006). Enlargement of spleen and widened red pulps area together with reduced white pulp area may indicate that NOR1 influences development of spleen during the fetal to adolescent stage and certain steps of hematopoiesis. Indeed, a previous report demonstrated translocation and deletion of the *NOR1* gene in leukemic cells from patients with acute myelogenous leukemia (Mullican, Zhang et al. 2007).

Consistently both in the TLR3 stimulation and the *T. Gondii* infection models, NOR1$^{-/-}$ mice exhibited attenuated induction of TNFα and IFNγ. DCs, macrophages and monocytes are the major sources of TNFα, and this cytokine is essential for full activation of the immune system upon pathogen infection (Trevejo, Marino et al. 2001; Bhopale 2003). For instance, phagocytosis and antigen presentation by DCs and macrophages are highly dependent on TNFα (Aderem and Underhill 1999; Trevejo, Marino et al. 2001). In the *T. Gondii* infection model, NOR1$^{-/-}$ mice demonstrated reduced production of IL-12 at day 4. This Th1 cytokine is essential to stimulate T cells and NK cells for their secretion of IFNγ, which play a pivotal role in the activation of the acquired immunity against *T. Gondii* (Aliberti, Jankovic et al. 2004). Further, NOR1$^{-/-}$ mice demonstrated reduced induction of IL-10, which is also produced mainly by DCs (Samarasinghe, Tailor et al. 2006). Although DCs are not the main producer of CCL2, they are attracted to the infected sites through this chemokine and they are also capable to secrete this

cytokine (Steinman 1991; Baggiolini, Dewald et al. 1997). Therefore, these results suggest that NOR1 plays a key role in induction of these inflammatory cytokines in DCs upon pathogen infection and subsequent activation of innate immunity.

Knockout of *NOR1* did not influence induction of IFNβ by Poly IC and *T. Gondii*, indicating that NOR1 has functional specificity for TNFα, IL-12 and other DC-related cytokines, but not for this type I IFN, which is also secreted from DCs.

Activation of TLR results in stimulation of downstream transcription factors, such as NF-κB, IRF3 and IRF7, which in turn activate the transcription of inflammatory cytokines, type I interferons and chemokines, such as TNFα, IL-12, IFNβ and CCL2 (Kawai and Akira 2009). Further cell-based experiments focusing on the specific regulatory mechanisms for induction of these molecules in DCs could reveal cellular roles and molecular mechanisms of NOR1 in the TLR-mediated signaling pathways and in the anti-pathogen immunity organized by these cells.

In conclusion, this part of my study showed that induction of DC-related cytokines was attenuated in NOR1$^{-/-}$ mice injected with TLR3 ligand Poly IC or infected with *T. Gondii*. These results indicate that the orphan receptor NOR1 is essential for anti-pathogen response of DCs. Examination for elucidating NOR1-directed molecular targets and signaling pathways is now under progress. I believe that such on-going research will provide important information on anti-pathogen immunity organized by DCs.

Chapter 4

Glucocorticoids Potently Enhance Virus-induced Interleukin-10 Production in Dendritic Cells

4.1 Introduction

Glucocorticoid hormones (GCs) are end-products of the hypothalamic-pituitary-adrenal (HPA) axis, secreted in a circadian fashion from the adrenal cortex under the regulation of upper centers, the brain hypothalamus and the pituitary gland. They are also secreted tremendously into circulation once individuals face physical, mental and/or psychological stress (Sapolsky, Romero et al. 2000). Under these stressed conditions, several physiological activities, including those regulating levels of blood glucose, fluid tonicity and blood pressure, are modulated, so that our bodies can be adjusted appropriately to cope (Sapolsky, Romero et al. 2000). Structural components of bacteria and viruses (such as bacterial cell wall-associated LPS and viral capsid proteins), as well as cytokines and inflammatory mediators released from the infected tissues are potent activators of the HPA axis, stimulating the brain hypothalamus and pituitary corticotrophs, and subsequently increasing the secretion of GCs from the adrenal cortex (Kino and Chrousos 2007). Secreted GCs then subside inflammation in the local tissues by suppressing various components of the immune system, functioning as a counter regulator for otherwise overshooting immune response (Chrousos and Kino 2005). It has long been reported that activation of the HPA axis by mental and/or physical stress and subsequent increase of circulating GCs are associated with increased susceptibility to infectious diseases, and prolong and worsen disease

course (Godbout and Glaser 2006). The underlying mechanism(s) of this well-known phenomenon, however, has (have) not been elucidated as yet.

Because of the potent and efficient suppressive effects of GCs on the immune system, natural and synthetic GCs are widely used in the treatment of allergic/autoimmune/inflammatory diseases, acute sepsis and shock, and in prevention of organ rejection (Boumpas, Chrousos et al. 1993). It is achieved by the dual effects of GCs on the immune system: GCs suppress the cellular immunity by reducing the production of T-helper 1 cytokines (such as the IL-12, TNFα and IFNγ). GCs on the other hand stimulate the humoral immunity and Th2-oriented anti-inflammatory cytokines (including IL-10, IL-4 and the transforming growth factor β) (Elenkov and Chrousos 1999; Elenkov 2004).

At the molecular levels, most of the known anti-inflammatory actions of GCs are mediated by the glucocorticoid receptor (GR), which belongs to the nuclear hormone receptor family (Lu, Wardell et al. 2006). According to Chapter 2 of this study, mRNA levels of GR were up-regulated in virus-infected DCs. Although DCs are most well-known in secreting type I IFNs and IL-12, they also produce anti-inflammatory cytokines including IL-10 upon pathogen infection (Samarasinghe, Tailor et al. 2006). Indeed, DCs are the major source of IL-10: They secrete IL-10 in the late phase of infection (peak production at around 24-48 hours) in contrast to the pro-inflammatory cytokines whose peak occurs in the early phase (few hours after the infection) (Ejrnaes, Filippi et al. 2006; Samarasinghe, Tailor et al. 2006; Medzhitov 2010). This characteristic cytokine secretory pattern indicates that DCs promote inflammation resolution through production of IL-10, in addition to stimulating the proinflammatory response, as excessive inflammation is harmful and detrimental (Steinman and Hemmi 2006).

Crosstalk between GR and TLRs is regarded as the major mechanism for mediating the immunosuppressive function of GCs in DCs. It is known that ligand-activated GR prevents the TLR-mediated phosphorylation of IRF3 and suppresses expression of the NF-κB-responsive genes (De Bosscher, Vanden Berghe et al. 2006; Reily, Pantoja et al. 2006), but exact mechanisms of how GR influences the TLR signaling pathways in DCs is still largely unknown. Due to the pivotal and indispensible role of DCs in anti-pathogen immunity, this part of my research aims to study how stress or GCs modulate the expression of TLR themselves, their signaling-intermediate molecules and target genes. Dexamethasone (DEX), one of the synthetic GCs was used to stimulate GR in bone marrow-derived DCs to mimic the elevated levels of circulating GCs observed in stressed conditions. Among the genes modulated by DEX in virus-infected DCs, I focused on the anti-inflammatory cytokine IL-10 as it demonstrated synergistic induction under viral infection and DEX treatment. I further examined the detailed time course of its synergistic secretion, and the possible signaling mechanism underlining such synergism. The described effect may explain in part the well-known phenomenon that individuals under stress have increased susceptibility to viral infection (Kino and Chrousos 2007).

4.2 Materials and Methods

4.2.1 Mice & DC culture

Male and female C57BL/6 mice at 6–8 week old were used as sources of the bone marrow-derived hematopoietic cells to generate mouse bone marrow-derived DCs *in vitro*. DCs were generated by culturing bone marrow cells in the presence of the FLT3L for 8 days, as described in Chapter 2.

4.2.2 Infection/stimulation of DCs

DCs (1 x 10^6 cells) were pre-treated with DEX (10^{-6} to 10^{-8} M; Sigma Aldrich St. Louis, MO), corticosterone (10^{-6} M; Sigma Aldrich) for 30 min. In some experiments, DCs were also treated with RU 486 (10^{-5} to 10^{-7} M), the specific ERK inhibitor U0129 (0.1 M to 10 M, EMD, Darmstadt, Germany), the specific p38 MAPK inhibitor SB203580 (10 μM, EMD) and MT4 (10 μM, EMD), the PKA inhibitor H-89 (12.5 nM, EMD) for 30 min prior to steroid treatment. Steroid/compound-treated cells were then incubated with NDV (Hertz strain, MOI=10) at 37°C for indicated time periods. Viability of DCs after the above treatments verified by trypan blue staining was always over 95%.

4.2.3 RNA Isolation and qPCR

Total RNA extraction, cDNA synthesis, qPCR and data calculation were described in Chapter 2. Primers were listed in Supplementary Table 1.

4.2.4 cDNA PCR array

DCs were treated with or without DEX (10^{-6} M) for 30 min before infected by NDV (MOI=10) for 6 h. Total RNA was isolated and 380 ng of the RNA was converted to cDNA for

PCR array quantification. The RT^2 Profile TLR signaling PCR Array (SA Bioscience, Frederick, MD) was used to examine simultaneously the mRNA levels of 89 genes including those associated with the TLR signaling pathways and five housekeeping genes (*Gusb, Hprt1, Hspcb, Gapdh* and *Actb*) in 96-well plates according to the protocol of the manufacturer. Each plate contained negative controls, such as those obtained in the absence of reverse transcription reaction or addition of template. Obtained C_t values were normalized by those of the five housekeeping genes, and fold changes were calculated using the comparative C_t method ($2^{-\Delta\Delta Ct}$ where $\Delta\Delta C_t = \Delta C_{t,sample} - \Delta C_{t,reference}$). The experiments were performed in triplicate.

4.2.5 ELISA

Media used for the culture of DCs (1×10^6 cells) under the specified treatments were collected at indicated time points. They were kept in -80 °C until measurement. Levels of IL-10 in the media were determined by using the murine IL-10 ELISA kit (R&D Systems Inc., Minneapolis, MN).

4.2.6 Preparation of cell extracts and immunoblot

DCs (1×10^7 cells) were stimulated with various compounds for indicated time periods. Whole cell extracts were prepared by using the nuclear protein extraction kit (Active Motif, Carlsbad, CA) according to manufacturer's instruction. Whole cell extracts (5 μg) were run on 8-12 % NuPAGE Bis-Tris gels (Invitrogen), transferred to nitrocellulose membranes and were immunoblotted with mouse anti-GR antibody (Abcam, Cambridge, CT) followed by treatment with the anti-rabbit horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnologies, Inc.).

4.2.7 Statistical analysis

The Student's t test was performed with the GraphPad Prism 5 software (GraphPad Inc, La Jolla, CA). *P*-Values less than 0.05 were considered as statistically significant.

4.3 Results

4.3.1 DEX modulates viral infection-induced mRNA expression of the molecules associated with the TLR signaling pathways in DCs

To study the effect of GCs on viral infection-induced alteration of DC activities, DCs were pre-treated with DEX and infected with NDV. The mRNA expression of the 89 target genes known to participate in the TLR signaling pathways were quantitatively examined by using the multiplex TLR pathway qPCR array plates. The genes included the TLRs (TLR1-10), TLR adaptor proteins (such as MyD88 and Tollip), downstream intermediate molecules (such as Irak1 and TAK1), effector molecules and the transcription factors (c-Jun N-terminal kinases (JNK), p38 mitogen-activated protein kinase (MAPK), components of the NF-κB and the IRFs), and target genes (IL-1β, IL-6, IL-10, IFNβ and CXCL10).

Twenty-six genes were found significantly modulated their expression after treatment. Genes were divided into three groups according to their responsiveness to DEX alone, NDV alone or combined treatment (Table 4.1, supplementary Table 2 for statistic values): Group 1 contains genes whose mRNA expression after NDV infection were either synergistically upregulated or diminished by DEX pre-treatment (*Il10*, *Clec4e, Ptgs*, *Nfkbib* and *Ifng*). Group 2 included the genes that responded to DEX but not to NDV infection (e.g., *Il1b*). Group 3 composed of the genes responsive to NDV but not to DEX pre-treatment (e.g., *Cd86* and *Ccl2*). The modulatory effect DEX on the four genes of Group 1 was verified by qPCRs with newly designed primers (Figure 4.1). In this re-constituted qPCRs, pre-treatment of DEX enhanced NDV-induced mRNA expression of *Il10* and *Clec4e* in DCs (Figure 4.1A), while suppressed the induction of *Ptgs* and *Ifng* mRNAs (Figure 4.1B).

Among the genes in Group 1, *Clec4e* is a lectin family PRR for recognizing carbohydrate antigens and mediates cell-cell interaction in antigen-presenting cells, such as macrophages and DCs (Matsumoto, Tanaka et al. 1999; Dam and Brewer 2010). *Ptgs2*, also named as the cyclooxygenase-2 (*Cox-2*) gene, expresses the rate-limiting enzyme for prostaglandin synthesis, which is essential for the maturation of DCs and their functions (Luft, Jefford et al. 2002; Jung, Jeong et al. 2010). IL-10 and IFNγ are the cytokines, which play important roles in the innate immunity (Bowie and Unterholzner 2008). Indeed, DCs are the major sources of IL-10 during viral infection (Murray 2006). DCs are also the prime targets of IL-10, as they express high amounts of the IL-10 receptor (Murray 2006). In contrast, majority of IFNγ is produced by the natural killer cells and T-cells, thus the contribution of DCs to its production is limited (Schroder, Hertzog et al. 2004). Because of a critical and major role of IL-10 in DC-mediated immune response, we further evaluated the details of synergistic induction of IL-10 by DEX and NDV and its underlying mechanism(s).

Table 4.1 DEX pre-treatment and NDV infection modulate mRNA expression of the TLR signaling-associated molecules in DCs.

Genes	Symbols	Treatment		
		DEX	NDV	DEX+NDV
Group 1: NDV responsive genes modulated by DEX pre-treatment				
(i) Up-regulated by DEX				
Interleukin 10	*Il10*	10.00	5.09	76.32
C-type lectin domain family 4 member e	*Clec4e*	14.90	15.03	41.41
(i) Suppressed by DEX				
Lymphotoxin A	*Lta*	-3.17	545.51	288.14
Interferon γ	*Ifng*	1.94	1453.69	77.38
Interleukin 6	*Il6*	-0.46	114.39	52.35
Prostaglandin-endoperoxide synthase 2	*Ptgs2*	-0.49	20.39	4.89
Group 2: Responsive gene of DEX pre-treatment				
Interleukin 1β	*Il1b*	-84.36	1.74	-14.39
Group 3: Responsive genes of NDV infection				
Interferon β1	*Ifnb1*	2.33	55873.05	37702.85
Chemokine (C-X-C motif) ligand 10	*Cxcl10*	-16.44	1336.89	1421.76
Heat shock protein 1A	*Hspa1a*	1.48	212.97	124.53
CD86 antigen	*Cd86*	0.41	18.03	16.27
Chemokine (C-C motif) ligand 2	*Ccl2*	-0.65	19.63	15.35
Interleukin 1α	*Il1a*	-0.25	21.31	14.02
Tumor necrosis factor	*Tnf*	-2.18	18.41	12.72
CD80 antigen	*Cd80*	-1.10	19.41	12.10
Tumor necrosis factor αinduced protein 3	*Tnfaip3*	-0.51	9.85	11.35
Eukaryotic translation initiation factor 2α kinase 2	*Eif2ak2*	-1.59	10.36	9.99
Nuclear factor of κ light chain gene enhancer in B-cells inhibitor, α	*Nfkbia*	3.24	6.10	8.22
Pellino 1	*Peli1*	-1.38	7.64	8.07
Interferon regulatory factor 1	*Irf1*	-1.86	8.43	6.96
Receptor (TNFRSF)-interacting serine-threonine kinase 2	*Ripk2*	-0.41	5.79	6.03
TANK-binding kinase 1	*Tbk1*	-1.64	5.40	5.78
Reticuloendotheliosis oncogene	*Rel*	-1.74	5.75	3.61
Interleukin 6 receptor α	*Il6ra*	2.11	-8.43	-3.33
ELK1, member of ETS oncogene family	*Elk1*	-0.62	-5.02	-4.16
Interleukin-1 receptor-associated kinase 1	*Irak1*	-2.11	-6.18	-4.89

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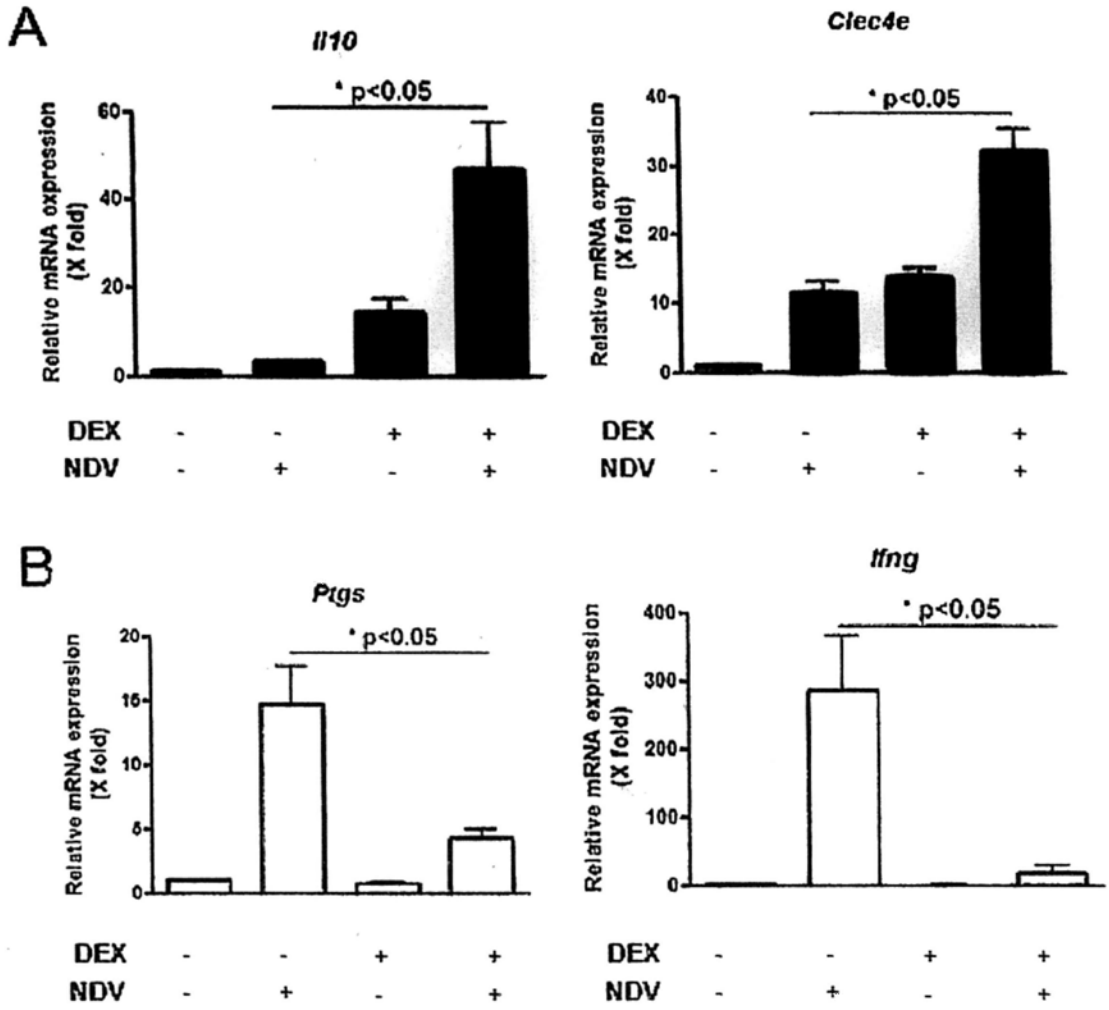

Figure 4.1 Verification of DEX-regulated genes in virus-infected DCs with reconstituted qPCR.

DCs were pre-treated with or without DEX (10^{-6} M) for 30 min, and were infected with NDV (MOI=10) for 6 h. The mRNA levels of the genes synergistically up-regulated by DEX and viral infection (A) and the genes suppressed by DEX (B) are shown. Data were normalized with Ct values of *Gapdh* and fold changes were calculated by comparing with untreated/uninfected control. Bars represent mean values ± SEM of fold changes for IL-10 mRNA expression obtained from three independent experiments. * $p < 0.05$, ** $p < 0.01$ comparing NDV infection alone with DEX+NDV.

4.3.2 DEX-induced synergistic induction of IL-10 in DCs: Time course, protein expression and contribution of GR

I first examined the time course of DEX- and NDV-induced synergistic effect on the mRNA expression of IL-10 in DCs. The effect was observed from 3 h after infection with NDV and sustained for 24 h (Figure 4.2A, left panel). The IL-10 secreted by DCs was measured in media with ELISA at three time points. Results showed that DEX and NDV synergistically increased IL-10 concentrations both at 12 h and at 24 h of post-infection (Figure 4.2A, right panel). Increasing concentrations of DEX enhanced NDV-stimulated IL-10 mRNA and protein expression in a dose-dependent fashion (Figure 4.2B).

Corticosterone (CORT), a major endogenous GC in mice, developed a synergistic effect with NDV on the IL-10 expression similar to DEX, although the effect of the former was less pronounced than that by the latter (Figure 4.2C). This could be explained by the difference in their potency of GR activation: CORT is ~20- to 30-times less active than DEX (Menconi, Gonnella et al. 2008). To examine if GR is required for this effect, DCs were treated with the GR receptor antagonist RU 486 prior to DEX treatment (Honer, Nam et al. 2003). RU 486 dose-dependently suppressed DEX-induced enhancement of IL-10 expression both at mRNA and protein levels (Figure 4.3A), indicating that GR mediates the synergistic effect of DEX and NDV on the IL-10 expression in DCs. Treatment of DCs with DEX and NDV did not increase GR protein levels, suggesting that the synergistic effect is not due to upregulation of GR expression (Figure 4.3B).

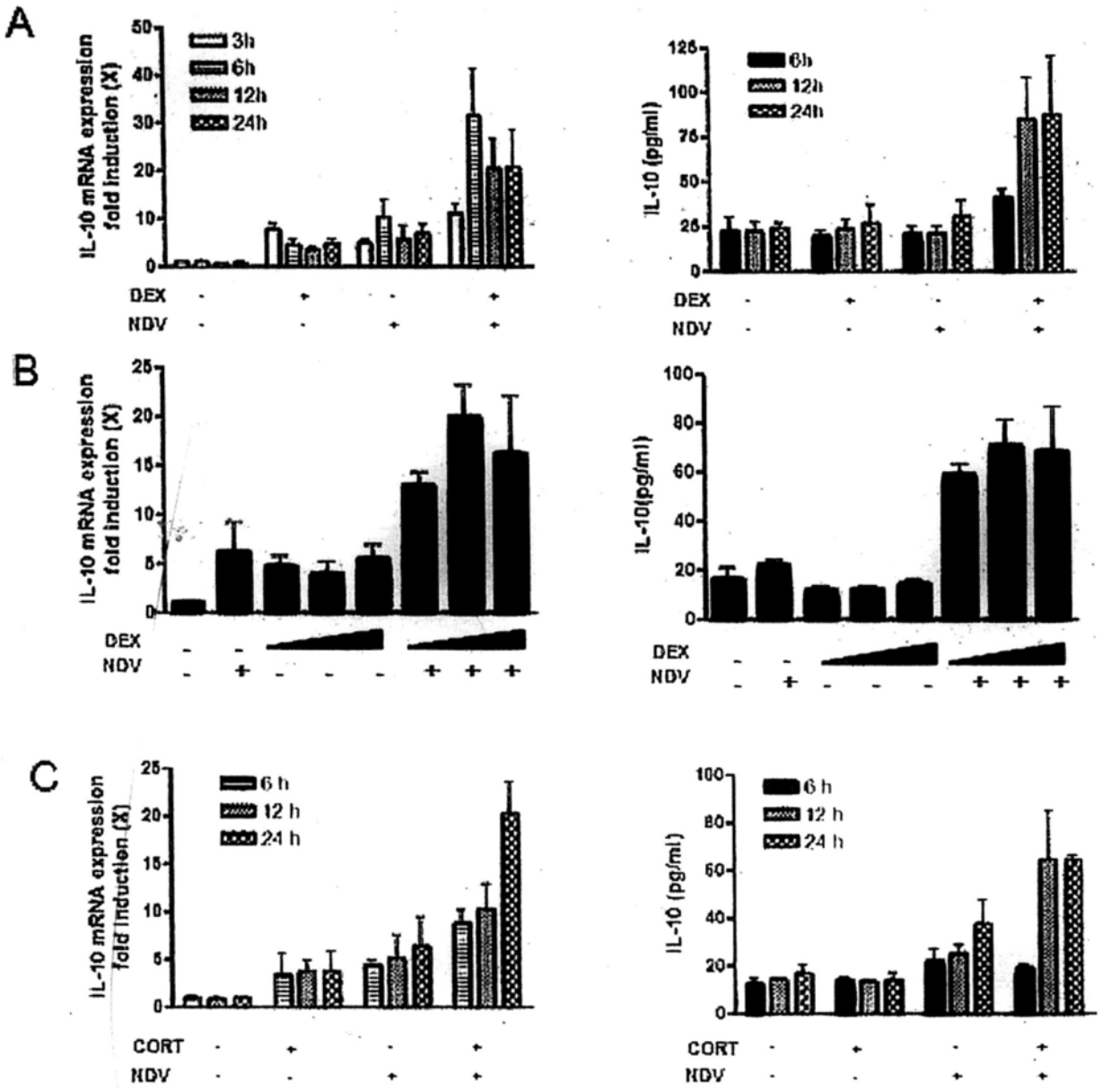

Figure 4.2 Time- and dose-dependent effects of DEX on IL-10 mRNA and protein expression in virus-infected DCs.

DCs were pre-treated with or without (A, B) DEX (10^{-6} to 10^{-8} M) or (C) CORT (10^{-6} M) for 30 min before NDV infection (MOI=10). mRNA expression of IL-10 and its concentrations in culture media were determined by qPCR and ELISA, respectively. Time-course and dose-dependent effects of DEX are shown in (A) and (B), respectively, while the effect of CORT on IL-10 mRNA expression (left column) and its protein concentrations (right column) are shown in (C). Bars represent mean values ± SEM of fold changes of IL-10 mRNA expression and protein concentrations obtained from three independent experiments.

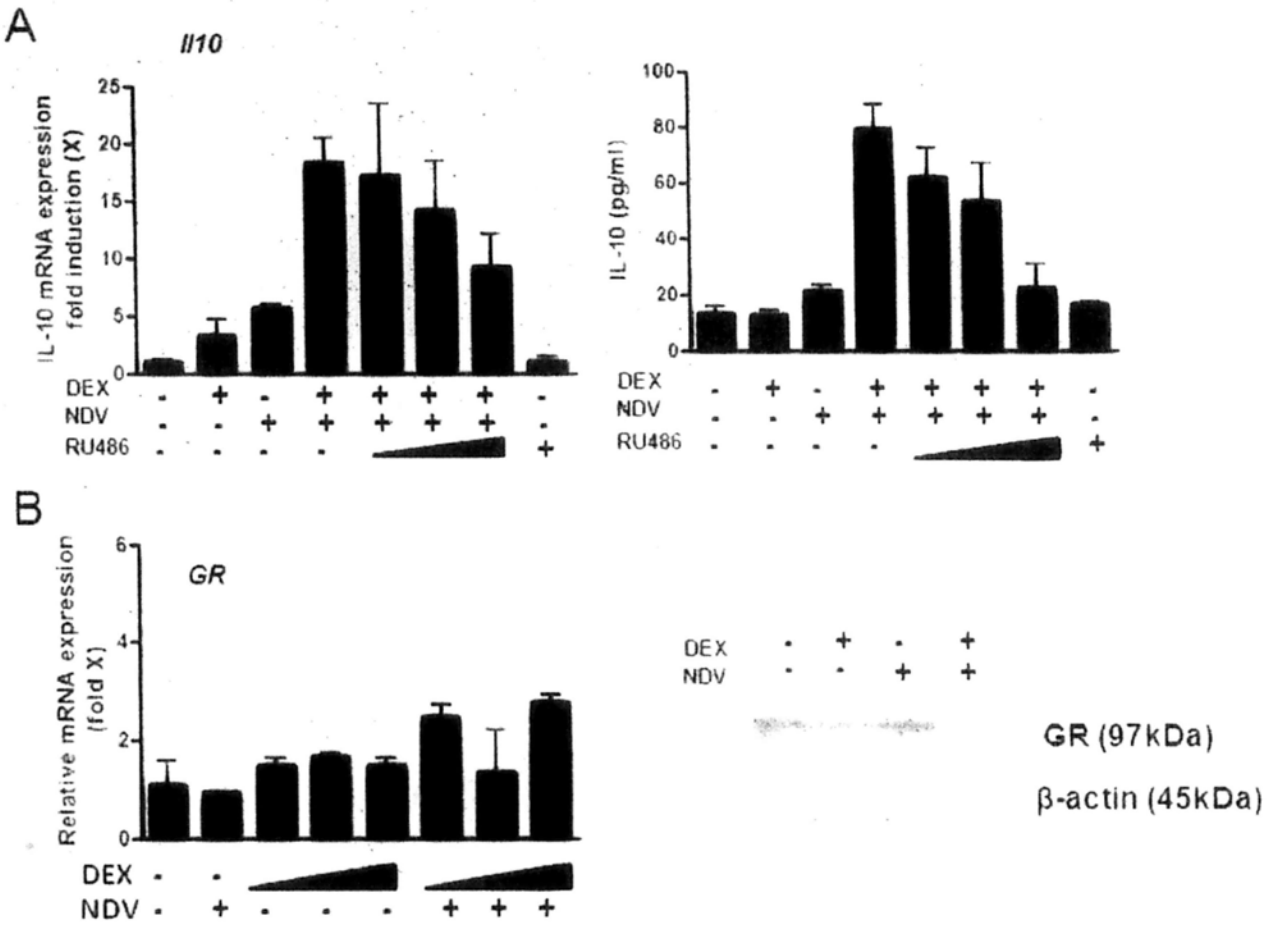

Figure 4.3 Synergistic IL-10 mRNA and protein expression caused by DEX treatment and viral infection is GR-dependent in DCs.

DCs were pre-treated with DEX (10^{-6} M) in the presence or absence of the GR antagonist RU 486 (10^{-5} to 10^{-7} M) for 30 min before NDV infection (MOI=10). (A) IL-10 mRNA expression (left column) and its protein concentrations (right column) in culture media are shown. (B) GR mRNA and protein levels were determined in DCs by qPCR and Western blot, respectively. Bars represent mean values ± SEM of fold changes of IL-10 mRNA expression or its protein concentrations. Data are obtained from three independent experiments.

4.3.3 ERK plays a key role in DEX-and NDV-induced synergistic IL-10 expression in DCs

Several distinct signaling pathways play important roles in the expression of IL-10 in various immune cells (Dillon, Agrawal et al. 2004; Park, Greten et al. 2005; Rogers, Slack et al. 2005; Goldsmith, Avni et al. 2009): These signaling pathways are represented by the PKA/Syk, p38 MAPK and ERK MAPK, and activation of these kinases converges in some part to the stimulation of the STAT3 to induce IL-10 transcription (Murray 2006). Thus, I examined the effect of chemical inhibitors for these kinases on DEX- and NDV-induced synergistic expression of IL-10 mRNA and protein in DCs. I found that U0126, a specific inhibitor for ERK1/2, almost abolished the synergistic effect, while the others failed to do so (Figure 4.4A). Dose-dependent inhibition of U0126 on the IL-10 expression was observed at mRNA and protein levels (Figure 4.4B). These results suggest that the cellular signaling pathway activated by DEX and NDV converges to this kinase for synergistically stimulating IL-10 expression in DCs.

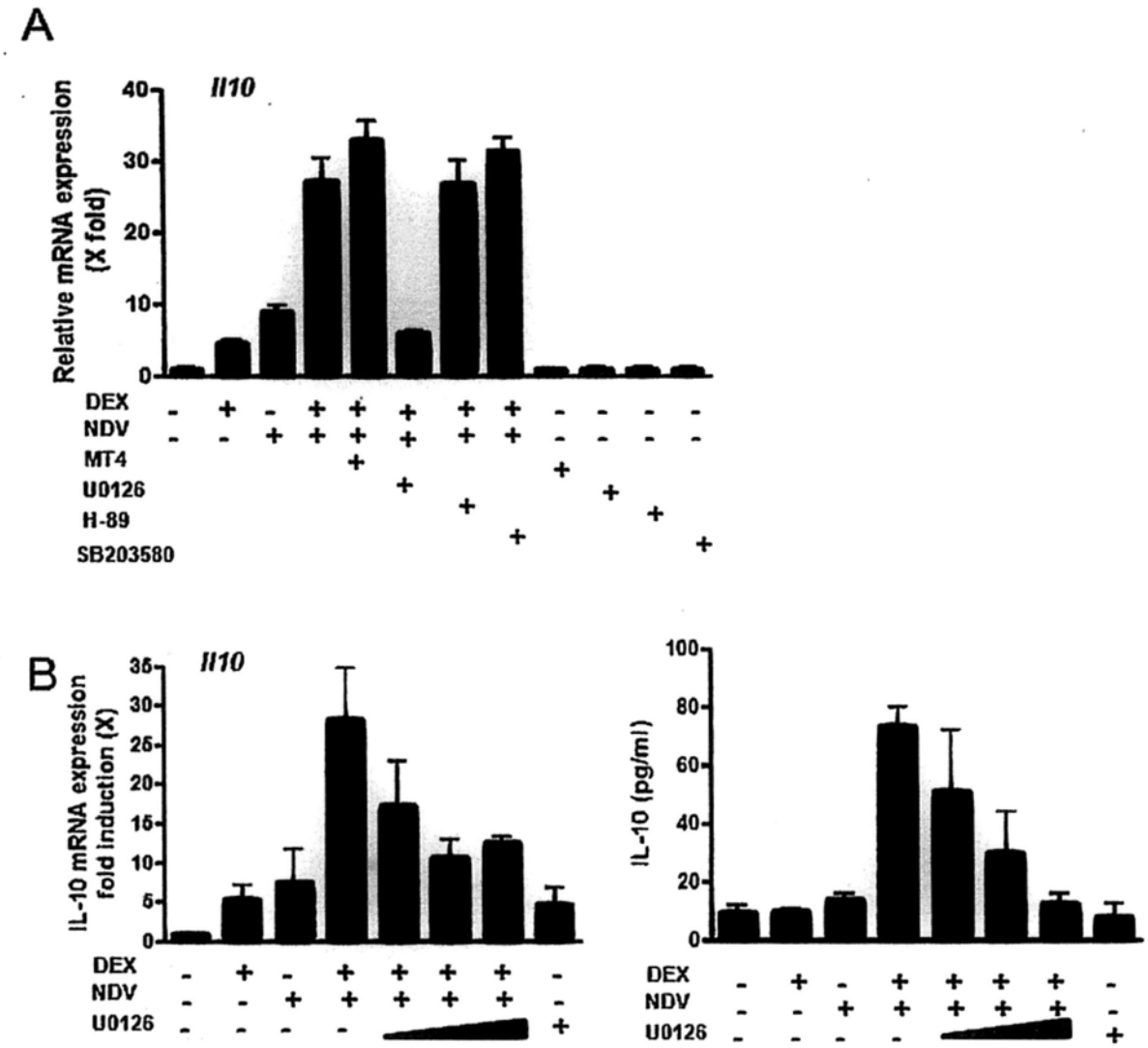

Figure 4.4 ERK1/2 play a key role in the synergistic induction of IL-10 mRNA and protein by DEX and NDV in DCs.

(A) DCs were pre-treated with or without indicated signaling inhibitors for 30 min (p38 inhibitors: MT4 (10 μM) and SB203850 (1 μM); ERK inhibitor: U0126 (0.1 M); PKA/Syk inhibitor: H-89 (12.5 nM)) before DEX (10^{-6} M) treatment and NDV infection (MOI=10). (B) DCs were treated with ERK inhibitor U0126 (0.1 M, 1M and 10M) for 30 min. DCs were then treated with DEX (10^{-6} M) for 30 min before NDV infection (MOI=10). IL-10 mRNA expression and its protein concentrations in culture media were determined by qPCR and ELISA, respectively. Bars represent mean values ± SEM of fold changes of IL-10 mRNA expression (left panel) and its protein concentrations (right panel) obtained from three independent experiments.

4.4 Discussion

In this study, I evaluated the effect of GCs on the mRNA expression of the 89 genes whose products play important roles in the TLR signaling pathways in mouse DCs infected with NDV. DEX pre-treatment enhanced or diminished the NDV-stimulated mRNA expression of four particular genes; they included the pathogen recognition receptor (*Clec1e*) and the enzyme important for prostaglandin synthesis (*Ptgs2*). Further, two TLR-target genes secreted from DCs, *Ifng* and *Il10*. I chose to focus on IL-10, as majority of this cytokine is secreted from DCs together with macrophages and B-cells, while it also has strong effects on DCs (Couper, Blount et al. 2008). DEX pre-treatment enhanced IL-10 protein production in addition to its mRNA expression in NDV-infected DCs: This effect of DEX persisted for 24 h after infection, and was mediated by GR. Interestingly, inhibition of ERK1/2 with its chemical inhibitor U0126 abolished DEX- and NDV-induced synergistic effect on the mRNA and protein expression of IL-10 in DCs.

The major function of IL-10 is to facilitate the resolution of inflammation caused by pro-inflammatory cytokines (Couper, Blount et al. 2008). This anti-inflammatory action of IL-10 is particularly important to prevent excessive and persistent immune activation, which would be detrimental to local inflammatory tissues (Medzhitov 2010). The levels and duration of IL-10 secretion are tightly regulated, as augmented and sustained increase of IL-10 production results in persistent infection, the worst case of which develops systemic anergy to viruses (Mege, Meghari et al. 2006). Indeed, persistent infection and reactivation of *M. tuberculosis* is associated with excessive production of IL-10 (Mege, Meghari et al. 2006). Further, some viral strains of the family of the herpes virus (including Epstein-Barr virus, poxvirus and cytomegalovirus) encode IL-10-like molecules, which share some immunosuppressive properties with the host IL-10, and increase infectivity of these viruses and induce latency of their infection

(Takayama, Tahara et al. 2001). Therefore, timely and appropriate production of IL-10 is indispensable for both resolution of infection and prevention of chronic infection of pathogens (Couper, Blount et al. 2008).

Although the current study did not examine the biological consequences of IL-10 synergistically increased by DEX and NDV infection, it is likely that viruses may increase their infectivity and pervasion to host tissues by potentiating production of IL-10 in DCs in the presence of GCs. Further, this cellular response against viral infection and GCs might underlie the clinical observation that mental/physical stress that elevates circulating GC levels increase susceptibility to viral infection and tendency to develop extended/pronounced disease course (Godbout and Glaser 2006). Of note, it is well known that psychological stress is involved in the induction and exacerbation of asthma where viral infection links to the development or worsening of its symptoms (Marshall and Agarwal 2000; Rosenkranz, Busse et al. 2005) (Mallia and Johnston 2006; Busse, Lemanske et al. 2010). The stress hormones induced systemic Th2 shift, which further induces and/or facilitates the Th2 condition typically observed in asthma (Agarwal and Marshall 1998; Xiang and Marshall 2011). Thus, these findings may reveal a new mechanism of how stress work in combination with viral infections and contribute to the pathogenesis of this airway disease.

My results demonstrated for the first time the synergistic effect of GCs and viral infection on the production of IL-10 in DCs. I found that ERK1/2 played a critical role in this synergism. GCs are known to regulate the ERK activity (Lowin, Straub et al. 2009; Newton, King et al. 2010; Yaniv, Lucki et al. 2010), while GCs promoted prolonged activation of ERK1/2 and increased phosphorylation of these molecules through activation of the adenosine receptor A3 through which GCs develop an anti-apoptotic effect in monocytes (Barczyk, Ehrchen et al. 2010).

GCs also activate ERK synergistically with norepinephrine in neuroblastoma cells, although exact signaling mechanisms for supporting this synergism were not demonstrated (Yaniv, Lucki et al. 2010). A previous report showed that viruses activate the ERK signaling, which is required for the production of inflammatory cytokines, such as IL-1 and IL-8 (Monick, Staber et al. 2001; Maggi, Moran et al. 2003; Monick, Cameron et al. 2005). Thus, it is likely that GCs and viral infection regulate the activity of ERK by targeting its upstream molecules, and synergistically stimulate the expression of IL-10 in DCs.

In conclusion, my results suggest a novel connection between viral infection and GCs on the production of IL-10 in DCs. Through this activity, viruses may increase their infectivity and invasion to organs/tissues of the individuals who have elevated levels of circulating GCs due to exposure to various stressors and/or therapeutic use of these hormones. In addition, this mechanism may also be involved in the pathophysiologic circuits contributing to the exacerbations of allergic/asthmatic conditions induced by stress and viral infections. Further intensive study is required for understanding pathophysiologic implication of this finding and underlying molecular mechanisms.

Chapter 5

General Discussion and Conclusions

From the modern era starting around mid-19th century, basic research and clinical studies have been conducted to look for rational therapeutic means to cure and eradicate previously fatal disorders as well as to shorten disease course and alleviate symptoms in all categories including infectious, genetic, autoimmune, metabolic and neoplastic diseases (Barnes 1998; Cha, Zhang et al. 2007; Narayanan, Mohler et al. 2008). For finding effective compounds and for achieving their optimal doses and timing of use, it is fundamental and essential to understand pathogenesis of such disorders and molecular mechanisms of the compounds used for their treatment. I focused on the viral infection and NRs and their ligands as representative models for the disorders and therapeutic compounds: The former is still a major threat for human health as evidenced in the recent pandemics of the human immunodeficiency virus type 1 and the highly virulent avian influenza virus, while the latters are emerging bioactive signaling pathways and compounds with numerous therapeutic potentials but have not been examined well in the immune field. I also worked on DCs, because they are key immune cells for recognizing pathogens and for regulating the subsequent immune response. Thus I designed a study to investigate the impact of NRs and their ligands on DCs immunity by infecting DCs with two viruses, NDV and MCMV or one protozoa, *T. gondii*.

In this viral infection model, I first examined mRNA expression of all NRs and their coregulators and protein expression of some of these molecules in DCs under the infection with NDV or MCMV, or treatment with TLR ligands. In Chapter 2, I demonstrated that expression of several metabolite-related NRs (LXRs and PPARγ) were significantly modulated upon viral

infection in DCs, suggesting that they are important regulators for the anti-viral response of these cells. It is also possible that their ligands introduced endogenously or exogenously could modulate DC-mediated immunity. Indeed, their synthetic ligands are already known to have anti-inflammatory activity, thus my findings may explain in part such known actions of these compounds (Chinetti, Fruchart et al. 2000; Patel and Forman 2004; Michalik, Auwerx et al. 2006; Moore, Kato et al. 2006; Zelcer and Tontonoz 2006; Hong and Tontonoz 2008; Szatmari and Nagy 2008).

Orphan receptors that account about half of the NR family members carry numerous functions important for embryonic development, cell differentiation, energy metabolism and organ homeostatsis (Benoit, Cooney et al. 2006). I found that about half of the orphan receptors were expressed in DCs at baseline and viral infection altered expression of some of them. Among the orphan receptors whose expression levels were regulated by viral infection in DCs, I found that the NR4A family members (NOR1, NUR77 and NURR1) demonstrated the most impressive and characteristic expression profiles in response to viral infection. The NR4A family proteins are interferon ligand binding (Benoit, Cooney et al. 2006). In contrast to other NRs, such as RXR, PPAR, LXR and FXR, which were previously recognized as orphan receptors, but were turned out to be "adopted" NRs with their specific ligands (Germain, Chambon et al. 2006; Germain, Chambon et al. 2006; Michalik, Auwerx et al. 2006; Moore, Kato et al. 2006). NR4A NRs act as constitutively active transcription factors, and their activity is mainly regulated at the levels of their expression and chemical modifications on their specific amino acid residues (Benoit, Cooney et al. 2006). Upon viral infection, expression of NOR1 was highly up-regulated (over 20-fold) in DCs, while that of NUR77 and NUR77 was significantly down-regulated (by 80-90%). Overexpression of NOR1 suppressed IRF3- and IRF7-induced transcriptional activity

of the IFNα promoter in HCT116 cells (from Chapter 2), while NOR1 knockout mice demonstrated blunted cytokine expression (IL-12, IFNγ, TNFα and CCL2) upon Poly IC (TLR3 ligand) injection or *T. Gondii* infection (from Chapter 4). These results suggest that NOR1 has multiple roles in the regulation of DC-mediated anti-pathogen activity. Viral-induced NOR1 appears to be required for the expression and secretion of the proinflammatory cytokines, which initiate inflammation of infected tissues, while it also suppresses type I IFN production indicating that NOR1 is essential for the fine-tuning of the inflammatory reaction against infected pathogens. The effect of this orphan receptor appears to be dependent also on the nature of pathogens, and degree and timing of their infection. Viral infection-associated chemical modification of this receptor protein, such as through phosphorylation, acetylation and ubiquitination, could also influence the activities of NRs. I am now working on elucidating the mechanisms and signaling pathways that underline the *in vivo* results demonstrated in Chapter 3. With such ongoing analyses on the action of NOR1 in DCs, it would be possible to provide more solid explanation to the roles of this receptor in the anti-pathogen immunity organized by these cells. Indeed, NOR1 might play a role in the regulation of other DC-mediated anti-pathogen activities, such as antigen presentation to downstream effector cells, migration of leukocytes and production of antibodies from B-cells/plasma cells (Wang, Jiang et al. 2009).

According to my results shown in Chapter 2 and 3, overexpression of NOR1 suppressed IRF-induced transcriptional activity of the IFNα promoter, while serum levels of IFNβ that belongs to the same type I IFN family did not differ between $NOR1^{-/-}$ and $NOR1^{+/+}$ mice after injection of Poly IC, a strong inducer of type I IFNs. Such discrepancy may be explained by the specificity of NOR1 to the signaling pathways that stimulate production of type I IFNs. Further studies are required.

Among members of the NR family, GR is the best studied in the immunology field, and its anti-inflammatory actions and their underlying mechanisms have been extensively elucidated (Glass and Ogawa 2006). Accordingly, its ligand GCs have been used as the first line immune-suppressive medicines in the treatment of allergic, inflammatory autoimmune diseases for over 3 decades, even though potent new immunosuppressive compounds, such as rapamycin, tacrolimus and cyclosporine, became available (Aberdein and Singer 2006; Collins, Fenwick et al. 2007; Salluh, Povoa et al. 2008). Serum levels of GCs are fluctuated in a circadian fashion and they are massively secreted into circulation in response to stress under the control of the HPA axis (Elenkov and Chrousos 1999). It has long been known that individuals under stress have high susceptibility to infectious diseases and tend to show longer disease course (Godbout and Glaser 2006). In Chapter 4, I demonstrated that GCs enhanced virus-induced production of the anti-inflammatory cytokine IL-10 in DCs by targeting the ERK signaling pathway in DCs. GR is known to stimulate secretion of this cytokine, but my results are the first report of synergistic enhancement of IL-10 by GCs and viral infection in DCs. It appears that virus increases its infectivity and chance of replication by modulating the host GC/GR system in key immune cells, DCs (Couper, Blount et al. 2008).

In conclusion, my thesis work demonstrated that NRs are integral components of the DC-mediated anti-pathogen immunity. Specifically, I found that one of the NR4A family protein NOR1 was highly expressed in DCs upon viral infection, and was essential for the cytokine production in animals infected with pathogens. I also demonstrated for the first time a novel synergism between viral infection and GCs in the production of the anti-inflammatory cytokine IL-10 from DCs that may account for the known increased susceptibility of stressed individuals to pathogen infection. In addition, my study suggests that DCs alter its metabolic activity and

energy expenditure by regulating expression of some NRs in response to pathogen infection, further indicating interplay between the immune and the endocrine/metabolic system.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1. List of primers for qPCR.

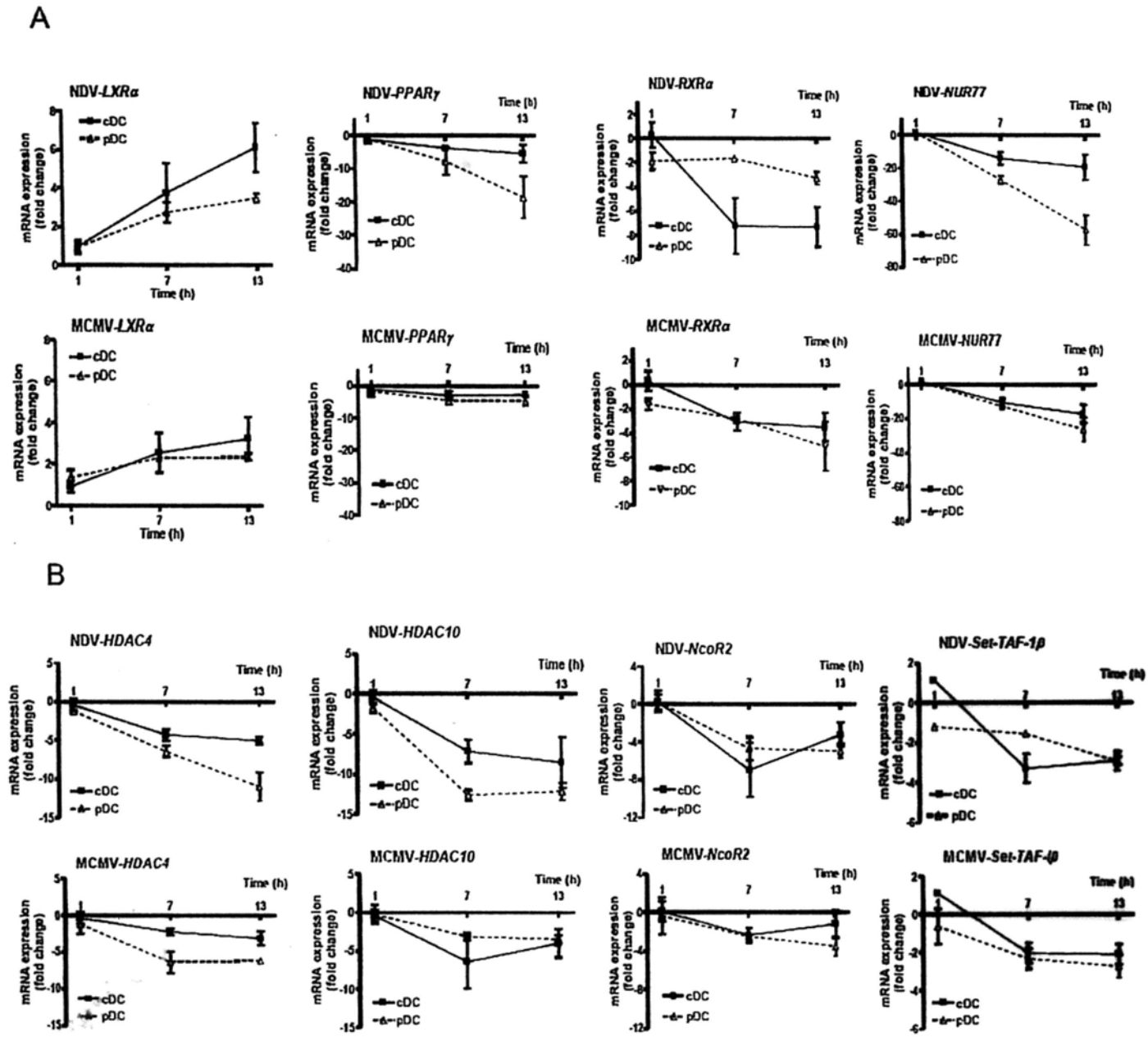
GenBank Accession no.	Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')
NM_008228	Hdac1	TCAAGCAGCGTCTCTTTGAG	ACCACCTTCTCCCTCCTCAT
NM_199198	Hdac10	ATGTCAGAGTGCCCTGGAGT	GGCTCAGTGAATCCTCTGCT
NM_207225	Hdac4	CAGACAGCAAGCCCTCCTAC	AGACCTGTGGTGAACCTTGG
NM_010504.2	Ifnα	CCTGAGAA/GAGAAGAAACACAGCC	GGCTCTCCAGAC/TTTCTGCTCTG
NM_010510.1	Ifnβ	GCTCCTGGAGCAGCTGAATG	CGTCATCTCCATAGGGATCTTGA
NM_001159424.1	Il12p40	TTTTGCTGGTGTCTCCACTCAT	GGTGAGGTTCACTGTTTCTCCA
NM_011424	Ncor2	GTGCTGAGAGGGACAGCACT	CCTGCTGATGGTACCCTTGT
NM_015743	Nr4a3 (NOR1)	AAGGGCTTCTTCAAGAGAACG	CGCACAACTTCCTTAACCATC
NM_013839	Nr1h3 (LXRα)	GCAGGACCAGCTCCAAGTAG	GAATGGACGCTGCTCAAAGT
NM_010444	Nr4a1 (NUR77)	CCTCATCACTGATCGACACG	CCTCCAACTTGAGGCAAAAG
NM_013613	Nr4a2 (NURR1)	CCAGGCAAACCCTGACTATC	CTGGGTTGGACCTGTATGCT
NM_011146	Pparγ	TCACAATGCCATCAGGTTTG	TCAGCGGGAAGGACTTTATG
NM_011305	Rxrα	GCTCACCAAATGACCCTGTT	GCCAGGAGAATCCCATCTTT
NM_023871	Set-TAF-Iβ	TGAACAAGCCAGTGAGGAAA	AGCAGTGCAGACACTTGTGG
NM_019948	Clec4e	TGGGGGCTCACCTGGTGGTT	CCATTGCCACTGACCCTCCACC
NM_0080173.3	GR	GTTCCTAAGGAAGGTCTGAAGAG	CAATTCTGACTGGAGTTTCC
NM_008084.2	Gapdh	GTGTTCCTACCCCCAATGT	TGTCATCATACTTGGCAGGTTTC
NM_008337.3	Ifnγ	GAGGAACTGGCAAAAGGATG	GCTGATGGCCTGATTGTCTT
NM_010548.2	Il10	CCTGGTAGAAGTGATGCCCC	TCCTTGATTTCTGGGCCATG
NM_011198	Ptgs2	AGGGCCCTTCCTCCCGTAGC	TGAGCCTTGGGGGTCAGGGA

Supplementary Table 2. Standard errors and statistical values of TLR PCR array.

PubMed reference sequence	Genes	Symbols	Treatment					
			DEX		NDV		DEX+NDV	
			SE	p-value	SE	p-value	SE	p-value
Group 1: NDV responsive genes modulated by DEX pre-treatment								
(i) Up-regulated by DEX								
NM_010548	Interleukin 10	Il10	1.19	0.0113	0.56	0.0334	15.13	0.0010
NM_019948	C-type lectin domain family 4 member e	Clec4e	0.13	0.0034	3.33	0.0029	11.00	0.0008
(ii) Suppressed by DEX								
NM_010735	Lymphotoxin A	Lta	1.24	0.2390	149.07	0.0007	74.39	0.0011
NM_008337	Interferon γ	Ifng	2.53	0.9671	1147.41	0.0016	62.63	0.0220
NM_031168	Interleukin 6	Il6	1.03	0.8780	24.42	0.0001	5.23	0.0002
NM_011198	Prostaglandin-endoperoxide synthase 2	Ptgs2	0.76	0.3773	2.86	0.0001	0.35	0.0004
Group 2: Responsive gene of DEX pre-treatment								
NM_008361	Interleukin 1β	Il1b	0.45	0.0024	0.23	0.0290	4.79	0.0053
Group 3: Responsive genes of NDV infection								
NM_010510	Interferon β1	Ifnb1	0.56	0.0715	6101.70	0	2978.58	0
NM_021274	Chemokine (C-X-C motif) ligand 10	Cxcl10	6.50	0.0035	90.09	3.50E-05	133.55	3.30E-05
NM_010479	Heat shock protein 1A	Hspa1a	0.29	0.1084	30.53	1.10E-05	23.72	2.50E-05
NM_019388	CD86 antigen	Cd86	0.72	0.9791	1.89	0.0002	2.03	0.0003
NM_011333	Chemokine (C-C motif) ligand 2	Ccl2	-0.38	0.9747	4.67	0.0033	2.99	0.0024
NM_010554	Interleukin 1α	Il1a	0.93	0.8200	12.02	0.0077	8.23	0.0215
NM_013693	Tumor necrosis factor	Tnf	0.41	0.2652	3.25	0.0052	1.75	0.0086
NM_009855	CD80 antigen	Cd80	0.06	0.7827	1.05	5.00E-06	1.34	0.0001
NM_009397	Tumor necrosis factor α induced protein 3	Tnfaip3	0.79	0.9747	1.21	0.0008	1.39	0.0006
NM_011163	Eukaryotic translation initiation factor 2α kinase 2	Eif2ak2	0.11	0.0017	1.21	0.0001	1.27	0.0001
NM_010907	Nuclear factor of k light chain gene enhancer in B-cells inhibitor, α	Nfkbia	0.45	0.0023	0.49	0.0001	0.99	0.0002
NM_023324	Pellino 1	Peli1	0.02	0.0036	0.90	4.20E-05	0.72	1.20E-05
NM_008390	Interferon regulatory factor 1	Irf1	0.16	0.0062	0.27	2.00E-05	1.21	0.0003
NM_138952	Receptor (TNFRSF)-interacting serine-threonine kinase 2	Ripk2	0.71	0.6876	1.23	0.0016	0.55	0.0001
NM_019786	TANK-binding kinase 1	Tbk1	0.07	0.0453	0.97	0.0006	0.74	0.0002
NM_009044	Reticuloendotheliosis oncogene	Rel	0.12	0.0263	1.23	0.0010	0.67	0.0021
NM_010559	Interleukin 6 receptor α	Il6ra	0.08	0.0036	0.90	0.0001	0.44	0.0027
NM_007922	ELK1, member of ETS oncogene family	Elk1	0.96	0.6582	1.40	0.0160	0.11	0.0045
NM_008363	Interleukin-1 receptor-associated kinase 1	Irak1	0.29	0.0106	0.16	0.0001	0.69	0.0007

Supplementary Table 3. Surface markers of leukocytes.

Tissue	Leukocyte Subsets	Surface Markers
Thymus	Double-negative thymocytes	CD4- CD8-
	Double-positive thymocytes	CD4+ CD8+
	CD4+ thymocytes	CD4+ CD8-
	CD8+ thymocytes	CD4- CD8+
	CD25+ regulatory T cells	CD4+ CD25+
Spleen & Lymph nodes	Total B cells	CD19+ B220+
	Plasma cells	CD19+ CD138+
	B-1a B cells	CD19+ CD5+
	Mature B cells	CD19+ B220+ IgM+
	Total T cells	CD3+
	CD4 T cells	CD3+ CD4+
	CD8 T cells	CD3+ CD8+
	CD25+ regulatory T cells	CD4+ CD25+
	NK cells	NK1.1+ CD3+
	Macrophages	CD11b+ F4/80+
	Granulocytes	CD11b+ F4/80- Gr-1 high
	Convention DCs	CD11c+ B220- CD19-
	Plasmacytoid DCs	CD11c+ B220+ CD19-



Supplementary Figure 1. Virus infection alters NR and coregulator mRNA expression in pDCs and cDCs.

The pDCs and cDCs isolated from total DCs were infected by NDV (MOI=10) or MCMV (MOI=10). Total RNA was extracted at 1 h, 7 h or 13 h post-infection. The mRNA levels of (A) NRs and (B) coregulators regulated by NDV and MCMV were measured by qPCR. Data were normalized with *Gapdh* mRNA abundance and the mean values +/- SEM of three independent experiments were shown.