SYMPATHETIC NEURAL CONTROL OF INFLAMMATION BY ADRB2-MEDIATED IL-10 SECRETION

APPROVED BY SUPERVISORY COMMITTEE

J. David Farrar, Ph.D. (mentor)

Lora Hooper, Ph.D. (chair)

Michelle Gill, M.D., Ph.D.

Michael Shiloh, M.D.

DEDICATION

To my beloved sister Büşra, for making everything better and brighter

ACKNOWLEDGEMENTS

I would like to thank to many individuals that have helped me be a better friend, a better person and a better scientist. Your support, care and compassion made this journey much easier, for that I am forever grateful.

First, I would like to thank my mentor, Dr. David Farrar. You have created a wonderful lab home to all of your trainees and supported us during the good and bad times. Thank you for your kindness and guidance when needed and thank you for giving me the independence to test my ideas and try the unlikely. Thank you!

Thank you, Drs. Hooper, Gill and Shiloh! You have challenged me to be a better scientist and supported me throughout this journey. I am happy to know all of you.

I also would like to thank to the members of the Department of Immunology and the Immunology Graduate Program. Andrea, Karen, Bettye, Cathy, Linda, Mariana, Angie, Christina and Harmony; your problem-solving abilities and you made my day-to-day life easier!

I had the best lab mates that anyone could have hoped for. Jon, Navin, Sarah, Leo and Regina, thank you for making the graduate school experience joyful. Thank you for your friendship and endless wisdom. You guys have been amazing.

I was lucky to meet with so many wonderful people during my time in graduate school. Egemen, Yasemin, Gizem, Duygu, Ece and many more friends. Thank you for your support and all the fun times!

Thank you Murat Can, for your unconditional support, guidance and love. Thank you for all the advices you gave, all the mischiefs, all the joy and laughter you brought to my life. You are the reason why every day is better than the day before. Thank you!

Annecim, babacım ve bir tanecik kardeşim Büşra…Hayatımın her aşamasında beni koşulsuz desteklediğiniz ve bana inandığınız için ve hayatıma kattığınız tüm güzellikler için teşekkürler! Sizlerin desteğini hissetmek doktoranın zor zamanlarını kolaylaştıran nadir şeylerden biriydi, sizi çok seviyorum.

SYMPATHETIC NEURAL CONTROL OF INFLAMMATION BY ADRB2-MEDIATED IL-10 SECRETION

by

DİDEM AĞAÇ

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2018

SYMPATHETIC NEURAL CONTROL OF INFLAMMATION BY ADRB2-MEDIATED IL-10 SECRETION

Didem AĞAÇ, Ph.D.

The University of Texas Southwestern Medical Center at Dallas, 2018

Supervising Professor: J. David FARRAR, Ph.D.

The nervous and immune systems reciprocally regulate their functions through the release of chemical messengers. Norepinephrine (NE), a neurotransmitter released by catecholaminergic nerve endings, allows the sympathetic nervous system to communicate with immune cells through adrenergic receptors (ADR). Although, the effects of adrenergic signaling has been studied in multiple cell types, its role in modulation of innate immune cells is relatively unknown. Here, I demonstrate a novel role for the β2-ADR (ADRB2) in controlling inflammation. NE suppresses pro-inflammatory cytokine secretion from primary macrophages in response to multiple TLR agonists, and ADRB2 signaling enhances early induction of IL-10. In addition to its *in vitro* affects, I have shown that ADRB2 signaling controls inflammation *in vivo.* The *in vivo* role of this pathway was assessed by using an

infection model, experimental colitis and LPS endotoxemia model. ADRB2$^{-/-}$ animals presented with splenomegaly and greater weight loss in infection and colitis, compared to ADRB2 sufficient animals, respectively. ADRB2$^{-/-}$ animals rapidly succumbed to a sub-lethal LPS challenge, which correlated with elevated serum levels of TNFα and reduced IL-10. Administration of exogenous IL-10 increased the survival of the ADRB2$^{-/-}$. Additionally, the ADRB2-specific agonist salmeterol rescued wild-type animals from a lethal LPS challenge, which was reversed by neutralizing anti-IL-10 antibody. These observations suggest that ADRB2 signaling is critical for controlling inflammation through the rapid induction of IL-10. Transcriptome analysis revealed that the NR4A nuclear orphan family members were induced by NE. The presence of several putative NR4A binding sites within the IL-10 promoter suggests that these factors may directly regulate IL-10 expression in response to ADRB2 signaling. Additionally, mice that deficient in NR4A1 are susceptible in LPS endotoxemia model. These results suggest a novel pathway for control of inflammation via neuroendocrine cues. Understanding this pathway will provide new insights into how the nervous and immune systems communicate through ADRB2 signaling.

TABLE OF CONTENTS

PRIOR PUBLICATIONS

Ağaç, D., Gill, MA., Farrar, JD. Adrenergic signaling at the interface of allergic asthma and viral infections. Frontiers in Immunology, 2018, https://doi.org/10.3389/fimmu.2018.00736

Estrada, LD., **Agac, D.**, Farrar, JD. Sympathetic neural signaling via the β2-adrenergic receptor suppresses T-cell receptor-mediated human and mouse CD8+ T-cell effector function. European Journal of Immunology, 2016, PMID:27222010

LIST OF FIGURES

LIST OF TABLES

LIST OF DEFINITIONS

BM – Bone marrow

BM-DC – Bone marrow-derived dendritic cell

BM-DM - Bone marrow-derived macrophage

cAMP – cyclic AMP

CD – Cluster of differentiation

cDNA – complementary DNA

ChIP – Chromatin immunoprecipitation

DAMP – Damage associate molecular patterns

DC – Dendritic cell

DNA – Deoxyribonucleic acid

DSS – dextran sodium sulfate

EDTA - Ethylenediaminetetraacetic acid

ELISA – Enzyme linked immunosorbent assay

FBS – Fetal bovine serum

GM-CSF - granulocyte-macrophage colony-stimulating factor

GPCR – G protein coupled receptor

HMGB1 – High mobility group box 1

IgE – Immunoglobulin E

IFN - Interferon

IκB – Inhibitor of κB

IKK - IκB kinase

IL – Interleukin

IRAK – Interleukin-1 associated kinase

LPS – Lipopolysaccharide

LM – Listeria monocytogenes

MAPK – Mitogen-activated protein kinase

M-CSF – Macrophage colony-stimulating factor

MHC – Major histocompatibility complex

MyD88 – Myeloid differentiation primary response 88

nAChR – Nicotinic acetylcholine receptor

NE - norepinephrine

NFκB – Nuclear factor κB

NLR – NOD-like receptors

NOS – Nitric oxide synthase

OMIM – Online Mendelian Inheritance in Man

PAMP – Pathogen associated molecular patterns

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate Buffered Saline

PCR – Polymerase chain reaction

PRR – Pattern recognition receptors

RLR – RIG-I-like receptor

RNA – Ribonucleic acid

qRT-PCR – Quantitative real time PCR

RPMI – Roswell Park Memorial Institute medium

TAK1 – Transforming growth factor beta-activated kinase 1

Th – T helper cell

TIR – Toll-IL-1 Receptor

TLR – Toll-like receptor

TNF – Tumor necrosis factor

TRAF – TNF receptor associated factor

Tpl2 – Tumor progression locus 2

UTR – Untranslated region

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Everyday exposure to millions of viruses and bacteria provides a challenging task to the immune system. To protect the host against various microbial insults, immune system is armed with germline encoded Pattern Recognition Receptors (PRR). PRRs either recognize the Pathogen Associated Molecular Patterns (PAMP) that are conserved structures on various microbes that cannot be altered by the organism, or recognize Danger Associated Molecular Patterns (DAMP) that are released by the host in response to tissue damage. The activation of PRRs on innate cells such as macrophages and dendritic cells promotes the release of several cytokines and chemokines, maturation of the innate immune cells and their migration to the lymph nodes where they lead the activation of the adaptive immune cells such as B and T lymphocytes. The priming of the adaptive immune system is particularly important to mount more robust immune responses upon re-encountering the same pathogen (Kawai and Akira, 2011; Pasare and Medzhitov, 2004). Detection of the pathogenic insult also changes the levels of neuroendocrine hormones such as norepinephrine or glucocorticoids in serum (Cain and Cidlowski, 2017; Webster et al., 2002). For many years, the immune and nervous systems have been studied independently; however, there is growing literature that shows a crosstalk between these systems (Chavan et al., 2017; Ferrari et al., 2016; Grace et al., 2014; Ji et al., 2016; Pavlov and Tracey, 2017; Steinman, 2004; Veiga-Fernandes and Mucida, 2016). The work presented here aims to reveal the impact of norepinephrine on innate

immune cell function at the cellular and molecular level as well as the *in vivo* consequences of the activation of this interaction in inflammation and infection.

Activation of macrophages and control of inflammation by IL-10

Macrophages: versatile immune cells

Macrophages are tissue resident cells that are involved in multiple processes: they sense the presence of PAMPs (bacterial, viral or fungal origin) and DAMPs through PRRs; additionally, they utilize phagocytic receptors and scavenger receptors to survey the environment. During homeostasis, macrophages are involved in clearance of apoptotic debris and, remodeling of the extracellular matrix, however; during infections these phagocytes are involved in the clearance of the pathogen by phagocytosis. Macrophages are also important to control metabolism and thermal adaptation (Amit et al., 2016; Glass and Natoli, 2016; Lavin et al., 2015; Okabe and Medzhitov, 2016).

During homeostasis, tissue resident macrophages are mostly maintained by the embryonic macrophage progenitors and do not rely on the circulating blood monocytes for their replenishment. However, in inflammatory conditions, monocytes migrate to injured or inflamed tissues and differentiate into macrophages and dendritic cells (Lavin et al., 2015).

Macrophages contribute to immune responses by clearance of pathogens via phagocytosis, secretion of cytokines and chemokines to modulate immune cell behavior and

act as antigen presenting cells to initiate adaptive immune responses (Mosser and Edwards, 2008).

Toll-like receptors

Toll gene in *Drosophila melanogaster* was shown to be important in production of the antifungal peptide drosomycin by Hoffmann and colleagues in 1996 (Lemaitre et al., 1996). A year later, the mammalian homologue of Toll was identified (Medzhitov et al., 1997). Since then, 13 members of the Toll-like receptor (TLR)-family have been identified. Of those, all TLRs except TLR10 can be found in mice; however human cells only express TLR1 through TLR10 (Kawasaki and Kawai, 2014). TLRs can sense bacterial, viral and fungal structures, as well as nucleic acids.

TLRs are composed of three conserved structural domains, (a) the leucine-rich repeats (N-terminal ligand binding domain), (b) transmembrane domain and (c) TIR (Toll-IL-1 Receptor) domain, the cytoplasmic signaling domain which is shared with IL-1R members. Upon ligand binding TLRs initiate signaling cascades through homotypic interactions with TLR associated adapter proteins (Botos et al., 2011). Most TLRs, except TLR3, depend on the adapter protein MyD88 (myeloid differentiation primary response 88) for signaling. In the MyD88-dependent pathway, MyD88 recruits IRAK4 to the signaling complex and IRAK4 activates IRAK1. IRAK1 associates with TRAF6, and later with TAK1 kinase. TRAF6-dependent polyubiquitination events initiate TAK1 activation. TAK1 binds to the IKK complex to facilitate the phosphorylation and activation of IKK, leading to phosphorylation and proteosomal degradation of IκBα, therefore promoting the nuclear

translocation of NFκB to induce gene expression. Additionally, TAK1 activation leads to the activation of MAPK family members including ERK1/2, JNK and p38 -eventually leading to AP-1 activation. In the MyD88-independent pathway, the adaptor molecule TRIF associates with TRAF6 and TRAF3 and recruits RIP-1, a molecule which interacts with TAK1 complex. TAK1 activation leads to NFκB and MAPK activation as described above. TRAF3 recruits TBK1 and IKKi, leading to IRF3 phosphorylation and dimerization and eventually to induction of interferon genes (Kawasaki and Kawai, 2014; Takeda and Akira, 2004).

TLR activation results in upregulation of co-stimulatory molecules, as well as production of pro- and anti-inflammatory cytokines in dendritic cells and in macrophages. Activation of the innate immune cells is an important step for the initiation of the adaptive immune responses (Mosser and Edwards, 2008; Pasare and Medzhitov, 2004).

Inflammation and IL-10 signaling in regulation of inflammatory responses

Inflammation is a protective host response that is initiated after infection or injury. Initiation of the inflammation is characterized by many changes in local and systemic processes, including changes in local vasculature, increase immune cell recruitment, release of mediators (e.g. cytokines, chemokines, complement), changes in energy expenditure and alterations of the body temperature. Although inflammation is necessary to eliminate the infecting pathogen or induce tissue repair after damage, it needs to be tightly controlled (Eming et al., 2017; Fullerton and Gilroy, 2016; Kotas and Medzhitov, 2015).

IL-10 is an anti-inflammatory cytokine that is produced by T and B lymphocytes, dendritic cells, monocytes, macrophages and granulocytes (Saraiva and O'Garra, 2010). IL-10 is an essential molecule to control inflammation; IL-10-deficient mice are susceptible to systemic LPS administration with worse survival outcomes (Berg et al., 1995) and infections (Gazzinelli et al., 1996; Hunter et al., 1997). Additionally, mice develop chronic intestinal inflammation in the absence of IL-10 (Kuhn et al., 1993) or IL-10Rb (Spencer et al., 1998).

IL-10 inhibits the production of several cytokines and chemokines from monocytes and macrophages, including but not limited to IL-1α, IL-1β, IL-6, IL-12, TNFα, RANTES, IL-8, IP-10 (de Waal Malefyt et al., 1991; Fiorentino et al., 1991; Kopydlowski et al., 1999; Marfaing-Koka et al., 1996; Nicod et al., 1995). Additionally, IL-10 can inhibit MHC Class II, CD80 and CD86 expression (de Waal Malefyt et al., 1991; Ding et al., 1993; Willems et al., 1994). Downregulation of these molecules can alter T cell responses. IL-10 can elicit its suppressive properties by destabilizing the target mRNA via AU-rich elements in the 3'UTR (Kishore et al., 1999). RNA-destabilizing factors such as tristetraprolin are involved in destabilization of mRNA species (Carballo et al., 1998; Schaljo et al., 2009).

Crosstalk between the nervous and immune systems

The nervous and immune systems were historically thought to be autonomous, however, growing evidence suggests that these systems reciprocally regulate each other's function to ensure homeostasis. Several neural cues (e.g. substance P, histamine) have been reported to modulate immune responses (Steinman, 2004). For example, neurons that initiate acute pain

(a cardinal sign of inflammation), nociceptor sensory neurons, can sense IL-1β (Binshtok et al., 2008). Similarly, in a dental injury model, sensory neurons that express TRPV1 were reported to express TLR4 and the downstream signaling molecules like MyD88, TRIF and NFκB (Lin et al., 2015). *S. aureus* infection was shown to directly activate sensory neurons to modulate inflammation (Chiu et al., 2013), suggesting that sensory neurons are capable of sensing the bacteria or the inflammatory mediators. Sensory neurons are thought to be important to modulate pathogen avoidance behavior (by initiation of social isolation due to pain) and itch (to remove the irritant or the pathogen) (Baral et al., 2016).

In addition to pain, fever (another cardinal sign of inflammation) and sickness syndrome can be thought as neuroimmune crosstalk instances. The pro-inflammatory cytokine IL-1β was described to affect thermal regulation centers in hypothalamus, leading to fever (Lesnikov et al., 1991; Malinowsky et al., 1995). Similarly, IL-1β signaling can induce anorexia and withdrawal leading to a behavior called the sickness syndrome (Elander et al., 2007; Gonzalez et al., 2006). Our understanding regarding the induction of fever has improved drastically; not only IL-1β, but other immune cell derived molecules prostaglandin E2, IL-6 and TNFα are also implied for generation of fever. The release of norepinephrine in response to fever has been implicated in adaptive thermogenesis to increase the body temperature and vasoconstriction to reduce heat loss. Additionally, the release of acetylcholine stimulates muscle cells to initiate shivering (Evans et al., 2015). These results suggest that the nervous and the immune cells work together to initiate fever responses.

Acetylcholine has gained interest for the last decade due to the description of the "inflammatory reflex". In this model, innate immune cell derived mediators (e.g. IL-1β)

stimulate vagus nerve fibers, leading to activation of the adrenergic splenic nerve. Norepinephrine released from the splenic nerve activates a subset of T cells via beta2-adrenergic receptors and leads to production of acetylcholine (Rosas-Ballina et al., 2011). Acetylcholine suppresses TNFα, IL6 and HMGB1 expression from macrophages by signaling through α7nAChR. The reduction in pro-inflammatory cytokines helps the host to control inflammation (Andersson and Tracey, 2012a, b; Pavlov and Tracey, 2017). Although this system suggests the suppression of pro-inflammatory cytokines in macrophages through an indirect mechanism, it fails to address if the adrenergic signaling on macrophages alter the cell behavior directly.

Adrenergic receptors and their impact on immune cells: a brief summary

Adrenergic receptors are G protein coupled receptors that recognize norepinephrine and epinephrine as their natural ligands. During homeostasis, adrenergic receptors are involved in several key physiological functions including the control of the heart rate, cardiac function, vasculature, muscle contractility. During stressful events, adrenergic receptors are involved in the fight or flight response.

The natural ligands of adrenergic receptors are synthesized through modification of tyrosine. There are 9 adrenergic receptors, either belonging to alpha or beta subtype. Alpha adrenergic receptors (α1A, α1B, α1D, α2A, α2B and α2C) can be coupled with G_i, G_q or G_s proteins, however, beta adrenergic receptors (β1, β2 and β3) preferentially bind to G_s protein (Hein and Kobilka, 1995; Philipp and Hein, 2004). Although all adrenergic receptors bind to

the same ligands, differences in their expression patterns and the G proteins they are coupled to determines the outcome of ligand binding.

Norepinephrine and epinephrine are released by adrenal glands or adrenergic neurons (marked by the expression of tyrosine hydroxylase). Adrenergic receptor expression on immune cells has been reported extensively (Maisel et al., 1989; Maisel et al., 1990; Moriyama et al., 2018). Due to innervation of lymphoid organs such as the thymus, lymph nodes, and spleen, it is likely that lymphocytes can receive adrenergic signals in tissue (Bellinger et al., 1992; Felten et al., 1985; Gabanyi et al., 2016; Kendall and al-Shawaf, 1991; Panuncio et al., 1999; Reilly et al., 1979).

Beta2-adrenergic receptor signaling is the most extensively studied adrenergic receptor, probably due to it being the first GPCR that is cloned (Dixon et al., 1986). Not surprisingly, the impact of adrenergic receptors on the immune system has been vastly examined as well. Stimulation of beta2 adrenergic receptors downregulated histamine (Chong et al., 1998; Church and Hiroi, 1987; Nials et al., 1994) and TNFα (Bissonnette Ey, 1997) release from mast cells. B lymphocytes increased the expression of CD86 and IgE in response to beta2-adrenergic receptor signaling (Kasprowicz et al., 2000). Adrenergic receptors have been shown to reduce IL-2 production from naïve CD4+ T cells (Swanson et al., 2001). IFNγ secretion from Th1 clones and Th1 cells was reduced, however Th2 cells were unaffected by adrenergic receptor ligands (Sanders et al., 1997; Swanson et al., 2001). This unresponsiveness was due to the reduction in beta2-adrenergic receptor expression in Th2 cells (McAlees et al., 2011). We have recently shown that beta2-adrenergic receptor signaling suppresses cytokine secretion and cytotoxic activity in CD8+ T cells (Estrada et al.,

2016). Similarly, Grebe *et al* reported that mice that underwent chemical sympathectomy had enhanced primary CD8+T cell responses against viral infections, suggesting that the sympathetic nervous system negatively regulates CD8+ T cell function (Grebe et al., 2009).

Adrenergic receptor signaling on macrophages and dendritic cells has been mostly reported during TLR4 ligand LPS stimulation. In these studies, signaling through beta2-adrenergic receptors downregulated LPS-induced TNFα production (Grailer et al., 2014; Izeboud et al., 1999; Monastra and Secchi, 1993; Spengler et al., 1994). However, it was unclear if TLR4 was the only TLR that can be modulated by adrenergic cues. Similar to suppression of TLR4 responses in macrophages, beta2-adrenergic receptor signaling downregulated LPS-induced IL-12 and IL-18 production (Mizuno et al., 2005). Beta2-adrenergic receptor agonists have been shown to decrease pro-inflammatory cytokine secretion from dendritic cells (Hu et al., 2012). Although adrenergic receptor signaling is mostly anti-inflammatory in nature, the treatment of dendritic cells with beta2-adrenergic receptor agonists has been reported to promote IL-17 producing CD4 T cells (Manni et al., 2011) or IL-4 producing Th2 cells (Nijhuis et al., 2014). Interestingly, Nijhuis *et al* also reported an increase in Foxp3+ IL-10+ regulatory T cells when the dendritic cells are exposed to adrenergic cues (Nijhuis et al., 2014). Although there have been some studies investigating the role of adrenergic receptors on macrophages and dendritic cells, these studies were mostly restricted to cell lines and the use of LPS as stimulation. Considering the role of adrenergic pathways on multiple cells, it was unlikely that the other TLR signaling pathways would be unaffected. This work aimed to determine how adrenergic receptors influence TLR signaling (how common is it for TLRs to be modulated by adrenergic

receptors, what cytokines were influenced and so on) and the *in vivo* consequences of adrenergic receptor signaling. The work presented here provides a comprehensive analysis of macrophage function in response to beta2-adrenergic receptor ligands *in vitro.* Additionally, we provide evidence for anti-inflammatory properties of beta2-adrenergic receptor signaling in multiple disease models.

CHAPTER II

MATERIALS AND METHODS

Human donors

Peripheral blood (5-10 mL) was obtained from consented human healthy adult donors by venipuncture according to University of Texas Southwestern Medical Center Institutional Review Board's guidelines. Blood was collected in 10 mL syringes prepared with 1 mL heparin at 5,000 U/mL (Sigma-Aldrich, Inc. St. Louis, MO).

Culturing whole human blood

Whole human blood was diluted 1:1 in RPMI and plated in 48 well plates (1 mL diluted blood/well). Diluted blood was stimulated in the presence or absence of ultra-pure lipopolysaccharide from *Escherichia coli* K12 at 10 μg/ml (Invivogen, San Diego, CA) and salbutamol sulfate (albuterol) at 50 nM (SelleckChem, Houston, TX) for 4 hours in 5% CO_2, 37^{o}C incubator. Plasma was harvested and stored at -80°C until further use.

Human monocyte-derived macrophage cultures

$CD14^{+}CD16^{-}$ human monocytes were isolated from healthy adult donor peripheral blood mononuclear cells (PBMC) using EasySep Human Monocyte Enrichment kit according to

manufacturer's instructions (StemCell, Vancouver, Canada). Cells were cultured with 5000 U/ml human M-CSF (R&D Systems, Minneapolis, MN) for 6 days to promote the differentiation of the monocytes to macrophages. Human monocyte-derived macrophages were stimulated for 2 hours and culture supernatant was harvested for ELISA and stored at -80°C until further use.

Animal subjects

All mouse experiments and mouse handling were conducted according to University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee's guidelines. All mice were housed in specific pathogen-free facilities, with *ad libitum* feeding at University of Texas Southwestern Medical Center. Wild-type C57BL/6 and Balb/cJ mice were purchased from the University of Texas Southwestern Medical Center Animal Research Facility. ADRB2 whole body knock-out mice, CD11c-cre mice, 10BiT (IL10 BAC-in transgene), ADRB2 fl/fl, Il10-deficient mice and LysM-cre mice were gifts from Dr. Virginia Sanders of the Ohio State University, Dr. Chandrashekhar Pasare of the University of Texas Southwestern Medical Center, Dr. Casey Weaver of the University of Alabama at Birmingham, Dr. Gerard Karsenty of the Columbia University Medical Center, Ann Stowe of the University of Texas Southwestern Medical Center and Dr. Tiffany Reese of the University of Texas Southwestern Medical Center respectively. Tail clips were used to genotype the mice.

Isolation of bone marrow cells

Femurs and tibiae were harvested from mice. Bones were submerged into RPMI. Contents were transferred to mortar and media was discarded. Bones were rinsed with sterile 70% ethanol and plain RPMI (twice each). Bones were crushed into finer pieces with the use of pestle and contents were passed through a 70-micron cell strainer (Fisher Scientific, Hampton, NH) to remove bone pieces from cells in solution. Bone marrow (BM) cells were obtained after red blood cell lysis and washes. BM cells were used for differentiation of several cell types including bone-marrow derived macrophages (BM-DM) and bone-marrow derived dendritic cells (BM-DC).

Differentiation and culture of bone-marrow derived macrophages

After obtaining BM cells (as described above), cells were resuspended in 10 ml macrophage media (30% v/v L929 supernatant (kindly provided by Dr. Chandrashekhar Pasare, University of Texas Southwestern Medical Center) and 70% v/v 10% FBS + cRPMI). These cells can release M-CSF and provide an inexpensive alternative to purified cytokines. Cells from each mouse were separately plated to tissue culture treated 10 cm-dishes. After overnight incubation (approximately 16 hours) in 5% CO_2, 37^{o}C incubator, non-adherent cells were harvested, washed with 10%FBS+cRPMI and counted. Cells were re-plated in macrophage media in 4 million cells per 10-cm Petri dish (non-TC treated). After 5 days of culture, non-adherent cells were discarded and adherent cells (macrophages) were removed from the Petri dishes by 2 mM EDTA/PBS by tapping at 4°C. Cells that were recovered were

85-98% F4/80 positive and were used as bone marrow-derived macrophages for downstream applications. Macrophages were stimulated at 0.5 million cells/ml concentration.

Differentiation of bone marrow-derived dendritic cells

After obtaining BM cells (as described above), cells were counted and resuspended in dendritic cell (DC) media (1% v/v J558 conditioned media (a cell line that produces GM-CSF, the supernatant kindly provided by Dr. Chandrashekhar Pasare) in 5% FBS + cRPMI). Cells were plated in TC-treated plates and incubated in 5% CO_2, 37˚C for 5 days. Half of the media was removed without touching the monolayer that forms at the bottom of the plate at days 2 and 4 and cells were provided with fresh DC media. Non-adherent cells were removed on day 5 and stained for CD11c and CD11c high cells were isolated through FACS and used as bone marrow-derived dendritic cells.

TLR agonists and reagents used in in vitro assays

The following TLR agonists were used to induce cytokine secretion in innate immune cells: TLR2:TLR1, Pam3CSK4; TLR3, polyI:C, TLR4, Ultrapure lipopolysachharide from *E. coli* K12, TLR7, R837 (Invivogen, San Diego, CA) and, TLR9, CpG (ODN1826, from Keck Oligonucleotide Synthesis Facility, New Haven, CT). The TLR ligands were initially provided as a gift for the preliminary experiments from Dr. Chandrashekhar Pasare of University of Texas Southwestern Medical Center, and later purchased from the abovementioned suppliers.

To stimulate adrenergic receptors, norepinephrine bitartrate salt (Sigma Aldrich, St. Louis, MO), salmeterol xinafoate and salbutamol sulfate (=albuterol) (SelleckChem, Houston, TX) were used. For pharmacological receptor antagonism experiments, phentolamine hydrochloride (pan-αAR antagonist), nadolol (pan-βAR antagonist), and atenolol (β1AR antagonist) (all from Sigma Aldrich, St. Louis, MO) were used. Additionally, ICI118,551 was used as β2AR antagonist (SelleckChem, Houston, TX).

The inhibitors used in Chapter V were as follows: p38 inhibitor (=SB203580) (Calbiochem, Kenilworth, NJ), Tpl2 inhibitor (Santa Cruz Biotechnology, Dallas, TX) and MEK1/2 inhibitor (=U0126). These inhibitors were resuspended in DMSO with final stock concentration of 10mM. All inhibitors were added at the same time as the stimulation reagents.

Rapamycin (a gift from Dr. Anwesha Ghosh from Dr. Melanie Cobb's laboratory) was used to inhibit mTOR complex and forskolin (Tocris Bioscience, Bristol, UK) was used to induce adenylyl cyclase activity.

RNA Isolation

Zymo Research's Quick-RNA MiniPrep kit was used for RNA isolation, according to the manufacturer's recommendations. Briefly, after removing the culture media, the macrophages were lysed with 300 µl of RNA lysis buffer. Cell lysates were stored at -80°C until further use or were used immediately. The cell lysate was passed through Spin-Away Filter to remove genomic DNA and the supernatant was obtained by 13000g, 1-minute

centrifugation. 300 µl 95-100% ethanol was added to the sample in RNA lysis buffer and the mixture was transferred to a Zymo-Spin IIICG Column and centrifuged for 30 seconds in 13000g. The flow-through was discarded. The column was washed with 400 µl RNA wash buffer and the flow-through was discarded. The column-bound sample was treated with DNase I in DNA digestion buffer (5 µl DNase I and 75 µl DNA digestion buffer per sample) for 15 minutes. 400 µl RNA Prep Buffer was added to the column and the column was centrifuged for 30 seconds at 13000g. After discarding the flow-through, 700 µl RNA wash buffer was added, and the column was centrifuged for 30 seconds at 13000g. As the last wash, 400 µl RNA wash buffer was added, and the column was centrifuged for 30 seconds at 13000g. After removing the flow-through, the column was centrifuged again for 1 minute at 13000 g to remove any remaining buffer. The column was inserted into a new RNase-free Eppendorf tube and air-dried for 15 seconds. 50 µl of DNase/RNase free water was added to the center of the column matrix and centrifuged for 30 seconds at 13000g. The eluted RNA can be stored at -80°C until further use.

qRT-PCR

Total RNA was isolated at the time points described in figure legends, using the protocol above. 40-100 ng of RNA was used to perform reverse transcription using ABI High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The cDNA was used as template for the qPCR reactions using Brilliant II SYBR Green Master Mix (Agilent Technologies, Santa Clara, CA) or the Maxima SYBR Green Master Mix

(Thermo Scientific, Waltham, MA) using ABI 7300 cycler or QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Foster City, CA). *Ppia* was used as reference gene in qPCRs. All primers used for qPCR assays can be found in Table 2.1. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schimttgen, 2001).

Luciferase Assays

RAW264.7 cells (kindly gifted by Dr. Chandrashekhar Pasare of UT Southwestern Medical Center) were grown in T150 flasks. Cells were counted and re-plated to 10-cm dishes (around 4-5 million cells/plate) and rested overnight. Next morning, the cells were transfected with the following procedure: DNA and Polyethylenimine (Linear, MW 25000, Transfection Grade (PEI 25K) from Polysciences, Warrington, PA) were mixed in 400 μL serum free-media (3 μg PEI for 1 μL DNA).

All conditions received a constitutively active renilla plasmid (3 μg). For luciferase expressing constructs, murine Il10 promoter truncations were purchased and used from Addgene (Cambridge, MA). These plasmids were constructed by Dr. Stephen Smale's group (Brightbill et al., 2000). All plasmids were in pGL2B backbones and the largest construct spans -1538/+64 of the Il10 promoter. Total of 7 plasmids were purchased (Plasmid #24942, #24943, #24944, #24945, #24946, #24947 and #24948). 3 μg DNA was used for the transfections.

After a brief vortex, the mixture was incubated at room temperature for 15 minutes to allow the lipid:DNA complexes to form. The mixture was added dropwise to the RAW264.7

cells and the plates were gently tilted to ensure proper distribution of the reagent. After 24 hours of incubation at 37°C, 5%CO_2, cells were taken off the plate, washed, counted and re-plated to 96-well plates. The cells were stimulated with ±NE and ±CpG for 3 hours. After the stimulation, the media was removed, and the cells were processed with the Dual-Glo Luciferase Reporter Assay Kit to quantify luciferase activity relative to renilla expression (Promega, Madison, WI) using the MicroBeta TriLux Counter (Perkin Elmer, Waltham, MA). The data was converted to arbitrary units, the ratio of luciferase to renilla signal; and for each truncation, the data was normalized to unstimulated control.

Chromatin Immunoprecipitation

Bone marrow-derived macrophages or RAW264.7 cells were plated (10-20 million cells per T75 flask) and rested before stimulation, to promote cell adhesion to the plates. Cells were stimulated with ±NE and/or ±CpG for 2 hours. Media was removed, and the cells were washed on the plate with 5 mL cold, sterile PBS. 5 mL PBS was added to the plate and 37% formaldehyde was added for a final concentration of 1% and the plates were tilted at room temperature for 10 minutes. 2M glycine was added for a final concentration of 0.125M and the plates were tilted at room temperature for 5 minutes to neutralize the formaldehyde. Cells were washed in 5 mL of cold PBS, twice and scraped off the plate. The samples were centrifuged at 400 g for 4 minutes at room temperature to pellet the cells. The cell pellets were resuspended in 1.5 mL of cell lysis buffer (5 mM Pipes, 85 mM KCl, 0.5% NP-40) and 1:100 protease inhibitors were added (P8340, Sigma Aldrich). Cells were incubated on ice

for 10 minutes with inversions of the samples at every 2.5 minutes. Nuclei were pelleted at 4000 rpm for 4 minutes at room temperature and cell lysis buffer was discarded. Nuclei were resuspended in 300 μL nuclear lysis buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 1% SDS) in the presence of 1:100 protease inhibitors for 10 minutes on ice. Chromatin was sonicated for 28 minutes using Bioruptor (Diagenode, Denville, NJ): samples were incubated for four-7-minute intervals, 30 seconds on and 30 seconds off, on "high" setting. The cellular debris was cleared by centrifugation at 14000 rpm for 10 minutes at room temperature and supernatants were transferred to new tubes. The concentration of the sonicated chromatin was measured by Nanodrop and aliquoted 3-10 μg chromatin per tube and stored at -80°C.

For immunoprecipitation, the sonicated samples were diluted 10-fold in ChIP dilution buffer (16.7 mM Tris-HCl pH 8.0, 1.2 mM EDTA, 1.1% TritonX-100, 0.01%SDS, 167 mM NaCl) in the presence of protease inhibitors. Protein A/G magnetic beads that were blocked with 1%BSA/PBS (with rotation) were incubated with specific antibody or isotype controls with sonicated chromatin, overnight at 4°C with rotation.

The following morning, the samples were placed on a magnet for 2 minutes and the supernatant was removed without touching the beads. Then the samples were washed sequentially for 5 minutes with 1 mL of the following solutions: Low Salt Buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 1% TritonX-100, 0.1% SDS, 150 mM NaCl), High Salt Buffer (20 mM Tris-HCl pH 8.0, 2 mM EDTA, 1% TritonX-100, 0.1% SDS, 500 mM NaCl), LiCl Buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% Deoxycholate, 1% NP-40, 0.25 M LiCl), and then twice in 1X Tris-EDTA (TE). All these steps were performed at 4°C. After removing all the TE, 150 μL Elution Buffer (0.1 M $NaHCO_3$, 1% SDS) was added and the

samples were vortexed for 10 minutes at room temperature at setting "3". After vortexing, the samples were placed in 37°C water bath for 10 minutes. Beads were placed on the magnet; the supernatant was saved in a new clean tube and the beads were subjected to the elution process one more time. The total volume of the elute will be 300 μL. Input samples were diluted in 300 μL elution buffer and 32 μL of 2.5 N NaCl and 1 μL Protease K (20 mg/mL) were added to all samples (input and ChIP samples). All samples were incubated at 37°C in water bath for 30 minutes and incubated at 62°C heat block overnight. The DNA was eluted from the samples using QIAgen MinElute PCR Purification Kit per manufacturer's recommendations (QIAGEN, Valencia, CA) and was quantified with Nanodrop. The eluted DNA was stored at -80°C until further use.

4 ng of IP DNA was used per PCR reaction and I have used 50 cycles. The list of the primers used for ChIP-qPCR can be found in Table 2.2. Each immunoprecipitated sample was compared to its own input sample using the formula below:

$$\%\text{ChIP Efficiency} = 2^{(\text{Input Ct-Sample Ct})} \text{ x dilution factor x100}$$

Next generation RNA-sequencing

Bone marrow-derived macrophages were stimulated with ± 5 μM NE and/or ± 1 μM CpG for 2 hours as described in the figure legends. Total RNA was isolated using QuickRNA Miniprep kit (ZymoResearch, Irvine, CA) or RNeasy Mini Kit (QIAGEN, Valencia, CA) following the manufacturer's recommendations. Poly(A) containing mRNA was enriched using NEBNext Poly(A) Magnetic Isolation Module (New England Biolabs, Ipswich, MA).

Using NEBNext mRNA Library Prep Reagent for Illumina, mRNA libraries were prepared. To summarize, mRNA was fragmented to approximately 200 bp pieces and cDNA synthesis was performed. The cDNA libraries were end-repaired and dA-tailed and for each sample, a unique adaptor was ligated to identify the reads and match with the corresponding samples. Samples were briefly amplified using universal primers. Upon size and quality assessment by Bioanalyzer (Agilent Genomics, Santa Clara, CA), the samples were submitted to the Genomics Core (University of Texas Southwestern Medical Center). The samples were single-end, 50 bp sequenced using Illumina HiSeq 1000 or 2000. I conducted a preliminary analysis using the GALAXY cluster which was hosted in University of Texas Southwestern Medical Center, Department of Molecular Biology; however, the data presented here were analyzed by Dr. Beibei Chen who was working in the University of Texas Southwestern Medical Center Bioinformatics Core Facility at the time of the analysis. She has generated the normalized DESeq quantification and some two-way differential expression analysis between samples that are discussed in Chapter V.

Quantification of cytokine secretion (ELISA and Mesoscale)

Both enzyme-linked immunosorbent assay (ELISA) and MesoScale analyses were used for quantification of cytokine secretion.

The following kits were used for ELISA: mouse IL-6 ELISA MAX (Biolegend, San Diego, CA), mouse TNFα ELISA MAX (Biolegend), mouse IL-1β ELISA MAX (Biolegend), mouse IL-10 ELISA MAX (Biolegend), mouse IL-10 DuoSet ELISA (R&D), mouse IL-1β DuoSet ELISA (R&D), human TNFα ELISA MAX (Biolegend) and human IL-

10 ELISA MAX (Biolegend). All the secondary antibodies used in these kits were biotinylated. Horseradish peroxidase (HRP) coupled with streptavidin (SA) was used to amplify the signal. BD OptEIA TMB Substrate Reagent Set was used as the detection agent for Biolegend kits and DuoSet ELISA Ancillary Reagent Kit was used for R&D kits. Manufacturer's instructions were followed for both human and mouse cytokine detection. Briefly, the desired number of wells and plates of NUNC Maxisorp 96-well ELISA plates were coated with primary antibody in bicarbonate coating buffer (8.4 g $NaHCO_3$, Na_2CO_3 in 1L of deionized water, pH 9.5) overnight, at 4ºC. Plates were washed with the wash buffer (0.05% Tween 20 in 1x PBS) and blocked with assay diluent (1%BSA in 1x PBS) for approximately 1 hour at room temperature. Samples and cytokine standards were prepared in assay diluent and transferred the plate after blocking step. Plates were incubated with samples and standards for approximately 2 hours at room temperature. Plates were washed with the wash buffer and incubated with the detection antibody (biotinylated anti-cytokine antibody) diluted in assay diluent for approximately 1 hour. Plates were then washed again and incubated with SA-HRP diluted in assay diluent for 20-30 minutes at room temperature. Plates were washed again, and HRP-substrate was added for developing color. This kit generates blue color, which darker blue indicates higher cytokine concentration. When the highest standards turn dark blue or 30 minutes after the color development starts, the reaction was stopped with 1M H_2SO_4. The absorbance of the plate was measured by iMark Microplate Absorbance Reader (Bio-Rad, Hercules, CA) at 450nm. The absorbance of the samples was determined based on a standard curve and the concentrations of the unknown were calculated by Beer's Law.

Additionally, Mouse Proinflammatory 7-Plex Tissue Culture Kit (MesoScale Discovery, Rockville, Maryland) was used to quantify the secreted cytokines. Wells of the plates in this kit were pre-coated in "spots", each spot corresponds to a different cytokine capture antibody. The kit allows detection of several pro- and anti-inflammatory cytokines (IFNγ, IL-1β, IL-6, IL-10, IL-12p70, KC and TNFα) at the same time. Manufacturer's instructions were followed. Briefly, samples and standards were added to plates and incubated for 2 hours at room temperature with constant shaking (400 rpm) to ensure equal sample distribution between different spots. Detection antibody was added to plates and incubated for 2 hours at room temperature with constant shaking. Plates were washed with 0.05% Tween 20 in 1x PBS, three times. Plates were read in MESO SECTOR S 600 (Department of Pathology) shortly after adding 2X Read Buffer T. MesoScale Discovery plates uses electrochemiluminescence; where the machine generates electricity that initiates red-ox reactions that causes light emission which was captured by a camera. Comparing the light intensities of samples to known standards for each spot, the program determines the concentration of an analyte in a given well.

Flow cytometry

Cells were plated in a V-bottom 96-well plate and centrifuged for 1500 rpm for 3 minutes. After aspirating the supernatant, cells were washed twice in 200 μL PBS. If the cells were stained for CD19, the staining protocol was conducted before fixing. Otherwise, the cells were resuspended in 50 μL 2.5% Formalin/PBS and incubated at room temperature for 20

minutes. After formalin-fixing, cells were washed with 150 μL PBS, followed by two additional 200 μL PBS washes to remove the excess formalin. Cells were later washed with 200 μL 1%BSA/PBS, twice. Samples were resuspended in 50 μL antibody cocktail for 20 minutes at room temperature, in dark. After two 200 μL 1%BSA/PBS washes, cells were resuspended in 200 μL 1%BSA/PBS and transferred to FACS tubes. LSR II and FACSCalibur were used to run the samples (BD Biosciences, Franklin Lakes, NJ) and FlowJo v8.87 was used to analyze the data (Ashland, Oregon). The list of all the antibodies used can be found in Table 2.3.

Listeria monocytogenes infections

Ovalbumin expressing *Listeria monocytogenes* (LM-OVA, gifted by Dr. James Forman of University of Texas Southwestern Medical Center, generated by Lefrançois group (Pope et al., 2001)) was cultured in streptomycin containing (1:1000) brain heart infusion (BHI) media for approximately 2 hours. Optical density (OD) of the cultures was measured, OD595 0.1 was roughly 225 million CFU/ml. The required volume of the bacterial culture was calculated depending on the OD of the cultures and the experimental design, the bacteria was injected intravenously in PBS to tail vein of the animals. Additionally, the injected bacteria were plated to Streptomycin plates to determine the actual CFU that was injected. Depending on the experiment, the livers and spleens were harvested at different time points. After weighing the organs, the spleen was divided into two pieces, one of the pieces was used for flow cytometry to assess the cellular composition of the organ. The other spleen piece and

livers were homogenized, and the homogenates were plated in 1:1, 1:10, 1:100, 1:1000 dilutions to streptomycin containing plates and cultured overnight. The CFU was determined next morning by counting the colonies.

In some experiments, salmeterol was administered to determine if adrenergic receptor signaling can alter the course of the infection. For those experiments, animals received 40 μg salmeterol (Tocris Bioscience, Bristol, UK) in PBS, intraperitoneally at days 0,1 and 2 of infection. The organs were harvested as described above.

Colitis studies

Adult male mice (6-8 weeks) were subjected to 3%DSS in drinking water for 7 days and the weight loss was assessed by daily measurements. At the end of the experiment, the colons were harvested, the length of the colons were measured, and the distal colon was processed in Bouin's solution overnight. The colons were submitted to University of Texas Southwestern Medical Center Pathology Core for sectioning and hematoxylin and eosin (H&E) staining. The histopathology was assessed by Dr. Purva Gupal of University of Texas Southwestern Medical Center, in a double-blinded fashion.

Endotoxemia studies

Endotoxemia was initiated by the intraperitoneal administration of LPS (*Escherichia coli* 055:B5, Sigma, St. Louis, MO) at the amounts indicated in figure legends, depending on the experiment. Recombinant murine IL-10 was administered at 1 μg/mouse intraperitoneally

(Biolegend, San Diego, CA) immediately after administration of LPS in some of the experiments. Additionally, in some experiments, 40 μg salmeterol, InVivoMab anti-mouse CD210 (IL-10R) or the isotype control (BioXCell, West Lebanon, NH) were administered intraperitoneally.

Survival of the animals was monitored every 3 hours for the first 4 days and every 6 hours for the rest of the experiment until day 7 post-LPS administration. In the studies that focus on the serum levels of cytokines, the animals were retro-orbitally bled at the indicated time points in the figure legends as a terminal procedure.

Statistical analysis

All data are represented as the mean ± standard error of the mean (SEM). Depending on the experiment, student's t-test, one-way or two-way ANOVA with a Bonferroni posthoc test, log-rank analysis was conducted as statistical analysis, using GraphPad Prism software. All p values$\leq$0.05 were considered significant.

Table 2.1 Primers used in qPCR assays

Gene	Direction	Sequence
Nr4a1	Forward	5'- GAGCCGGCTGGAGATGC -3'
	Reverse	5'- AGGAACAAGCTGAGGAGCAC -3'
Nr4a2	Forward	5'- TAAACAAAGGCACATTGGCGG -3'
	Reverse	5'- AGATCTCCTTGTCCGCTCTCT -3'
Nr4a3	Forward	5'- AATCCAGATTTCGGGGTCGC -3'
	Reverse	5'- ACGCAGGGCATATCTGGAGG -3'
Ppia	Forward	5'- TTATTCCAGGATTCATGTGCCAGGG -3'
	Reverse	5'- TCATGCCTTCTTTCACCTTCCCAA -3'
Il6	Forward	5'- GCCTTCTTGGGACTGATGCT -3'
	Reverse	5'- TGCCATTGCACAACTCTTTTC -3'
Tnf	Forward	5'- CTGTAGCCACGTCGTAGCA -3'
	Reverse	5'- AGCAAATCGGCTGACGGTGT -3'
TTP	Forward	5'- CATCTACGAGAGCCTCCAGTC -3'
	Reverse	5'- CAAAGGTGCAAAACCAGGGG -3'
Il10	Forward	5'- GGTTGCCAAGCCTTATCGGA -3'
	Reverse	5'- ACCTGCTCCACTGCCTTGCT -3'

Table 2.2 Primers used in ChIP-qPCRs

Gene	**Direction**	**Sequence**
Foxp3 promoter	Forward	5'- ACCTTTTACCTCTGTGGTGAG -3'
	Reverse	5'- GTAGTTTTTTTTTCTTTGCTCTC -3'
Il10 promoter site #1	Forward	5'- TAAATGTAGACCTCCTGTTCTTGGT -3'
	Reverse	5'- CATAGAACAGCTGTCTGCCTCAG -3'
Il10#2 promoter site #2	Forward	5'- AGAGGCCCTCATCTGTGGATTCCAT -3'
	Reverse	5'- CTGACCAACTGCCCCACAGCACACA -3'
Il10 promoter site #3	Forward	5'- CTCAGACTTCTGGGAGGCTTG -3'
	Reverse	5'- CAAACATTCCCTGGTCAACAGG -3'
Il10 promoter site #4	Forward	5'- AAGTAGACCCATGTAGAGGGTACAC -3'
	Reverse	5'- GTTGCTTCTGCTGTTGGAAACG -3'

Table 2.3 Antibodies used for flow cytometry

Target	**Fluorochrome**	**Clone**	**Company**
CD4	PE	RM4-5	Biolegend
CD8	FITC	Unknown	Caltag
CD11b	PE Cy7	M1/70	BD Biosciences
CD11c	Pacific Blue	N418	Biolegend
CD19	Brilliant Violet 510	6D5	Biolegend
Ly6C	PerCP	HK1.4	Biolegend
Ly6G	APC	1A8	Biolegend
TCRβ	APC	H57-597	BD Biosciences
F4/80	APC	BM8	Biolegend
F4/80	PE	BM8	Biolegend
CD11b	PE	M1/70	Tonbo
Nur77 (NR4A1)	PE	12.14	Molecular Probes

CHAPTER III

BETA2-ADRENERGIC RECEPTOR SIGNALING ALTERS CYTOKINE PRODUCTION IN INNATE IMMUNE CELLS

The data presented in this chapter is unpublished. Didem Ağaç executed all the experiments in this chapter unless otherwise indicated in figure legends and/or text.

Introduction

Macrophages are sentinel innate immune cells that sense and respond the external cues (tissue damage, presence of microbial insult etc.) by germline encoded receptors (e.g. NLRs, TLRs, RLRs). Functions of macrophages include clearance of cellular debris, apoptotic bodies or pathogens by phagocytosis; remodeling of the extracellular matrix; promoting tissue healing and repair; and production of growth factors, cytokines and chemokines. Therefore, the regulation of macrophage function is essential to ensure homeostasis (Lavin et al., 2015; Okabe and Medzhitov, 2016; Wynn and Vannella, 2016).

Beta2-adrenergic receptor agonists were demonstrated to block LPS-induced TNFα and IL-6 secretion from murine and human macrophage cell lines (Hasko et al., 1998; Izeboud et al., 1999; Keränen et al., 2016; Verhoeckx et al., 2005; Wang et al., 2009). Kizaki *et al* reported that forced-expression of beta2-adrenergic receptor inhibits LPS-induced NOS2 expression and NFκB activation through β-arrestins in RAW264 cells (Kizaki et al.,

2008). Additionally, Spengler and colleagues showed that LPS induced TNFα release can be inhibited by isoproterenol in peritoneal macrophages (Spengler et al., 1994). Although there have been many papers demonstrating that TLR4-induced TNFα and IL-6 secretion can be suppressed by beta2-adrenergic receptor agonists, there has been no study to our knowledge that investigates if the effects of adrenergic receptor signaling are limited to TLR4 signaling or can be observed in primary murine and human cells. As part of this study, I have found that norepinephrine (NE) suppressed the production of pro-inflammatory cytokines (not only TNFα and IL-6, but many more) and induced the IL-10 production via the beta2-adrenergic receptor. The acute repression of TNFα did not rely on the induction of IL-10 and the alterations of cytokines were also present in human monocyte-derived macrophages, suggesting an evolutionary conservation. In summary, we have identified that beta2-adrenergic receptors are general negative regulators of TLRs and we have described a novel pathway for IL-10 production *in vitro* by beta2-adrenergic receptor signaling.

Results

Inhibition of pro-inflammatory cytokine secretion by norepinephrine

LPS-induced TNFα has been shown to be inhibited by norepinephrine in murine and human monocytic cell lines (Hasko et al., 1998; Izeboud et al., 1999; Keränen et al., 2016; Verhoeckx et al., 2005; Wang et al., 2009). I sought to determine if norepinephrine can alter cytokine secretion from murine macrophages and dendritic cells that were stimulated with a TLR agonist that was not signaling through TLR4. I chose to use CpG to stimulate cytokine

production by macrophages and assessed if norepinephrine altered the cytokine production. The first thing to note is that in the absence of TLR9 stimulation, norepinephrine alone was unable to induce pro-inflammatory cytokine secretion (Figure 3.1). However, when cells were exposed to NE in the presence of TLR-stimulation, the pro-inflammatory cytokines TNFα, IL-6, IL-12p70, KC, and IFNγ levels were reduced (Figure 3.1). The effects of NE were long-lasting, even though the half-life of NE is short (e.g. estimated to be 2-2.5 minutes in blood, (Beloeil et al., 2005)); the level of cytokines remained low at later hours of stimulation (e.g. 24-48 hours). The reduction of pro-inflammatory cytokines can be detected around 2-4 hours post stimulation. Interestingly, at 2 hours, NE upregulated IL-6 production (for comparison with TNFα, see Figure 3.2) but later on the samples that were treated with NE had less IL-6 than their TLR-alone counterparts. Similar to our observation, Tan *et al* reported that RAW264 cells treated with salmeterol, a beta2-adrenergic receptor agonist, expressed higher levels of IL-6 mRNA, 45 minutes after stimulation (Tan et al., 2007). However, this study no protein data from this time point was reported in this study. Because the classical adrenergic signaling utilizes adenylyl cyclase-protein kinase A pathway to upregulate cAMP (Hein and Kobilka, 1995), it is possible that the temporal increase in cAMP can upregulate IL-6 production (Hershko et al., 2002). Additionally, it is important to note that although most pro-inflammatory cytokines that have been measured were reduced in response to NE, the extent of the suppression was dependent on the cytokine; the reduction in TNFα secretion was the greatest.

The reduction in pro-inflammatory cytokines in response to NE was not restricted to the TLR9 signaling pathway but was common across multiple TLR pathways. Although

TLR2:TLR6, TLR5, TLR8, TLR11, TLR12, and TLR13 ligands were not tested, NE suppressed TNFα in response to TLR1:TLR2 (Pam3CSK4), TLR3 (polyI:C), TLR4 (LPS), TLR7 (R837) and TLR9 (CpG) ligands (Figure 3.1 and Figure 3.3). For these experiments in Figure 3.3, 2, 8 and 48-hour time points were selected as reflective of early-, peak-, and late-phases of the stimulation. NE suppressed the TLR-induced pro-inflammatory cytokine production in response to all the agonists that were tested (Figure 3.4).

Innate immune cells have been reported to express high number of adrenergic receptors. Maisel *et al* reported that approximately 1500 beta-adrenergic receptors were expressed on the surface of monocytes (Maisel et al., 1989). To test how sensitive the macrophages were to NE's suppressive ability, a dose-titration assay was conducted. When CpG-treated macrophages were exposed to NE, TNFα was suppressed in a dose-dependent manner, and suppressive effects of NE were observed in the nM range (Figure 3.5), suggesting that cytokine expression in macrophages is sensitive to low concentrations of NE.

Norepinephrine mediates its suppressive effects via ADRB2

Norepinephrine and epinephrine are the natural ligands of adrenergic receptors. As described in *Chapter 1*, adrenergic receptors are comprised of 5 sub-classes, namely α1-, α2-, β1-, β2-, and β3-adrenergic receptors. In order to determine the adrenergic receptor that mediates the pro-inflammatory cytokine secretion in response to NE, macrophages were pre-incubated with pan α- or pan β-adrenergic receptor antagonists, phentolamine and nadolol respectively. Blocking β-adrenergic receptors, but not α-adrenergic receptors reversed TNFα suppression

by NE (Figure 3.6). Additionally, blocking β2-adrenergic receptors but not β1-adrenergic receptors reversed the suppression of TNFα and IL-12p70 (Figure 3.7). This suggests that NE's suppressive effects were mediated by β2-adrenergic receptors.

Use of pharmacological antagonists suggested a role for β2-adrenergic receptors in suppression of TLR-dependent pro-inflammatory cytokine secretion. ADRB2-deficient mice were used to confirm this observation. NE was unable to suppress TLR-induced TNFα secretion in ADRB2-deficient macrophages, showing that NE mediates its suppressive effects via ADRB2 (Figure 3.8). Similarly, TNFα was inhibited when ADRB2-sufficient macrophages were stimulated with increasing concentrations of albuterol (a short-acting ADRB2 agonist, Figure 3.9) and salbutamol (a long-acting ADRB2 agonist, Figure 3.10), however ADRB2-deficient macrophages were resistant to the effects of these beta2-adrenergic receptor agonists.

Adrenergic receptor signaling enhances TLR-induced IL-10 secretion

Previous studies suggested an anti-inflammatory role for adrenergic signaling, since the pro-inflammatory cytokines were inhibited in the presence of adrenergic ligands. However, the status of the anti-inflammatory cytokines in acute phases of stimulation has not been investigated before. Engagement of adrenergic receptor signaling induced rapid production of IL-10 in all TLRs that were tested (Figure 3.11). However, when the kinetics of IL-10 production was examined, several differences were noted: In Pam3CSK4, polyI:C and LPS treated samples the presence of norepinephrine resulted in increased IL-10 production

throughout the culture period (up to 48 hours). On the other hand, in R837 treated samples, norepinephrine treatment induced acute IL-10 production at 2 hours as reported in Figure 3.11. However, in later phases of stimulation (8 and 48 hours), increased IL-10 production was not sustained and samples that did not receive norepinephrine had higher IL-10 levels. Interestingly, in CpG-treated samples, the presence of norepinephrine only increased IL-10 production at 2 hours. IL-10 production between norepinephrine treated and untreated samples was comparable at 8 and 48 hours. This observation suggests that although the induction of IL-10 in response to norepinephrine is a common feature of all TLRs, the kinetics of IL-10 are dependent on the TLR signaling pathway (Figure 3.12). It is possible that the nature of the pathogen that the cell encounters could determine the kinetics of IL-10 and how norepinephrine would influence the secretion of the cytokine. Lastly, the IL-10 upregulation was dependent on ADRB2; macrophages that were deficient in ADRB2 expression were unable to induce IL-10 in response to NE (Figure 3.13).

IL-10 is dispensable for early suppression of TNFα

IL-10 can downregulate pro-inflammatory cytokines, including TNFα. In order to investigate the role of IL-10 in acute suppression of pro-inflammatory cytokines, ADRB2-sufficient macrophages were pre-treated with an IL-10R (CD210) neutralizating antibody or an isotype control. The antibody would bind to the IL-10R and would prevent the binding of IL-10 to its receptor even though IL-10 might be in the supernatant. Additionally, although the antibody is a neutralizing antibody, its binding does not initiate signaling. After 30 minutes of

incubation, the macrophages were treated with TLR agonists R837 or CpG to stimulate TLR7 and TLR9 receptors, respectively. Additionally, the cells were treated with or without norepinephrine for 2 or 8 hours. When cytokines were assessed at 2 hours post-stimulation, IL-10R neutralization did not reverse the suppressive effects of norepinephrine on pro-inflammatory cytokines (Figure 3.14). Additionally, IL-10 levels were unaffected in IL-10R blocked samples at 2 hours. These observations were independent of the TLR agonist that was used. However, when the cytokines were measured at 8 hours, cells that were stimulated with R837 and CpG behaved differently. For example, in R837 treated samples, blocking the autocrine IL-10 signaling partially rescued the suppression of TNFα. On the other hand, IL-10R blocking completely reversed the suppression of TNFα in CpG treated cells. In both TLR stimulations, IL-10R blocking resulted in enhanced IL-10 secretion at 8 hours, suggesting that NE-induced IL-10 could inhibit IL-10 production in autocrine manner. There were discrepancies in IL-12p70 and IL-1β as well: in R837 treated samples, IL-10R blocking completely reversed the suppression of these cytokines, whereas in CpG treated samples, IL-10R blocking resulted in cytokine production even more than CpG alone. Interestingly, IL-10R neutralization reversed the suppression of KC and IL-6 similarly in both R837 and CpG treated samples (Figure 3.14). These observations suggest that adrenergic receptor signaling elicits a complex regulatory cascade, where IL-10 signaling affects the pro-inflammatory cytokine production depending on the TLR that the cells were stimulated with, the duration of the stimulation and the cytokine that is in question for suppression. Based on the results of our IL-10R neutralization experiments, IL-10 elicits its suppressive effects in later hours of stimulation. Additionally, upregulation of IL-10 in response to norepinephrine does not

explain why some cytokines were suppressed at 8 hours (e.g. TNFα), since IL-10R neutralization was unable to reverse this suppression completely. However, this data also suggests that the early suppression of the pro-inflammatory cytokines was not regulated by IL-10, since the IL-10R neutralization did not alter the suppression at 2 hours.

Using a genetic model, the requirement of IL-10 for the suppression of TNFα at early phases of stimulation was tested. The cytokine expression profiles of IL-10-deficient and -sufficient macrophages were compared in response to TLR ligands and norepinephrine. IL-10 was not necessary for the suppression of TNFα at early phases of stimulation (2 hours) (Figure 3.15). This data suggests that the induction of IL-10 and suppression of TNFα at 2 hours relies on two distinct pathways. Additionally, higher TNFα secretion was observed in IL-10 deficient macrophages, although norepinephrine was still able to suppress TNFα (Figure 3.16).

Norepinephrine can suppress cytokine production in dendritic cells

Our observations on macrophages show that the presence of norepinephrine in the context of TLR stimulation could down regulate the secretion of pro-inflammatory cytokines and upregulate the production of IL-10 acutely. I wanted to investigate if this phenomenon was restricted to macrophages or it can be observed in other innate immune cells, such as dendritic cells.

Similar to macrophages, bone marrow derived dendritic cells (BM-DC) had reduced TNFα production when exposed to NE. This suppression was a general phenomenon across

multiple TLRs and was dependent on the expression of ADRB2 (Figure 3.17). Additionally, IL-10 secretion was higher in cells that were exposed to NE at 2 hours post-stimulation (Figure 3.18). These observations suggest that adrenergic receptor engagement alters TLR-induced cytokine production in multiple innate immune cells.

Norepinephrine alters cytokine mRNA levels

Alterations in multiple cellular processes could yield to reduction in protein detected in the supernatant: the protein production or secretion could be altered, the mRNA stability could be different in response to norepinephrine, or the gene transcription might be different in response to adrenergic receptor signaling.

In order to determine if adrenergic receptor signaling alters the transcript levels, the mRNA levels of *Tnf*, and *Il10* were assessed in different time points. Although the presence of NE significantly induced *Il10* transcript in the absence of TLR stimulation, no difference was observed in *Tnf* transcript at 2 hours post stimulation (Figure 3.19a). NE alone could upregulate *Il10* transcript but, in the absence of TLR signaling, it couldn't induce protein secretion (Figure 3.12). However, when cells were exposed to TLR stimulation, an increase in *Tnf* and *Il10* transcripts were observed. Additionally, exposure of macrophages to TLR in the presence of NE reduced *Tnf* transcript and increased the *Il10* transcript, in a similar pattern to protein expression (Figure 3.19b for Pam3CSK4 and Figure 3.19c for LPS). The alterations in mRNA levels were not observed in ADRB2-deficient macrophages (Figure 3.20), again, showing that the expression of the gene was under direct control of the ADRB2 signaling. Additionally, the *Tnf* transcript remained lower in samples treated with TLR+NE

compared to TLR treated samples at 8 hours post-stimulation, however, *Il10* transcript remained unchanged between TLR and TLR+NE treated samples (Figure 3.21), suggesting that the regulation of cytokine transcripts happens in cytokine-specific manner.

Evolutionary conservation of the suppressive effects of NE

Adrenergic receptors are highly conserved between mouse and human (Figure 3.22). In order to test if adrenergic receptor's ability to alter cytokine production was conserved in humans, whole blood from consenting adults was stimulated with LPS in the presence of albuterol for 4 hours. The exposure to albuterol reduced LPS-induced TNFα secretion in all donors tested. The acute nature of the response and the stimulation method suggested that the suppression occurred in an innate immune cell, most likely monocytes. To determine if human macrophages could alter TLR-induced cytokine secretion, monocyte-derived macrophages were subjected to LPS and NE. Similar to our observations in murine macrophages, exposure to NE down-regulated TNFα and upregulated IL-10 (Figure 3.23). This suggests that the control of cytokine production by NE in innate immune cells is conserved across species.

Discussion

To my knowledge the present study is the first to demonstrate that norepinephrine suppresses the pro-inflammatory cytokines in response to multiple TLRs. I showed that cytokine secretion from both endosomal TLRs (TLR3, TLR7 and TLR9) and cell-surface TLRs

(TLR2:TLR1 and TLR4) was altered during adrenergic signaling. Furthermore, I showed that the canonical anti-inflammatory cytokine, IL-10 was acutely induced in response to TLR/adrenergic receptor co-stimulation. Grailer and colleagues recently reported similar observations on induction of IL-10 by norepinephrine and epinephrine via beta2-adrenergic receptor signaling in LPS treated cells, at 18 hours post-stimulation (Grailer et al., 2014). This observation is similar to my time-course experiments for IL-10 production (Figure 3.12c), I have also observed higher IL-10 in norepinephrine treated groups. However, to my knowledge, the acute induction of IL-10 has not been reported before.

Adrenergic receptor stimulation does not induce M2 lineage, rather it causes an acute alteration of cytokine milieu since the norepinephrine treated macrophages do not upregulate the canonical M2 markers Ym1 or arginase1 (Chapter 5). These alterations in cytokine production were dependent on the expression of ADRB2. Additionally, a recent study showed that beta2-adrenergic receptor signaling can suppress LPS-induced IL-27 (Roewe et al., 2017), suggesting that there might be additional cytokines that are affected by this pathway waiting to be studied.

Additionally, I showed that IL-10 is dispensable for the acute suppression of TNFα, suggesting that beta2-adrenergic receptor signaling controls the acute IL-10 induction and the suppression of TNFα through two different pathways. Based on my *in vitro* observations, I propose a model in Figure 3.24. TLR signaling induces the expression of both pro-inflammatory (e.g. *Tnf* and *Il12*) and anti-inflammatory (e.g. *Il10*) cytokines (left panel). The engagement of ADRB2 during TLR stimulation (right panel) leads to a reduction on the pro-inflammatory cytokines that are produced (both transcripts as well as the proteins). This is

accompanied by a rapid induction of IL-10. Since IL-10 is not required for the acute suppression of the pro-inflammatory effectors, I propose that the induction of IL-10 and the suppression of the pro-inflammatory cytokines operated through different signaling pathways. In monocytic cell lines, arrestins are involved in the suppression of TLR-induced NOS2 and pro-inflammatory effectors in response to ADRB2 signaling (Kizaki et al., 2008; Wang et al., 2009). I have not ruled out the involvement of arrestins in murine BM-DMs and BM-DCs, it remains as a possible mechanism. The molecular pathway leading to IL-10 induction will be discussed in Chapter 5.

Previously our laboratory reported that CD8+ T cell reduce IFNγ and TNFα secretion in response to norepinephrine (Estrada et al., 2016). However, this suppression was not accompanied with an induction of IL-10. This suggests that the regulation of cytokine expression by norepinephrine was controlled differently between macrophages and T cells.

Our observations on changes in cytokine production in response to beta2-adrenergic receptors is also observed in dendritic cells (DCs). In the scope of this dissertation, I have not investigated other functions of dendritic cells (or macrophages), such as phagocytosis and T cell priming. However, studies by other groups indicated that adrenergic receptor signaling can influence those function as well. Kim *et al* reported that epinephrine can increase the expression of the maturation markers MHCII, CD80 and CD86 and cytokines IL-12p35 and IL-23p19 in dendritic cells. When epinephrine pre-treated BM-DCs are co-cultured with purified CD4+ T cells, T cell-derived IFNγ was reduced but IL-4 and IL-17A were increased (Kim and Jones, 2010). Similarly, Nijhuis *et al* reported an increase in MHCII, CD80 and CD86 in dendritic cells in response to epinephrine. In this study, the investigators also

showed an increase in IL-4+ T cells, as well as Foxp3+IL-10+ T cells after co-culturing epinephrine treated DCs with naïve T cells. The presence of epinephrine increased the phagocytosis capacity of dendritic cells (Nijhuis et al., 2014). Grailer *et al* reported that macrophages increase their ability to phagocytose, similar to DCs, when epinephrine is present (Grailer et al., 2014). These observations suggest that the presence of adrenergic signals can influence DC-priming of T cells; favoring Th2, Th17 or Treg lineages – but *in vivo* demonstration is needed to make conclusive conclusions.

Although I have investigated how pro-inflammatory cytokines are affected by beta2-adrenergic receptor signaling, I have not investigated if adrenergic receptor signaling can alter the anti-viral cytokines. Hilbert *et al* showed that in human PBMCs, epinephrine treatment decreased IFNA1 production in response to CpG (Hilbert et al., 2013). Likewise, Cole *et al* observed an increase in p24 protein in PBMCs infected with HIV, in response to norepinephrine. These observations suggest that adrenergic receptor signaling down-regulates anti-viral responses (Cole et al., 1998). The molecular mechanisms leading to interferon suppression need to be investigated.

The data presented here suggests that neuroendocrine cues can alter pro- and anti-inflammatory cytokine production, which could play an integral role in controlling inflammation *in vivo.*

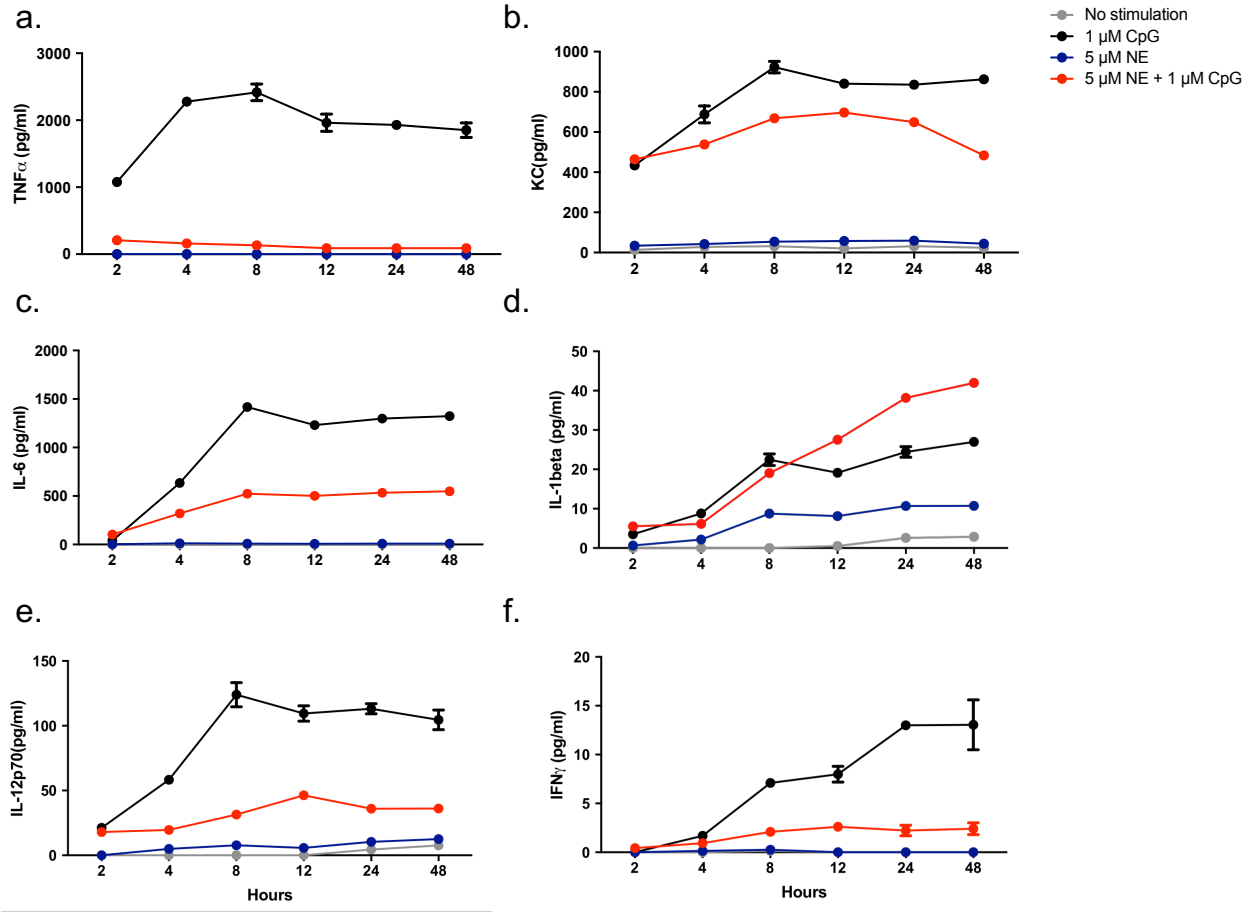

Figure 3.1. Norepinephrine inhibits pro-inflammatory cytokine secretion in bone marrow-derived macrophages (BM-DM). BM-DMs from C57Bl/6J mouse were stimulated with the conditions listed above for 2-48 hours. Supernatants were harvested at each time point and secreted cytokines were assessed using Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery (n=1, error bars represent the technical error from triplicates)

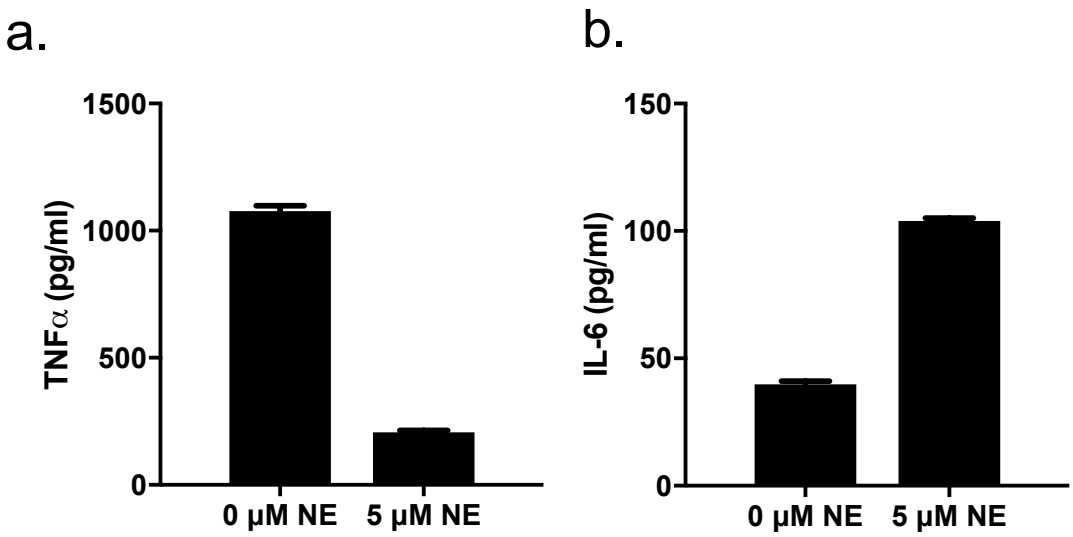

Figure 3.2. Norepinephrine selectively suppresses TNFα and enhances IL-6 production at 2 hours. BM-DMs from C57Bl/6J mouse were stimulated with 1 μM CpG for 2 hours in the presence or absence of 5 μM NE. (a) TNFα and (b) IL-6 that was secreted in the supernatant was assessed by Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery (n=1, error bars represent the technical error from triplicates).

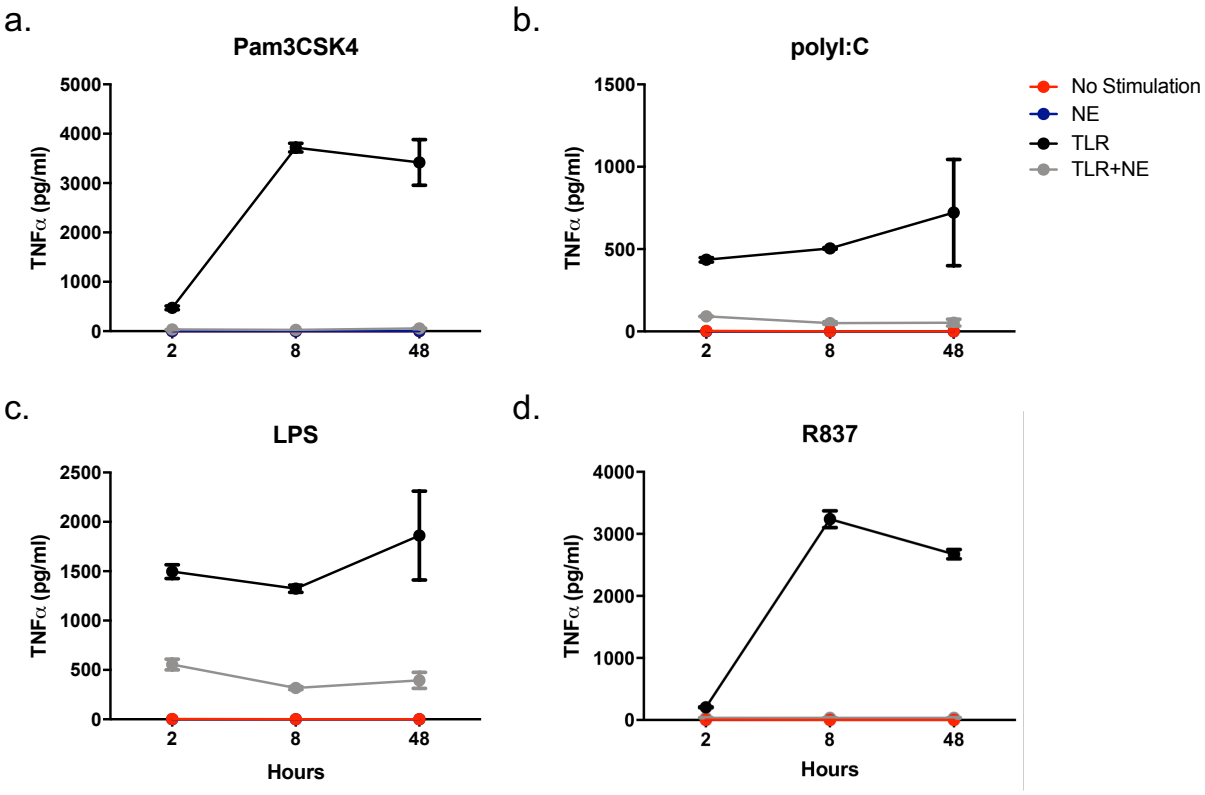

Figure 3.3. Norepinephrine suppresses TNFα secretion in response to multiple TLRs. BM-DMs from 2 female Balb/cJ mice were combined and stimulated with (a) 100 ng/mL Pam3CSK4, (b) 10 μg/mL poly I:C, (c) 100 ng/mL LPS, and (d) 1 μg/mL R837 in the absence or presence of 5 μM NE for 2, 8 and 48 hours. Supernatants were harvested at each time point and secreted cytokines were assessed using Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery (n=2, error bars represent the technical error from triplicates for each mouse).

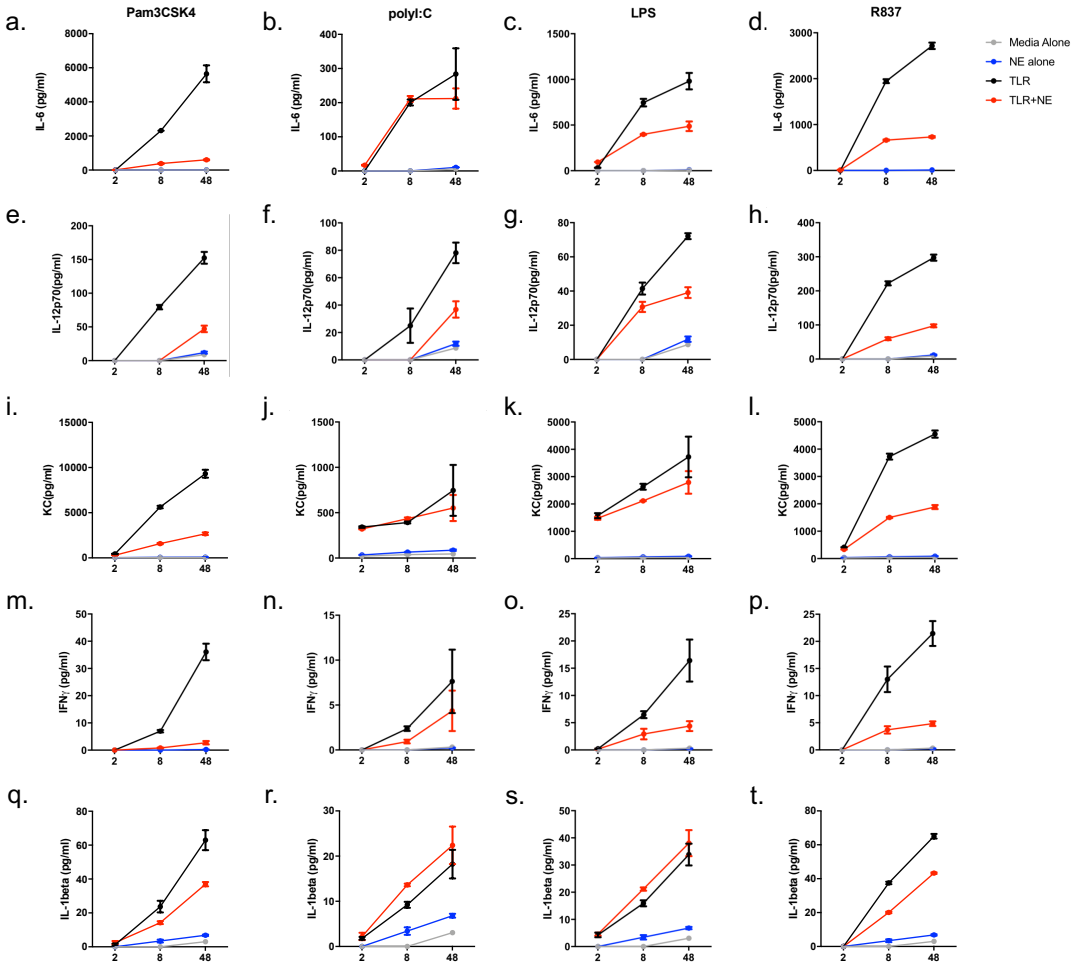

Figure 3.4. Norepinephrine alters pro-inflammatory cytokine secretion in response to multiple TLRs. BM-DMs from 2 female Balb/cJ mice were combined and stimulated with (a, e, i, m and q) 100 ng/mL Pam3CSK4, (b, f, j, n and r) 10 μg/mL poly I:C, (c, g, k, o and s) 100 ng/mL LPS, and (d, h, l, p and t) 1 μg/mL R837 in the absence or presence of 5 μM NE for 2, 8 and 48 hours. Supernatants were harvested in each time point and secreted cytokines were assessed using Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery.

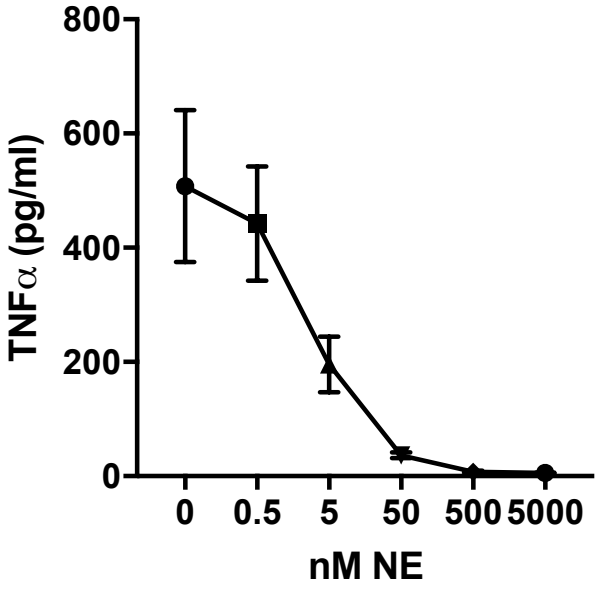

Figure 3.5. BM-DMs are sensitive to norepinephrine. BM-DMs from Balb/cJ mice were stimulated for 8 hours with 1 μM CpG in the presence of NE (0-5000 nM). Supernatants were harvested and TNFα in the supernatant was quantified using Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery. Error bars represent the standard error of the mean from n=4 mice.

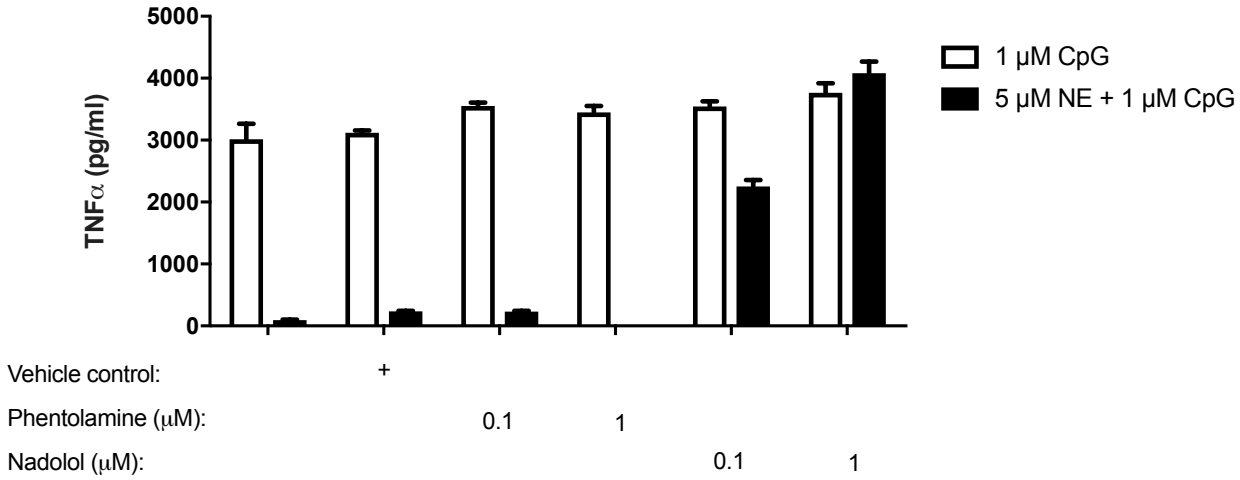

Figure 3.6. Suppression of TNFα by norepinephrine depends on beta-adrenergic receptors. Phentolamine (pan α-adrenergic receptor antagonist) or nadolol (pan β-adrenergic receptor antagonist) was added to Balb/cJ BM-DM cultures which were stimulated with 1 μM CpG and 5 μM NE for 24 hours. Supernatants were harvested and TNFα in the supernatant was quantified by ELISA. Error bars represent the standard error of the mean from n=3 mice.

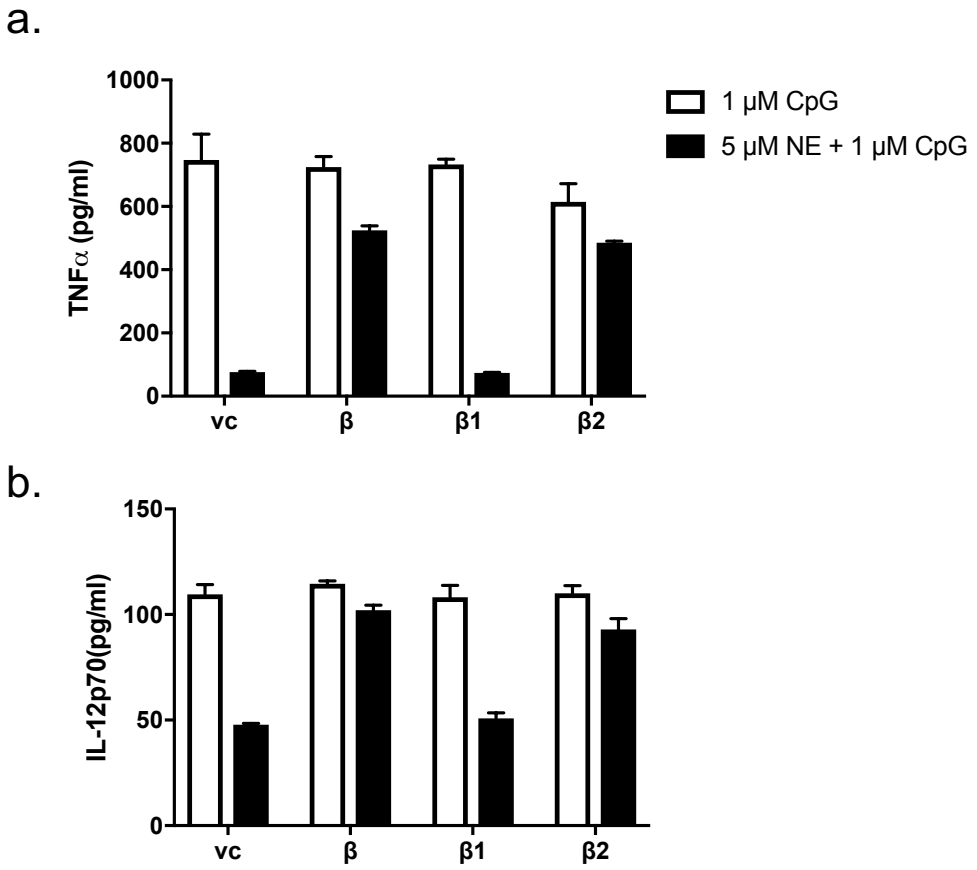

Figure 3.7. Suppression of the pro-inflammatory cytokines depends on signaling via the beta2-adrenergic receptor. BM-DMs from C57Bl/6J mice were stimulated with norepinephrine and CpG for 24 hours in the presence of pan β-adrenergic receptor antagonist (nadolol), or β1-adrenergic receptor antagonist (atenolol), or β2-adrenergic receptor antagonist (ICI118, 551). Supernatants were harvested and secreted cytokines (a) TNFα and (b) IL-12p70 were assessed using Mouse Pro-inflammatory 7-Plex Tissue Culture Kit. Error bars represent the technical error from the triplicate wells. n=3 mice were used to prepare BM-DMs but macrophages were combined to have enough cells for each condition. vc: vehicle control.

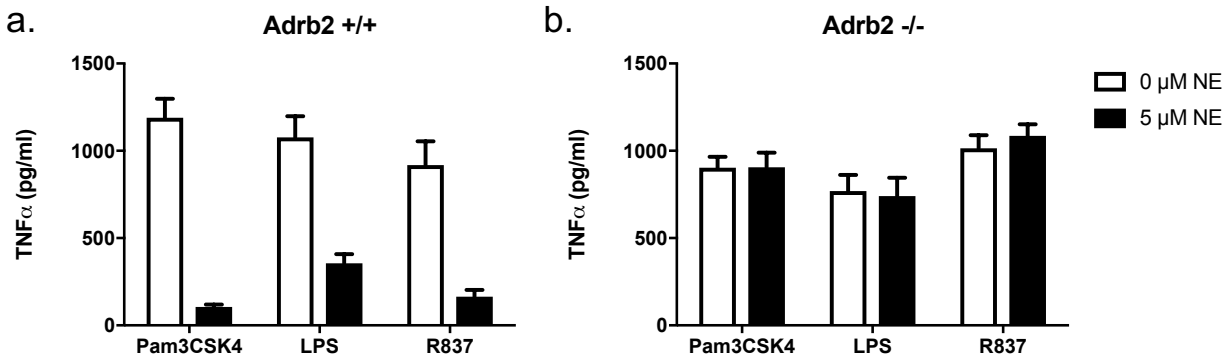

Figure 3.8. ADRB2-deficient macrophages are resistant to suppression of TNFα by norepinephrine. (a) ADRB2- sufficient and (b) ADRB2-deficient BM-DMs were stimulated with different TLR ligands in the presence or absence of norepinephrine for 24 hours. Supernatants were harvested and TNFα in the supernatant was quantified by ELISA. Error bars represent the standard error of the mean from n=3 mice per strain.

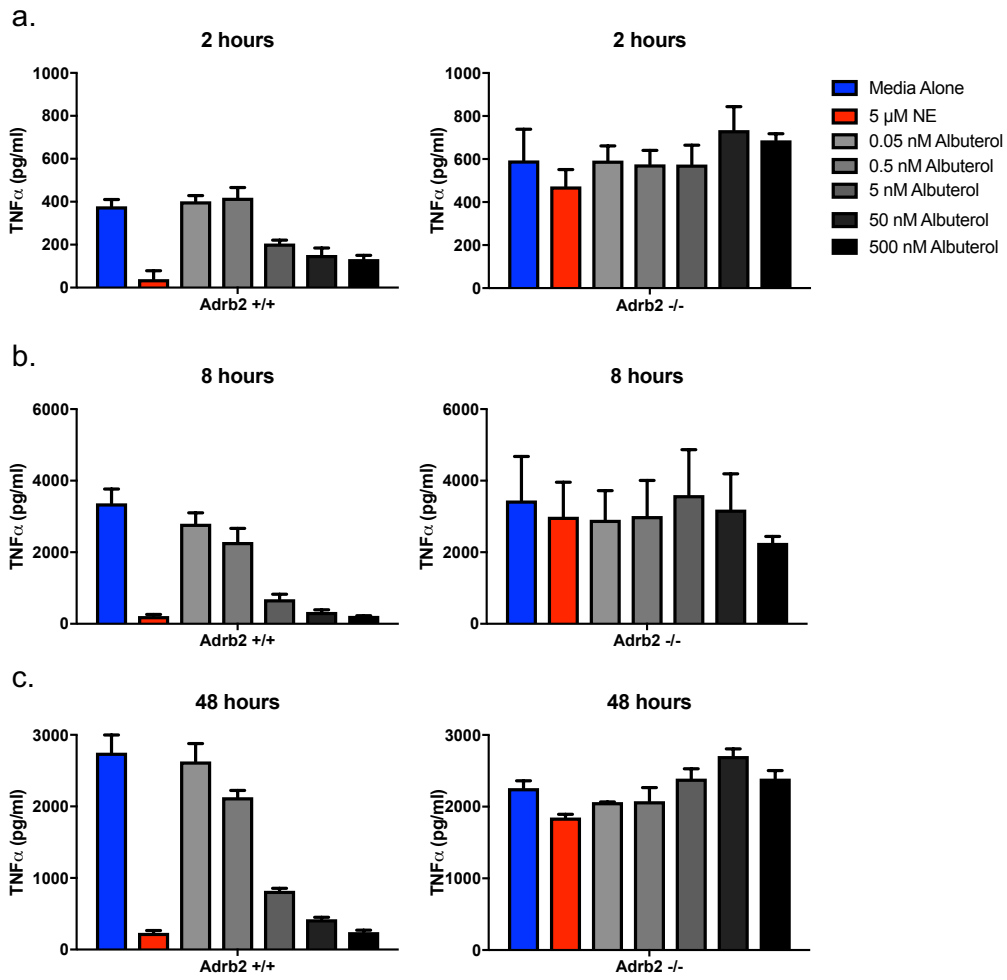

Figure 3.9. Albuterol, a short-acting beta2-adrenergic receptor specific agonist, downregulates CpG-induced TNFα production. ADRB2-sufficient (left column) and ADRB2-deficient (right column) BM-DMs were stimulated with 1 μM CpG for (a) 2 hours, (b) 8 hours, and (c) 48 hours, in the presence of norepinephrine (red bar) or increasing concentrations of albuterol (grey bars). While downregulation of TNFα was observed in ADRB2-sufficient BM-DMs, ADRB2-deficient macrophages were resistant to albuterol's suppressive effects. Error bars represent the standard error of the mean from n=3 mice per strain.

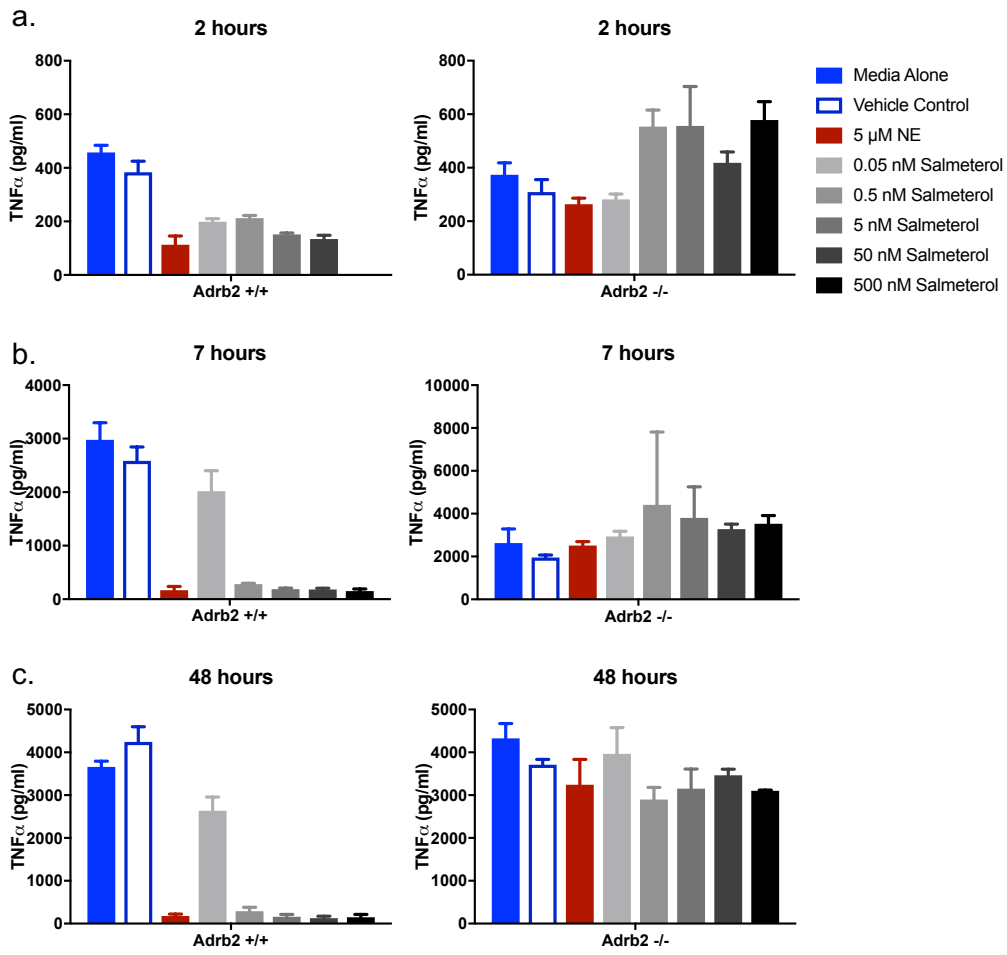

Figure 3.10. Salmeterol, a long-acting beta2-adrenergic receptor specific agonist, downregulates CpG-induced TNFα production. ADRB2-sufficient (left column) and ADRB2-deficient (right column) BM-DMs were stimulated with 1 μM CpG for (a) 2 hours, (b) 7 hours, and (c) 48 hours, in the presence of norepinephrine (red bar) or increasing concentrations of salmeterol (grey bars). While downregulation of TNFα was observed in ADRB2-sufficient BM-DMs, ADRB2-deficient macrophages were resistant to salmeterol's suppressive effects. Error bars represent the standard error of the mean from n=2 mice per strain.

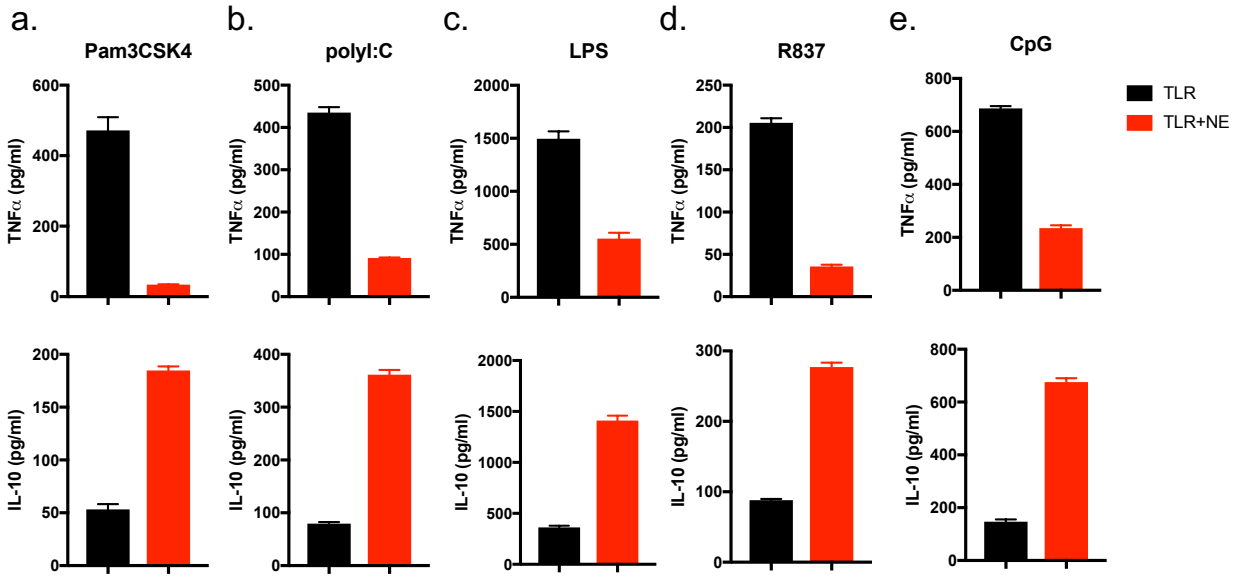

Figure 3.11. Adrenergic receptor signaling induces rapid IL-10 production. BM-DMs were stimulated with (a) 100 ng/mL Pam3CSK4, (b) 10 μg/mL poly I:C, (c) 100 ng/mL LPS, (d) 1 μg/mL R837, and (e) 1 μM CpG for 2 hours in the absence (black bars) or presence (red bars) of 5 μM NE. The cytokines released in the supernatant were quantified using Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery.

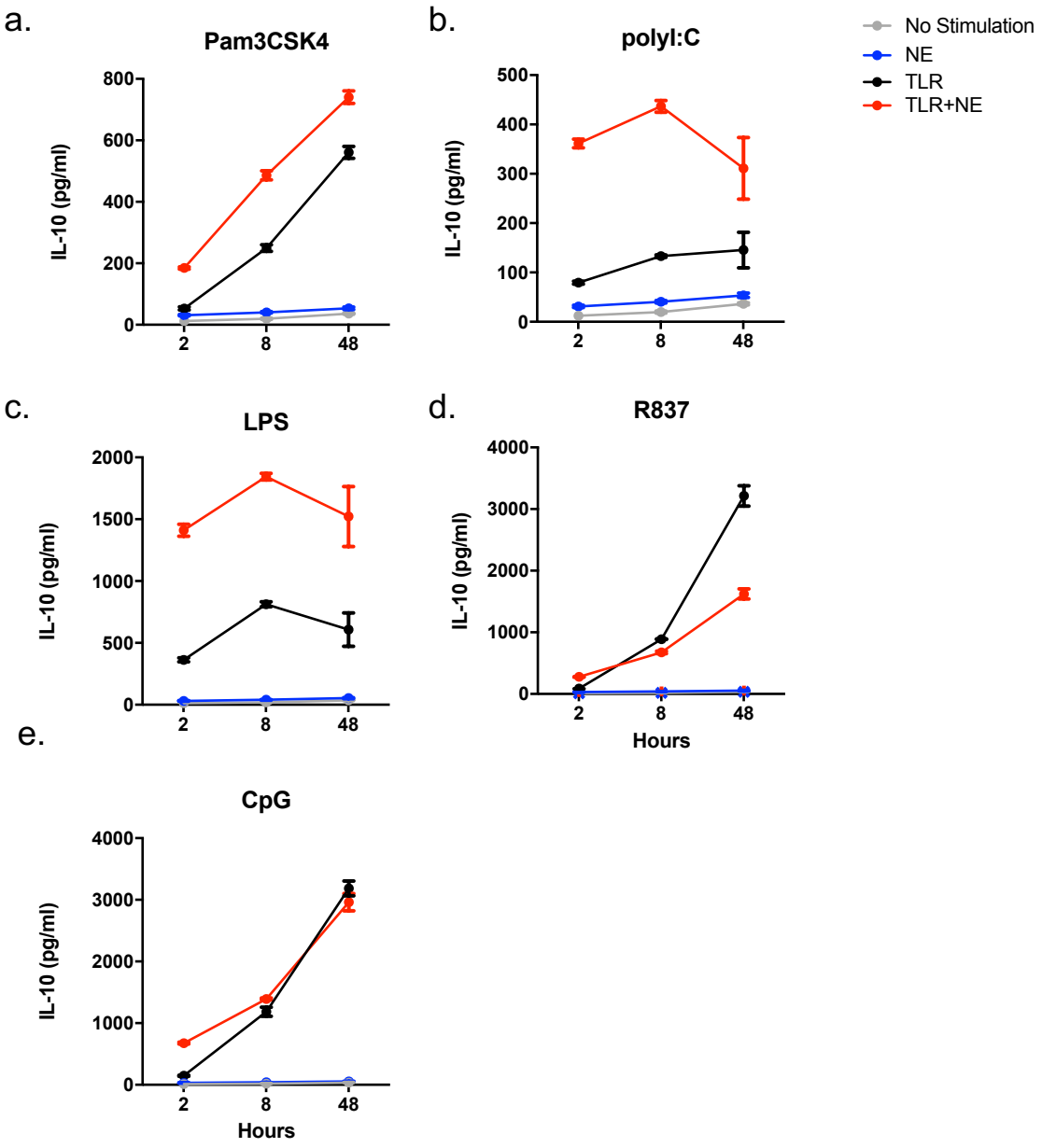

Figure 3.12. Norepinephrine alters IL-10 production in response to multiple TLRs. BM-DMs were stimulated with (a) 100 ng/mL Pam3CSK4, (b) 10 μg/mL poly I:C, (c) 100 ng/mL LPS, (d) 1 μg/mL R837, and (e) 1 μM CpG in the absence or presence of 5 μM NE for 2, 8 or 48 hours and the IL-10 released in the supernatants was quantified using Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery.

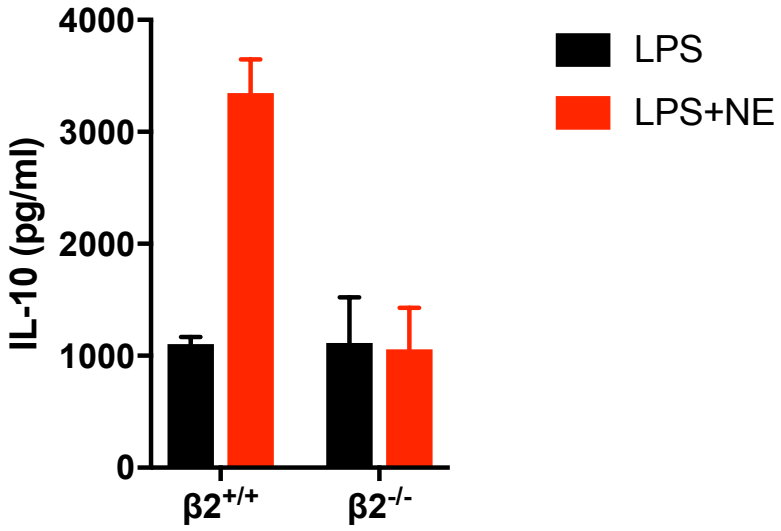

Figure 3.13. Upregulation of IL-10 in response to norepinephrine depends on the presence of beta2-adrenergic receptor. ADRB2-sufficient and -deficient macrophages were stimulated with 100 ng/mL LPS in the absence or presence of 5 μM NE for 8 hours. IL-10 in the supernatant was assessed by Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery. Error bars represent the standard error of the mean from n=3 mice per strain.

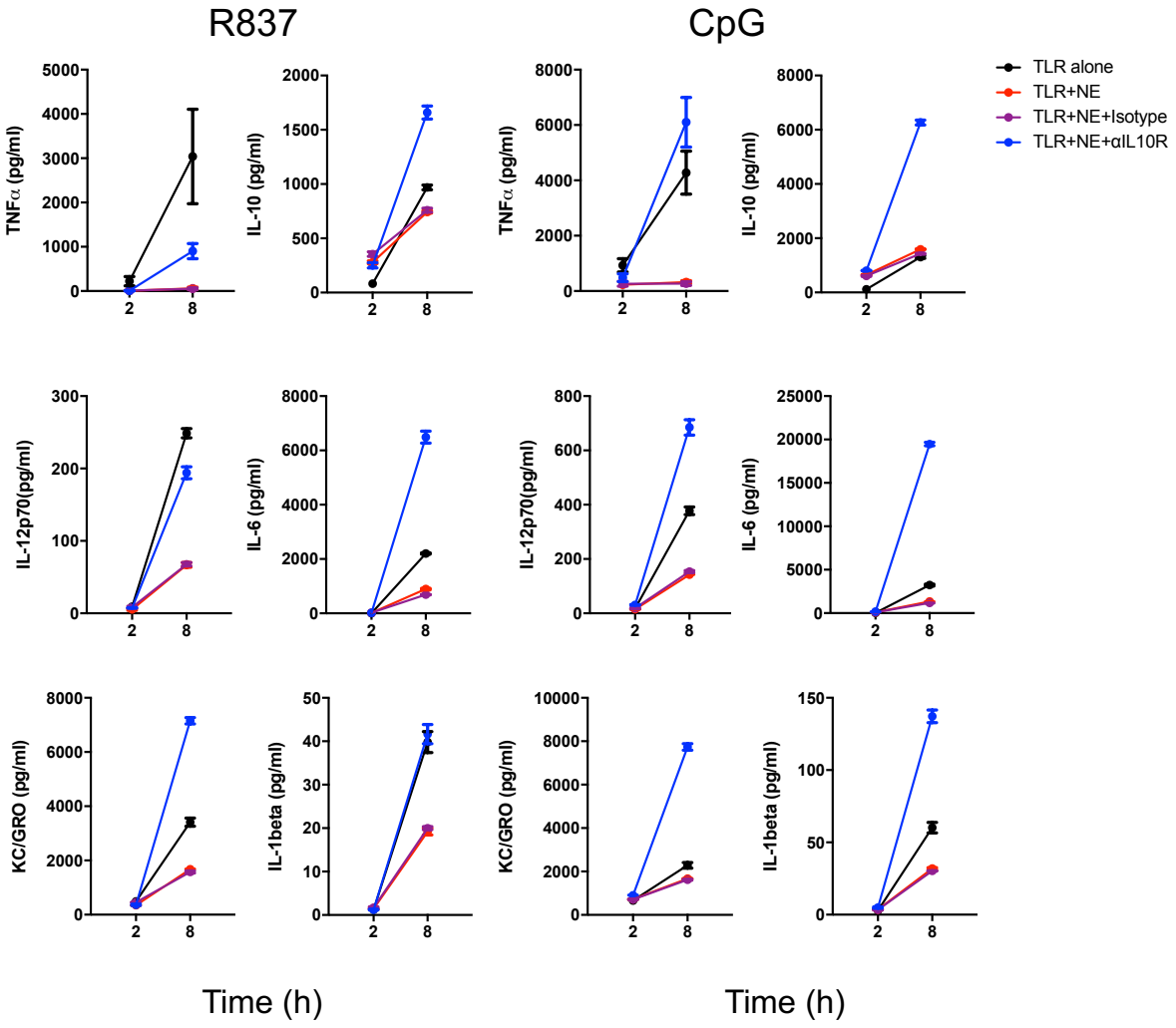

Figure 3.14. Effects of IL-10R neutralization on cytokine production. ADRB2-sufficient macrophages were pre-treated with antiCD210 (IL-10R) antibody or an isotype control for 30 minutes. The cells were stimulated with 1 μg/mL R837 or 1 μM CpG in the absence or presence of 5 μM NE for 2 or 8 hours. The cytokines that were secreted in the supernatant were assessed by Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery. Error bars represent average results and SEM from n=4 mice per time point for TNFα and n=1 for the other cytokines.

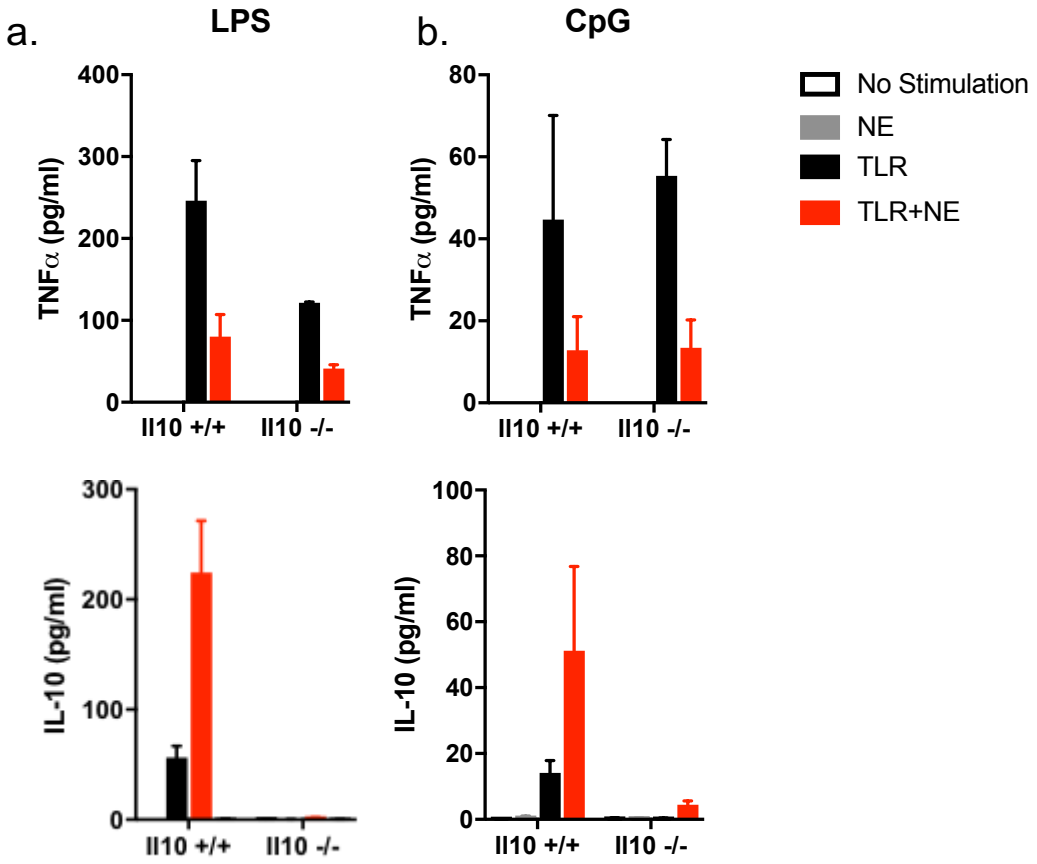

Figure 3.15. IL-10 is dispensable in suppression of TNFα in response to adrenergic receptor stimulus. IL-10-sufficient and -deficient BM-DMs were stimulated with (a) 100 ng/mL LPS or (b) 1 μM CpG in the presence or absence of 5 μM norepinephrine for 2 hours. Presence of norepinephrine during TLR stimulation was able to suppress TNFα, suggesting that acute induction of IL-10 and suppression of the pro-inflammatory cytokines depends on two independent pathways. The cytokine concentrations were determined using Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery. Error bars represent the standard error of the mean from n=3 mice per strain.

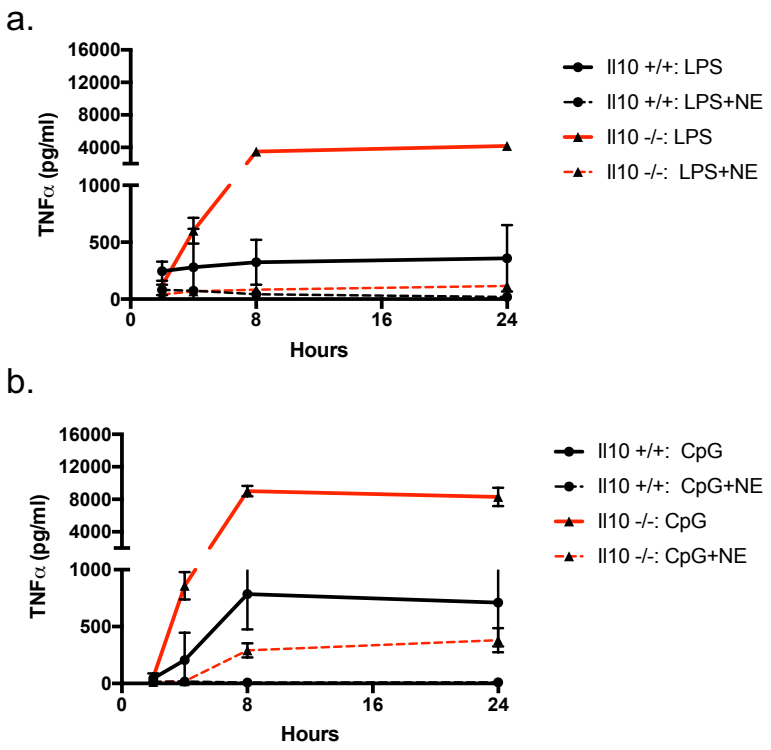

Figure 3.16. Time course for TNFα secretion in IL-10-sufficient and -deficient macrophages. IL-10-sufficient (black lines) and -deficient BM-DMs (red lines) were stimulated with (a) 100 ng/mL LPS or (b) 1 μM CpG in the presence or absence of 5 μM norepinephrine for 2, 4, 8 and 24 hours. Presence of norepinephrine (dashed lines) during TLR stimulation was able to suppress TNFα, suggesting that the acute induction of IL-10 and the suppression of the pro-inflammatory cytokines depends on two independent pathways. Although the TNFα concentration was higher in IL-10-deficient cells, norepinephrine was yet able to suppress TNFα throughout the time course. The cytokine concentrations were determined using Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery. Error bars represent the standard error of the mean from n=2-3 mice per strain.

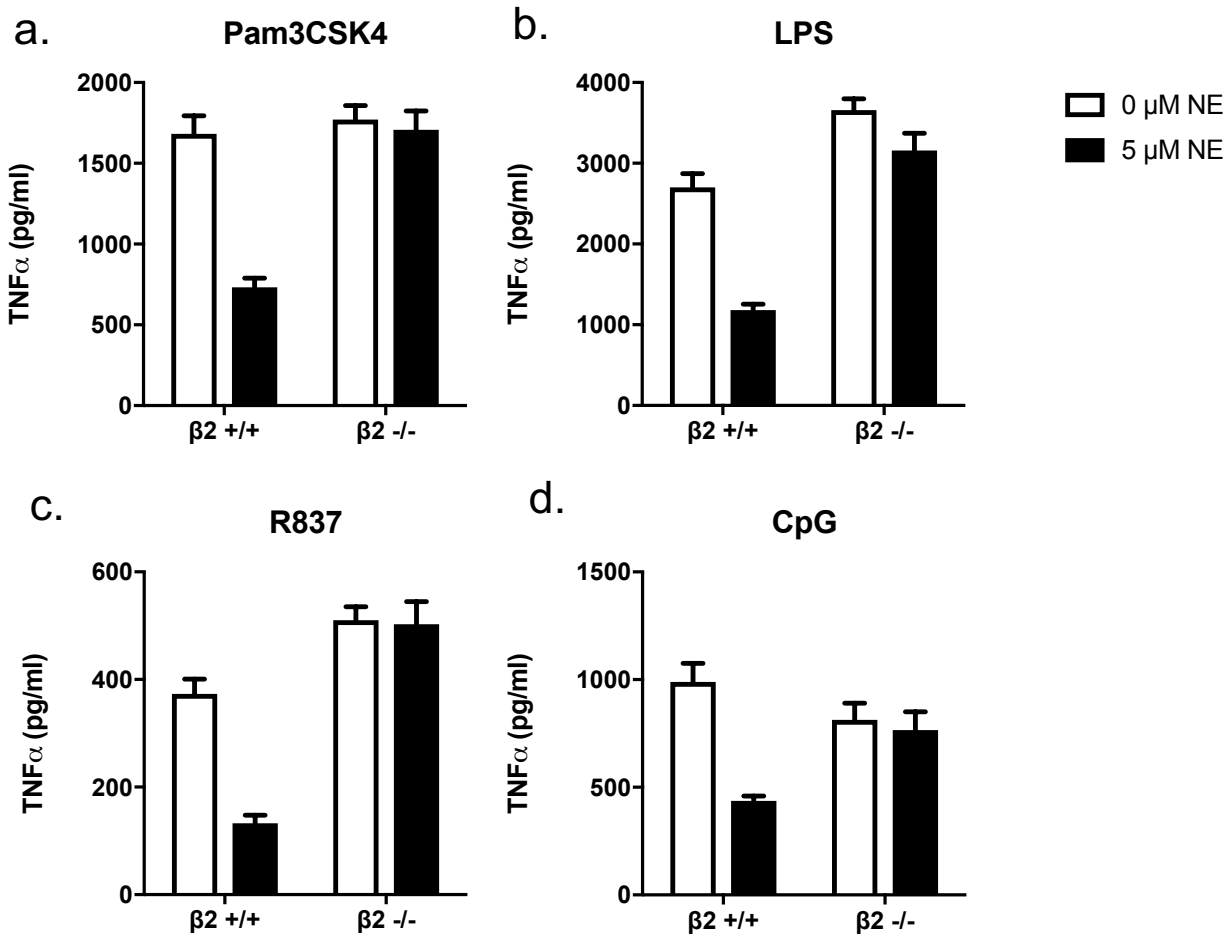

Figure 3.17. Norepinephrine suppresses TLR-induced TNFα secretion in bone marrow-derived dendritic cells via the beta2-adrenergic receptor. ADRB2- sufficient and ADRB2-deficient BM-DCs were stimulated with (a) 100 ng/mL Pam3CSK4, (b) 100 ng/mL LPS, (c) 1 μg/mL R837, and (d) 1 μM CpG in the absence (white bars) or presence (black bars) of 5 μM NE for 2 hours and TNFα levels were assessed by ELISA. Error bars represent the standard error of the mean from n=2 mice per strain.

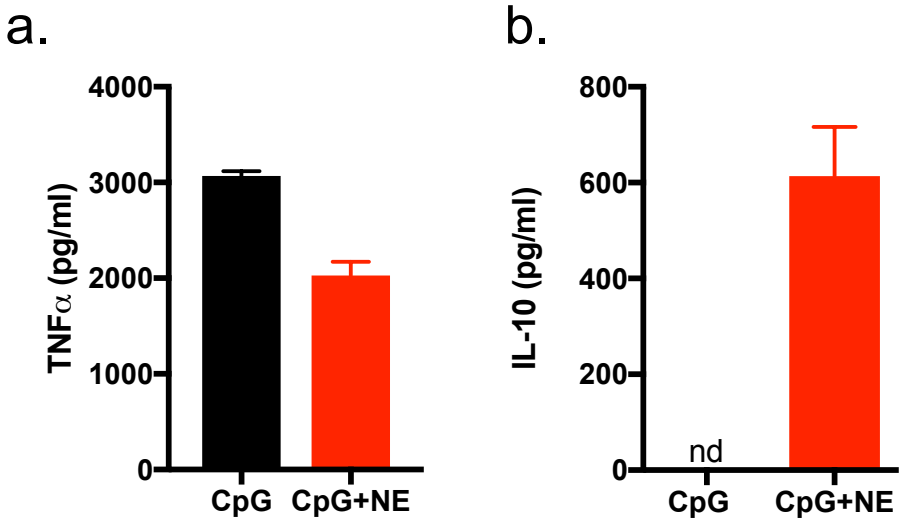

Figure 3.18. Presence of norepinephrine during TLR stimulation induces acute IL-10 production in bone marrow-derived dendritic cells. BM-DCs from Balb/cJ mouse were stimulated with 1 μM CpG in the absence or presence of 5 μM NE for 2 hours and TNFα and IL-10 levels were assessed by ELISA. n.d. not detected.

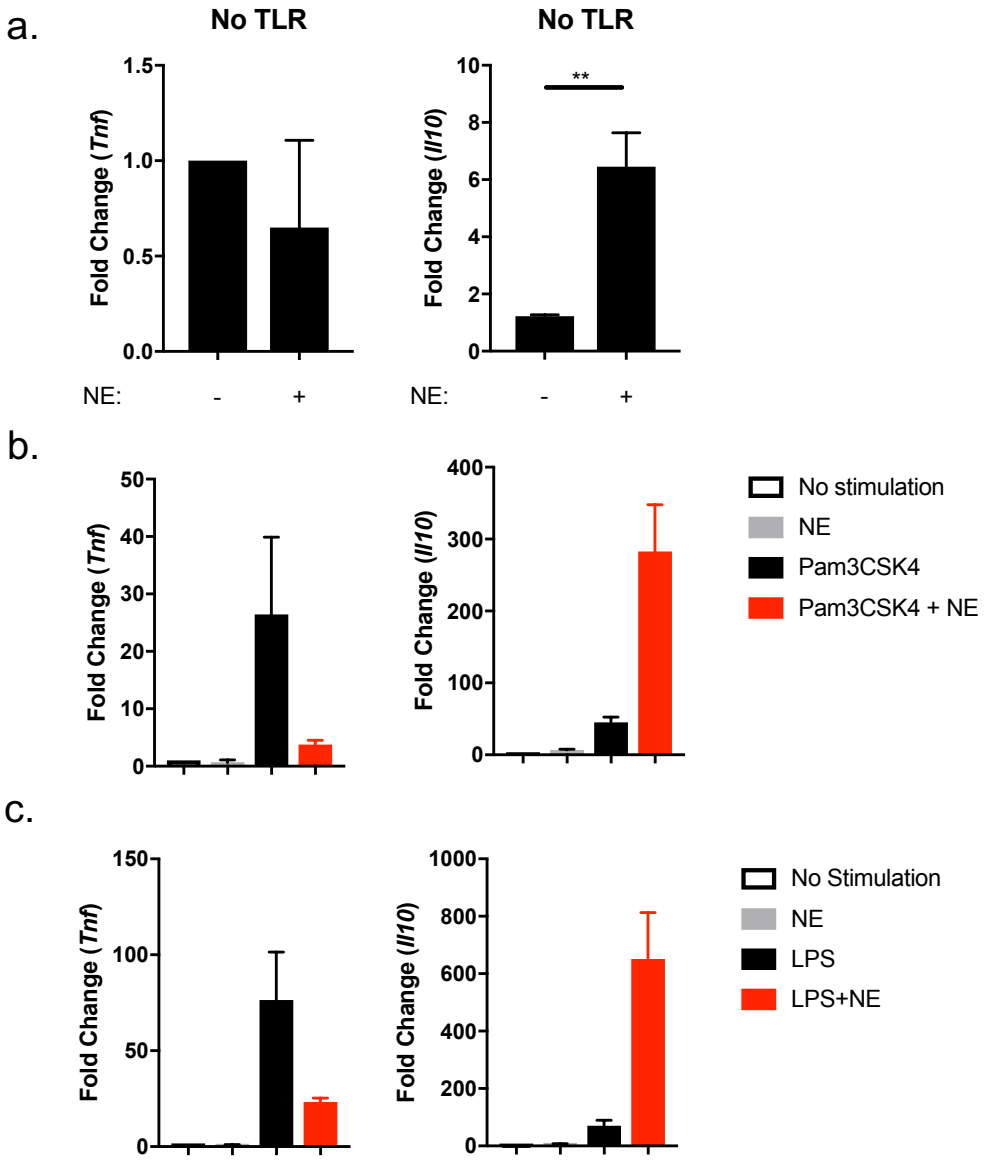

Figure 3.19. Norepinephrine can alter cytokine mRNA levels. BM-DMs were stimulated (a) in the absence of TLR agonists, (b) in the presence of 100 ng/mL Pam3CSK4, or (c) in the presence of 100 ng/mL LPS, with or without 5 μM NE for 2 hours and *Tnf* and *Il10* transcripts were measured by qRT-PCR. Error bars represent the standard error of the mean from n=4 mice.

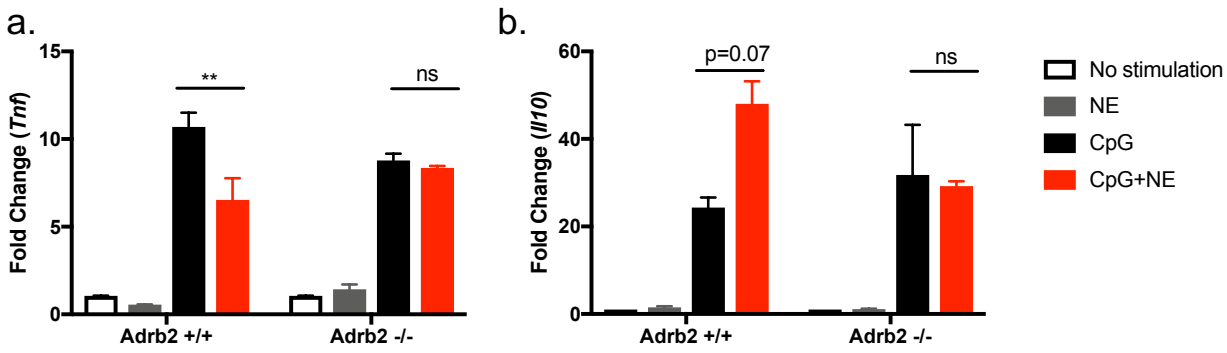

Figure 3.20. Norepinephrine alters cytokine mRNA levels via beta2-adrenergic receptor. ADRB2-sufficient or -deficient macrophages were stimulated with or without 1 μM CpG in the absence or presence of 5 μM NE for 2 hours. (a) *Tnf* and (b) *Il10* transcript levels were quantified by qRT-PCR. Error bars represent the standard error of the mean from n=2 mice per strain.

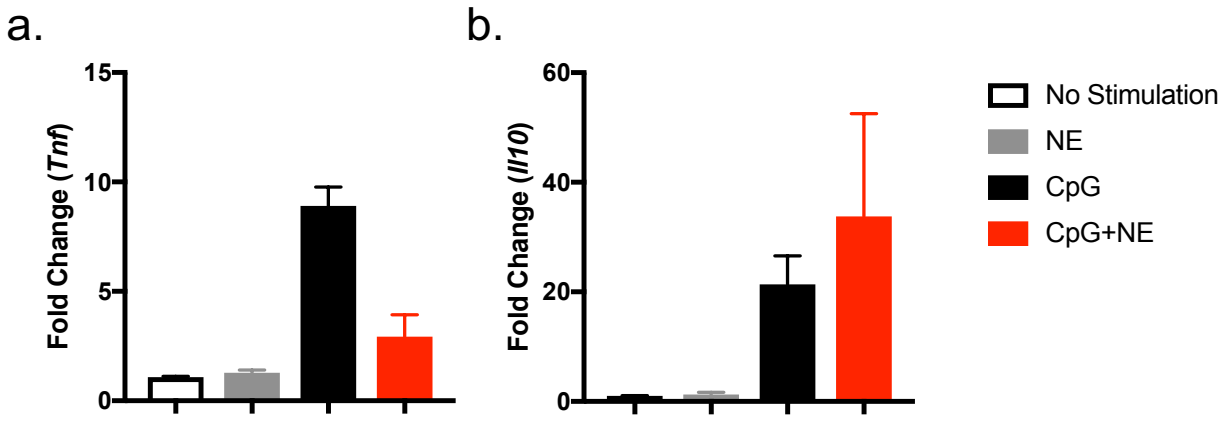

Figure 3.21. Tnf and Il10 transcripts at 8 hours post-stimulation. BM-DMs were stimulated with or without 1 μM CpG in the absence or presence of 5 μM NE for 8 hours. (a) *Tnf* and (b) *Il10* transcript levels were quantified by qRT-PCR. Error bars represent the standard error of the mean from n=3 mice per strain.

```
Hu_ADRB2          1 MGQPGNGSAFLLAPNRSHAPDHDVTQQRDEVWVVGMGIVMSLIVLAIVFG     50
                    ||..||.|.||||||.|.||||||||:|||.||||||.|:||:||||||||
Ms_ADRB2          1 MGPHGNDSDFLLAPNGSRAPDHDVTQERDEAWVVGMAILMSVIVLAIVFG     50

Hu_ADRB2         51 NVLVITAIAKFERLQTVTNYFITSLACADLVMGLAVVPFGAAHILMKMWT    100
                    |||||||||||||||||||||.||||||||||||||||||:|||||||.
Ms_ADRB2         51 NVLVITAIAKFERLQTVTNYFIISLACADLVMGLAVVPFGASHILMKMWN    100

Hu_ADRB2        101 FGNFWCEFWTSIDVLCVTASIETLCVIAVDRYFAITSPFKYQSLLTKNKA    150
                    ||||||||||||||||||||||||||||||.||||||||||||||||||
Ms_ADRB2        101 FGNFWCEFWTSIDVLCVTASIETLCVIAVDRYVAITSPFKYQSLLTKNKA    150

Hu_ADRB2        151 RVIILMVWIVSGLISFLPIQMHWYRATHQEAINCYANETCCDFFTNQAYA    200
                    ||:||||||||||.||||||||||||||::||:||..||||||||||||||
Ms_ADRB2        151 RVVILMVWIVSGLTSFLPIQMHWYRATHKKAIDCYTEETCCDFFTNQAYA    200

Hu_ADRB2        201 IASSIVSFYVPLVIMVFVYSRVFQEAKRQLQKIDKSEGRFHVQNLSQVEQ    250
                    ||||||||||||:||||||||.||||||||||||||||.||||||||
Ms_ADRB2        201 IASSIVSFYVPLVVMVFVYSRVFQVAKRQLQKIDKSEGRFHAQNLSQVEQ    250

Hu_ADRB2        251 DGRTGHGLRRSSKFCLKEHKALKTLGIIMGTFTLCWLPFFIVNIVHVIQD    300
                    |||:||||||||||||||||||||||||||||||||||||||||||||:|
Ms_ADRB2        251 DGRSGHGLRRSSKFCLKEHKALKTLGIIMGTFTLCWLPFFIVNIVHVIRD    300

Hu_ADRB2        301 NLIRKEVYILLNWIGYVNSGFNPLIYCRSPDFRIAFQELLCLRRSSLKAY    350
                    |||.|||||||||:|||||.||||||||||||||||||||||.|.|
Ms_ADRB2        301 NLIPKEVYILLNWLGYVNSAFNPLIYCRSPDFRIAFQELLCLRRSSSKTY    350

Hu_ADRB2        351 GNGYSSNGN-----TGEQSGYHVEQEKENKLLCEDLPGTEDFVGHQGTVP    395
                    |||||||.|     |||.:...:.||:|.:||||||.||.|.||..|||||
Ms_ADRB2        351 GNGYSSNSNGRTDYTGEPNTCQLGQEREQELLCEDPPGMEGFVNCQGTVP    400

Hu_ADRB2        396 SDNIDSQGRNCSTNDSLL    413
                    |.::||||||||||||.|
Ms_ADRB2        401 SLSVDSQGRNCSTNDSPL    418
```

Figure 3.22. Alignment of human and mouse ADRB2. Human (AAB82151.1) and mouse (NP_031446.2) ADRB2 were aligned using EMBOSS Needle pairwise sequence alignment tool. Proteins share 86.4% identity and 90.7% similarity. | represents conservation in that base, : represents amino acid conservation with strong similarity, . represents amino acid conservation with weak similarity.

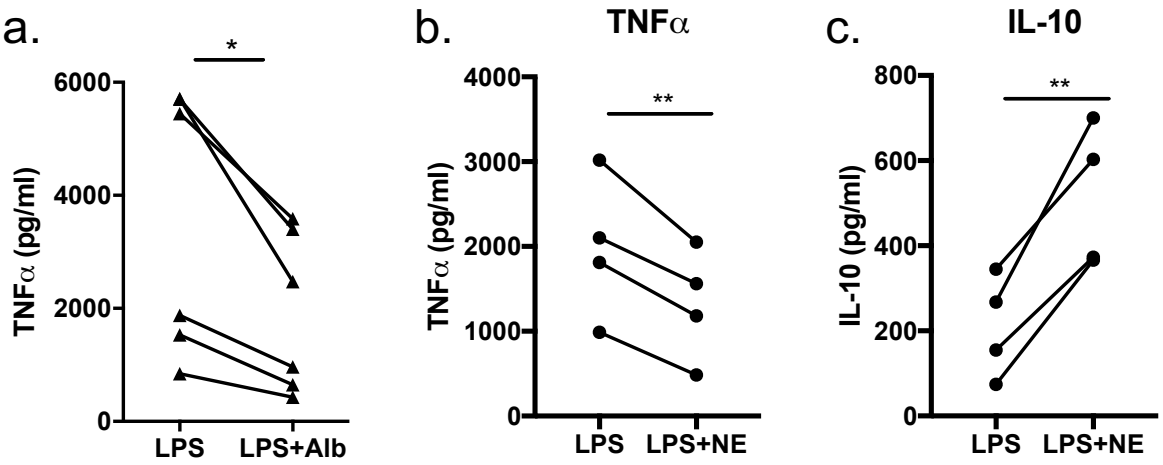

Figure 3.23. Adrenergic receptor engagement alters cytokine production in human cells. (a) Whole blood from healthy adult donors was stimulated with 10 μg/mL LPS in the presence or absence of 50 nM albuterol for 4 hours. Plasma TNFα was assessed by ELISA. The data represents 6 donors. (b-c) Human monocyte-derived macrophages were stimulated with 10 μg/mL LPS in the presence or absence of 5 μM NE for 2 hours. Secreted TNFα and IL-10 were quantified by ELISA. The data represents 4 donors. Monocytes were isolated by Dr. Regina Rowe. The data is significant ($p<0.05$) by paired t-test analysis.

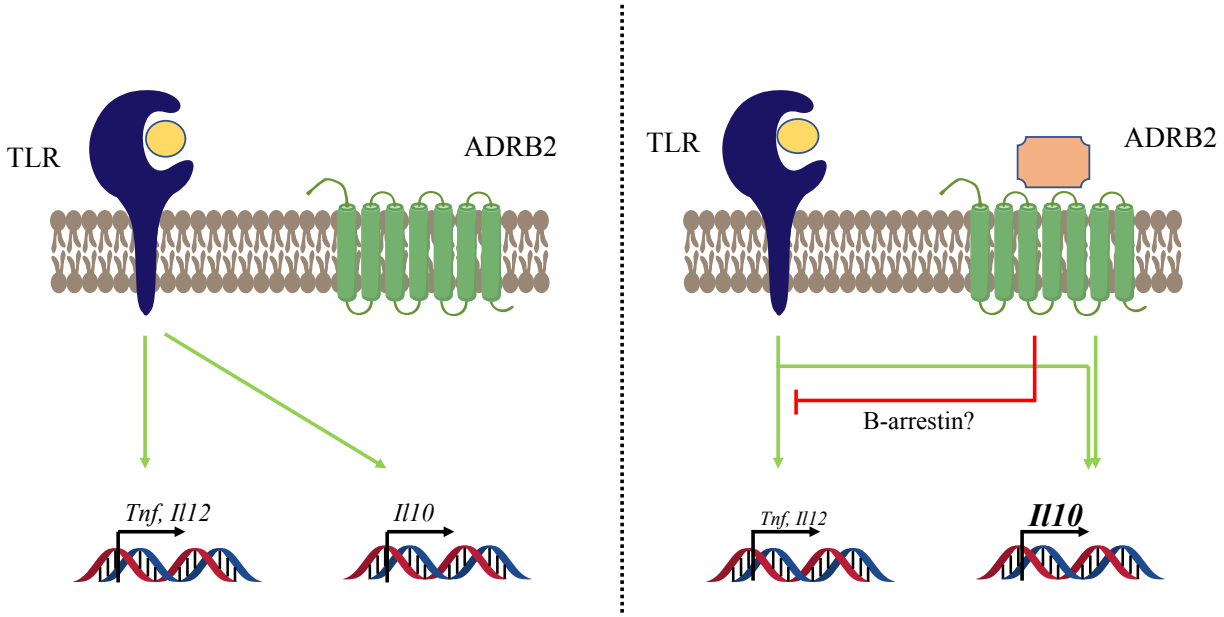

Figure 3.24. ADRB2-TLR crosstalk model. (Left) In the absence of adrenergic stimulus, TLR signaling can induce both pro-inflammatory (e.g. *Tnf*, *Il12*) cytokines and the anti-inflammatory cytokine *Il10*. (Right) Presence of adrenergic stimulus during TLR signaling results in upregulation of *Il10* and suppression of pro-inflammatory cytokines by two independent pathways. The role of β-arrestin on suppression of the pro-inflammatory cytokines needs further investigation.

CHAPTER IV

BETA2-ADRENERGIC RECEPTOR CONTROLS HYPERINFLAMMATION IN VIVO

The work presented here is unpublished data. Didem Ağaç conducted all the studies described here. Leonardo Estrada provided technical help, Sean Murray injected mice i.v. in some of the experiments, Robert Maples helped during colitis experiments and Kelly Ruhn taught me how to harvest colons. The colitis experiments were scored by Purva Gopal.

Introduction

To recognize potential threats and mount appropriate responses, the immune system and the nervous system need to cooperate. The nervous system can sense alterations in the environment, such as temperature and mechanosensory inputs. The immune system orchestrates inflammatory responses to recruit immune cells to site of infection or injury. However, tight spatial and temporal controls are required to limit inflammation. In addition, the degree of inflammation needs to be modulated: too little, and the organism risks inefficient elimination of the danger; too much, there is the risk of organ damage and autoimmune disease.

The cross-talk between the nervous and the immune systems enable the host to mount proper responses. The nervous system can sense the presence of inflammation by pattern

recognition receptors (Leow-Dyke et al., 2012; Lin et al., 2015; Xu et al., 2015) and cytokine receptors (Sawada et al., 1993). On the other hand, the immune cells express several neurotransmitter receptors, such as adrenergic receptors (Abrass et al., 1985; Liggett, 1989; Maisel et al., 1989) and acetylcholine receptors (Borovikova et al., 2000). Recent advances also suggest that the immune cells can express chemicals that are historically designated as neurotransmitters, including acetylcholine (Jiang et al., 2017; Rosas-Ballina et al., 2011) and dopamine (Papa et al., 2017). Lymphoid organs, such as thymus, lymph nodes and, spleen are highly innervated (Felten et al., 1985; Kendall and al-Shawaf, 1991; Panuncio et al., 1999; Reilly et al., 1979) to further enable this cross-talk. Additionally, immune cells in circulation can be exposed to neuroendocrine effectors and change their behavior. In summary, these observations suggest a complex cross-regulation between the nervous and the immune system to ensure proper inflammatory responses.

In Chapter III, I described the observation in which beta2-adrenergic receptor signaling resulted in an acute induction of IL-10 and the suppression of a plethora of pro-inflammatory cytokines in macrophages and dendritic cells *in vitro*. I was interested in exploring the *in vivo* role of this pathway that can alter several cytokines that were implicated in disease. Here, I utilized many murine models and identified that ADRB2 signaling is important to control inflammation *in vivo*, where in the absence of this signaling, mice develop hyper-inflammation and worse disease outcomes. Additionally, I showed that beta2-agonists can be used to control diseases like sepsis and infection. These results demonstrate how ADRB2 signaling is key in creating proper levels of inflammation in disease setting.

Based on our observations, we hypothesize that the beta2-adrenergic receptor signaling is a key regulator of inflammation.

Results

Our *in vitro* studies revealed that beta2-adrenergic receptor signaling is important to determine pro-and anti-inflammatory cytokine production from innate immune cells, namely dendritic cells and macrophages. I wanted to determine if adrenergic receptor signaling or the lack of this signal can alter organism-level immune responses. To address this, I conducted *in vivo* studies using 3 model systems: infections with *Listeria monocytogenes*, DSS-colitis and, LPS-induced endotoxemia. The common conclusions of all these studies were that (a) mice deficient in *Adrb2* present with hyperinflammation and (b) ADRB2-specific ligands, such as salmeterol, can alter the course of inflammation. This opens a new window of opportunity for the use of beta2-agonists, where beta2-agonists can be used as first-line therapeutics for many inflammatory disorders. In the following sections, I will present and discuss our *in vivo* results.

ADRB2-deficient animals are sensitive to Listeria monocytogenes infections

To determine if ADRB2 signaling is important in immune responses against bacterial infection, ADRB2-sufficient and -deficient mice were challenged with *Listeria monocytogenes* that express ovalbumin (LM-OVA). In our initial experiments, lethal doses of LM-OVA were used to determine if the presence of ADRB2 signaling was important for

the host survival. Mice that lack ADRB2 succumbed to death faster than the animals with intact ADRB2 signaling (the average time for survival was 73 hours for the ADRB2-deficient mice and 88 hours for the ADRB2-sufficient mice) (Figure 4.1). When both groups of animals were challenged with sub-lethal doses of LM-OVA, ADRB2-deficient mice developed splenomegaly in response to infection at day 3 (Figure 4.2a) and day 7 (Figure 4.2c) post-infection. This was not accompanied by hepatomegaly (Figure 4.2b and 4.2d).

When cellular distribution in the spleen was assessed by flow cytometry at day 3 post-infection, most splenic populations had slightly higher cell numbers in ADRB2-deficient animals, however, the differences between ADRB2-sufficent and -deficient animals did not reach statistical significance (Figure 4.3). This suggests that adrenergic receptor signaling did not alter the cellular recruitment of a select group of cells, rather all immune cells were affected similarly. When cellular distribution was assessed at day 7; the total number of splenocytes and the total number of CD11b low (non-myeloid) cells were significantly higher in ADRB2-deficient animals (Figure 4.4a and 4.4c). Within CD11b low cells, there were more T cells in ADRB2-deficient animals, but B cell numbers were unchanged (Figure 4.4d and 4.4e). The CD11b high, myeloid cells were slightly higher in ADRB2-deficient animals (Figure 4.4b), however, this did not reach statistical significance. Overall, these data suggest that lack of ADRB2-signaling increased cellularity in the spleen at different times after infection and the increase of the cell number could not be attributed to a single subset but rather it was a change in multiple populations.

Interestingly, the increase in spleen size and cellularity did not correlate with better clearance of the pathogen (Figure 4.5). ADRB2-deficiency did not alter the number of viable

bacteria recovered from spleen or liver, either at day 3 (Figure 4.5a and 4.5b) or day 7 (Figure 4.5c and 4.5d). Additionally, animals from both ADRB2-sufficient and -deficient groups were able to clear the infection at day 7. This suggests that the lack of ADRB2-signaling did not impair clearance of this particular pathogen. Although the clearance of LM was comparable between ADRB2-sufficient and -deficient animals, ADRB2-deficient animals had more cells in their spleens. This observation raises the possibility of an intrinsic defect in pathogen clearance (per cell basis) in ADRB2-deficient cells; to reach comparable pathogen clearance in organism level, ADRB2-deficient cells would be recruited (or retained in spleen) more, leading the splenomegaly. However, I have not formally addressed this aspect.

ADRB2-agonists can alter the splenomegaly in LM-infections

To determine if beta2-adrenergic receptor agonists could change an organism's response to infection, animals were treated with salmeterol, a long-acting beta2-adrenergic receptor agonist, during LM-OVA infection. ADRB2-sufficient mice were challenged with LM-OVA at day 0. Additionally, the animals were administered salmeterol or vehicle control at days 0, 1 and 2 post-infection. The mice that received salmeterol had a reduction in their spleen weight at day 7, suggesting that the engagement of the beta2-adrenergic receptor signaling could reduce the inflammation. As expected, there were no difference in liver size (Figure 4.6). Interestingly, engagement of beta2-adrenergic receptor slightly reduced total cell numbers of many immune cell populations, rather than affecting one cell type. The minor

differences in all groups reflected as a reduction in total spleen size (Figure 4.7). The data presented here suggest that adrenergic receptor signaling could modulate inflammatory responses during an infection.

ADRB2-deficient mice lose more weight in DSS-colitis

We wanted to determine if the effects of beta2-adrenergic receptor signaling were limited to infection or can be observed in a tissue-damage insult model. We have used DSS (dextran sodium sulfate) to initiate chemically induced acute intestinal inflammation. DSS induced colitis is one of many animal models that are developed to understand human inflammatory bowel diseases (IBD) such as Crohn's Disease and Ulcerative Colitis. Although the mechanism of DSS-induced inflammation is unclear, the current understanding is likely due to the disruption of the epithelial lining in large intestine. Once the barrier is breached, intestinal contents would disseminate to the colonic tissue and induce inflammation. DSS-induced colitis remains a common model in IBD research due to its simplicity and controllability of the inflammation by modification of the dose of DSS. Interestingly, adaptive immune cells are not required for initiation of disease, therefore, DSS stands as a good model to understand the role of innate immune cells in inflammation. The inflammation and the disease outcome could be assessed several ways: weight loss, alterations in mucosal tissue (e.g. inflammatory cell infiltration, goblet cell dysplasia, muscle thickening, disruption of the epithelial lining), stool consistency, and rectal bleeding (Chassaing et al., 2014; Kim et al., 2012; Wirtz et al., 2017).

IL-10 signaling is important to control intestinal inflammation. In humans, IL10 and IL10R polymorphisms are associated with IBD (Franke et al., 2008; Moran et al., 2013). Additionally, IL-10-deficient animals are reported to develop spontaneous colitis (Kuhn et al., 1993). Li and colleagues showed that macrophage specific deletion of IL10Rα resulted in similar weight loss to IL-10-deficient mice in DSS-induced colitis (Li et al., 2014). Similar results have been reported by other groups (Zigmond et al., 2014). These results suggest a role for macrophages in control of inflammation through IL-10 signaling.

ADRB2-sufficient, -heterozygous and -deficient animals were challenged with 3%DSS in drinking water for 7 days. The mice were weighed each day and the weight loss was assessed over the course of the experiment. ADRB2-deficient mice but not the control groups lost weight compared to their original body weights (Figure 4.8a). Although the ADRB2-deficient mice were more sensitive to the DSS, the colon length (Figure 4.8b) or histopathology scores (Figure 4.9) were unchanged between groups. The criterion for the scoring can be found in Table 4.1.

ADRB2-deficient mice are sensitive in LPS-endotoxemia

Norepinephrine and epinephrine, natural ligands for adrenergic receptors, are commonly used as the first line therapeutics for septic shock syndrome (De Backer et al., 2010). We wanted to investigate if the clinical benefit of norepinephrine/epinephrine administration has any relevance to beta2-adrenergic receptor's role in the control of inflammation.

There are two main animal models of sepsis, cecal ligation puncture (CLP) and LPS-endotoxemia. CLP relies on the initiation of inflammation due the dissemination of intestinal contents to tissue after physical intestinal injury ("puncture" by a needle). This method is technically challenging, and the inflammation cannot be controlled by the experimenter. LPS endotoxemia is a reproducible and simple method, where the investigator administers LPS to intraperitoneal cavity and relies on the systemic activation of the immune system. This model recapitulates some of the human disease; increased circulating levels of TNFα, IL-6 and HMGB1, hypotension and aneroxia (Fink, 2014). It is also possible to adjust the dose of LPS to control the inflammation and the survival of the animals. This would enable the experimenter to assess the LPS sensitivity of different stains or help to test if an intervention would alter survival.

We have challenged the ADRB2-sufficent and -deficient mice with intraperitoneal administration of LPS. ADRB2-deficient mice were sensitive to LPS. It is important to note that the dose that the animals were challenged was a non-lethal dose for ADRB2-sufficient mice, yet the ADRB2-deficient animals succumbed to death (Figure 4.10).

Our *in vitro* studies suggested a role for beta2-adrenergic signaling to regulate cytokine production. Additionally, IL-10 has been shown to be an important cytokine for recovery from sepsis. Latifi *et al* showed that IL-10-deficient mice were susceptible to cecal-ligation puncture, a murine model of endotoxemia (Latifi et al., 2002). We sought to determine if the ADRB2-deficient animals develop dysregulation in serum cytokines. After challenging the ADRB2-sufficient and -deficient mice with LPS, the cytokines present in serum were assessed by ELISA. 30 minutes and 2 hours were chosen as points of

investigation, since changes in cytokine secretion *in vitro* were acute, and neither group presented lethality in the abovementioned time points. LPS challenge induced an increase in TNFα, IL-10 and, IL-6 (Figure 4.11a-c), but not in IL-1β (Figure 4.11d). It is possible that IL-1β was regulated at later time points, however, I have not assessed this possibility. Although TNFα and IL-10 levels were different between the two groups at 2 hours post-challenge, no difference was observed in IL-6 at 2 hours. It is possible that the IL-6 levels were different between ADRB2-sufficient and -deficient mice between 30 minutes and 2 hours and our assays were unable to catch this since we have not sampled in between. The most exciting result from this assay was the difference between ADRB2-sufficient and -deficient mice in TNFα and IL-10 production. ADRB2-deficient mice had significantly higher TNFα in their serum at 2 hours compared to ADRB2-sufficient mice (Figure 4.11a). This was also accompanied by the reduction in IL-10 in ADRB2-deficient mice (Figure 4.11b). The changes were limited to TNFα and IL-10, there was no difference in serum levels of KC/GRO, IL-12p70 or IFNγ (Figure 4.12). Our data is in agreement with previous observations where propranolol (a beta blocker) enhanced TNFα, IL-6 and, MCP-1 in BAL in mice that are intranasally treated with LPS (Giebelen et al., 2008). These observations suggest that beta2-adrenergic signaling promotes an anti-inflammatory state *in vivo*.

ADRB2 signaling controls IL-10 production in vivo

We hypothesized that ADRB2-deficient mice are susceptible to endotoxemia due to their ability to induce adequate levels of IL-10. If this was the case, supplementation of IL-10 should be able to rescue the lethality of ADRB2-deficient animals. To test this idea, 1 μg recombinant murine IL-10 was administered to ADRB2-deficient mice, 30 minutes after LPS administration. Mice that received exogenous IL-10 survived better during endotoxemia (Figure 4.13).

Next, we wanted to determine if engaging the beta2-adrenergic receptors would enhance IL-10 production *in vivo*. Administration of salmeterol during LPS endotoxemia (salmeterol was injected 30 minutes after LPS injection) reduced the serum TNFα levels (Figure 4.14a) and drastically enhanced serum IL-10 levels (Figure 4.14b). However, IL-6 and IL-1β levels were unaffected in response to salmeterol (Figure 4.14c-d).

To test if engagement of beta2-adrenergic receptors could increase the survival, the ADRB2-sufficient animals were challenged with a high dose of LPS in the presence or absence of salmeterol. Administration of salmeterol increased the survival of the animals in endotoxemia. The effects of salmeterol depended on IL-10 signaling, as the animals that received IL-10R neutralization antibody succumbed even when treated with salmeterol (Figure 4.15). These data suggest that beta2-adrenergic receptor signaling *in vivo* controls IL-10 production.

Myeloid cell-specific deletion of Adrb2 phenocopy the endotoxemia sensitivity

Our *in vitro* studies suggested that macrophages and dendritic cells alter their cytokine profiles in response to adrenergic receptor signaling (Chapter III). In my initial endotoxemia experiments, I utilized whole-body knockouts and reported sensitivity of ADRB2-deficient animals to endotoxemia. It is important to determine if this sensitivity was derived from innate immune cells. I crossed Adrb2 fl/fl animals (that allows the deletion of Adrb2 under the control of the Cre allele that is provided) with LysM-cre animals. Although LysM-cre line is considered a monocyte/macrophage specific line, it is actually a myeloid specific line. In addition to macrophages, neutrophils, granulocytes and to a lesser extend dendritic cells have been reported to be targeted by LysM-cre allele (Clausen et al., 1999). Although there is not a single cre-line that is "specific" to macrophages; even the use of LysM-cre would allow us to identify if the LPS sensitivity was derived from innate immune cells. LysM-cre, Adrb2 fl/fl animals and the control mice were challenged with LPS (Figure 4.16). The sensitivity towards LPS was increased in conditional knockout animals, suggesting that the innate immune cells were the primary cells that respond to adrenergic cues to help the host survival.

Discussion

Data presented here suggest that ADRB2-signaling influences different inflammatory settings, both systemic (LM-infection and endotoxemia) and local inflammations (colitis). I have additionally shown that administration of beta2-agonists can reduce the inflammation and give the animals survival benefits. Similar to our *in vitro* observations, salmeterol can

induce IL-10 and reduce TNFα *in vivo*. This raises the possibility that if beta2-agonists, a drug class commonly used for symptom treatment of COPD and asthma, can influence local lung environment, as well. Our observations suggest a general anti-inflammatory role for beta2-adrenergic receptor signaling *in vivo*.

It has been previously reported that acute cold/restrain stress inhibited the clearance of LM infection via beta1-adrenergic receptors. In the same study, administration of beta2-adrenergic receptor antagonist ICI118,551 did not alter the LM burden (Cao et al., 2003a). In a follow-up study, Cao *et al* showed that acute cold/restrain stress did not alter the spleen weight or cellularity in response to LM infection (Cao et al., 2003b). In our experiments, we did not observe differences in LM-OVA burden between ADRB2-sufficient and deficient animals but observed an increase in spleen weight in ADRB2-deficient animals. It is possible that different adrenergic receptors control different cascades of anti-bacterial response; maybe beta2-signaling is important for cellular recruitment, and beta1-signaling is important for bactericidal properties. Also, Cao *et al* utilized 5-6 times fewer bacteria than what we used in our study, thus the severity of the infection may change the level of the catecholamines that are released and may influence which receptor would have more ligand availability.

In agreement with our observations, Seeley and colleagues reported that ablation of adrenergic neurons by 6-OHDA increases survival of the animals during *K. pneumoniae* induced peritonitis. This phenotype was attributed to enhanced monocyte recruitment and bacteria clearance via increased MCP-1, in the absence of adrenergic neurons (Seeley et al., 2013). Based on our studies, in the absence of epinephrine/norepinephrine production, we

expect to see a more pro-inflammatory state, potentially leading to more efficient bacterial clearance.

A study by Heidt and colleagues reported that chronic stress increases norepinephrine levels in bone marrow and decrease neutrophil numbers in the blood. This was accompanied by a reduction in CXCL12, a chemokine that inhibits hematopoietic stem cell proliferation and migration and is important for neutrophil retention in bone marrow. However, CXCL12 levels were restored when beta3-adrenergic receptor blocker was administered, suggesting a role for adrenergic receptor signaling and leukocyte trafficking (Heidt et al., 2014). A similar role has been suggested for beta2-adrenergic receptor signaling for as well (Katayama et al., 2006). ADRB2 was additionally shown to regulate diurnal oscillation of B and T cell numbers in circulation and lymph nodes (Suzuki et al., 2016). In line with these observations, our study suggests that the engagement of beta2-adrenergic receptors via salmeterol can influence spleen weight by modestly altering different immune cell populations. However, the effect of beta2-adrenergic receptor agonists or antagonists on diurnal oscillations of immune cells need to be investigated further.

There are not many studies that investigate the role of adrenergic receptor signaling in intestinal homeostasis. Gabanyi *et al* recently suggested that the intestinal muscularis macrophages resided in close proximity to the enteric nervous system and were polarized towards M2 macrophages via ADRB2 (Gabanyi et al., 2016). However, it is unclear how different intestine resident immune cells are affected by neuroendocrine signaling (not just adrenergic) and the consequences of this in disease conditions and homeostasis.

Sepsis and severe sepsis (sepsis that involves acute organ function) are a common cause of mortality in the United States and pose an annual burden of $14 billion. Although Gram-negative species are commonly implicated, Gram-positive bacteria, fungi or parasites are also cultured from patients with sepsis. However, Gram-negative bacteria are associated with higher mortality compared to Gram-positive bacteria (Mayr et al., 2014). In our studies, we have mimicked Gram-negative bacteremia by administering LPS derived from *E.coli.* However, it would be interesting to test if ADRB2 has a role in protecting against Gram-negative bacteremia.

Early studies showed that the mortality during bacteremia and sepsis are not completely dependent on the presence of bacteria. In mice that were challenged with *E. coli, P.mirabilis* or *K.pneumoniae,* administration of antibiotics at the time of pathogen administration caused less mortality than administration of antibiotics at later time points (e.g. 4-8 hour after pathogen challenge). This data suggests that the bacteremia associated mortality is not completely dependent on the presence of the pathogen (Greisman et al., 1979). With our increased understanding of immune responses to pathogens, this data can be explained further by the upregulation of the pro-inflammatory cytokines and their downstream effects, a cascade that no longer relies on the presence of bacteria. Therefore, understanding the regulation of cytokines can provide insights to disease progression and offer new therapies.

In animal studies, endotoxin administration has been shown to rapidly increase plasma norepinephrine and epinephrine (as early as 90 seconds) (Spink et al., 1966). Similar observations were confirmed by Rosenberg and colleagues, where they observed increased

plasma catecholamines (norepinephrine and epinephrine) 5-minutes after LPS challenge in dogs (Rosenberg et al., 1961) however the serotonin levels remain unchanged. In human endotoxemia models, the subjects increased the plasma epinephrine and cortisol after 3 hours of LPS challenge (Calvano and Coyle, 2012). However, the level of norepinephrine was not measured in this study. Lastly, plasma concentrations of epinephrine were reported to be upregulated when mice were challenged with LPS, compared to unchallenged groups (Lukewich and Lomax, 2014). The data summarized here suggests that presence of bacteria can be sensed by the neuroendocrine system and this causes rapid upregulation of catecholamines, which can influence immune cell behavior.

In human endotoxemia models, infusion of epinephrine for 4 hours (starting the time of LPS administration) reduced the plasma TNFα levels. When epinephrine was used as a prophylactic agent (e.g. administered t= -3 hours to +6 hours of LPS challenge), the subjects had more IL-10 and less TNFα in their plasma samples (van der Poll et al., 1996b). This suggests that adrenergic signaling can reduce inflammation, either as a prophylactic agent or after exposure to LPS. It is possible that being able to alter serum cytokines is one of the benefits of norepinephrine/epinephrine usage in septic shock treatment.

A meta-analysis of shock data of 3,544 patients from 32 trials suggested that norepinephrine should be chosen as the first line vasopressor in septic shock treatment. Patients that received norepinephrine had reduced adverse events (tachyarrhythmias, organ ischemias etc.) compared to patients that received dopamine. Patients that received epinephrine or norepinephrine, but no other vasopressors had reduced mortality at day 28 but there was no benefit of epinephrine over norepinephrine (Avni et al., 2015). If our *in vivo*

experiments reflect the biology of sepsis, this result can be explained by the effect of norepinephrine or epinephrine on beta2-adrenergic receptors and control of inflammation by IL-10. It would be interesting to investigate if administration of beta2-agonists would have benefits in human septic shock patients and if the benefits reported by Avni *et al* correlate with serum cytokine change alterations (for example more IL-10) that can contribute to better disease outcomes. It is also interesting to note that SNPs on *Adrb2* are associated with increased mortality and more organ dysfunction in sepsis (Nakada et al., 2010).

Similar to our observations, Vida *et al* showed that administration of salmeterol, 24 hours after the initiation LPS endotoxemia can increase survival. In these studies, the investigators gave 6-15 mg salmeterol/kg to mice, which was a higher dose than what we used (1.6 mg/kg). In addition to LPS endotoxemia, this study also showed that administration of salmeterol can increase survival in cecal-ligation puncture model (Vida et al., 2011).

Manipulation of the neuroendocrine system for treatment of inflammatory diseases is an emerging idea. Stimulation of the vagus nerve (VNS) in a group of rheumatoid arthritis patients reduced the TNFα production in response to LPS, compared to time points before VNS (Koopman et al., 2016). Based on work by Tracey lab, VNS can elicit its anti-inflammatory effects on macrophage cytokine production through the release of acetylcholine (Andersson and Tracey, 2012a, b). However, the role of VNS on the adrenergic system and downstream effects on macrophages have not been formally addressed.

Adrenergic receptors are important in a myriad of physiological functions and their capacity to alter/control immune responses is a fast-growing interest. Although both agonists and antagonists of adrenergic receptors are used in medicine, we still lack a comprehensive

understanding of how adrenergic receptors affect disease and what is the role that adrenergic receptors embark during homeostasis. There are many unanswered questions: do beta-blockers or beta-agonists alter the risk of infections? Do SNPs in *Adrb2* gene change disease susceptibility? Can we use adrenergic receptor agonists on the treatment of new inflammatory diseases (e.g. colitis)? In addition to these organism-level questions, we still do not know how adrenergic receptors elicit their anti-inflammatory properties in different immune cells. What are the underlying signaling cascades? How does the signaling change between different cells? Chapter 5 will be a humble attempt to describe some of the molecular players that are involved in upregulation of IL-10 and how adrenergic receptor signaling alters macrophage transcriptome.

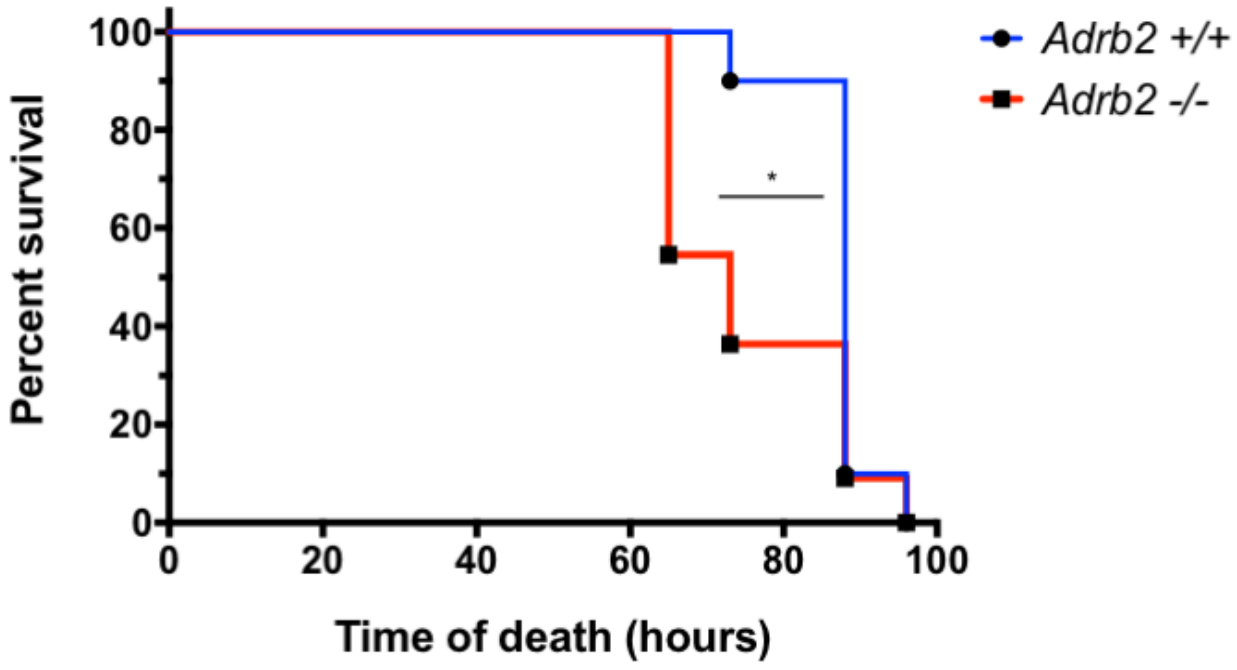

Figure 4.1. ADRB2-deficient mice succumb to lethal LM-OVA infection earlier than ADRB2-sufficient mice. ADRB2-sufficient and -deficient mice were challenged with 277K CFU LM-OVA per mouse i.v. and the survival of the animals was monitored for 7 days. Data is significant with Mantel-Cox statistical test. ($p<0.05$, n=10-11 mice per group)

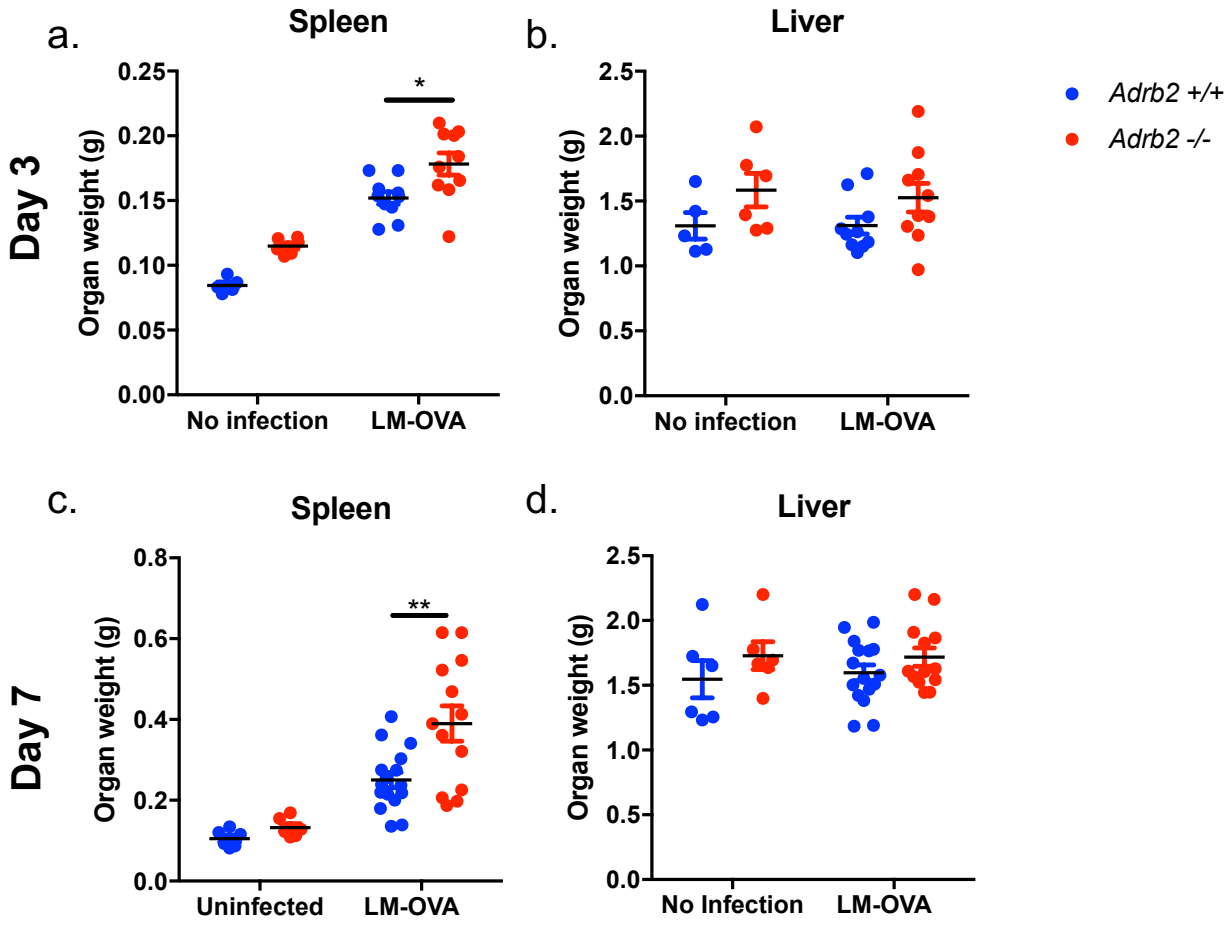

Figure 4.2. ADRB2-deficient mice present with splenomegaly in response to LM-infection. ADRB2-sufficient and -deficient mice were challenged with approximately 20K CFU LM-OVA per mouse i.v. Spleens (a and c) and livers (b and d) were harvested at days 3 (a and b) and day 7 (c and d) post infection. Organ weighs were assessed using 2-way ANOVA. ($p<0.05$, uninfected: n=5-6, infected n=10-16 mice per group per time point).

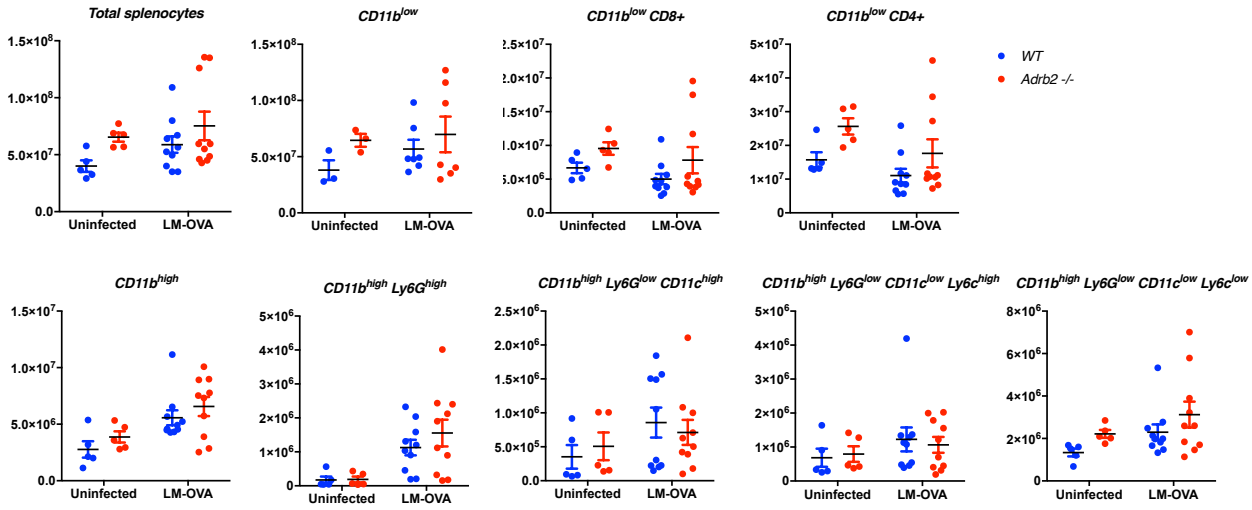

Figure 4.3. Quantification of immune cell subsets in spleen in response to LM-OVA infection, 3 days post-infection. ADRB2-sufficient and -deficient mice were challenged with approximately 20K CFU LM-OVA per mouse i.v. The number of cells in each subset was back calculated and the absolute number of cells was reported. Data was assessed using 2-way ANOVA. ($p<0.05$, uninfected: n=5, infected n=3-10 mice per group).

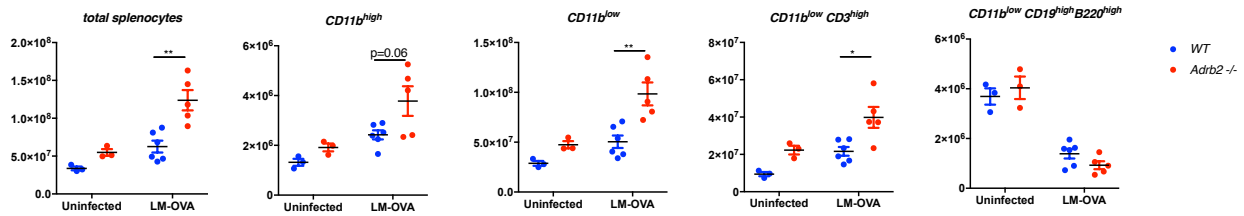

Figure 4.4. Quantification of immune cell subsets in spleen in response to LM-OVA infection, 7 days post-infection. ADRB2-sufficient and -deficient mice were challenged with approximately 20K CFU LM-OVA per mouse i.v. The number of cells in each subset was back calculated and the absolute number of cells was reported. Data was assessed using 2-way ANOVA. ($p<0.05$, uninfected: n=3, infected n=5-6 mice per group).

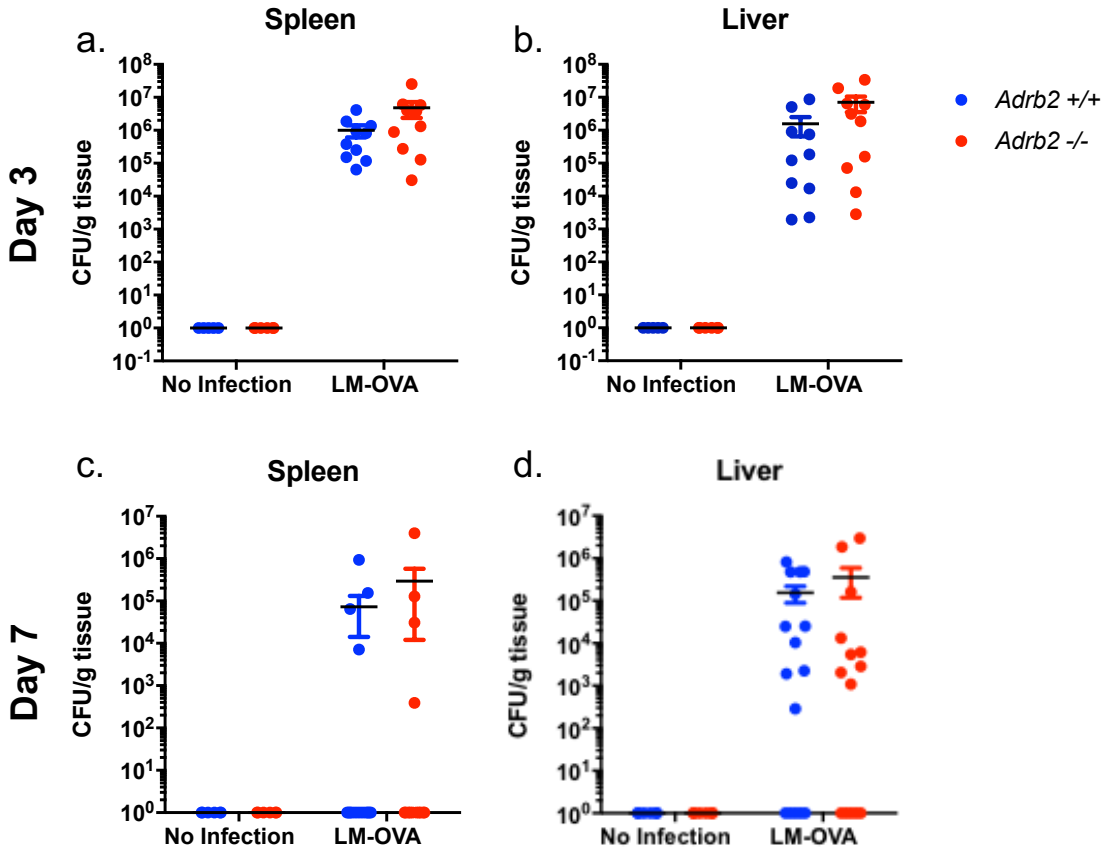

Figure 4.5. ADRB2-deficient mice clear LM-OVA in comparable levels to ADRB2-sufficient mice. ADRB2-sufficient and -deficient mice were challenged with approximately 20K CFU LM-OVA per mouse i.v. Spleens (a and c) and livers (b and d) were harvested at days 3 (a and b) and day 7 (c and d) post infection. After homogenizing the organs, serial dilutions of the homogenate were spread to streptomycin containing BHI-agar plates and colonies were grown overnight in 37°C. Colonies were counted the next morning and viable CFU was back calculated and normalized to weight of the organs. The colony counts were assessed using 2-way ANOVA. (uninfected: n=5-6, infected n=10-16 mice per group per time point).

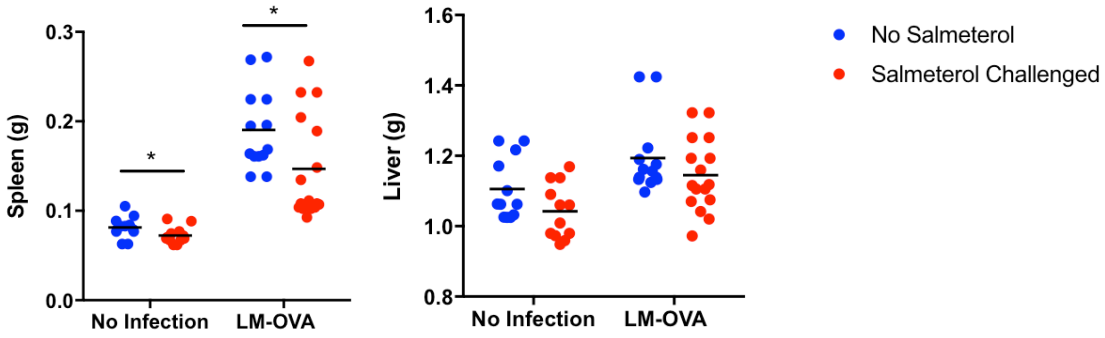

Figure 4.6. Administration of salmeterol reduces splenomegaly. ADRB2-sufficient mice were challenged with approximately 20K CFU LM-OVA per mouse i.v. The animals received 40 μg salmeterol i.p. in 0, 1 and 2 days post-infection. Spleens and livers were harvested at post infection. Organ weighs were assessed using Mann-Whitney U test. ($p<0.05$, uninfected: n=12, infected n=13-16 mice per group).

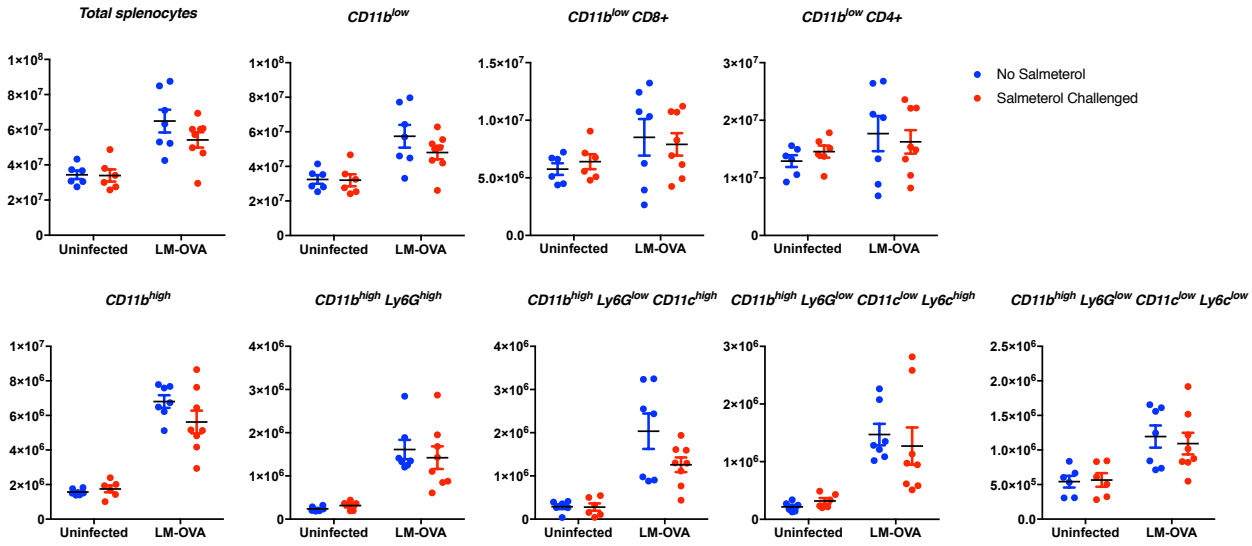

Figure 4.7. Quantification of immune cell subsets in spleen in response to salmeterol during LM-OVA infection, 7 days post-infection. ADRB2-sufficient mice were challenged with approximately 20K CFU LM-OVA per mouse i.v. The animals received 40 µg salmeterol i.p. in 0, 1 and 2 days post-infection. The number of cells in each subset was back calculated and the absolute number of cells was reported. Data was assessed using 2-way ANOVA (uninfected: n=6, infected n=7-8 mice per group).

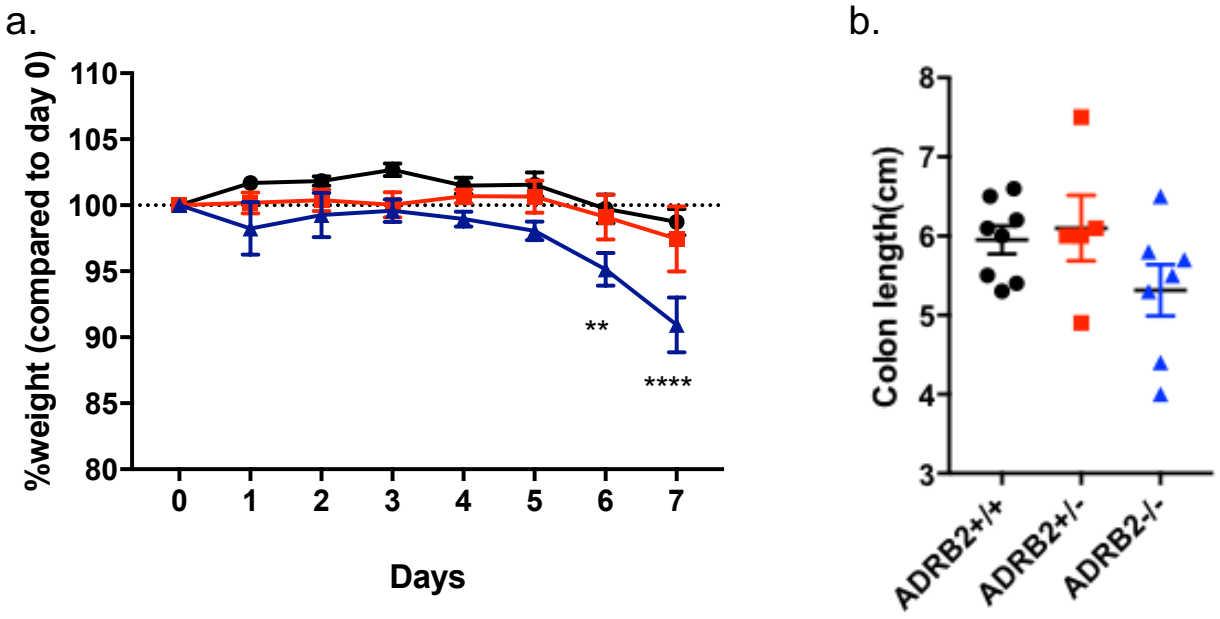

Figure 4.8. ADRB2-deficient animals significantly lose weight during DSS treatment. ADRB2-sufficient, -heterozygous and –knockout animals were subjected to 3%DSS for 7 days. (a) Animals were weighed every day and the weight of the animals were reported as normalized values to day 0, (b) Length of the colon (cecum to rectum) at day 7 (n=5-8 animals per strain, data represents two experiments that are combined). The data presented in (a) was assessed using 2-way ANOVA and (b) was assessed using 1-way ANOVA.

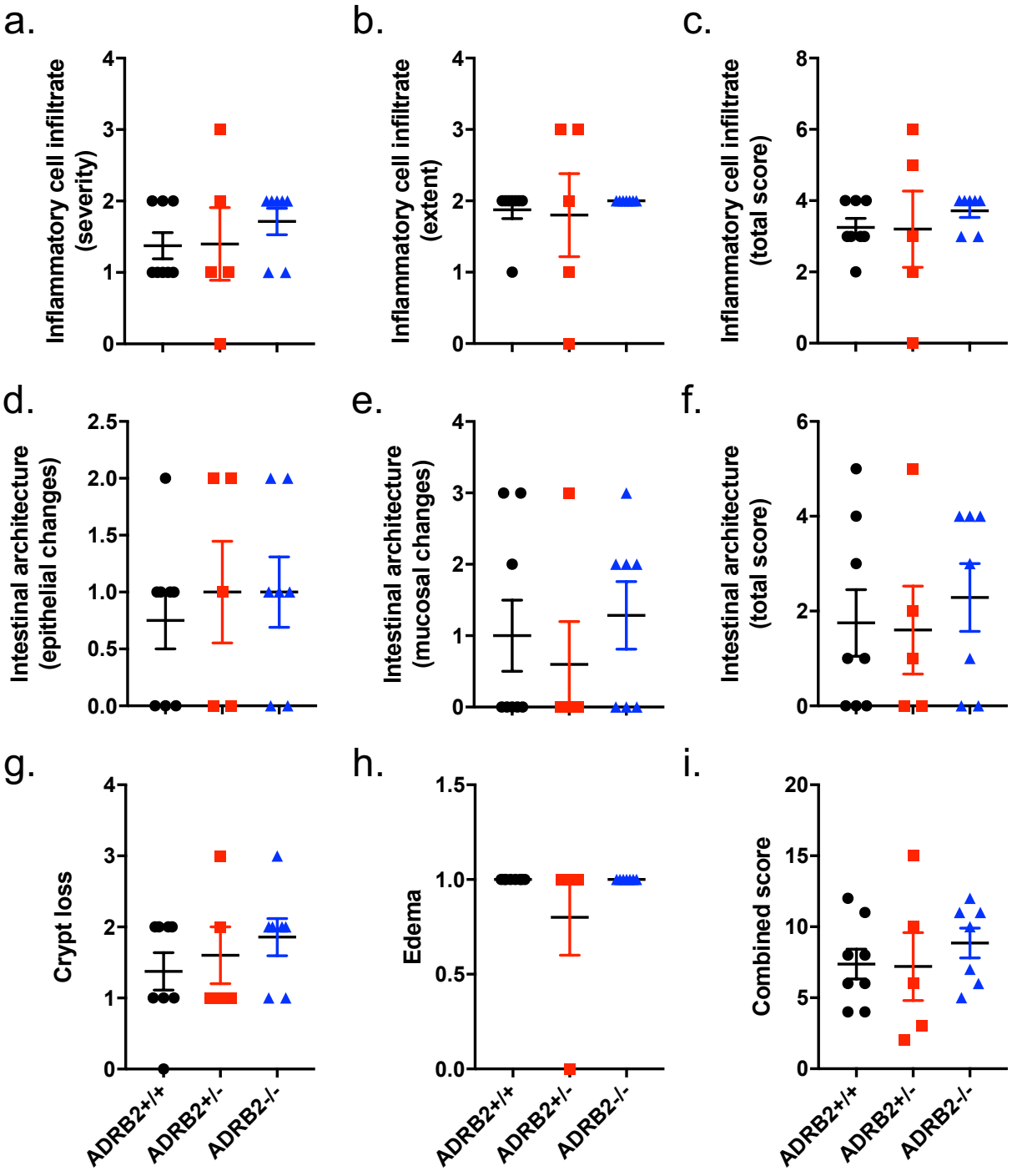

Figure 4.9. Histopathology scores for DSS experiments (presented in Figure 4.8.).

ADRB2-sufficient, -heterozygous and –knockout animals were subjected to 3%DSS for 7 days (n=5-8 animals per strain, data represents two experiments that are combined). Distal colon was embedded in paraffin, and sections of the colon were stained with hematoxylin&eosin by the UT Southwestern Pathology Core. Dr. Purva Gopal (UT Southwestern, Department of Pathology) scored the slides in a double-blinded fashion, based on the criteria in Table 4.1. (a) represents the degree of the inflammatory cell infiltrate (none to severe, 0-3), (b) represents the extent of the inflammatory infiltrate (none to transmural, 0-3), (c) is a combined score of (a) and (b). (d) represents changes in the epithelium (none to erosions, 0-2), (e) represents changes in the mucosal structure (focal ulcerations, pseudopolyps etc, 2-3), (f) is a combined score of (d) and (e). (g) represents the degree of the crypt loss (none to severe, 0-3), (h) represents the absence (0) or presence (1) of edema. (i) is the combined histopathology score of the slides, obtained by summation of (c), (f), (g) and (i).

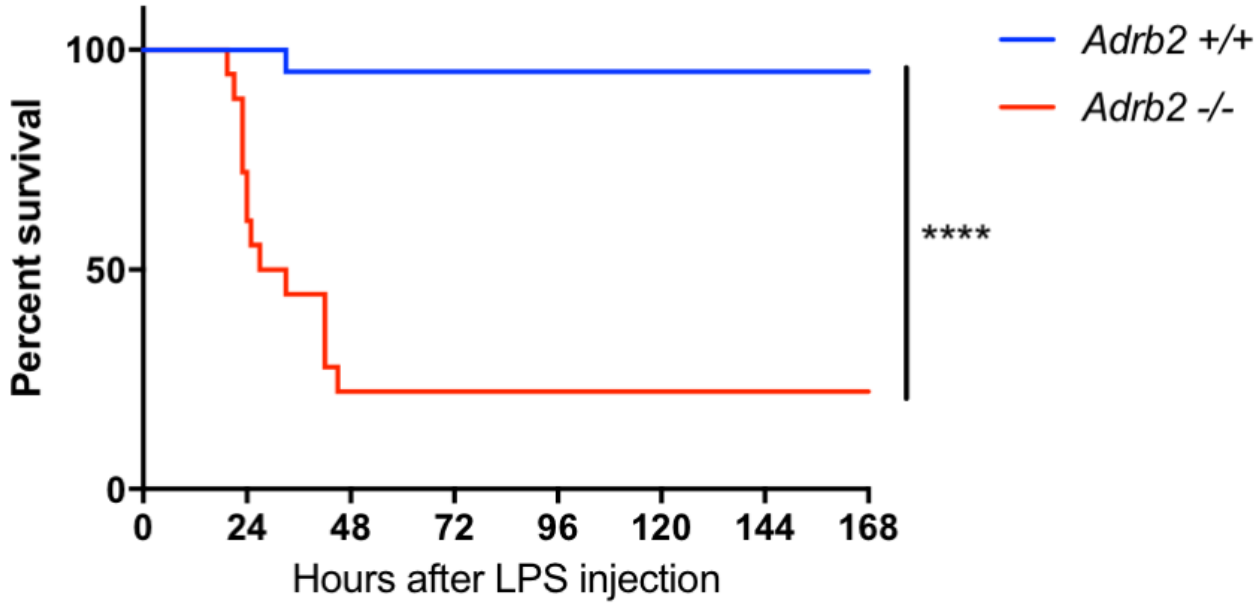

Figure 4.10. ADRB2-deficient mice are susceptible to LPS-endotoxemia. ADRB2-sufficient and –deficient mice were challenged i.p. with 300 µg LPS and the survival of the animals was monitored for 7 days. Data is significant with Mantel-Cox statistical test. ($p<0.0001$, n=18-20 mice per strain)

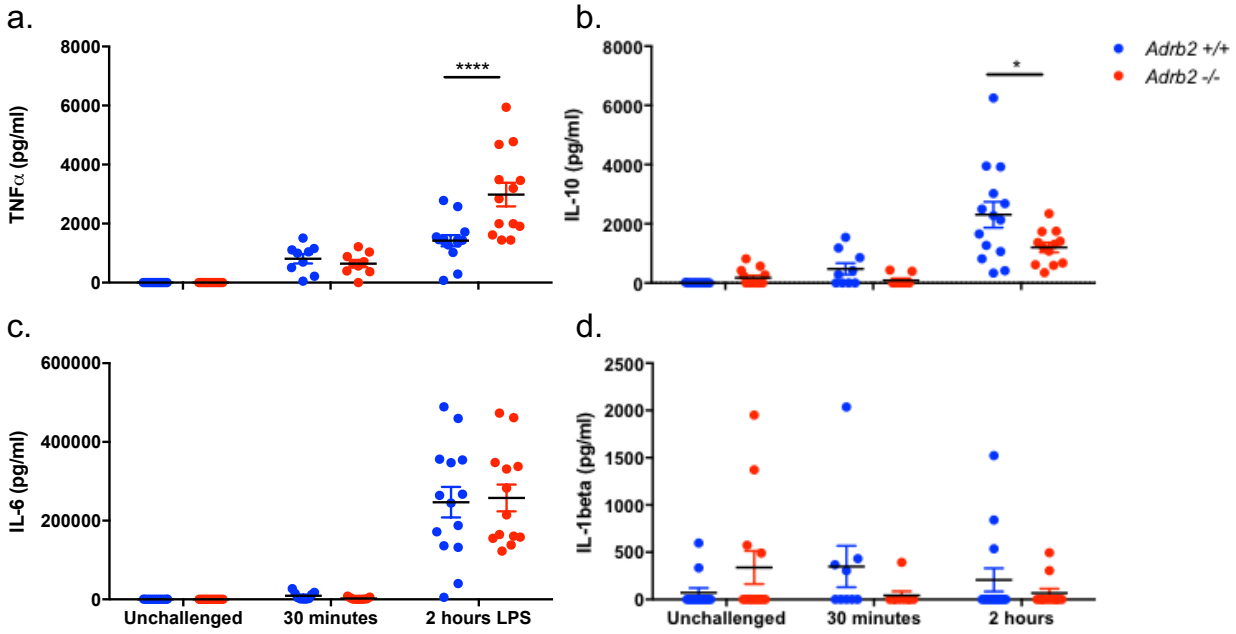

Figure 4.11. ADRB2-deficient animals develop serum cytokine dysregulation. ADRB2-sufficient and –deficient mice were challenged i.p. with 300 µg LPS and the serum cytokines: (a) TNFα, (b) IL-10, (c) IL-6 and (d) IL-1β were assessed by ELISA ($p<0.05$, n= 9-13 mice per strain, per condition). 2-way ANOVA is used to assess statistical significance.

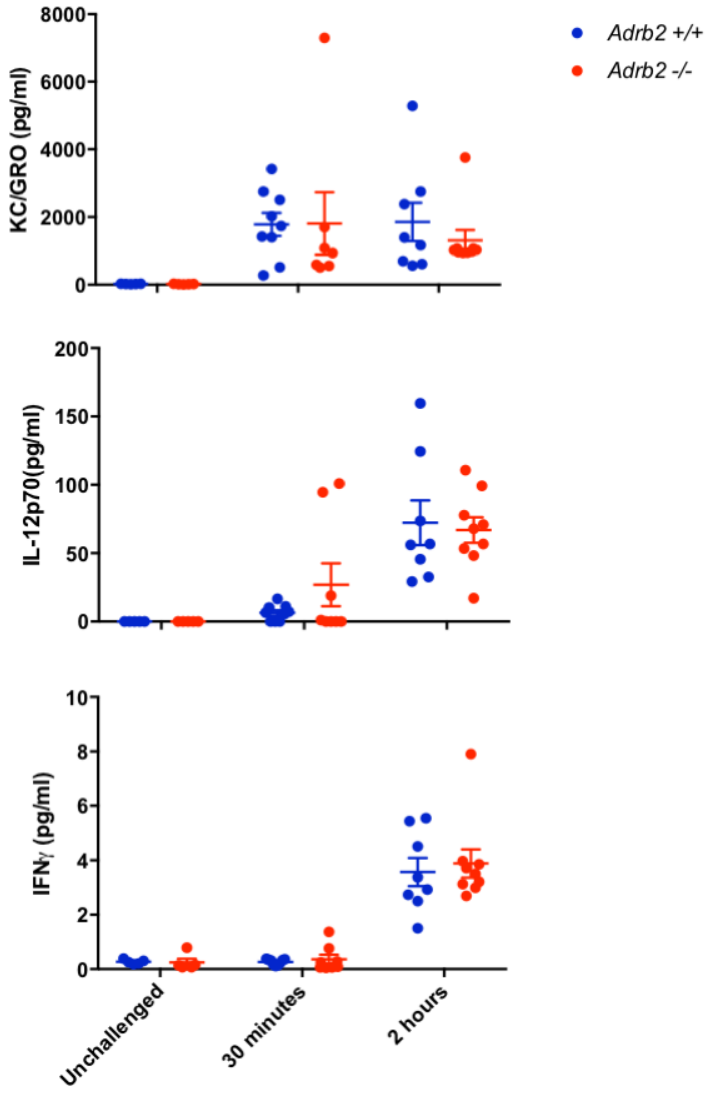

Figure 4.12. ADRB2-deficiency does not alter serum levels of KC/GRO, IL-12p70 and IFNγ. Subset of serum samples from Figure 4.11 were assessed with Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery. Levels of KC/GRO, IL-12p70 and IFNγ were assessed and found not to be statistically different between ADRB2-sufficient and -deficient mice using 2-way ANOVA. (n=5 for unchallenged, n=8-9 for LPS challenged per strain)

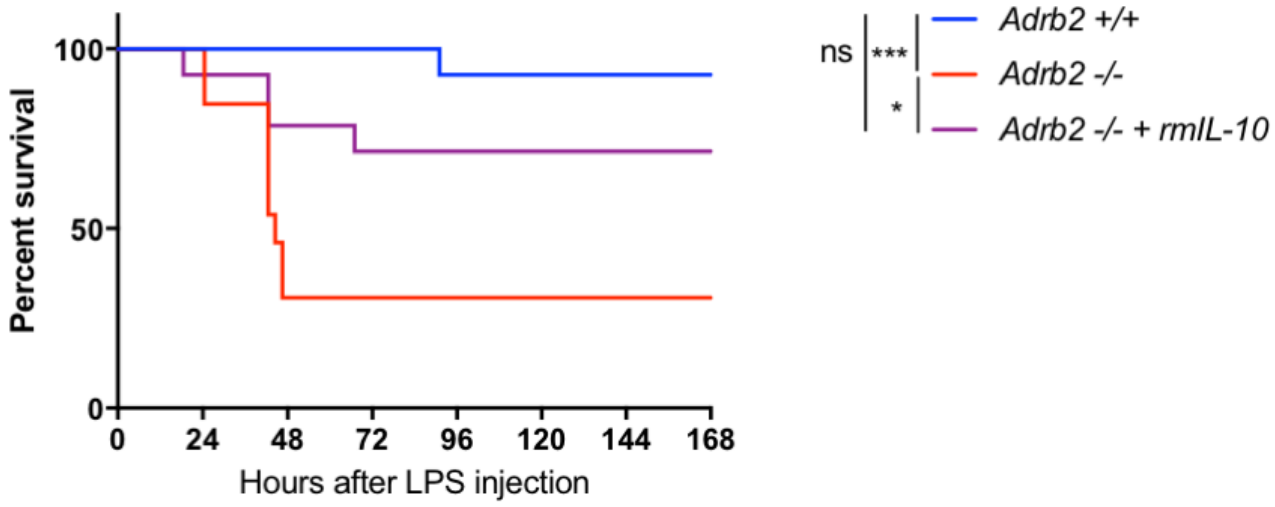

Figure 4.13. Exogenous IL-10 rescues the ADRB2-deficient animals. ADRB2-sufficient and –deficient mice were challenged i.p. with 200-250 µg LPS and were administered 1 µg recombinant murine IL-10 i.p. immediately after LPS challenge. The survival of the animals was monitored for 7 days. The data is assessed with Mantel-Cox statistical test (n=13-14 mice per condition)

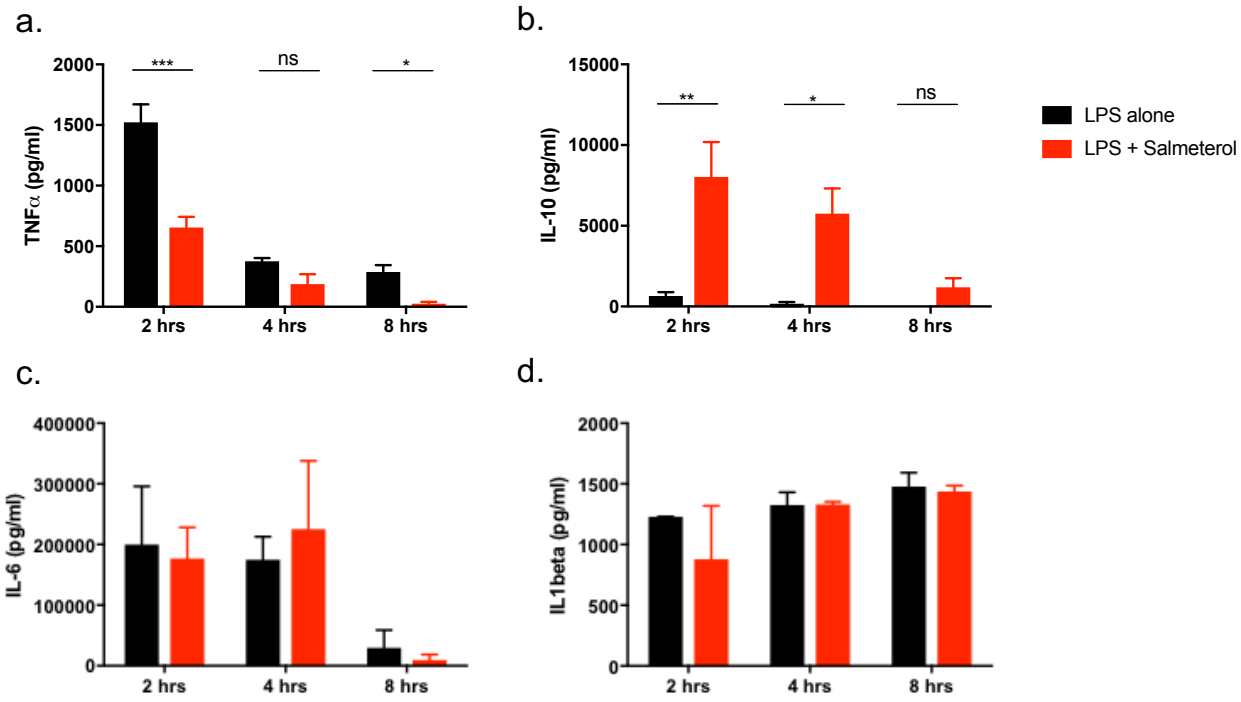

Figure 4.14. Beta2-adrenergic receptor agonists salmeterol alters serum cytokine levels during endotoxemia. ADRB2-sufficient mice were challenged with 300 µg LPS in the absence or presence of 40 µg salmeterol. Blood was harvested at indicated time points and serum cytokines were quantified by ELISA. t-test was conducted between black and red bars for each time point and each cytokine. (n=3-10 mice per time point)

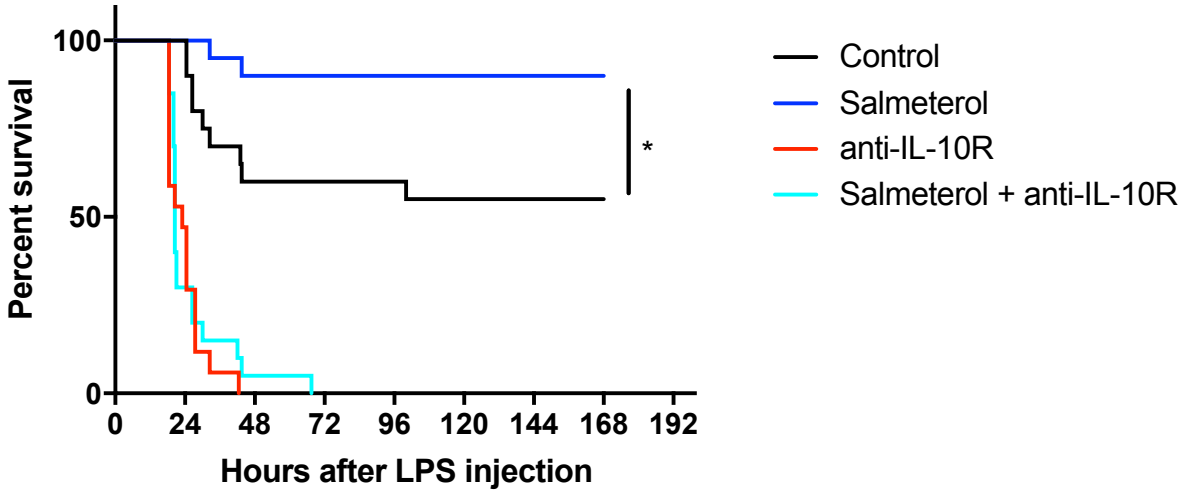

Figure 4.15. Beta2-adrenergic receptor signaling enhances survival through IL-10. Mice were challenged with 750 µg LPS, in the presence of 40 µg salmeterol (or vehicle control) and/or antiCD210 (IL-10R) antibody (or isotype control). The survival of animals was monitored for 7 days. (n=16-20 mice per treatment group). The data is significant with Mantel-Cox statistical test.

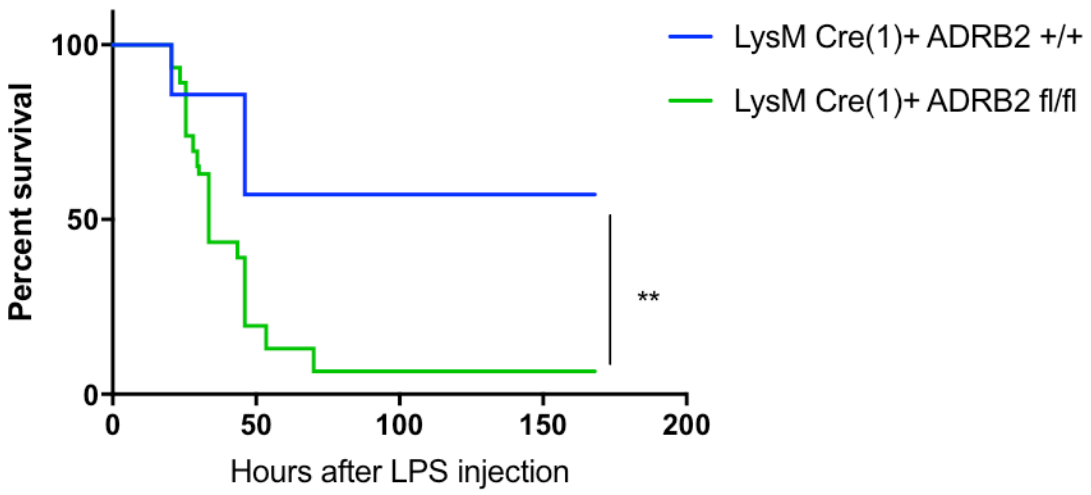

Figure 4.16. Myeloid cell-specific deletion of Adrb2 leads to susceptibility in LPS-endotoxemia. Myeloid specific conditional knockout mice (LysM Cre+ Adrb2 fl/fl) and the controls were challenged with 250 µg LPS per mouse and the survival of the animals were monitored for 7 days. The data is assessed with Mantel-Cox statistical test. ($p<0.05$, n=7 for Adrb2+/+, n=7 for Adrb2+/- and n=46 for Adrb2 fl/fl)

Inflammatory cell infiltrate (Severity)	*None*	0
	Mild	1
	Moderate	2
	Severe	3
Inflammatory cell infiltrate (Extent)	*None*	0
	Mucosa	1
	Submucosa	2
	Transmural	3
Epithelial changes	*No erosion*	0
	Focal erosion	1
	Erosions	2
Mucosal architecture	*Focal ulceration*	2
	Extended ulcerations	3
	Granulation	3
	Pseudopolyps	3
Crypt loss	*None*	0
	Mild	1
	Moderate	2
	Severe	3
Edema	*Absent*	0
	Present	1

Table 4.1. The criteria used in DSS-colitis experiments.

CHAPTER V

ACUTE ADRENERGIC SIGNALING ALTERS THE TRANSCRIPTOME OF MACROPHAGES

The data presented here is unpublished. Didem Ağaç executed all the experiments in this chapter unless otherwise indicated in figure legends and/or text. Beibei Chen calculated the DESeq normalized expression values and ARC technicians administered LPS in endotoxemia experiments.

Introduction

The adrenergic receptor signaling has been reported to alter the production of cytokines in the literature, however, the underlying cellular mechanism is mostly unknown. The data presented in Chapter III demonstrates that in response to beta2-adrenergic receptor signaling, IL-10 is acutely upregulated in macrophages and dendritic cells. This is accompanied by the suppression of the pro-inflammatory cytokines, but acute IL-10 production is not required for this to occur.

I wanted to determine the pathways that are involved in either early upregulation of IL-10 or acute suppression of the pro-inflammatory cytokines. First, I ruled out the involvement of adenylyl cyclase and cAMP activation by using inhibitors or activators of

the classical adrenergic receptor signaling pathway. Second, I showed that the p38 pathway is involved in the suppression of TNFα in later stages of stimulation. Third, I showed that the adrenergic receptors alter macrophage transcriptome and the effects of this pathway were not limited to cytokines. Lastly, I propose that NR4A family of transcription factors that are regulated by adrenergic receptor signaling are important in controlling *Il10* and mice are susceptible to endotoxemia in the absence of NR4A1.

Results

Inhibitor studies

In order to have a better understanding of the molecular pathways that are involved in suppression of TNFα and upregulation of IL-10, activators and inhibitors of several pathways were used.

In classical adrenergic receptor signaling, adrenergic receptor undergoes conformational changes upon ligand binding which enables the binding on G protein heterotrimer to the receptor. Once the complex is formed, GDP that is associated with the G protein is released and GTP associates with the free alpha-subunit. This triggers the dissociation of alpha and beta-gamma subunits from the adrenergic receptor and initiate downstream signaling cascades. Adenylyl cyclase (AC) is activated by the alpha subunit, leading to an increase in intracellular cAMP levels. Protein kinase A (PKA) is a cAMP regulated protein and increased cAMP leads to activation of downstream PKA-MEK1/2-

ERK pathway. Hydrolysis of GTP to GDP by the alpha unit reassembles the heterotrimeric G-protein (Rasmussen et al., 2011).

In order to determine if the classical adrenergic receptor signaling is utilized in suppression of TNFα, several chemical agonists/antagonists were utilized. First, by AC activation via forskolin treatment the following hypotheses were tested: (a) can increasing the intracellular cAMP levels would lead to reduction in TNFα levels in TLR-stimulated ADRB2-sufficient macrophages and (b) in the absence of NE and bypassing the need for ADRB2-NE binding; can forskolin treatment reduce TNFα levels in ADRB2-deficient macrophages?

The level of TNFα secretion was comparable between CpG treated/vehicle control cells and CpG treated/forskolin treated macrophages. Additionally, forskolin was unable to reduce TNFα in ADRB2-deficient macrophages in all the time points that were tested (Figure 5.1). As a complementary experiment, ADRB2-sufficient macrophages were treated with AC inhibitor to investigate if the inhibition of the AC can reverse the suppression of TNFα. AC inhibitor treatment did not ameliorate the suppression of TNFα in the time points that we have tested (Figure 5.2). This data suggests that the classical adrenergic receptor signaling, and upregulation of cAMP are not involved in suppression of TNFα. Unfortunately, I have not measured IL-10 in these experiments, the role of AC in IL-10 production needs to be investigated.

Several TLR signaling pathways utilize MEK and Erk molecules (Kogut et al., 2007; Pourrajab et al., 2015). To test the involvement of MEK/Erk, macrophages were treated with U126, an inhibitor for MEK1/2. If MEK is involved, inhibition of the

protein should reverse the inhibition of TNFα. Although the presence of the inhibitor reduced TNFα levels in CpG treated samples, suppression of the TNFα by norepinephrine was still present (Figure 5.3). This result suggests that TLR-induced TNFα production relies on MEK, as reported in the literature, however, the suppression of TNFα by adrenergic receptors does not.

Grailer et al showed that wortmannin could inhibit epinephrine induced IL-10 production and argued that catecholamine enhanced IL-10 production operates through the PI3K pathway (Grailer et al., 2014). However, wortmannin is known to inhibit both PI3K and mTOR pathways (Feldman and Shokat, 2010), and this was not elucidated by the abovementioned study. To test if mTOR signaling is involved in suppression of TNFα, macrophages were stimulated with rapamycin. Presence of rapamycin did not alter TNFα secretion, suggesting that mTOR pathway was not involved (Figure 5.4). However, I have not tested if rapamycin treatment alters IL-10 secretion at the time of experimentation. This data does not exclude the possibility of PI3K or mTOR pathways for IL-10 production in response to adrenergic receptor signaling, rather suggests that mTOR pathway was not involved in suppression of TNFα.

Engagement of TLRs initiates p38 activation (Kopp and Medzhitov, 2003) and p38 activation is important for TNFα production (Pattison et al., 2016). To test if p38 activation is involved in suppression of TNFα, macrophages were treated with increasing concentrations of p38 inhibitor during TLR and/or NE stimulation. In the acute phases of stimulation, increasing concentrations of the p38 inhibitor decreased TLR-induced TNFα production. This observation was in concordance with the previously reported roles of

p38 in TLR-induced TNFα production. However, in late phases of stimulation (24 hours), with increased concentrations of p38 inhibitor, norepinephrine was unable to suppress TNFα (Figure 5.5a). This data suggests a role for p38 in adrenergic receptor dependent suppression of TNFα. In order to determine the kinetics of the p38 inhibitor's effect, a time-course experiment is needed.

To test if IL-10 was also affected by the p38 inhibitor, secreted IL-10 was measured. At 2 hours, in the absence of p38 inhibition, IL-10 was upregulated in response to NE. However, we observed little to no IL-10 when samples were treated with p38 inhibitor (Figure 5.5b), supporting the role of p38 signaling in induction of IL-10 (Horie et al., 2007; Hou et al., 2012). It would have been a better idea to test multiple time points to see the effects of p38 inhibition on IL-10 production. At later phases of stimulation, samples had comparable IL-10 production between norepinephrine treated and untreated groups, irrespective of the presence of the p38 inhibitor (Figure 5.5b).

Transcriptomic changes in murine macrophages in response to adrenergic signals

Our inhibitor experiments were not very informative in revealing potential pathways that were involved in suppression of TNFα or upregulation of IL-10. An unbiased transcriptomics approach was employed to have a global understanding of the changes that occur in response to adrenergic receptor signaling. Although adrenergic receptor signaling has been shown to alter expression of genes in many different cell types (Gabanyi et al., 2016; Podojil and Sanders, 2003; Tan et al., 2007), there has not been a

study that investigates the acute changes in transcriptome of murine macrophages, in response to adrenergic cues during TLR stimulation.

Our study was not the only study that describes the transcriptomic changes upon adrenergic receptor stimulation. In fact, when the Gene Expression Omnibus was searched with the keyword 'adrenergic', there were 219 distinct datasets, cell types ranging from adipocytes to cardiomyocytes (as of March 2018). In contrast, the keywords 'adrenergic macrophage' only led to 7 entries of which only one was reporting the effect of adrenergic signaling on macrophages. In this study, Lamkin *et al* used bone marrow derived macrophages that were stimulated with isoproterenol (a beta1- and beta2-adrenergic receptor agonist) for 24 hours and compared the transcriptome of these cells to macrophages that have been stimulated with IL-4, IFNγ or selective ADRB1, ADBR2 and ADRB3 antagonists. Their results suggest that isoproterenol treatment upregulates M2-like genes such as *Arg1* and *Il10* (Lamkin et al., 2016), however, they fail to comment on global changes in transcriptome and rather limit themselves to a select set of genes associated with the M2-phenotype.

Based on the results that were presented in Chapter III, beta2-adrenergic receptor signaling rapidly alters cytokine secretion. The changes in *Tnf* and *Il10* transcripts were observed as early as 2 hours. By conducting transcriptomic analysis, I anticipated to find a gene or family of genes that are responsive to adrenergic receptor signaling and could explain the induction of *Il10* during adrenergic receptor stimulation; if the regulator that controls *Il10* upregulation is controlled transcriptionally. To this end, murine bone-marrow derived macrophages were stimulated in the presence or absence of NE and

TLR9 agonist CpG for 2 hours. Additionally, some of the samples were pre-treated with anti-CD210 (IL-10R) antibody or an isotype control for 30 minutes prior to stimulation. The antibody treatment aimed to neutralize autocrine IL-10 signaling. At the time of the experimentation, I have not ruled out if *early* IL-10 induction could explain the suppression of the pro-inflammatory cytokines. This hypothesis was incorrect, since TNFα production from IL-10- deficient macrophages were acutely suppressed in response to NE (Figure 3.15).

After stimulation of bone marrow derived macrophages for 2 hours, cDNA libraries were prepared, and the samples were sequenced as described in Chapter II. After generation of FastQ files using Galaxy Server, the raw data was sent to UT Southwestern Bioinformatics Core Facility. In this exploratory analysis, Dr. Beibei Chen assessed the differentially expressed genes using EdgeR. The comparisons that were made and the number of genes that were differentially expressed can be found in Table 5.1.

It is interesting to note that the highest number of differentially expressed genes was found in between Isotype_No CpG_No NE vs Isotype_No CpG_Plus NE and Isotype_No CpG_No NE vs Isotype_Plus CpG_No NE (Table 5.1). These comparisons represent the genes that are differentially expressed in response to adrenergic receptor signaling alone or TLR signaling alone. However, when the differences in gene expression between Isotype_Plus CpG_No NE vs Isotype_Plus CpG_Plus NE were assessed, there were only 20 genes that were differentially expressed. This suggests that when both signals are present, only a small portion of the transcriptome was affected, and potentially, there was specificity in genes that were altered. It is also important to note

that by using less stringent cut-offs, more "differentially expressed" genes could be acquired.

Our analysis was able to capture some known targets. For example, several cytokine genes (e.g. *Tnf*, *Ifnb1*, *Il12b* and *Il1b*) and chemokine genes (e.g. *Ccl3*, *Cxcl2* and *Cxcl5*) were upregulated in response to TLR stimulation. I have provided the list of top differentially expressed genes (up or down regulated) in Tables 5.2, 5.3, 5.4 and 5.5. Additionally, DESeq values (normalized expression values) of different classes of genes were represented in Figure 5.6 through Figure 5.12 in heat map format.

Our *in vitro* (Chapter III) and *in vivo* (Chapter IV) data suggest that norepinephrine elicits its effects on macrophages by beta2-adrenergic receptor signaling. When the expression of different adrenergic receptor transcripts was compared across treatments, *Adrb2* was highlighted as the most expressed adrenergic receptor (in transcript level) (Figure 5.6). Although there were transcripts of *Adra1a* and *Adrb1* (alpha1a- and beta1-adrenergic receptors, respectively), they were significantly lower than *Adrb2* transcript expression, even in the absence of any stimulation. Treatment of macrophages with NE downregulated the *Adrb2* transcript, this was expected in protein levels since GPCR-treatment initiates receptor internalization by endocytosis (Magalhaes et al., 2012) but unexpected in the transcript of *Adrb2.*

Macrophages can express transcripts of all the TLRs. Although *Tlr2* transcript was highly expressed, the presence of norepinephrine upregulated the expression even higher. TLR2 can recognize several bacterial and viral structures (Uematsu and Akira, 2008). It is possible that the upregulation of the transcript in response to a "stress"

hormone is a way in which the cell becomes more available and sensitive for upcoming danger. I have not formally tested if pre-treatment with NE yields more cytokines in response to TLR2 stimulation or even if the TLR2 protein is also upregulated in response to adrenergic cues; but this remains an interesting and understudied observation.

Our *in vitro* studies suggest that adrenergic receptor signaling alters cytokine production. Some of our *in vitro* observations were recapitulated in the RNA-seq (Figure 5.8a): *Tnf* transcript was reduced in response to norepinephrine in CpG treated samples (however there was an increase in *Tnf* transcript in the absence of CpG), *Il10* and *Il6* were upregulated in response to norepinephrine. Similar to a recent publication (Roewe et al., 2017), we have also observed an increase in *Il27* transcript in response to norepinephrine (Figure 5.8b). Interestingly, *Il1b* transcript was upregulated in response to norepinephrine (Figure 5.8c): this is a unique, and to my knowledge, the first ever occurrence of TLR-independent *Il1b* upregulation. More studies need to be conducted to determine if inflammasome can be assembled by adrenergic stimulation too.

Most interferon genes had little to no detectible transcripts. As expected, *Ifnb* was upregulated in response to CpG; however, the presence of norepinephrine did not alter the expression of *Ifnb* (Figure 5.9).

When chemokines (Figure 5.10a) and their receptors (Figure 5.10b) were examined, most of the transcripts were minimally affected. However, there were select genes that were upregulated (*Ccl2, Ccl3, Ccl7, Ccr1, Cxcr4* etc.) and downregulated (*Ccr2, Cx3cr1* etc.). These observations suggest that adrenergic receptor signaling may alter the macrophage migration to certain cues. It is also likely that the recruitment of

other cells by macrophage derived chemokines are altered in response to adrenergic signals.

Stimulation of macrophages in the presence or absence of norepinephrine and CpG for 2 hours did not alter MHC Class I, MHC Class II, *Cd80* and *Cd86* transcripts, however, it is possible that with longer stimulation, these transcripts might change. Canonical M1 (*Nos2*) and M2 (*Arg1*) markers were unchanged, however *Mrc1* (mannose receptor, CD206) was upregulated in response to norepinephrine (Figure 5.11). These observations and the cytokine data suggest that presence of norepinephrine does not induce a differentiation pathway alteration, rather it acutely changes macrophage behavior.

The number of genes that were differentially expressed were quite low, independent of the comparison that was made, and this was especially true when we look at the subset of genes that were differentially expressed after FDR correction to account for multiple hypothesis testing. Since this was an exploratory analysis and there were not many genes that passed the FDR correction, I investigated the genes that were differentially expressed before FDR correction. My goal was to find genes that change in response to adrenergic receptor signaling (also, since I had such low number of genes that are differentially expressed in other comparisons, I was limited in things I could do with this data), I have mainly focused on the following two conditions: (1) Isotype_No CpG_No NE vs Isotype_No CpG_Plus NE and (2) Isotype_Plus CpG_No NE vs Isotype_Plus CpG_Plus NE. These comparisons could give us the list of genes that are differentially expressed in response to adrenergic receptor signaling in the absence or

presence of TLR stimulation, respectively. For each set, the '$\log_2$(Fold Change)' was sorted for genes that are highly up- or down-regulated. The top 50 up- or downregulated genes can be in Tables 5.2, 5.3, 5.4 and 5.5.

Ideally, the transcriptomic analysis of cells should give the investigator sets of genes that are co-regulated or identify potential pathways that are influenced by the experimental treatment. Since, there were not many genes that were differentially expressed; network or pathway analysis tools or IPA analysis did not yield any further information. The list of top differentially expressed genes were searched in the literature to generate hypotheses. From that attempt, a family of nuclear orphan transcription factors, NR4A family were found to be interesting and were followed up experimentally, since these genes were differentially upregulated in response to norepinephrine but not CpG (Table 5.2 and Figure 5.12).

NR4A family member of transcription factors are induced in response to norepinephrine

NR4A1, NR4A2 and NR4A3 are nuclear orphan receptors and there are no known ligands for these molecules. NR4A family of receptors can bind to DNA as monomers, homodimers or heterodimers (Glass and Saijo, 2010). Unlike other nuclear receptors, NR4A family is proposed to be constitutively active (Martínez-González and Badimon, 2005). Additionally, NR4A family members were suggested to be ligand independent, due to differences in their tertiary structures (Wang et al., 2003).

In muscle cells, beta-adrenergic receptor signaling (via isoprenaline treatment) induced *Nr4a1* transcript (Maxwell et al., 2005). Similarly, the simulation of muscle cells with beta2-adrenergic receptor agonist formoterol increased mRNA expression of *Nr4a1, Nr4a2* and *Nr4a3.* Induction of NR4A members were dependent on the activation of adenylyl cyclase, protein kinase A, MEK1/2 and p38 activation (Pearen et al., 2008). NR4A family members were upregulated in multiple organs when animals were challenged with isoprenaline. It was interesting to note that the induction of the NR4A family was rapid; the transcripts were significantly upregulated in multiple organs as early as 1 hour (Myers et al., 2009). These results suggest that NR4A family are upregulated in response to adrenergic signals and they are some of the early-response genes.

NR4A family members were followed up further since these transcripts were some of the most differentially expressed transcripts (Table 5.12) and were sensitive to norepinephrine (Figure 5.12). Additionally, according to JASPAR database, the proximal *Il10* promoter includes several putative binding sites (Figure 5.13). In addition to RNA-seq results, the upregulation of *Nr4a1, Nr4a2* and *Nr4a3* were confirmed by qPCR (Figure 5.14). I hypothesized that macrophages upregulate NR4A family in response to norepinephrine and macrophages use these transcription factors to poise the *Il10* promoter for rapid activation. When TLR signaling is provided, *Il10* gene is activated and that leads to the upregulation of the IL-10 protein, as described in Chapter III.

In order to test which region in *Il10* promoter is responsive to norepinephrine, luciferase assays were conducted using vectors that carry truncations of *Il10* promoter

coupled with luciferase gene (For regions that are truncated and their positions with respect to NR4A1/NR4A2 binding sites, see Figure 5.15a). The signal that was derived from the luciferase activity is used as a correlate to the activity of the *Il10* promoter. In Figure 5.15b, the presence of norepinephrine in the longest transcript (Il10#1) increased the relative luciferase activity. With the truncations of the promoter construct, the norepinephrine dependent signal is lost (between plasmids #2 and #3 in both TLR treated and untreated samples). This data suggests that there exists a norepinephrine sensitive site between -938/-638 of *Il10* TSS. As a complementary approach, *Nr4a1* overexpression plasmid was co-transfected with luciferase plasmids to test if the force expression of *Nr4a1* can induce luciferase activity. In Figure 5.15c, the overexpression of *Nr4a1* increased the signal that was coming from CpG treated samples (in Il10#1); suggesting that *Nr4a1* overexpression can phenocopy and maybe surpass norepinephrine's effects. The overexpression's effect disappeared with the first truncation, suggesting that the NR4A1 sensitive region is between plasmids #1 and #2 (between -1536 and -938 of *Il10* TSS).

Role of Nr4a1 in regulation of inflammation

In order to test the role of NR4A1 on cytokine modulation, NR4A1-deficient mice were purchased through Jackson Laboratory. In the *in vitro* experiments, NR4A1-sufficient and -deficient macrophages were stimulated in the presence or absence of CpG and NE. Presence of norepinephrine during TLR stimulation upregulated IL-10 in both strains at 2

hours. The suppression of TNFα in response to NE was not significant in NR4A1-deficient macrophages. Although there was a slight decrease in TNFα production in NR4A1-deficient CpG treated samples compared to NR4A1-sufficient samples, this did not reach statistical significance (Figure 5.16). Irrespective of the cytokine that was assessed, the pro-inflammatory cytokine release from NR4A1-sufficient and -deficient macrophages were comparable (Figure 5.16 and Figure 5.17). Similarly, across CpG treated samples, there was no statistical difference in both time points and all the cytokines (Figure 5.16 and Figure 5.17). Between the cytokines that were assessed, NR4A1-sufficient macrophages statistically significantly upregulated IL-6, IFNγ and IL-1β, but NR4A1-deficient macrophages could not (Figure 5.17). However, the concentration of these cytokines was quite low, it is unclear if there is any effect in the absence of NR4A1 *in vitro.* Similar results have been reported for TNFα, IL-6 and IL-12 (Chao et al., 2013).

When *Tnf* and *Il10* transcripts were measured, induction of *Il10* and the suppression of the *Tnf* transcript were observed in NR4A1-sufficient and -deficient macrophages. However, the *Tnf* upregulation in response to CpG was significantly lower in NR4A1-deficient macrophages (Figure 5.18). This observation is in line with the previous publication of Li et al, where they have identified a role for NR4A1 to block NFκB activity (Li et al., 2015). Additionally, *Il10* transcript in response to NE was enhanced in NR4A1-deficient cells (Figure 5.18). This data suggests that NR4A1 can modulate the transcript levels of cytokines in response to norepinephrine, but this alteration was not enough to change the protein levels. It is possible that NR4A2, NR4A3

or some unknown protein is involved in controlling the translation of the cytokine transcripts.

Nr4a1-deficiency confers susceptibility in LPS-endotoxemia

In order to determine the role of Nr4a1 *in vivo,* male mice that were NR4A1-sufficient and -deficient were challenged with LPS. NR4A1-deficient mice were susceptible to endotoxemia (Figure 5.19a). Additionally, the serum cytokines were assessed to determine if the susceptibility correlates with a dysbalance in TNFα and IL-10. Interestingly, NR4A1-deficient mice had lower TNFα and IL-6 in their serum. IL-10 was unchanged between NR4A1-deficient and sufficient mice and IL-1β levels were reduced in NR4A1-deficient animals although this did not reach statistical significance (Figure 5.19b). This is in contrast to the reported literature where the pro-inflammatory cytokines in serum (TNFα, IL-6 and IL-1β) are increased in NR4A1-deficient mice during endotoxemia (Li et al., 2015). It is hard to explain the discrepancy between our results and the previous literature. However, the differences in the animal housing or the LPS that was used in these experiments can contribute to this. In our animals the LPS sensitivity in NR4A1-deficient mice cannot be explained by pro-and anti-inflammatory cytokine dysbalance. It is possible that there are additional pathways that Nr4a1 controls where further experimentation is needed.

Discussion

The data presented in this chapter demonstrate that murine macrophages change their transcriptome in response to adrenergic signaling. This was in line with the data presented in Figure 3.19 and Figure 3.20, where the changes in *Tnf* and *Il10* transcripts were reported.

When inhibitors or agonists of several cellular pathways were utilized (Figure 5.1 -Figure 5.5), I was unable to find a pathway that could account for the acute suppression of TNFα or upregulation of IL-10. Of the pathways that were assessed, the p38 pathway seemed to be important for the suppression of TNFα in later phases of stimulation, however, IL-10 was not affected (Figure 5.5). More experiments are needed to investigate the role of the p38 pathway.

Since I was unable to determine a candidate pathway for regulation of cytokines, we decided to perform RNA sequencing to have a better understanding of the transcriptomic landscape. In response to adrenergic signaling and TLR9 signal, there was little change in the transcriptome (Table 5.1). This data suggested that acute effects of these signaling pathways were limited on a small, select set of genes. This result could be partially explained by the low number of subjects that were sequenced (3 mice). It is possible that with more subjects, additional genes with low signals would make it to the significance cut off.

It was interesting to observe that the dominant adrenergic receptor (at least in terms of transcript) was *Adrb2* (Figure 5.6). Macrophages were previously shown to

express alpha1- and alpha 2-adrenergic receptors (Muthu et al., 2007), beta1- and beta2-adrenergic receptors (Sigola and Zinyama, 2000) as well as beta 3-adrenergic receptor (Hadi et al., 2017). RNA-seq results were intriguing in showing that the *Adrb2* transcript is highly expressed and is downregulated by its ligand norepinephrine. Although the receptor downregulation is a common feature of GPCRs, we did not measure ADRB2 on macrophages to see if it happened on protein level too. Additionally, although the transcripts of other adrenergic receptors are expressed in low levels, it is possible that some of these genes have more transcript stability and yield stable expression of the receptor.

When the cytokine transcripts were assessed (Figure 5.8 and Figure 5.9), we only observed alteration in expression in a small subset of cytokines. Of these, *Il1b* transcript upregulation by norepinephrine was a really intriguing. We observed very little to no IL-1β protein in response to adrenergic stimulation. However, *Il1b* transcript was highly upregulated. I hypothesize that adrenergic receptor stimulation induces *Il1b* transcripts to prepare the cells ready for rapid activation. Possibly, in the absence of a danger signal (ATP, bacterial toxin etc.) the assembly of the inflammasome will not occur, therefore no IL-1β protein will be released. Unfortunately, I have not formally tested this TLR-independent upregulation of inflammasome hypothesis.

There was no difference in MHC Class I and Class II genes in response to adrenergic signals (Figure 5.11). Similarly, *Cd80* and *Cd86* transcripts were unaffected by norepinephrine. I have not tested if acute adrenergic signals can alter priming of adaptive immune cells, but it would be an interesting area of research. Additionally, there

are reports suggesting that adrenergic signaling alters macrophages to M2-lineage (Grailer et al., 2014). I disagree with the "lineage" descriptions of macrophages. It is hard and not possible to categorize the macrophage behavior just as M1 or M2. The complexity of the macrophage behavior and the lack of defined master regulators make the arguments of "lineage" questionable in macrophages. I rather consider our experiments as "snapshots"; for example, in CpG treated samples, the presence of norepinephrine acutely downregulates pro-inflammatory cytokines and upregulate IL-10 (Chapter III). Similar observations can be made for multiple TLRs. However, these macrophages are unable to upregulate M2 genes, such as *Arg1*, *Il4* or *Il13* (Figure 5.8 and Figure 5.11). Do we still categorize these cells as M2, if M2 was a defined lineage? If not, what are those cells? These macrophages do not express *Nos2* (Figure 5.11), have little IFNγ production (Chapter III): it is hard to call these cells M1 too. I consider that the adrenergic receptor signaling does not differentiate macrophages to known "lineages", rather it acutely changes behavior of the cell. It would be interesting to determine if the effects of adrenergic receptor signaling can be reversed when adrenergic receptor agonist was removed, and the cells were restimulated.

NR4A family of transcription factors were highly sensitive to adrenergic receptor signaling (Figure 5.12 and Figure 5.14). When I have searched for the putative binding sites for NR4A1 and NR4A2 (NR4A3 was not present in Contra database), I found 10 NR4A1 and 17 NR4A2 putative binding sites in mouse. There were 7 NR4A1 and 10 NR4A2 putative binding sites conserved between mouse and human IL-10 promoter (Figure 5.13). Although there has been no report describing the role of NR4A family on

IL-10 upregulation, NR4A triple deficient mice were susceptible to systemic lethal autoimmunity due to loss of Treg cells and succumbed around 3 weeks of age (Sekiya et al., 2013). This publication suggested a role for NR4A family in control of inflammation, however studies from this group focused on Treg cells, presenting NR4A2 as an important factor for induction and function of Treg cells (Sekiya et al., 2011; Sekiya et al., 2015).

Additionally, there have been studies in macrophages that investigates the role of NR4A family. LPS treatment induced NR4A1, NR4A2 and NR4A3 transcripts in human macrophages (Pei et al., 2005). We have not observed a robust upregulation of these molecules in response to CpG (Figure 5.12 and Figure 5.14). In THP-1 cells, knockdown of NR4A1 or NR4A3 resulted in increased IL-1β and IL-8 in response to LPS (Bonta et al., 2006). When NR4A2 was overexpressed in peritoneal macrophages, Arginase-1, mannose receptor and Ym1 was upregulated; IL-10 was induced and TNFα and IL-12 were suppressed (Mahajan et al., 2015). NR4A1 was recently identified as a key molecule for development of $Ly6c^{low}$ monocyte population, where the lack of NR4A1 resulted in reductions in this population (Chao et al., 2013; Thomas and Smale, 2016). In summary, these results suggest that in macrophages NR4A family members are important in control of the pro-inflammatory cytokines and differentiation of certain subsets.

We did not observe differences in cytokine production *in vitro*, in response to adrenergic receptor signaling between NR4A1-sufficient and -deficient macrophages. NR4A family members do not share high homology when the entire protein is considered: NR4A1 and NR4A2 has %46.48 identity, NR4A1 and NR4A3 has %43.09

identity and NR4A2 and NR4A3 has %51.50 identity (based on CLUSTALW analysis of the following sequences: CAJ18495.1 for NR4A1, NP_001132981.1 for NR4A2 and EDL02347.1 for NR4A3). However, the NR4A family members share high homology in their zinc finger domain as well as ligand binding domain of nuclear hormone receptors (These regions are highlighted in yellow and teal respectively, Figure 5.20). Based on this preliminary analysis, it is possible that these molecules share targets and the presence of NR4A2 and NR4A3 *in vitro* might explain why we do not observe differences in cytokine production in macrophages.

When we assess the role of this pathway *in vivo* in endotoxemia setting, we observed that NR4A1-deficient mice were susceptible to endotoxemia (Figure 5.19a). However, this was not due to the dysregulation of serum IL-10/TNFα. In fact, with reduction of TNFα and IL-6 in serum (Figure 5.19b), we expect to see comparable, if not better survival in NR4A1-deficient mice. Similar observations for LPS sensitivity was demonstrated in the literature. However, in contrast to our findings, increased IL-6, IL-1β and TNFα in serum was reported (Li et al., 2016). The discrepancy might be due to the source of LPS, the dose or the facility that the animals were housed in. In our experiments, the sensitivity to LPS may not involve cytokines, but rather might be due to alteration in other physiological pathways (heart rate, vasoconstriction etc). These animals need to be further characterized in their responses to endotoxemia. Additionally, the alterations in serum TNFα would suggest that a non-macrophage source of TNFα might have dependency to NR4A1 for TNFα production.

In summary, this chapter suggests that the adrenergic stimulation alters gene expression in a small subset of genes in macrophages. Additionally, NR4A family of transcription factors were proposed to be regulators of *Il10* in response to adrenergic signals. However, potentially due to the redundancy between NR4A members, we did not observe differences in cytokine production in NR4A1-deficient macrophages. Deletion of the other NR4A family members (to create single, double or triple knockouts) by CRISPR would be a viable approach to directly test the involvement of this pathway in induction of IL-10, in response to adrenergic signaling.

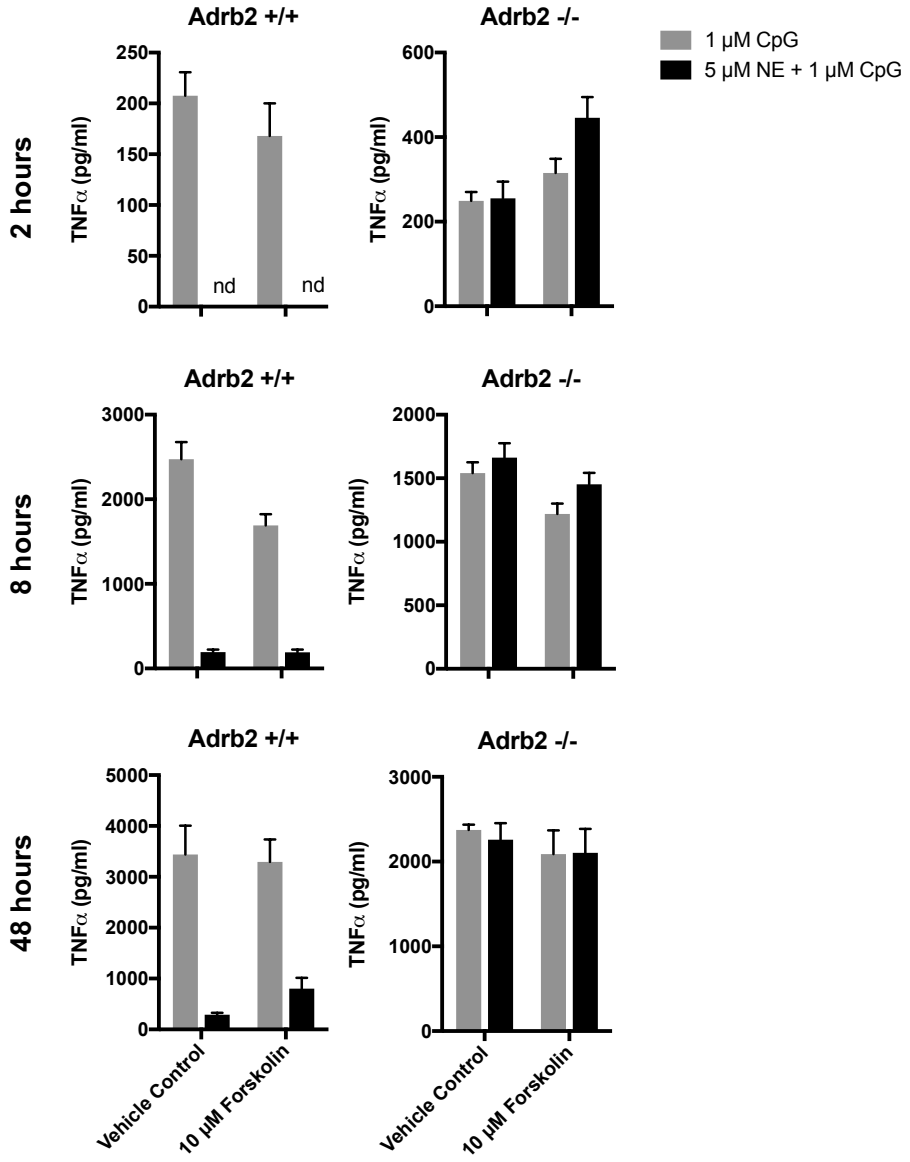

Figure 5.1 Effects of forskolin in TNFα secretion in macrophages. ADRB2-sufficient and -deficient macrophages were stimulated with 10 μM forskolin or vehicle control in the presence or absence of 1 μM CpG and 5 μM NE for 2, 8 or 48 hours. Secreted TNFα was measured by ELISA. The data represents mean ± SEM for 2 mice per strain.

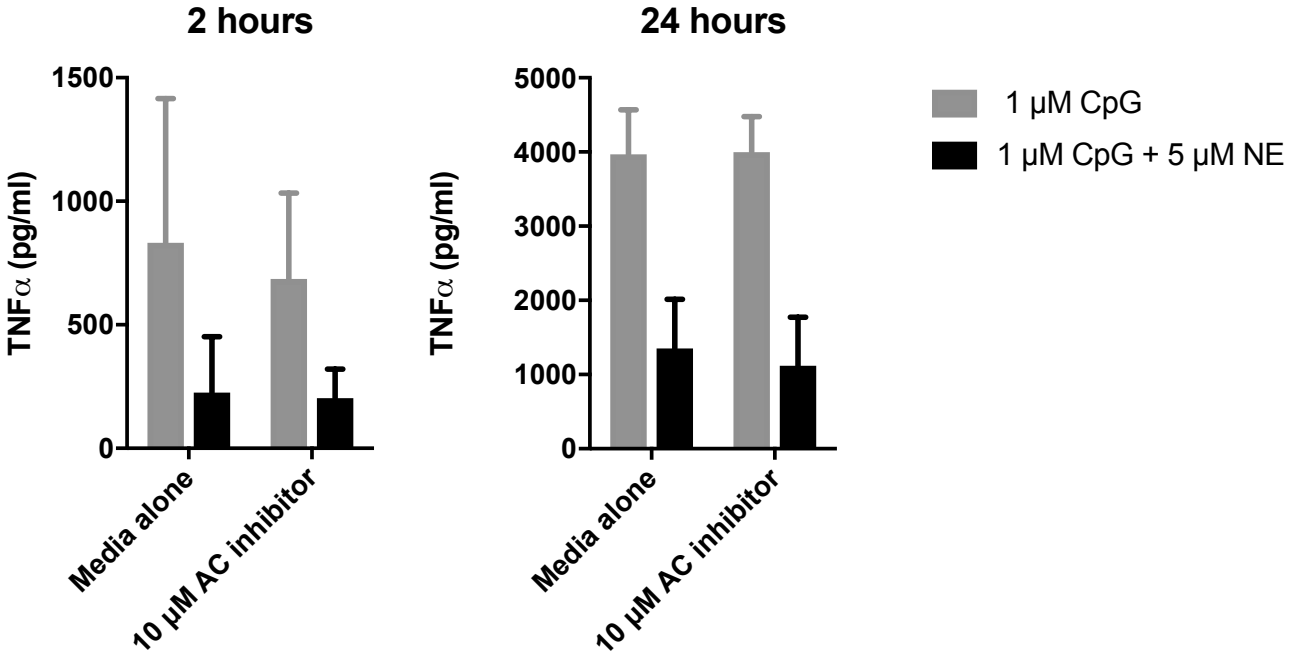

Figure 5.2. Effects of adenylyl cyclase inhibitor in TNFα secretion in macrophages. ADRB2-sufficient macrophages were stimulated with 10 μM AC inhibitor in the presence or absence of 1 μM CpG and 5 μM NE for 2 or 24 hours. Secreted TNFα was measured by ELISA. The data represents as mean ± SEM for 2 mice.

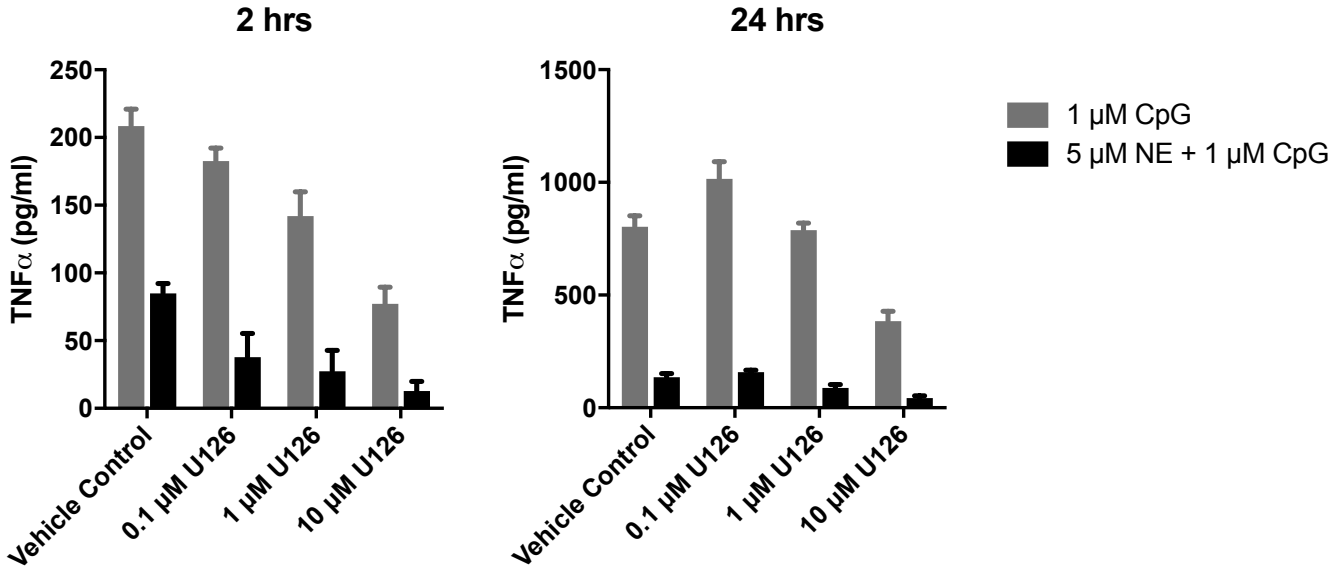

Figure 5.3. Effects of MEK inhibition in TNFα secretion in macrophages. ADRB2-sufficient macrophages were stimulated with increasing concentrations of MEK inhibitor U126 in the presence or absence of 1 μM CpG and 5 μM NE for 2 or 24 hours. Secreted TNFα was measured by ELISA. The data represents mean ± SEM for 2 mice.

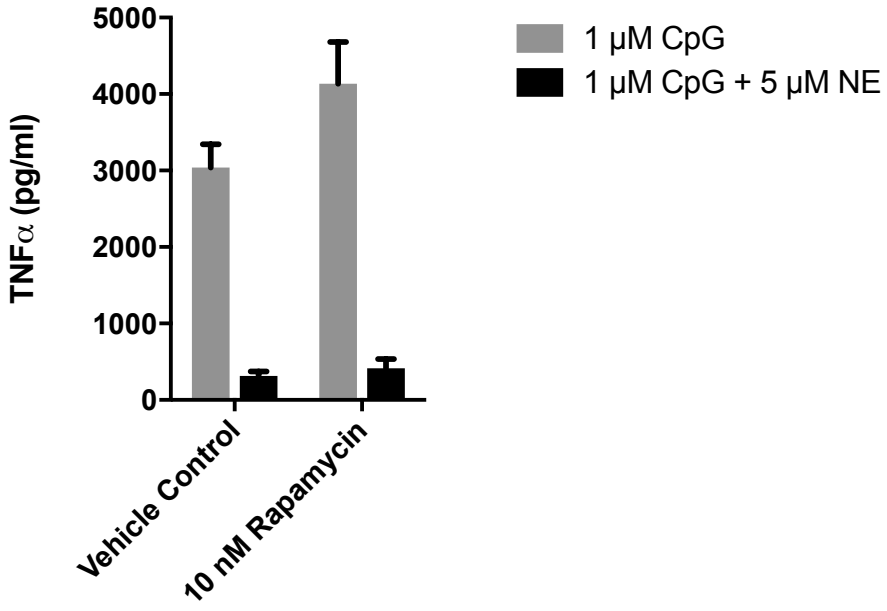

Figure 5.4. Effects of rapamycin in TNFα secretion in macrophages. ADRB2-sufficient macrophages were stimulated with 10 μM rapamycin in the presence or absence of 1 μM CpG and 5 μM NE for 8 hours. Secreted TNFα was measured by ELISA. The data represents mean ± SEM for 4 mice.

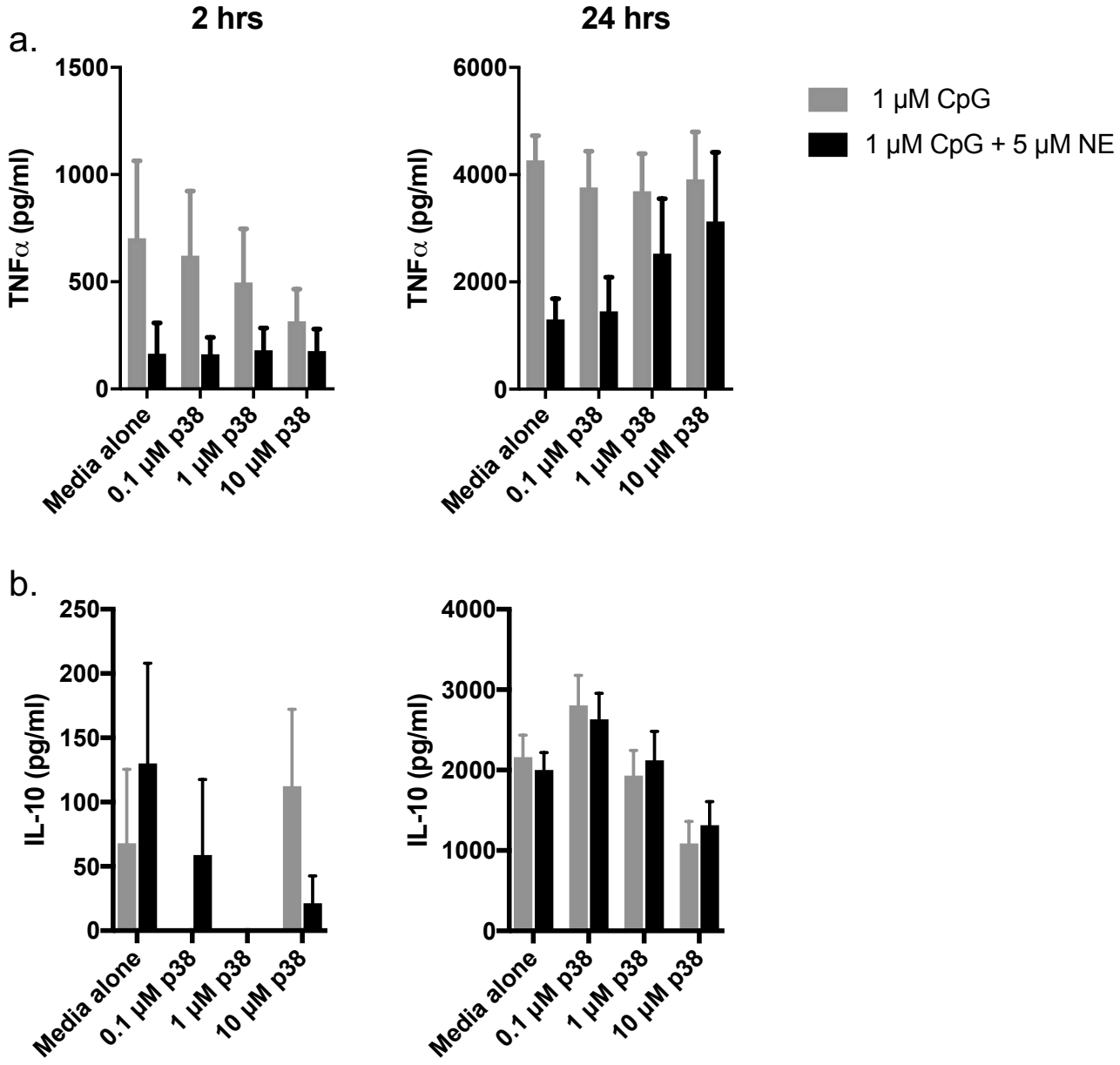

Figure 5.5. Effects of p38 inhibitor in TNFα secretion in macrophages. ADRB2-sufficient macrophages were stimulated with increasing concentrations of the p38 inhibitor in the presence or absence of 1 μM CpG and 5 μM NE for 2 or 24 hours. Secreted TNFα was measured by ELISA. The data represents mean ± SEM for 3 mice.

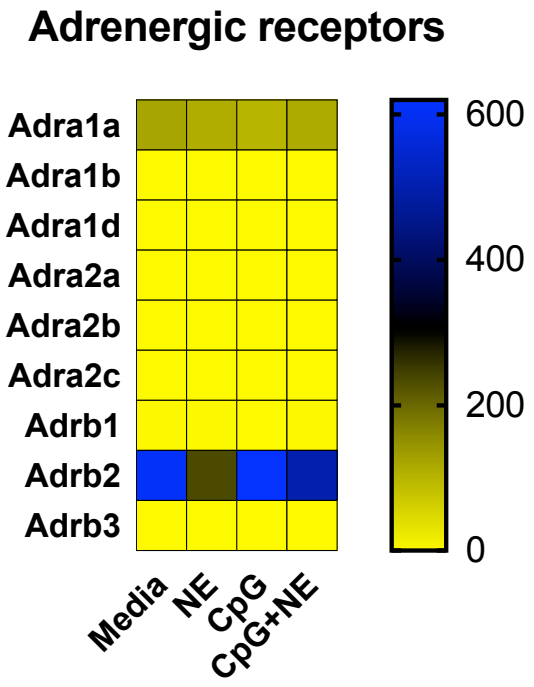

Figure 5.6. Expression levels of adrenergic receptors. ADRB2-sufficient macrophages were stimulated in the presence or absence of 1 μM CpG and 5 μM NE for 2. The transcriptome was analyzed by RNA-Seq. Normalized DESeq values were calculated by Beibei Chen. This heat map represents the DESeq values of abovementioned genes. n=2-3 mice per condition.

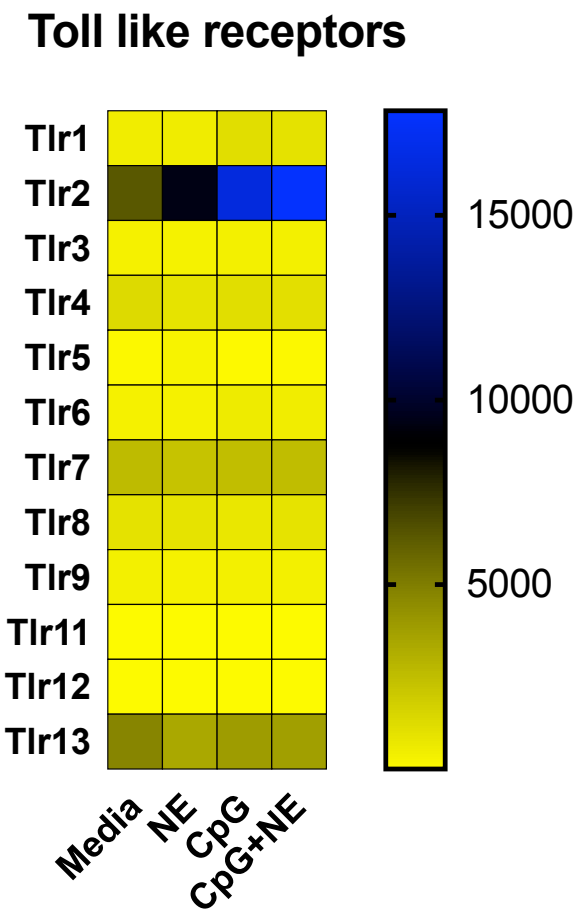

Figure 5.7. Expression levels of Toll-like receptors. ADRB2-sufficient macrophages were stimulated in the presence or absence of 1 μM CpG and 5 μM NE for 2. The transcriptome was analyzed by RNA-Seq. Normalized DESeq values were calculated by Beibei Chen. This heat map represents the DESeq values of abovementioned genes. n=2-3 mice per condition.

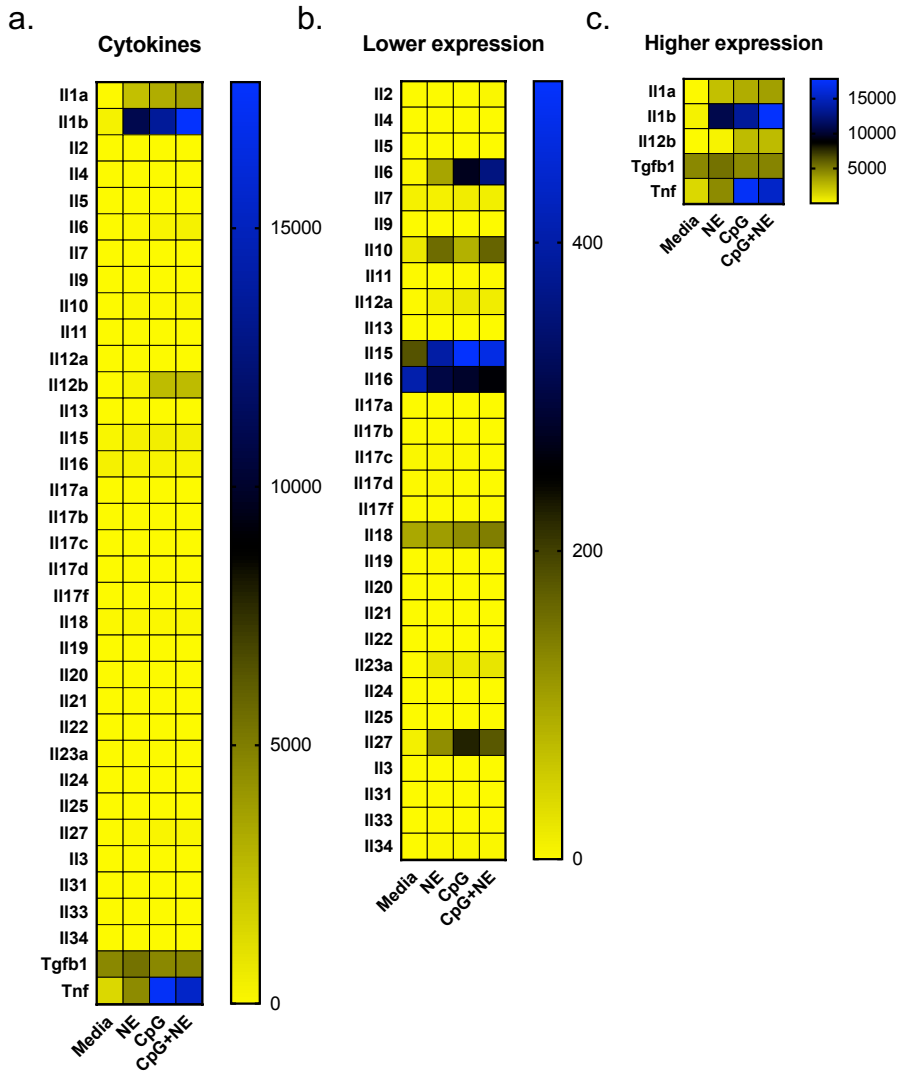

Figure 5.8. Expression levels of cytokines. ADRB2-sufficient macrophages were stimulated in the presence or absence of 1 μM CpG and 5 μM NE for 2. The transcriptome was analyzed by RNA-Seq. Normalized DESeq values were calculated by Beibei Chen. This heat map represents the DESeq values of abovementioned genes. (a) heat map of all the cytokines that were assessed, (b) cytokines with low transcripts were replotted to observe the differences, (c) cytokines with high transcript expression. n=2-3 mice per condition.

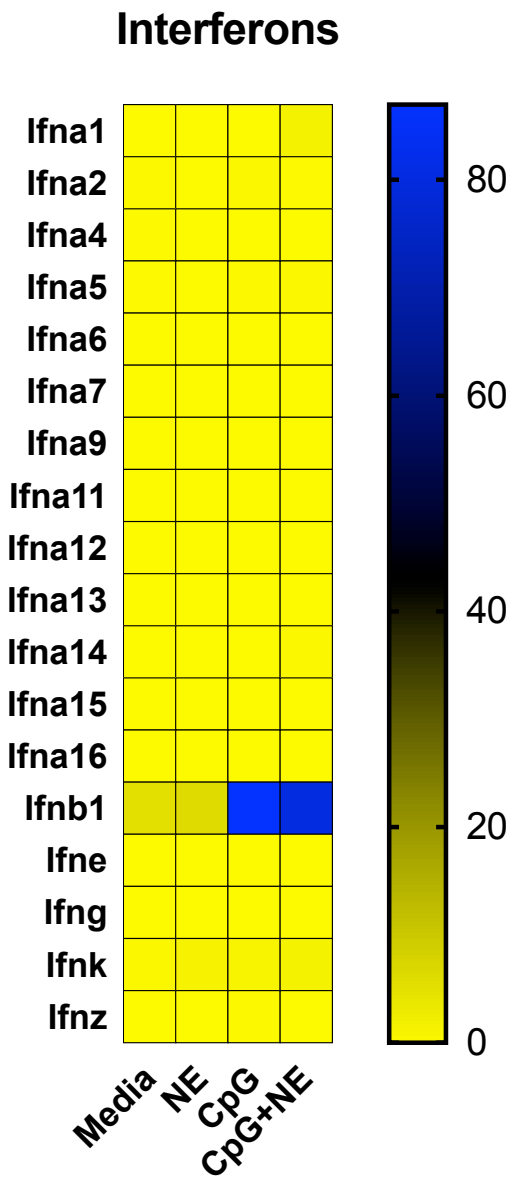

Figure 5.9. Expression levels of interferons. ADRB2-sufficient macrophages were stimulated in the presence or absence of 1 μM CpG and 5 μM NE for 2. The transcriptome was analyzed by RNA-Seq. Normalized DESeq values were calculated by Beibei Chen. This heat map represents the DESeq values of abovementioned genes. n=2-3 mice per condition.

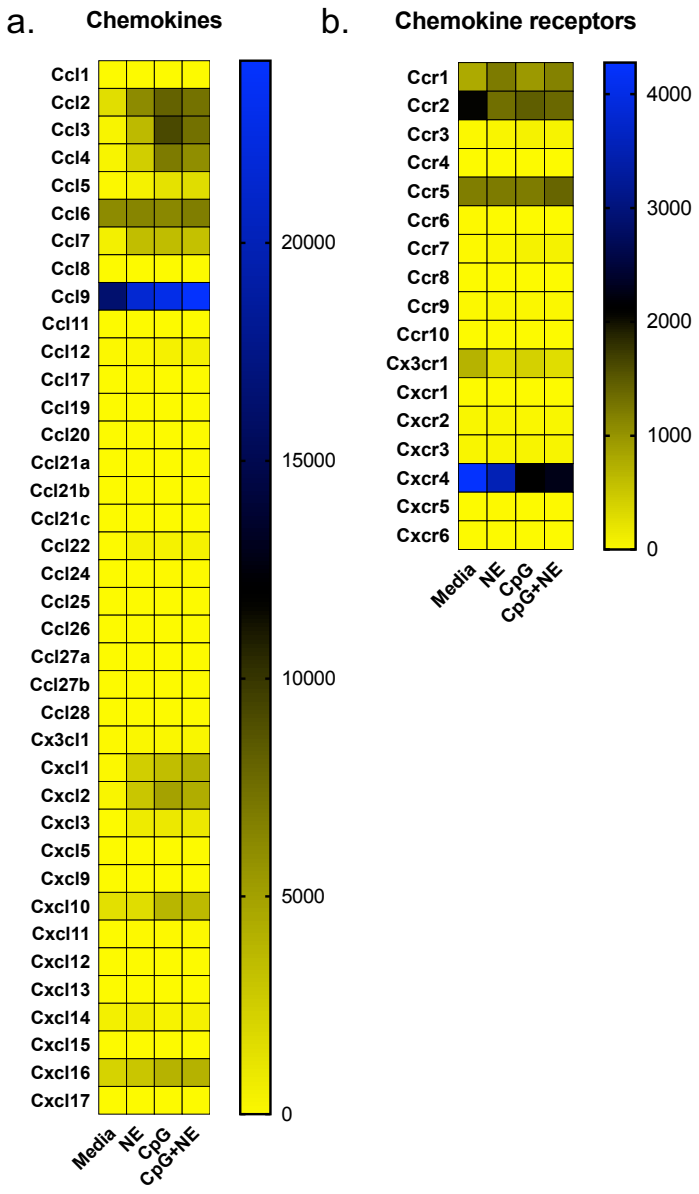

Figure 5.10. Expression levels of chemokines and chemokine receptors. ADRB2-sufficient macrophages were stimulated in the presence or absence of 1 μM CpG and 5 μM NE for 2. The transcriptome was analyzed by RNA-Seq. Normalized DESeq values were calculated by Beibei Chen. This heat map represents the DESeq values of (a) chemokine genes and (b) chemokine receptor genes. n=2-3 mice per condition.

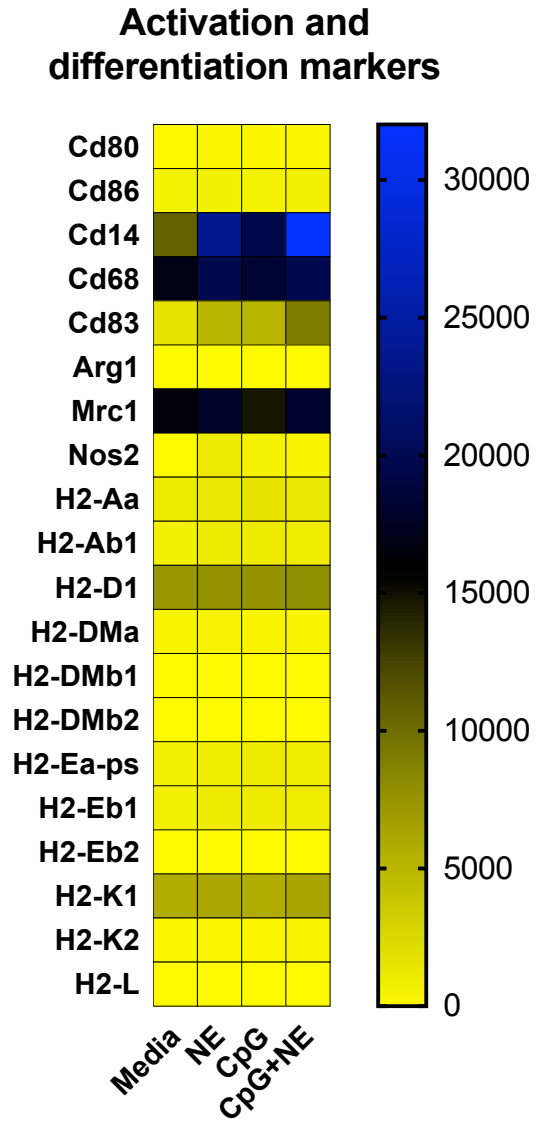

Figure 5.11. Expression levels of activation and differentiation markers. ADRB2-sufficient macrophages were stimulated in the presence or absence of 1 μM CpG and 5 μM NE for 2. The transcriptome was analyzed by RNA-Seq. Normalized DESeq values were calculated by Beibei Chen. This heat map represents the DESeq values of abovementioned genes. n=2-3 mice per condition.

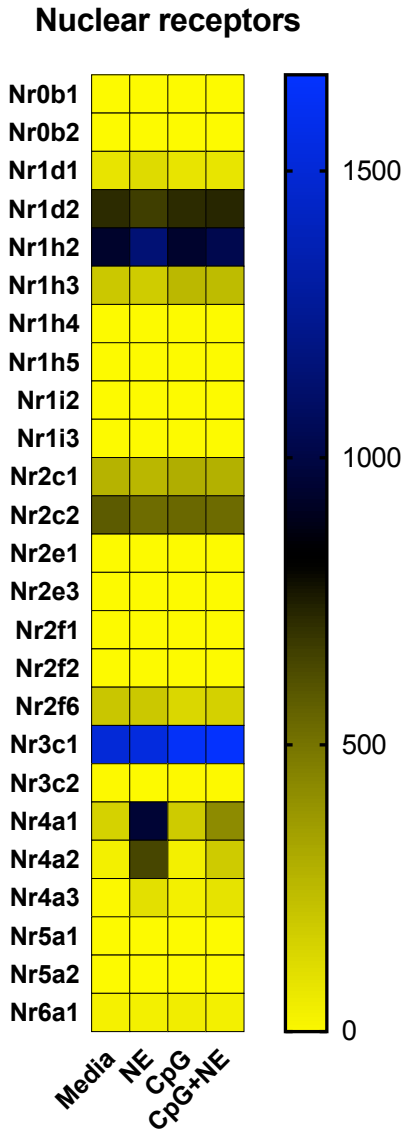

Figure 5.12. Expression levels of nuclear receptors. ADRB2-sufficient macrophages were stimulated in the presence or absence of 1 μM CpG and 5 μM NE for 2. The transcriptome was analyzed by RNA-Seq. Normalized DESeq values were calculated by Beibei Chen. This heat map represents the DESeq values of nuclear receptors. n=2-3 mice per condition.

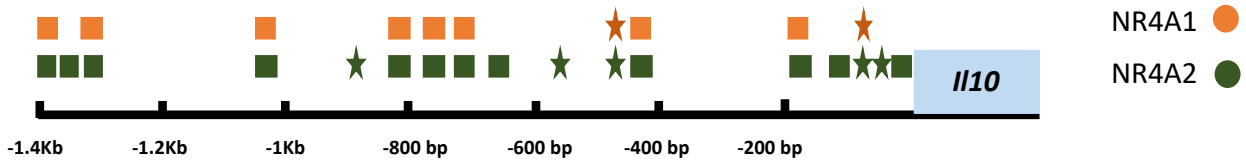

Figure 5.13. Il10 promoter carries multiple putative NR4A1 and NR4A2 binding sites. Mouse and human upstream *Il10* promoter was examined for the presence of NR4A1 and NR4A2 putative binding sites in Contra database. Squares represent conserved putative binding sites between mouse and human. Stars represent sites that are putative binding sites in mouse *Il10* promoter only.

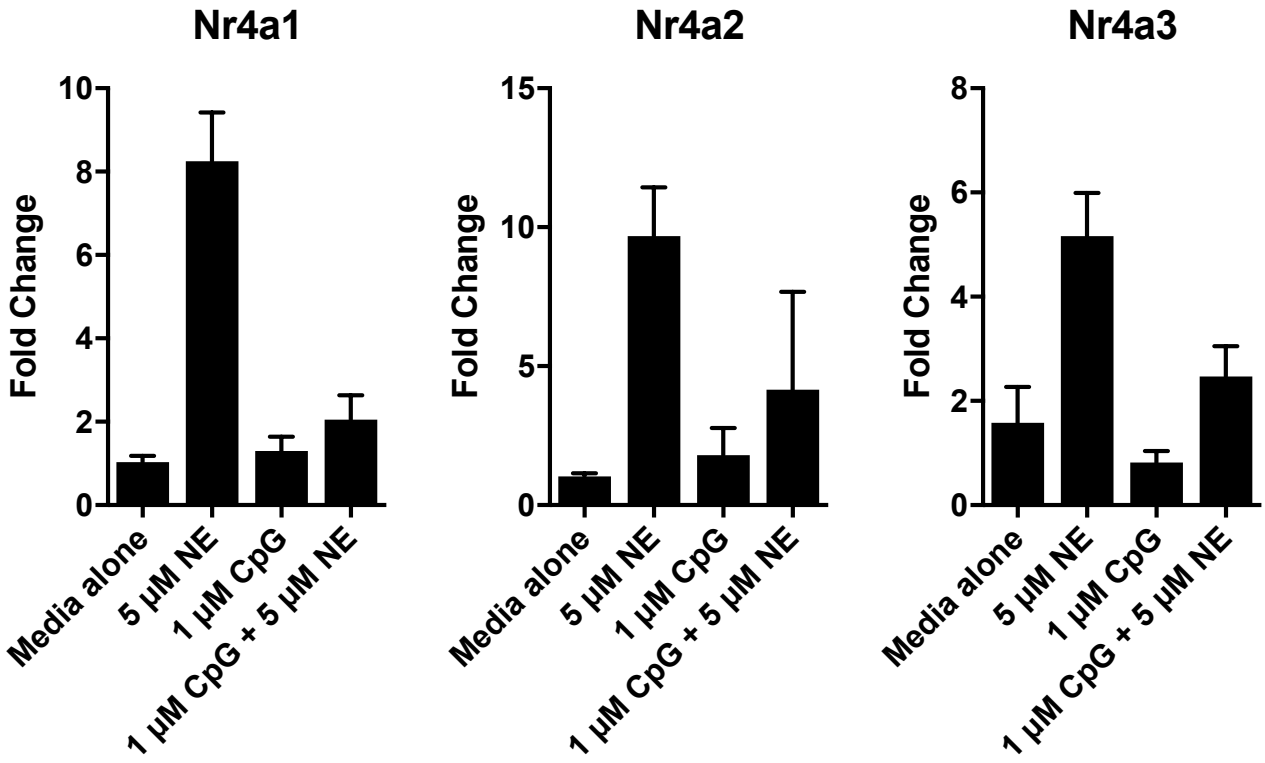

Figure 5.14. NR4A family members are upregulated in response to norepinephrine. ADRB2-sufficient macrophages were stimulated with CpG and norepinephrine in the concentrations listed above for 2 hours. *Nr4a1, Nr4a2* and *Nr4a3* transcripts were assessed by qPCR. Data represents mean ± SEM for 2 mice.

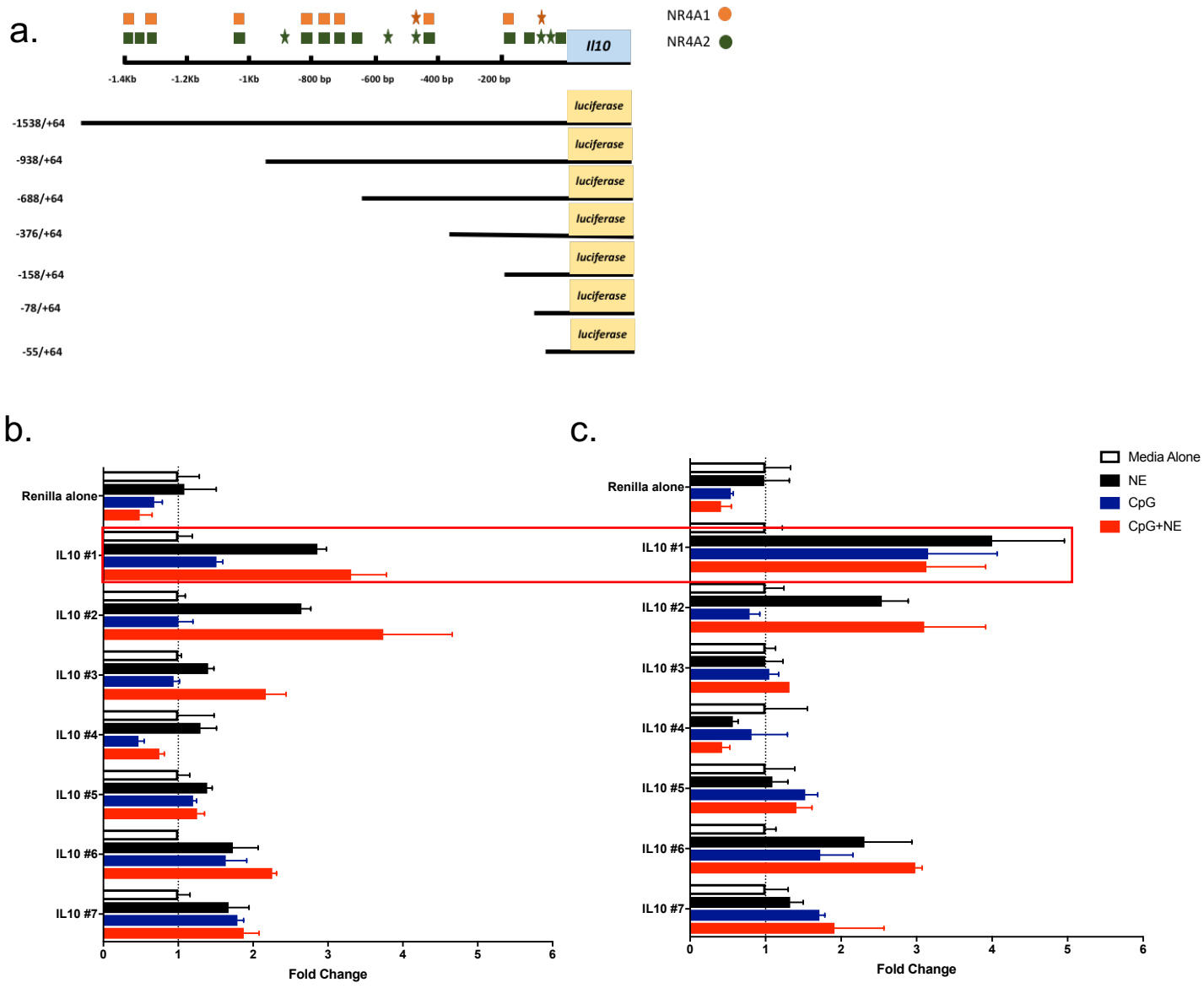

Figure 5.15. Il10 promoter contains norepinephrine and Nr4a1 sensitive regions. (a) RAW264.7 cells were transfected with luciferase reporter constructs that correspond to regions. 24 hours after transfection, the cells were stimulated in the presence or absence of 1 μM CpG and 5 μM norepinephrine for 3 hours. Signal is normalized to untreated sample in each construct in the absence (b) or presence (c) of NR4A1 overexpression.

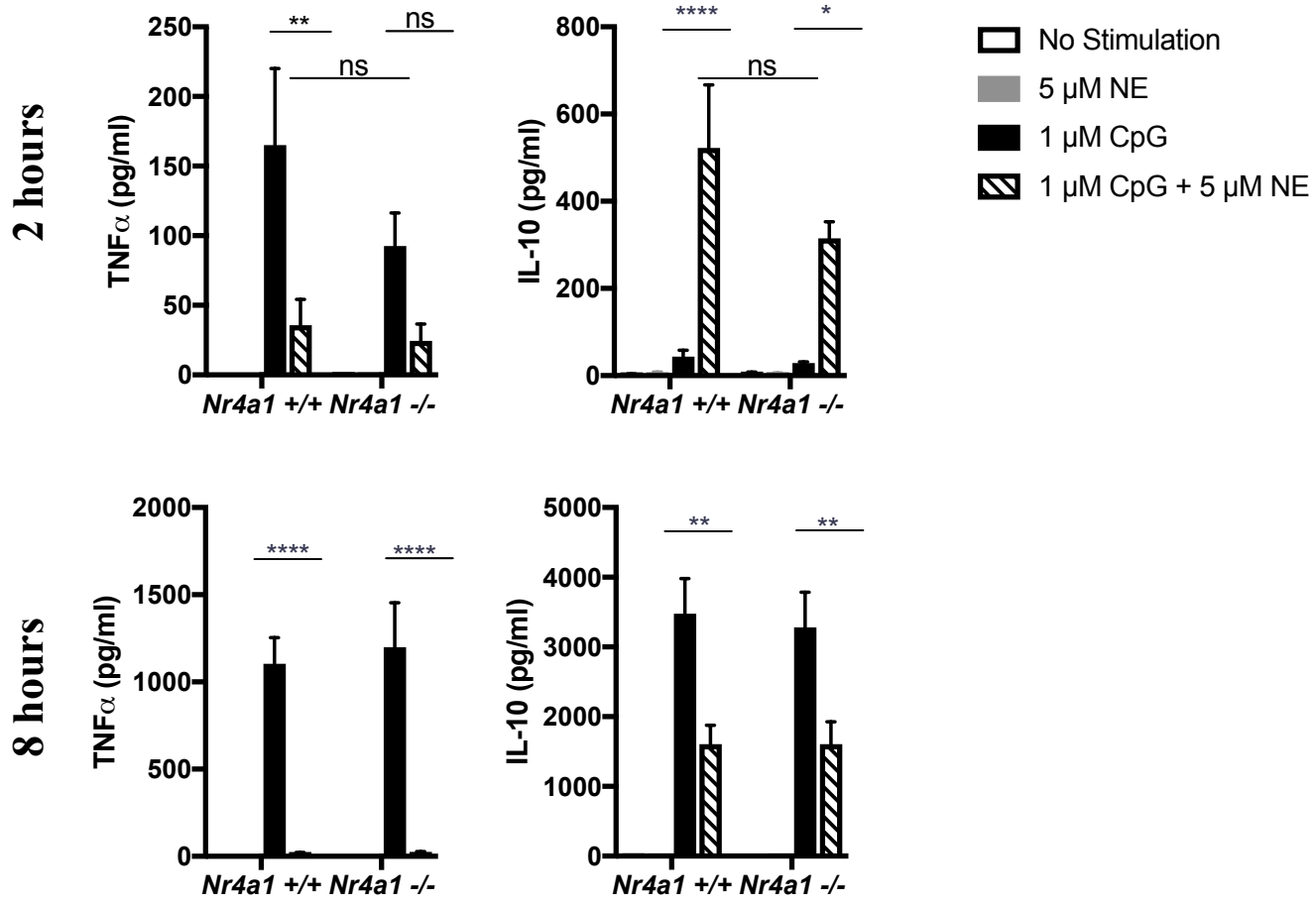

Figure 5.16. TNFα and IL-10 expression in NR4A1-sufficient and -deficient macrophages. NR4A1-sufficient and -deficient macrophages were stimulated in the presence or absence of 1 μM CpG and 5 μM norepinephrine for 2 (top panels) or 8 hours (bottom panels). Cytokine expression was assessed using Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery. The data represents mean ± SEM for 5 mice per strain.

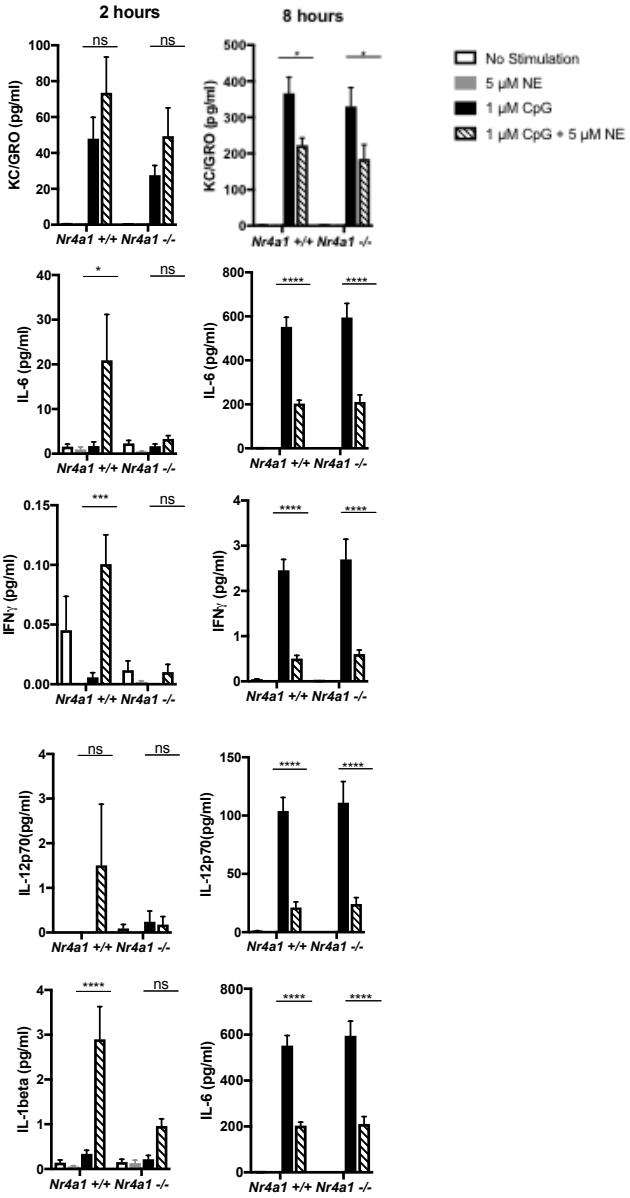

Figure 5.17. Pro-inflammatory cytokine expression in NR4A1-sufficient and -deficient macrophages. NR4A1-sufficient and -deficient macrophages were stimulated in the presence or absence of 1 μM CpG and 5 μM norepinephrine for 2 or 8 hours. Cytokine expression was assessed using Mouse Pro-inflammatory 7-Plex Tissue Culture Kit from Mesoscale Discovery. The data represents mean ± SEM for 5 mice per strain.

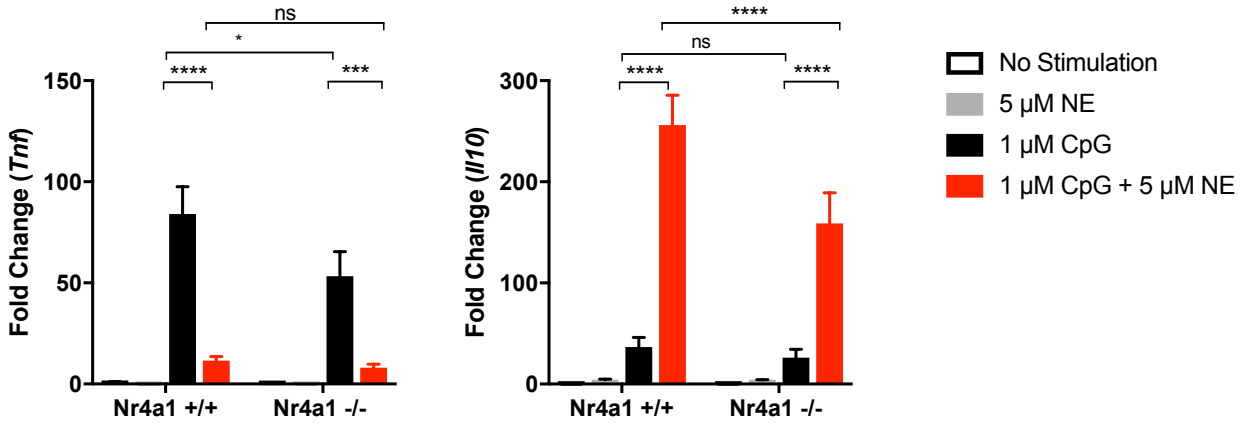

Figure 5.18. Expression of Tnf and Il10 in NR4A1-sufficient and -deficient macrophages. NR4A1-sufficient and -deficient macrophages were stimulated in the presence or absence of 1 μM CpG and 5 μM norepinephrine for 2 hours. Cytokine transcripts were assessed by qPCR. Data represents mean ± SEM for 6-7 mice.

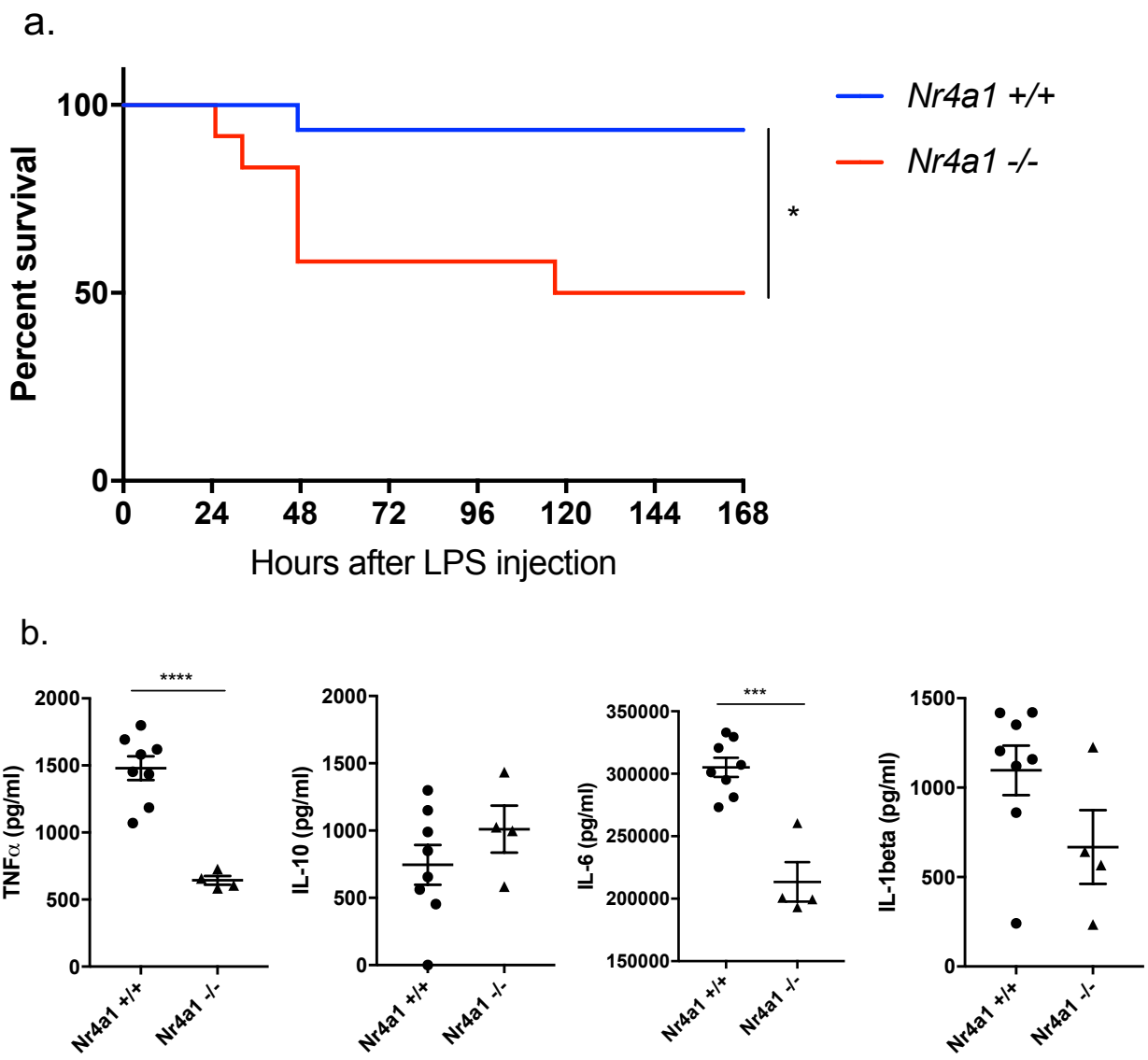

Figure 5.19. NR4A1-deficient mice are susceptible to LPS endotoxemia. (a) NR4A1-sufficient and -deficient male mice were challenged with 250 µg LPS intraperitoneally and the survival of the animals were monitored for 7 days (n=12-15 mice per strain). (b) NR4A1-sufficient and -deficient mice were challenged with 250 µg LPS intraperitoneally and the serum cytokines were assessed at 2 hours post-challenge by ELISA (n=4-8 mice per strain).

Figure 5.20. NR4A family members are highly homologous in zinc finger and ligand binding domain (LBD) of nuclear hormone receptors. Murine NR4A1, NR4A2 and NR4A3 protein sequences were aligned using multiple sequence alignment tool CLUSTALW. Yellow and teal highlighted regions are predicted zinc finger and LBD of nuclear hormone receptors, respectively; where there are many amino acids that are shared between the members. The domain prediction was achieved using PFAM database. Legend: * = amino acids that are conserved in all the samples, : = conservation of amino acids with highly similar properties and . = conservation of amino acids with weakly similar properties.

Table 5.1. The number of differentially expressed genes across treatment comparisons

Treatment 1	Treatment 2	# of genes differentially expressed: Fold Change>1.5 and p<0.05	# of genes differentially expressed: corrected q<0.25
Isotype_No CpG_No NE	Isotype_No CgG_Plus NE	286	2
Isotype_No CpG_Plus NE	Isotype_CpG_Plus NE	154	1
Isotype_No CpG_Plus NE	Anti IL10R_No CpG_Plus NE	155	2
Isotype_No CpG_No NE	Isotype_CpG_No NE	397	14
Isotype_CpG_Plus NE	Anti IL10R_CpG_Plus NE	24	0
Isotype_CpG_No NE	Anti IL10R_CpG_No NE	47	0
Isotype_CpG_No NE	Isotype_CpG_Plus NE	20	0
Anti IL10R_CpG_ No NE	Anti IL10R_CpG_Plus NE	22	1

Table 5. 2. The top 50 upregulated genes in the presence of adrenergic receptor signaling (in the absence of TLR)

Gene name	DESeq value: Isotype_No CpG_No NE	DESeq value: Isotype_No CpG_Plus NE	Fold Change	log_2(Fold Change)	p_{val}
Il12b	2.838692586	306.8200916	108.0850012	6.325328561	6.68E-05
Il1a	50.35898376	2510.018559	49.84251809	5.611512225	0.003354701
Shisa3	2.642726706	144.9838714	54.8614698	5.324646414	0.031223556
Ptgs2	51.15562778	1984.25135	38.78852507	5.250354934	0.01573337
Il1b	379.4188407	11018.77791	29.04119861	4.856362639	0.000114836
Il6	2.887183035	102.1111395	35.36704749	4.72933135	0.000590172
Cxcl3	33.69034561	908.2246753	26.95801004	4.712030719	0.003561528
Cxcl1	112.3130397	2549.778768	22.70242864	4.492551982	0.001873536
Il23a	0.592187077	27.69892362	46.77394133	4.171914865	0.01101824
Nr4a2	39.83043805	650.9013738	16.34180807	3.996936765	0.012425482
Nfkbiz	429.8489576	5808.366804	13.51257622	3.753126826	2.80E-05
Cxcl5	0.939917826	24.15603804	25.7001595	3.696837275	0.002035178
Cish	23.26287678	288.2948846	12.39291629	3.575718292	0.031390654
Nr4a3	8.479230855	103.9927978	12.26441403	3.469376553	0.000380392
Socs3	602.2145358	6452.455205	10.71454577	3.419328693	0.008578719
Csf2	0.207580153	11.59489826	55.85745113	3.382648612	0.009789519
Ch25h	103.4031139	1048.699617	10.14185722	3.329739897	0.043706841
Il27	12.17779539	125.5179399	10.30711519	3.263161033	0.029774229
Cxcl2	320.9396784	3076.595723	9.586211771	3.256941428	0.01162464
Dusp5	46.41515431	430.0460303	9.265207381	3.184421803	0.001433831
Bcl2a1a	3.399294967	37.18218782	10.93820577	3.11755553	0.014386991
Tnfsf9	57.93430583	509.9747093	8.802637781	3.116072306	0.007513718
Flrt3	69.10976405	573.5943339	8.299758244	3.034856482	0.026015169
Serpine1	308.5383525	2321.488552	7.524149051	2.907481407	0.007194577
Ramp3	4.895555009	41.47536318	8.472045174	2.848926743	0.03828167
Thbs1	1965.215177	14037.87852	7.143176322	2.835934572	0.008524451
Gm14023	1.672255499	17.43999055	10.4290227	2.786708059	0.019881139
Fosl1	0.799767231	10.98173953	13.73116966	2.734955141	0.002169651
Sele	0.695461497	9.881247507	14.20818773	2.68209404	0.000914215
Il12a	1.250772398	13.20876055	10.56048292	2.65828863	0.008199845
Sik1	61.98814868	395.4462402	6.379384586	2.65397293	0.041772243
Gfpt2	5.627892682	38.91000286	6.913778401	2.590128235	0.023630181
Gm15056	0	4.878850772	Inf	2.555534157	0.021561957

Nr4a1	163.9908936	966.0058822	5.890606857	2.551138266	0.027883562
Adora2a	34.85474935	207.3061599	5.947716275	2.538469366	0.032959683
Dusp2	104.5550069	599.0569662	5.729586595	2.507104455	0.013557017
Tpbg	20.43780255	120.169577	5.879769936	2.498798578	0.040086878
Trem1	106.5472996	601.1422915	5.642022782	2.485133149	0.018349709
Vegfa	264.925225	1484.499446	5.603465829	2.481855528	0.040000743
Cytip	379.3593936	2118.514979	5.584453727	2.478299022	0.046163999
Prickle1	4.070525876	25.16653192	6.182624233	2.367515439	0.000311668
Il17rd	1.702808881	12.72456413	7.472690726	2.344228927	0.000668782
Errfi1	332.1842831	1682.557042	5.065131398	2.33712035	0.001254676
Dusp1	494.0137158	2452.301956	4.964036174	2.309184409	0.020452315
Zc3h12a	509.9761746	2525.472102	4.952137428	2.30579632	0.012738029
Gm6377	169.5236452	827.3360172	4.88035764	2.280244319	0.037655519
Rab11fip1	442.111306	2150.708143	4.864630499	2.279741362	0.006331167
Adora2b	177.7588067	854.7524004	4.808495378	2.259179115	0.001073039
Fosl2	627.7682086	2946.952997	4.694332967	2.229113342	0.004036646
Bcl2a1d	28.40405434	130.1664086	4.582670031	2.157311298	0.01207754

Table 5. 3. The top 50 downregulated genes in the presence of adrenergic receptor signaling (in the absence of TLR)

Gene name	DESeq value: Isotype_No CpG_No NE	DESeq value: Isotype_No CpG_Plus NE	Fold Change	$\log_2$(Fold Change)	p_{val}
Spry3	15.67607534	3.117556856	0.198873557	-2.017919319	0.02973339
Catsperg2	4.522562358	0.369723672	0.081750928	-2.011452931	0.044359565
Mir27a	4.034681014	0.322080507	0.079827998	-1.929090344	0.041137114
Cct6b	5.048351192	0.720063813	0.142633463	-1.814079822	0.032390523
Sesn1	2131.843245	649.3920173	0.304615275	-1.713396482	0.011065122
Adcy5	4.730142511	0.767706977	0.162301025	-1.696691873	0.042849494
Jam3	10.15045504	2.851995617	0.280972193	-1.53342462	0.01561906
Samd15	12.9575922	3.894139937	0.300529595	-1.511922829	0.025308368
Trpc4	6.73748317	1.800975194	0.267306819	-1.46593516	0.040876951
Gpr155	197.986457	72.06185579	0.363973662	-1.44547994	0.003928728
Adrb2	609.0487025	236.2335174	0.387872951	-1.362616568	0.012578441
5031425F14Rik	23.95630492	8.870105471	0.370261837	-1.338266935	0.0101095
Aox1	14.00384912	5.078299533	0.362635979	-1.303592984	0.04999673
Fam46b	11.75424967	4.445560163	0.378208758	-1.227825662	0.037797838
Slc26a1	14.47279135	5.742758295	0.396796869	-1.1983227	0.040833426
Cx3cr1	724.7767898	316.6221558	0.436854712	-1.192214369	0.008733966
Fam214a	188.1859595	84.7679506	0.450447796	-1.141294469	0.020077554
Smad9	11.67623715	4.843543468	0.41482058	-1.117211179	0.047446781
Bik	29.73064172	13.31220588	0.447760462	-1.102431848	0.013339873
Med12l	27.89606767	12.64069944	0.453135531	-1.082955554	0.026252285
Rtkn2	35.68000121	16.55676845	0.464034975	-1.062966364	0.048338534
Arvcf	61.83331915	29.98618973	0.484951967	-1.01990443	0.031713813
S1pr2	1344.301984	666.2749644	0.495628938	-1.011576774	0.013766872
Usp2	815.848714	410.9572522	0.503717473	-0.987574266	0.010033074
Nuak1	247.1358161	124.6428297	0.504349518	-0.981801649	0.018146488
Trp53cor1	19.89207257	9.579661856	0.481581888	-0.981662103	0.038187656
9930014A18Rik	52.34668236	26.40199155	0.504368001	-0.961117802	0.010805
Zdhhc15	25.3060623	12.71731935	0.502540427	-0.939396734	0.029329631
Oxld1	48.79771443	24.99899966	0.512298577	-0.937623413	0.016181314
Tfap4	113.6150511	58.93297779	0.518707488	-0.935374548	0.011652673
Pif1	187.8915945	97.90857313	0.521090757	-0.933391024	0.013561123
Map3k9	506.9114854	267.5592821	0.527822489	-0.919336512	0.02172662
Rnf144b	636.5704356	338.4398491	0.531661274	-0.90942879	0.000389654

Abcd2	273.0651763	146.0330261	0.534791833	-0.898378781	0.019479627
Chd3os	56.26224379	29.73766359	0.528554526	-0.897576695	0.013032623
Ppargc1b	109.2135946	58.30585187	0.533869909	-0.894055817	0.006632194
Mettl20	71.40417415	38.17153869	0.534584135	-0.886267072	0.011819095
Rab40c	472.6171742	258.6342197	0.547238302	-0.867240854	0.006575325
Fam84b	446.2669002	244.5409189	0.547970102	-0.86517253	0.036702041
Fam13a	57.66052089	32.07935447	0.556348676	-0.826458801	0.028066689
Zbtbd6	42.28672124	23.86045769	0.564254144	-0.800071671	0.043740117
Srl	67.59604167	39.09216259	0.578320292	-0.774805089	0.022936517
Kitl	315.0732072	184.0959618	0.584295832	-0.771985326	0.041147878
Klhl23	80.95834613	47.17517755	0.582709255	-0.766600888	0.03989168
Meis1	32.56830299	18.92691608	0.581145296	-0.752381151	0.040784338
Mertk	337.9339269	200.7486745	0.594047115	-0.748444862	0.023777454
Bhlhe41	2121.217521	1271.841623	0.599580953	-0.737519617	0.038438468
Rab39	154.0767792	92.15733295	0.598126034	-0.735241436	0.014566195
Plekhm1	1294.299901	781.3634051	0.603695793	-0.727375369	0.004819778
Apobec1	11457.98727	7021.104608	0.612769454	-0.706504153	0.013468118

Table 5. 4. The top upregulated genes in the presence of adrenergic receptor signaling (in the presence of TLR)

Gene name	DESeq value: Isotype_Plus CpG_No NE	DESeq value: Isotype_Plus CpG_Plus NE	Fold Change	$\log_2$(Fold Change)	p_{val}
Gcm1	0	5.981965563	Inf	2.803633241	0.036787277
Myl2	0.424934514	6.190680677	14.56855228	2.335232726	0.043775826

Table 5.5. The top downregulated genes in the presence of adrenergic receptor signaling (in the presence of TLR)

Gene name	DESeq value: Isotype_Plus CpG_No NE	DESeq value: Isotype_Plus CpG_Plus NE	Fold Change	$\log_2$(Fold Change)	p_{val}
Mir6973a	4.034120614	0	0	-2.331739783	0.033329392
Kcnk7	5.728344138	0.384759053	0.067167587	-2.280616528	0.00896078
Prph	3.81338156	0	0	-2.267050791	0.029173799
Mybpc2	3.598157038	0	0	-2.201055738	0.037707633
Sdsl	6.357474111	0.651196204	0.102430021	-2.155699002	0.027309212
Serpine3	9.332015707	1.450507657	0.155433478	-2.075969182	0.03419373
4930444G20Rik	4.459055128	0.384759053	0.086287126	-1.979016296	0.045841013
A230103J11Rik	7.207343138	1.420714309	0.197120393	-1.761482448	0.037110173
Adcy2	15.89919981	5.018122433	0.315621069	-1.489569573	0.037068068
Chkb	22.23461579	7.623920416	0.342885188	-1.429860019	0.027871424
Phospho1	14.82859173	4.928742389	0.332381016	-1.416734888	0.037815183
Ppp1r26	17.18503241	6.426311311	0.373948164	-1.292033798	0.03087621
Lhx2	11.87610826	4.294814103	0.361634806	-1.282044673	0.036692743
Pira2	40.93724893	16.90519895	0.412953957	-1.227853676	0.014590659
Ccdc39	22.27321751	9.060889474	0.406806492	-1.209912819	0.031992205
Mdk	30.10969063	14.24455675	0.473088778	-1.029069844	0.017876088
Tmem86b	29.48056065	14.28687553	0.484620211	-0.995595874	0.033622883
Kcnq1ot1	172.9652063	100.0098307	0.578207796	-0.784303081	0.024910484

CHAPTER VI

DISCUSSION AND CONCLUSIONS

Summary

Adrenergic receptor signaling mediates many biological pathways through different receptors expressed on immune and non-immune cells. Although the role of this pathway has been studied in physiology, its role in immune cells is less understood. This is a result of the diverse signaling pathways that different adrenergic receptors operate and the context-specific role that adrenergic receptors play in each target cell. Previous studies have reported the ability of norepinephrine to suppress TNFα production, however, the kinetics of this suppression and the underlying molecular processes are largely unknown. The study presented here demonstrates a role for beta2-adrenergic receptor signaling in controlling the pro-inflammatory cytokine production in response to multiple TLRs and in enhancing TLR-induced IL-10 production *in vitro* (Chapter III). Additionally, I have demonstrated that beta2-adrenergic receptor signaling is crucial for setting proper inflammatory responses *in vivo* and beta2-agonists can be used for modulation of inflammation (Chapter IV). Lastly, I have demonstrated that the adrenergic receptor signaling alters the transcriptome of macrophages and we propose that the NR4A family of transcription factors as mediators of enhanced IL-10 production (Chapter V). This work provides insights to the control of inflammation by adrenergic receptors.

In Chapter III, I expanded our understanding of the effects of adrenergic signaling on control of pro-inflammatory cytokines. Previous studies mostly relied on the use of cell lines and rarely utilized primary murine cells. The majority of these studies utilized the TLR4 ligand LPS to activate cells; it was difficult to discern if the effects of adrenergic receptor signaling were limited to TLR4 signaling or if this was a general conserved phenomenon across multiple TLRs. The work presented here demonstrated that norepinephrine suppressed TNFα production from both endosomal (TLR3, TLR7 and TLR9) and cell surface TLRs (TLR2:TLR1 and TLR4).

There have been reports in the literature that suggest that adrenergic receptor signaling can enhance IL-10 production (Grailer et al., 2014; Nijhuis et al., 2014; van der Poll et al., 1996a). These aforementioned studies measured cytokine expression at 18-24 hours post stimulation in LPS treated cells (Grailer et al., 2014; Nijhuis et al., 2014). In our experiments, norepinephrine can enhance IL-10 production in LPS treated cells in later phases of stimulation too. However, there was only one publication to my knowledge that studied the acute effects of adrenergic receptor signaling on IL-10 production. In that work, epinephrine and norepinephrine induced LPS-induced IL-10 production at 4 hours post stimulation (Grailer et al., 2014) but the investigators did not study the role of adrenergic signaling in IL-10 production in other TLRs. We have shown that norepinephrine upregulates IL-10 production in response to multiple TLR agonists at 2 hours post-stimulation. This observation highlights the acute nature of the synergism between adrenergic and TLR signaling pathways.

I have observed the down-regulation of multiple pro-inflammatory cytokines in response to norepinephrine (Chapter III). These findings suggest that the effects of adrenergic receptor signaling are not limited to TNFα and IL-6 as reported before. Taken together, these results provide evidence that adrenergic receptor signaling can alter cytokine production and promote an anti-inflammatory setting by both suppressing the pro-inflammatory cytokines, as well as, increasing IL-10. Using IL-10- deficient macrophages, I have demonstrated that the acute suppression of TNFα does not depend on IL-10. However, by IL-10R neutralization experiments, we have demonstrated a role for IL-10 induced suppression of pro-inflammatory cytokines in a TLR- and cytokine-specific manner in later phases of the stimulation. Although IL-10 can partially explain the suppression of the pro-inflammatory cytokines at 8 hours, I did not provide an explanation as to how adrenergic receptor signaling suppresses pro-inflammatory cytokines in acute phases of the stimulation. As described in the introduction, adrenergic receptors, as GPCRs, could be under the control of arrestins (Kizaki et al., 2008). Additionally, beta-arrestin 2 deficient animals were susceptible to cecal ligation puncture models and presented with increased serum IL-6 (Fan et al., 2010). These results suggest a role for arrestins in control of inflammation, however I have not tested the involvement of this pathway in modulation of cytokines. Although arrestins might be involved in suppression of the pro-inflammatory cytokines, this signaling pathway cannot fully explain our observations. If arrestins were involved in suppression of pro-inflammatory cytokines, neutralization of IL-10R would not be able to reverse the phenotype. It is likely that the arrestins control the *acute* suppression of the pro-inflammatory cytokines,

but that hypothesis was not formally tested throughout this work. Additionally, arrestins are proposed to regulate LPS-induced nitric oxide production by stabilization of NFκB. If this was the case, we would expect to detect less IL-10 in response to adrenergic signals, since IL-10 is also controlled by NFκB signaling (Saraiva and O'Garra, 2010).

It is possible that the regulation of cytokines in response to adrenergic cues depends on the TLR the macrophages were stimulated with, the time in which the cytokine secretion was assessed, the adrenergic receptor ligand that was used and maybe even the cell type that is studied. Although my work was unable to address to the full complexity of this pathway, I have provided evidence to show the complexity of this system.

In Chapter IV, I have demonstrated that the adrenergic receptor signaling controls inflammation in many disease settings. In LM-OVA infections, ADRB2-deficient mice developed splenomegaly and succumbed faster compared to the ADRB2-sufficient cohort. In colitis setting, ADRB2-deficient animals lost more weight. Lastly, in endotoxemia models, ADRB2-deficiency resulted in sensitivity to LPS and dysbalance in serum cytokines with more TNFα and reduced IL-10. In line with these observations, the beta2-adrenergic receptor agonist salmeterol increased serum IL-10 and reduced TNFα. Complementary to our studies, adrenergic receptor signaling was reported to be protective in endotoxemia setting (Deng et al., 2004; Grailer et al., 2014; Monastra and Secchi, 1993; van der Poll et al., 1996b; Verhoeckx et al., 2005). These observations show that beta2-adrenergic receptor signaling is important to control inflammation *in vivo*.

In Chapter V, we demonstrated the changes in macrophage transcriptome in response to adrenergic signals. Adrenergic receptors alter expression of several cytokine and chemokine transcripts. Additionally, we identified NR4A family of transcription factors as potential candidates for IL-10 production. *Il10* promoter contains several putative binding sites for NR4A1 and NR4A2. NR4A1 overexpression induced Luciferase activity in TLR stimulated cells, to comparable levels with TLR+NE treated cells. Additionally, NR4A1-deficient animals were sensitive to LPS endotoxemia. Although I did not demonstrate a complete signaling pathway, NR4A family members are potential candidates for regulation of *Il10*.

Beta2-adrenergic regulation of other immune cells

Expression of adrenergic receptors on immune and non-immune cells have been reported extensively. For example, Maisel *et al* identified the expression of beta-adrenergic receptor density on lymphocytes as ranging 1000 - 2000 receptors/cell (Maisel et al., 1989). The functional consequence of adrenergic receptor activation in most cells has been reported to be anti-inflammatory. For example, in human neutrophils, the respiratory burst and leukotriene B4 were suppressed by beta2-adrenergic receptor signaling (Brunskole Hummel et al., 2013; Nielson, 1987). Munoz and colleagues reported that human peripheral blood eosinophils reduced secretion of leukotriene C4 and eosinophil peroxidase in response to beta2-adrenergic receptor signaling (Munoz et al., 1994). Further, beta2-agonists inhibit the release of histamine and leukotriene from mast

cells *in vitro* and *in vivo* (Chong et al., 1998; Church and Hiroi, 1987; Nials et al., 1994). Similarly, beta2-agonists reduce histamine release from human lung mast cells when co-cultured with airway smooth muscle cells (Lewis et al., 2016). IgE-mediated release of TNFα is also reduced in response to beta2-agonists (Bissonnette Ey, 1997). These suggest that mast cell mediators that are involved in acute inflammatory responses can be controlled by adrenergic receptor agonists.

In IFNγ primed human dendritic cells, salbutamol inhibited IL-12, IL-1α, IL-1β, IL-6 and, TNFα in a dose-dependent manner, however, IL-10 was unaffected. When naive T cells are primed with dendritic cells exposed to salbutamol, commitment to the Th1 lineage was significantly reduced (possibly due to the reduction in IL-12) (Panina-Bordignon et al., 1997). This was accompanied by an increase in IL-4+ Th2 cells in the co-culture. This suggested that use of beta2-agonists may skew T cells to the pathogenic Th2 lineage. Similarly, in murine bone-marrow derived dendritic cells, epinephrine enhanced differentiation of IL-4 and IL-17A producing T cells (Kim and Jones, 2010). In addition to T cell priming, beta2-agonists also altered phagosomal degradation of antigens and cross-presentation of dendritic cells (Hervé et al., 2013).

We and others have recently demonstrated that CD8+ T cell function is impaired in response to adrenergic receptor signaling (Estrada et al., 2016; Grebe et al.; Kalinichenko et al., 1999). Beta2-agonists reduced TCR-induced IFNγ and TNFα production, as well as cytolytic activity of the human and murine T cells (Estrada et al., 2016). Similarly, the use of beta-blockers increased the frequency of intratumoral CD8+T

cells and increased the efficacy of anti-PD-1 treatment in murine tumor models (Bucsek et al., 2017).

In CD4+ T cells, the presence of norepinephrine increases IFNγ production from Th1 cells (Swanson et al., 2001). Although Th1 cells have been reported to be affected by NE, Th2 cells are unresponsive to NE, this is due to the reduction of ADRB2 expression during differentiation and lack of the receptor expression on mature Th2 cells (McAlees et al., 2011; Sanders et al., 1997). Lastly, beta2-adrenergic receptor signaling enhanced the suppressive function of Treg cells (Guereschi et al., 2013).

Recently, ILC2 cells have been reported to express beta2-adrenergic receptor. The ADRB2 agonist salmeterol inhibited of anti-helminth responses (via reduction of IL-5 and IL-13 in ILC2 cells), as well as decreased ILC2 frequency. In contrast, ADRB2 deficient mice had increased ILC2 cells and eosinophils. ADRB2-deficiency resulted in reduced worm load in *N. brasiliensis* infection (Moriyama et al., 2018).

In summary, both innate and adaptive immune cells change their behavior in response to adrenergic receptor signaling. Although the changes in each cell type are different from each other, adrenergic receptor engagement promotes an anti-inflammatory signal across multiple cells types and multiple cellular contexts.

Adrenergic receptor agonists for the treatment of inflammatory diseases

Beta2-adrenergic receptor agonists are commonly used for COPD and asthma treatment. Beta2-agonists are utilized for long-term control of the disease (via long-acting agonists)

or alleviation of the bronchoconstriction by the short-acting agonists. The mechanism of action is thought to be mostly through the relaxation of the smooth muscle cells. However, based on our and other groups' observations, it is also possible that beta2-agonists benefit patients due to their anti-inflammatory properties. Many immune cells such as mast cells, T cells, macrophages and B cells are involved in the pathogenesis of asthma and beta2-adrenergic signaling was shown to modulate inflammatory responses in all these cells (Broadley, 2006; Norris et al., 2006). Although the role of adrenergic signaling on lymphocyte trafficking to lung has not been studied, ADRB2 signaling was shown to be important for lymphocyte egress from lymph nodes during diurnal oscillations (Suzuki et al., 2016). It would be an interesting area of research to determine if chronic use of beta2-agonists would influence the recruitment of immune cells to the lungs.

The natural ligands of adrenergic receptors, epinephrine and norepinephrine, are commonly used as first line vasopressors in septic shock treatment and improve the long-term disease outcomes (Avni et al., 2015; De Backer et al., 2010). In line with our observations, epinephrine infusion reduced plasma TNFα and increased IL-10 levels in response to LPS in human donors (van der Poll et al., 1996b). However, these studies did not address if the cytokine alteration is dependent on beta2-adrenergic receptors. Interestingly, in van der Poll study (van der Poll et al., 1996b), the epinephrine was administered continuously for 9 hours (starting t=-3 hours with respect to LPS administration). We were able to detect differences in serum cytokines in our animal models by single administration of salmeterol; this was potentially due to the half-life of

salmeterol: 5.5 hours (Food and DrugAdministration, 2006). To my knowledge, no study in humans tested if beta2-adrenergic receptor agonists are beneficial in the treatment of sepsis.

Although I have studied the role of beta2-adrenergic receptors on control of inflammation during my dissertation, antagonists and agonists of other adrenergic receptors are also used in medicine. For example, beta3-adrenergic receptor agonists are proposed in treatment of diabetes and obesity; due to their effects on lipolysis and adipose tissues (de Souza and Burkey, 2001; Weyer et al., 1999). Beta1- agonists (e.g. xamoterol) are used in cases of heart failure as cardiac stimulant (Marlow, 1989; Snow, 1989). Although the effectiveness of the drugs is recently questioned, beta-blockers are used to treat hypertension (Ram, 2010; Wiysonge et al., 2017) and proposed to reduce mortality in traumatic brain injury (Schroeppel et al., 2010).

Adrenergic receptor agonists as well as antagonists are used to treat multiple diseases. In this body of work, we propose that the beta2-adrenergic receptor agonists can be used for the management of infections, sepsis and colitis due to their anti-inflammatory properties. It is possible that the ligands of the beta2-adrenergic receptor have broader effects than what has been reported in the literature and herein. It would be a fruitful area of research to investigate the role of adrenergic receptor signaling in regulating other inflammatory conditions (e.g. autoimmune diseases). Additionally, it is intriguing to think why this neuro-immune connection has evolved and its role during homeostasis. Although not tested, I hypothesized that the exposure of lymphocytes to adrenergic signals in circulation or in tissue could be immunoregulatory, even in the

absence of a disease setting. It is possible that the immune cells evolved to express the adrenergic receptors to receive tonic neuroendocrine signals to protect the host from autoreactivity. These questions are beyond the scope of this dissertation and will be investigated in the future.

Future directions

Throughout my time working on this project, I have tried to understand how beta2-adrenergic receptor signaling affects the macrophage function *in vitro* and the role of this pathway *in vivo* in various disease settings in murine models. Although I provided a comprehensive analysis of changes that occurs in response to adrenergic receptor signaling (or the lack of), there are many questions remaining.

I have used TLR agonists to activate macrophages and dendritic cells, but what would have happened if the cells received RIG-I-like receptor agonists, or NOD-like receptor agonists instead of TLR agonists? It would be interesting to investigate how other pathogen sensing pathways are affected by adrenergic signals. Understanding the role of adrenergic receptor signaling on regulation of different pathogen sensing pathways could provide novel therapeutic targets for infectious diseases.

We propose NR4A family members to be important for the acute induction of *Il10* at 2 hours. However, to definitively show that, additional experiments utilizing macrophages from triple knockout mice or CRISPR lines need to be completed. Further, we need to determine the underlying signaling pathways for suppression of the pro-

inflammatory cytokines. As discussed in Chapter V, the involvement of arrestins is likely, although it is not enough to explain how downregulation of NFκB signaling does not affect *Il10*.

Although albuterol altered TNFα and IL-10 production in human monocyte derived macrophages, it would be interesting to determine what other differences occur in response to beta2-adrenergic receptor signaling. I would like to assess the transcriptomic changes in response to albuterol. Additionally, cytokine secretion should be assessed using a multiplexed ELISA or a Luminex platform to determine which of the transcriptomic changes are reflected at the protein level.

Our *in vivo* infection experiments suggested that adrenergic receptor signaling is important to control the cellular recruitment of lymphocytes to spleen. It would be interesting to assess if serum cytokines/chemokines were different between adrenergic receptor sufficient and deficient mice. *Listeria* is a Gram-positive pathogen, we have not assessed if ADRB2 (or another adrenergic receptor) is important to control Gram-negative infections, viral infections or helminth infections. Additionally, we have shown that ADRB2-deficient mice lost more weight during DSS-colitis, however, we have not tested if beta2-adrenergic receptor agonists can be used to alleviate colitis symptoms.

Although this work has been mostly in murine systems, our increased understanding of adrenergic receptor – immune cell interactions might be applicable to human disease. One implication of our study is the induction of an anti-inflammatory environment when adrenergic signaling is engaged. Work from other groups suggested that in addition to the suppression of pro-inflammatory cytokines, the anti-viral

interferons were downregulated in response to adrenergic receptor signaling (Collado-Hidalgo et al., 2006; Hilbert et al., 2013). Social stress has been reported to be a key risk factor in reactivation of latent herpes simplex virus (HSV) (Jenkins and Baum, 1995; Padgett et al., 1998) and in murine models, stress (heat, fatigue or cold) was shown to reactivate latent HSV (Huang et al., 2011). It would be an interesting area of research to test if latent infections that are known to be reactivated after stress (e.g. HSV and varicella zoster (shingles)) could be alleviated by the use of beta-blockers?

Work by Repasky' group has shown that when mice were exposed to cold stress, the increased serum norepinephrine and excessive beta-adrenergic receptor signaling caused the animals to be more resistant to GVHD (Leigh et al., 2015). In line with this observation, in murine tumor models, cold stress increased the tumor burden. However, the administration of beta-adrenergic receptor antagonist propranolol reduced the tumor volume, increased the frequency of intratumoral CD8+ T cells that can express either Granzyme B or IFNγ and enhanced the efficacy of anti-PD-1 therapy (Bucsek et al., 2017). These results suggested that the reduction of beta-adrenergic receptor signaling during cold stress can enhance immune responses to tumors. Considering the ever-increasing number of immunotherapy patients, it would be crucial to determine the effects of stress on immunotherapy (probably not cold stress for humans, but rather psychological stress). Additionally, considering the suppressive role of beta2-adrenergic receptor signaling on many cell types, it is intriguing to consider that the use of beta-blockers might be a viable combination therapy for cancer patients.

Lastly, another interesting area of research regarding our studies is depression. There are several reports suggesting that the patients with major depressive disorder present with increased pro-inflammatory cytokines in their serum. Thinking depression as an inflammatory disease is an emerging idea (Miller and Raison, 2016; Wohleb et al., 2016). It would be interesting to determine if engagement of beta2-adrenergic receptor signaling would alleviate the symptoms and the inflammatory characteristic of depression.

Concluding remarks and implications

The data presented here has provided insight into the role of adrenergic receptors during inflammatory settings. Despite being used for treatment of many diseases, the mechanisms by which the adrenergic receptor signaling alter immunity are largely unknown. Here, we demonstrate that the beta2-adrenergic receptor signaling is important to modulate IL-10 production both *in vivo* and *in vitro.* Additionally, we propose that beta2-agonists, which are commonly used to treat COPD and asthma, can be used to dampen inflammation in various settings. If similar observations occur in humans, beta2-adrenergic receptor agonists could potentially be used for many more inflammatory settings.

REFERENCES

Abrass, C.K., O'Connor, S.W., Scarpace, P.J., and Abrass, I.B. (1985). Characterization of the beta-adrenergic receptor of the rat peritoneal macrophage. J Immunol *135*, 1338-1341.

Administration, F.a.D. (2006). SEREVENT® DISKUS®

Amit, I., Winter, D.R., and Jung, S. (2016). The role of the local environment and epigenetics in shaping macrophage identity and their effect on tissue homeostasis. Nat Immunol *17*, 18-25.

Andersson, U., and Tracey, K.J. (2012a). Neural reflexes in inflammation and immunity. J Exp Med *209*, 1057-1068.

Andersson, U., and Tracey, K.J. (2012b). Reflex principles of immunological homeostasis. Annu Rev Immunol *30*, 313-335.

Avni, T., Lador, A., Lev, S., Leibovici, L., Paul, M., and Grossman, A. (2015). Vasopressors for the Treatment of Septic Shock: Systematic Review and Meta-Analysis. PLoS One *10*, e0129305.

Baral, P., Mills, K., Pinho-Ribeiro, F.A., and Chiu, I.M. (2016). Pain and Itch: Beneficial or Harmful to Antimicrobial Defense? Cell Host Microbe *19*, 755-759.

Bellinger, D.L., Lorton, D., Felten, S.Y., and Felten, D.L. (1992). Innervation of lymphoid organs and implications in development, aging, and autoimmunity. Int J Immunopharmacol *14*, 329-344.

Beloeil, H., Mazoit, J.X., Benhamou, D., and Duranteau, J. (2005). Norepinephrine kinetics and dynamics in septic shock and trauma patients. Br J Anaesth *95*, 782-788.

Berg, D.J., Kuhn, R., Rajewsky, K., Muller, W., Menon, S., Davidson, N., Grunig, G., and Rennick, D. (1995). Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance. J Clin Invest *96*, 2339-2347.

Binshtok, A.M., Wang, H., Zimmermann, K., Amaya, F., Vardeh, D., Shi, L., Brenner, G.J., Ji, R.-R., Bean, B.P., Woolf, C.J.*, et al.* (2008). Nociceptors are interleukin-1beta sensors. J Neurosci *28*, 14062-14073.

Bissonnette Ey, B.A.D. (1997). Anti-inflammatory effect of b-agonists: Inhibition of TNF- release from human mast cells. J ALLERGY CLIN IMMUNOL.

Bonta, P.I., van Tiel, C.M., Vos, M., Pols, T.W.H., van Thienen, J.V., Ferreira, V., Arkenbout, E.K., Seppen, J., Spek, C.A., van der Poll, T.*, et al.* (2006). Nuclear receptors Nur77, Nurr1, and NOR-1 expressed in atherosclerotic lesion macrophages reduce lipid loading and inflammatory responses. Arterioscler Thromb Vasc Biol *26*, 2288-2294.

Borovikova, L.V., Ivanova, S., Zhang, M., Yang, H., Botchkina, G.I., Watkins, L.R., Wang, H., Abumrad, N., Eaton, J.W., and Tracey, K.J. (2000). Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. Nature *405*, 458-462.

Botos, I., Segal, D.M., and Davies, D.R. (2011). The structural biology of Toll-like receptors. Structure *19*, 447-459.

Brightbill, H.D., Plevy, S.E., Modlin, R.L., and Smale, S.T. (2000). A prominent role for Sp1 during lipopolysaccharide-mediated induction of the IL-10 promoter in macrophages. J Immunol *164*, 1940-1951.

Broadley, K.J. (2006). Beta-adrenoceptor responses of the airways: for better or worse? Eur J Pharmacol *533*, 15-27.

Brunskole Hummel, I., Reinartz, M.T., Kälble, S., Burhenne, H., Schwede, F., Buschauer, A., and Seifert, R. (2013). Dissociations in the effects of β2-adrenergic receptor agonists on cAMP formation and superoxide production in human neutrophils: support for the concept of functional selectivity. PLoS One *8*, e64556.

Bucsek, M.J., Qiao, G., MacDonald, C.R., Giridharan, T., Evans, L., Niedzwecki, B., Liu, H., Kokolus, K.M., Eng, J.W.L., Messmer, M.N.*, et al.* (2017). β-Adrenergic Signaling in Mice Housed at Standard Temperatures Suppresses an Effector Phenotype in CD8+ T Cells and Undermines Checkpoint Inhibitor Therapy. Cancer Res *77*, 5639-5651.

Cain, D.W., and Cidlowski, J.A. (2017). Immune regulation by glucocorticoids. Nat Rev Immunol *17*, 233-247.

Calvano, S.E., and Coyle, S.M. (2012). Experimental human endotoxemia: a model of the systemic inflammatory response syndrome? Surg Infect *13*, 293-299.

Cao, L., Hudson, C.A., and Lawrence, D.A. (2003a). Acute cold/restraint stress inhibits host resistance to Listeria monocytogenes via beta1-adrenergic receptors. Brain Behav Immun *17*, 121-133.

Cao, L., Hudson, C.A., and Lawrence, D.A. (2003b). Immune changes during acute cold/restraint stress-induced inhibition of host resistance to Listeria. Toxicol Sci *74*, 325-334.

Carballo, E., Lai, W.S., and Blackshear, P.J. (1998). Feedback inhibition of macrophage tumor necrosis factor-alpha production by tristetraprolin. Science *281*, 1001-1005.

Chao, L.C., Soto, E., Hong, C., Ito, A., Pei, L., Chawla, A., Conneely, O.M., Tangirala, R.K., Evans, R.M., and Tontonoz, P. (2013). Bone marrow NR4A expression is not a dominant factor in the development of atherosclerosis or macrophage polarization in mice. J Lipid Res *54*, 806-815.

Chassaing, B., Aitken, J.D., Malleshappa, M., and Vijay-Kumar, M. (2014). Dextran sulfate sodium (DSS)-induced colitis in mice. Curr Protoc Immunol *104*, Unit 15.25.

Chavan, S.S., Pavlov, V.A., and Tracey, K.J. (2017). Mechanisms and Therapeutic Relevance of Neuro-immune Communication. Immunity *46*, 927-942.

Chiu, I.M., Heesters, B.A., Ghasemlou, N., Von Hehn, C.A., Zhao, F., Tran, J., Wainger, B., Strominger, A., Muralidharan, S., Horswill, A.R.*, et al.* (2013). Bacteria activate sensory neurons that modulate pain and inflammation. Nature *501*, 52-57.

Chong, L.K., Cooper, E., Vardey, C.J., and others (1998). Salmeterol inhibition of mediator release from human lung mast cells by β-adrenoceptor-dependent and independent mechanisms. British journal of.

Church, M.K., and Hiroi, J. (1987). Inhibition of IgE-dependent histamine release from human dispersed lung mast cells by anti-allergic drugs and salbutamol. Br J Pharmacol *90*, 421-429.

Clausen, B.E., Burkhardt, C., Reith, W., Renkawitz, R., and Forster, I. (1999). Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res *8*, 265-277.

Cole, S.W., Korin, Y.D., Fahey, J.L., and Zack, J.A. (1998). Norepinephrine accelerates HIV replication via protein kinase A-dependent effects on cytokine production. J Immunol *161*, 610-616.

Collado-Hidalgo, A., Sung, C., and Cole, S. (2006). Adrenergic inhibition of innate anti-viral response: PKA blockade of Type I interferon gene transcription mediates catecholamine support for HIV-1 replication. Brain Behav Immun *20*, 552-563.

De Backer, D., Biston, P., Devriendt, J., Madl, C., Chochrad, D., Aldecoa, C., Brasseur, A., Defrance, P., Gottignies, P., Vincent, J.-L.*, et al.* (2010). Comparison of dopamine and norepinephrine in the treatment of shock. N Engl J Med *362*, 779-789.

de Souza, C.J., and Burkey, B.F. (2001). Beta 3-adrenoceptor agonists as anti-diabetic and anti-obesity drugs in humans. Curr Pharm Des *7*, 1433-1449.

de Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C.G., and de Vries, J.E. (1991). Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. J Exp Med *174*, 1209-1220.

Deng, J., Muthu, K., Gamelli, R., Shankar, R., and Jones, S.B. (2004). Adrenergic modulation of splenic macrophage cytokine release in polymicrobial sepsis. Am J Physiol Cell Physiol *287*, C730-736.

Ding, L., Linsley, P.S., Huang, L.Y., Germain, R.N., and Shevach, E.M. (1993). IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. J Immunol *151*, 1224-1234.

Dixon, R.A., Kobilka, B.K., Strader, D.J., Benovic, J.L., Dohlman, H.G., Frielle, T., Bolanowski, M.A., Bennett, C.D., Rands, E., Diehl, R.E.*, et al.* (1986). Cloning of the gene and cDNA for mammalian beta-adrenergic receptor and homology with rhodopsin. Nature *321*, 75-79.

Elander, L., Engstrom, L., Hallbeck, M., and Blomqvist, A. (2007). IL-1beta and LPS induce anorexia by distinct mechanisms differentially dependent on microsomal prostaglandin E synthase-1. Am J Physiol Regul Integr Comp Physiol *292*, R258-267.

Eming, S.A., Wynn, T.A., and Martin, P. (2017). Inflammation and metabolism in tissue repair and regeneration. Science *356*, 1026-1030.

Estrada, L.D., Ağaç, D., and Farrar, J.D. (2016). Sympathetic neural signaling via the β2-adrenergic receptor suppresses T-cell receptor-mediated human and mouse CD8(+) T-cell effector function. Eur J Immunol *46*, 1948-1958.

Evans, S.S., Repasky, E.A., and Fisher, D.T. (2015). Fever and the thermal regulation of immunity: the immune system feels the heat. Nat Rev Immunol *15*, 335-349.

Fan, H., Bitto, A., Zingarelli, B., Luttrell, L.M., Borg, K., Halushka, P.V., and Cook, J.A. (2010). Beta-arrestin 2 negatively regulates sepsis-induced inflammation. Immunology *130*, 344-351.

Feldman, M.E., and Shokat, K.M. (2010). New inhibitors of the PI3K-Akt-mTOR pathway: insights into mTOR signaling from a new generation of Tor Kinase Domain Inhibitors (TORKinibs). Curr Top Microbiol Immunol *347*, 241-262.

Felten, D.L., Felten, S.Y., Carlson, S.L., Olschowka, J.A., and Livnat, S. (1985). Noradrenergic and peptidergic innervation of lymphoid tissue. J Immunol *135*, 755s-765s.

Ferrari, D., McNamee, E.N., Idzko, M., Gambari, R., and Eltzschig, H.K. (2016). Purinergic Signaling During Immune Cell Trafficking. Trends Immunol *37*, 399-411.

Fink, M.P. (2014). Animal models of sepsis. Virulence *5*, 143-153.

Fiorentino, D.F., Zlotnik, A., Mosmann, T.R., Howard, M., and O'Garra, A. (1991). IL-10 inhibits cytokine production by activated macrophages. J Immunol *147*, 3815-3822.

Franke, A., Balschun, T., Karlsen, T.H., Sventoraityte, J., Nikolaus, S., Mayr, G., Domingues, F.S., Albrecht, M., Nothnagel, M., Ellinghaus, D*., et al.* (2008). Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. Nat Genet *40*, 1319-1323.

Fullerton, J.N., and Gilroy, D.W. (2016). Resolution of inflammation: a new therapeutic frontier. Nat Rev Drug Discov *15*, 551-567.

Gabanyi, I., Muller, P.A., Feighery, L., Oliveira, T.Y., Costa-Pinto, F.A., and Mucida, D. (2016). Neuro-immune Interactions Drive Tissue Programming in Intestinal Macrophages. Cell *164*, 378-391.

Gazzinelli, R.T., Wysocka, M., Hieny, S., Scharton-Kersten, T., Cheever, A., Kuhn, R., Muller, W., Trinchieri, G., and Sher, A. (1996). In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha. J Immunol *157*, 798-805.

Giebelen, I.A.J., Leendertse, M., Dessing, M.C., Meijers, J.C.M., Levi, M., Draing, C., von Aulock, S., and van der Poll, T. (2008). Endogenous beta-adrenergic receptors inhibit lipopolysaccharide-induced pulmonary cytokine release and coagulation. Am J Respir Cell Mol Biol *39*, 373-379.

Glass, C.K., and Natoli, G. (2016). Molecular control of activation and priming in macrophages. Nat Immunol *17*, 26-33.

Glass, C.K., and Saijo, K. (2010). Nuclear receptor transrepression pathways that regulate inflammation in macrophages and T cells. Nat Rev Immunol *10*, 365-376.

Gonzalez, P.V., Cragnolini, A.B., Schioth, H.B., and Scimonelli, T.N. (2006). Interleukin-1 beta-induced anorexia is reversed by ghrelin. Peptides *27*, 3220-3225.

Grace, P.M., Hutchinson, M.R., Maier, S.F., and Watkins, L.R. (2014). Pathological pain and the neuroimmune interface. Nat Rev Immunol *14*, 217-231.

Grailer, J.J., Haggadone, M.D., Sarma, J.V., Zetoune, F.S., and Ward, P.A. (2014). Induction of M2 regulatory macrophages through the β2-adrenergic receptor with protection during endotoxemia and acute lung injury. J Innate Immun *6*, 607-618.

Grebe, K.M., Hickman, H.D., Irvine, K.R., Takeda, K., Bennink, J.R., and Yewdell, J.W. Sympathetic nervous system control of anti-influenza CD8 T cell responses.

Grebe, K.M., Hickman, H.D., Irvine, K.R., Takeda, K., Bennink, J.R., and Yewdell, J.W. (2009). Sympathetic nervous system control of anti-influenza CD8+ T cell responses. Proc Natl Acad Sci U S A *106*, 5300-5305.

Greisman, S.E., DuBuy, J.B., and Woodward, C.L. (1979). Experimental gram-negative bacterial sepsis: prevention of mortality not preventable by antibiotics alone. Infect Immun *25*, 538-557.

Guereschi, M.G., Araujo, L.P., Maricato, J.T., Takenaka, M.C., Nascimento, V.M., Vivanco, B.C., Reis, V.O., Keller, A.C., Brum, P.C., and Basso, A.S. (2013). Beta2-adrenergic receptor signaling in CD4+ Foxp3+ regulatory T cells enhances their suppressive function in a PKA-dependent manner. Eur J Immunol *43*, 1001-1012.

Hadi, T., Douhard, R., Dias, A.M.M., Wendremaire, M., Pezze, M., Bardou, M., Sagot, P., Garrido, C., and Lirussi, F. (2017). Beta3 adrenergic receptor stimulation in human macrophages inhibits NADPHoxidase activity and induces catalase expression via PPARgamma activation. Biochim Biophys Acta *1864*, 1769-1784.

Hasko, G., Nemeth, Z., Szabo, C., Zsilla, G., Salzman, A.L., and Sylvester Vizi, E. (1998). Isoproterenol Inhibits IL-10, TNF-a, and Nitric Oxide Production in RAW 264.7 Macrophages. Brain Res Bull *45*, 183-187.

Heidt, T., Sager, H.B., Courties, G., Dutta, P., Iwamoto, Y., Zaltsman, A., von Zur Muhlen, C., Bode, C., Fricchione, G.L., Denninger, J.*, et al.* (2014). Chronic variable stress activates hematopoietic stem cells. Nat Med *20*, 754-758.

Hein, L., and Kobilka, B.K. (1995). Adrenergic receptor signal transduction and regulation. Neuropharmacology *34*, 357-366.

Hershko, D.D., Robb, B.W., Luo, G., and Hasselgren, P.O. (2002). Multiple transcription factors regulating the IL-6 gene are activated by cAMP in cultured Caco-2 cells. Am J Physiol Regul Integr Comp Physiol *283*, R1140-1148.

Hervé, J., Dubreil, L., Tardif, V., Terme, M., Pogu, S., Anegon, I., Rozec, B., Gauthier, C., Bach, J.-M., and Blancou, P. (2013). β2-Adrenoreceptor agonist inhibits antigen cross-presentation by dendritic cells. J Immunol *190*, 3163-3171.

Hilbert, T., Bongartz, J., Weisheit, C., Knüfermann, P., Baumgarten, G., Hoeft, A., and Poth, J.M. (2013). Beta2-adrenoceptor stimulation suppresses TLR9-dependent IFNA1 secretion in human peripheral blood mononuclear cells. PLoS One *8*, e65024.

Horie, K., Ohashi, M., Satoh, Y., and Sairenji, T. (2007). The role of p38 mitogen-activated protein kinase in regulating interleukin-10 gene expression in Burkitt's lymphoma cell lines. Microbiol Immunol *51*, 149-161.

Hou, J., Wang, L., Quan, R., Fu, Y., Zhang, H., and Feng, W.H. (2012). Induction of interleukin-10 is dependent on p38 mitogen-activated protein kinase pathway in macrophages infected with porcine reproductive and respiratory syndrome virus. Virol J *9*, 165.

Hu, Z., Chen, R., Cai, Z., Yu, L., Fei, Y., Weng, L., Wang, J., Ge, X., Zhu, T., Wang, J.*, et al.* (2012). Salmeterol attenuates the inflammatory response in asthma and decreases the pro-inflammatory cytokine secretion of dendritic cells. Cell Mol Immunol *9*, 267-275.

Huang, W., Xie, P., Xu, M., Li, P., and Zao, G. (2011). The influence of stress factors on the reactivation of latent herpes simplex virus type 1 in infected mice. Cell Biochem Biophys *61*, 115-122.

Hunter, C.A., Ellis-Neyes, L.A., Slifer, T., Kanaly, S., Grunig, G., Fort, M., Rennick, D., and Araujo, F.G. (1997). IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi. J Immunol *158*, 3311-3316.

Izeboud, C.A., Monshouwer, M., van Miert, A.S., and Witkamp, R.F. (1999). The beta-adrenoceptor agonist clenbuterol is a potent inhibitor of the LPS-induced production of TNF-alpha and IL-6 in vitro and in vivo. Inflamm Res *48*, 497-502.

Jenkins, F.J., and Baum, A. (1995). Stress and reactivation of latent herpes simplex virus: a fusion of behavioral medicine and molecular biology. Ann Behav Med *17*, 116-123.

Ji, R.-R., Chamessian, A., and Zhang, Y.-Q. (2016). Pain regulation by non-neuronal cells and inflammation. Science *354*, 572-577.

Jiang, W., Li, D., Han, R., Zhang, C., Jin, W.-N., Wood, K., Liu, Q., Shi, F.-D., and Hao, J. (2017). Acetylcholine-producing NK cells attenuate CNS inflammation via modulation of infiltrating monocytes/macrophages. Proc Natl Acad Sci U S A *114*, E6202-E6211.

Kalinichenko, V.V., Mokyr, M.B., Graf, L.H., Cohen, R.L., and Chambers, D.A. (1999). Norepinephrine-mediated inhibition of antitumor cytotoxic T lymphocyte generation involves a β-adrenergic receptor mechanism and decreased TNF-α gene expression. The Journal of Immunology *163*, 2492-2499.

Kasprowicz, D.J., Kohm, A.P., Berton, M.T., Chruscinski, A.J., Sharpe, A., and Sanders, V.M. (2000). Stimulation of the B cell receptor, CD86 (B7-2), and the beta 2-adrenergic receptor intrinsically modulates the level of IgG1 and IgE produced per B cell. J Immunol *165*, 680-690.

Katayama, Y., Battista, M., Kao, W.-M., Hidalgo, A., Peired, A.J., Thomas, S.A., and Frenette, P.S. (2006). Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell *124*, 407-421.

Kawai, T., and Akira, S. (2011). Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. Immunity *34*, 637-650.

Kawasaki, T., and Kawai, T. (2014). Toll-like receptor signaling pathways. Front Immunol *5*, 461.

Kendall, M.D., and al-Shawaf, A.A. (1991). Innervation of the rat thymus gland. Brain Behav Immun *5*, 9-28.

Keränen, T., Hömmö, T., Hämäläinen, M., Moilanen, E., and Korhonen, R. (2016). Anti-Inflammatory Effects of β2-Receptor Agonists Salbutamol and Terbutaline Are Mediated by MKP-1. PLoS One *11*, e0148144.

Kim, B.-J., and Jones, H.P. (2010). Epinephrine-primed murine bone marrow-derived dendritic cells facilitate production of IL-17A and IL-4 but not IFN-γ by CD4+ T cells. Brain Behav Immun *24*, 1126-1136.

Kim, J.J., Shajib, M.S., Manocha, M.M., and Khan, W.I. (2012). Investigating intestinal inflammation in DSS-induced model of IBD. J Vis Exp.

Kishore, R., Tebo, J.M., Kolosov, M., and Hamilton, T.A. (1999). Cutting edge: clustered AU-rich elements are the target of IL-10-mediated mRNA destabilization in mouse macrophages. J Immunol *162*, 2457-2461.

Kizaki, T., Izawa, T., Sakurai, T., Haga, S., Taniguchi, N., Tajiri, H., Watanabe, K., Day, N.K., Toba, K., and Ohno, H. (2008). Beta2-adrenergic receptor regulates Toll-like receptor-4-induced nuclear factor-kappaB activation through beta-arrestin 2. Immunology *124*, 348-356.

Kogut, M.H., Genovese, K.J., and He, H. (2007). Flagellin and lipopolysaccharide stimulate the MEK-ERK signaling pathway in chicken heterophils through differential activation of the small GTPases, Ras and Rap1. Mol Immunol *44*, 1729-1736.

Koopman, F.A., Chavan, S.S., Miljko, S., Grazio, S., Sokolovic, S., Schuurman, P.R., Mehta, A.D., Levine, Y.A., Faltys, M., Zitnik, R.*, et al.* (2016). Vagus nerve stimulation inhibits cytokine production and attenuates disease severity in rheumatoid arthritis. Proc Natl Acad Sci U S A *113*, 8284-8289.

Kopp, E., and Medzhitov, R. (2003). Recognition of microbial infection by Toll-like receptors. Curr Opin Immunol *15*, 396-401.

Kopydlowski, K.M., Salkowski, C.A., Cody, M.J., van Rooijen, N., Major, J., Hamilton, T.A., and Vogel, S.N. (1999). Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. J Immunol *163*, 1537-1544.

Kotas, M.E., and Medzhitov, R. (2015). Homeostasis, inflammation, and disease susceptibility. Cell *160*, 816-827.

Kuhn, R., Lohler, J., Rennick, D., Rajewsky, K., and Muller, W. (1993). Interleukin-10-deficient mice develop chronic enterocolitis. Cell *75*, 263-274.

Lamkin, D.M., Ho, H.-Y., Ong, T.H., Kawanishi, C.K., Stoffers, V.L., Ahlawat, N., Ma, J.C.Y., Arevalo, J.M.G., Cole, S.W., and Sloan, E.K. (2016). β-Adrenergic-stimulated

macrophages: Comprehensive localization in the M1-M2 spectrum. Brain Behav Immun *57*, 338-346.

Latifi, S.Q., O'Riordan, M.A., and Levine, A.D. (2002). Interleukin-10 controls the onset of irreversible septic shock. Infect Immun *70*, 4441-4446.

Lavin, Y., Mortha, A., Rahman, A., and Merad, M. (2015). Regulation of macrophage development and function in peripheral tissues. Nat Rev Immunol *15*, 731-744.

Leigh, N.D., Kokolus, K.M., O'Neill, R.E., Du, W., Eng, J.W.L., Qiu, J., Chen, G.L., McCarthy, P.L., Farrar, J.D., Cao, X.*, et al.* (2015). Housing Temperature–Induced Stress Is Suppressing Murine Graft-versus-Host Disease through β2-Adrenergic Receptor Signaling. The Journal of Immunology *195*, 5045-5054.

Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.-M., and Hoffmann, J.A. (1996). The Dorsoventral Regulatory Gene Cassette spatzle/Toll/cactus Controls the Potent Antifungal Response in Drosophila Adults. Cell *86*, 973-983.

Leow-Dyke, S., Allen, C., Denes, A., Nilsson, O., Maysami, S., Bowie, A.G., Rothwell, N.J., and Pinteaux, E. (2012). Neuronal Toll-like receptor 4 signaling induces brain endothelial activation and neutrophil transmigration in vitro. J Neuroinflammation *9*, 230.

Lesnikov, V.A., Efremov, O.M., Korneva, E.A., Van Damme, J., and Billiau, A. (1991). Fever produced by intrahypothalamic injection of interleukin-1 and interleukin-6. Cytokine *3*, 195-198.

Lewis, R.J., Chachi, L., Newby, C., Amrani, Y., and Bradding, P. (2016). Bidirectional Counterregulation of Human Lung Mast Cell and Airway Smooth Muscle β2 Adrenoceptors. J Immunol *196*, 55-63.

Li, B., Alli, R., Vogel, P., and Geiger, T.L. (2014). IL-10 modulates DSS-induced colitis through a macrophage-ROS-NO axis. Mucosal Immunol *7*, 869-878.

Li, L., Liu, Y., Chen, H.-Z., Li, F.-W., Wu, J.-F., Zhang, H.-K., He, J.-P., Xing, Y.-Z., Chen, Y., Wang, W.-J.*, et al.* (2015). Impeding the interaction between Nur77 and p38 reduces LPS-induced inflammation. Nat Chem Biol *11*, 339-346.

Li, X.-M., Zhang, S., He, X.-S., Guo, P.-D., Lu, X.-X., Wang, J.-R., Li, J.-M., and Wu, H. (2016). Nur77-mediated TRAF6 signalling protects against LPS-induced sepsis in mice. J Inflamm *13*, 4.

Liggett, S.B. (1989). Identification and characterization of a homogeneous population of beta 2-adrenergic receptors on human alveolar macrophages. Am Rev Respir Dis *139*, 552-555.

Lin, J.-J., Du, Y., Cai, W.-K., Kuang, R., Chang, T., Zhang, Z., Yang, Y.-X., Sun, C., Li, Z.-Y., and Kuang, F. (2015). Toll-like receptor 4 signaling in neurons of trigeminal ganglion contributes to nociception induced by acute pulpitis in rats. Sci Rep *5*, 12549.

Lukewich, M.K., and Lomax, A.E. (2014). Endotoxemia enhances catecholamine secretion from male mouse adrenal chromaffin cells through an increase in Ca(2+) release from the endoplasmic reticulum. Endocrinology *155*, 180-192.

Magalhaes, A.C., Dunn, H., and Ferguson, S.S. (2012). Regulation of GPCR activity, trafficking and localization by GPCR-interacting proteins. Br J Pharmacol *165*, 1717-1736.

Mahajan, S., Saini, A., Chandra, V., Nanduri, R., Kalra, R., Bhagyaraj, E., Khatri, N., and Gupta, P. (2015). Nuclear Receptor Nr4a2 Promotes Alternative Polarization of Macrophages and Confers Protection in Sepsis. J Biol Chem *290*, 18304-18314.

Maisel, A.S., Fowler, P., Rearden, A., Motulsky, H.J., and Michel, M.C. (1989). A new method for isolation of human lymphocyte subsets reveals differential regulation of beta-adrenergic receptors by terbutaline treatment. Clin Pharmacol Ther *46*, 429-439.

Maisel, A.S., Knowlton, K.U., Fowler, P., Rearden, A., Ziegler, M.G., Motulsky, H.J., Insel, P.A., and Michel, M.C. (1990). Adrenergic control of circulating lymphocyte subpopulations. Effects of congestive heart failure, dynamic exercise, and terbutaline treatment. J Clin Invest *85*, 462-467.

Malinowsky, D., Chai, Z., Bristulf, J., Simoncsits, A., and Bartfai, T. (1995). The type I interleukin-1 receptor mediates fever in the rat as shown by interleukin-1 receptor subtype selective ligands. Neurosci Lett *201*, 33-36.

Manni, M., Granstein, R.D., and Maestroni, G. (2011). β2-Adrenergic agonists bias TLR-2 and NOD2 activated dendritic cells towards inducing an IL-17 immune response. Cytokine *55*, 380-386.

Marfaing-Koka, A., Maravic, M., Humbert, M., Galanaud, P., and Emilie, D. (1996). Contrasting effects of IL-4, IL-10 and corticosteroids on RANTES production by human monocytes. Int Immunol *8*, 1587-1594.

Marlow, H.F. (1989). Xamoterol, a beta 1-adrenoceptor partial agonist: review of the clinical efficacy in heart failure. Br J Clin Pharmacol *28 Suppl 1*, 23S-30S.

Martínez-González, J., and Badimon, L. (2005). The NR4A subfamily of nuclear receptors: new early genes regulated by growth factors in vascular cells. Cardiovasc Res *65*, 609-618.

Maxwell, M.A., Cleasby, M.E., Harding, A., Stark, A., Cooney, G.J., and Muscat, G.E.O. (2005). Nur77 regulates lipolysis in skeletal muscle cells. Evidence for cross-talk between the beta-adrenergic and an orphan nuclear hormone receptor pathway. J Biol Chem *280*, 12573-12584.

Mayr, F.B., Yende, S., and Angus, D.C. (2014). Epidemiology of severe sepsis. Virulence *5*, 4-11.

McAlees, J.W., Smith, L.T., Erbe, R.S., Jarjoura, D., Ponzio, N.M., and Sanders, V.M. (2011). Epigenetic regulation of beta2-adrenergic receptor expression in T(H)1 and T(H)2 cells. Brain Behav Immun *25*, 408-415.

Medzhitov, R., Preston-Hurlburt, P., and Janeway, C.A., Jr. (1997). A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. Nature *388*, 394-397.

Miller, A.H., and Raison, C.L. (2016). The role of inflammation in depression: from evolutionary imperative to modern treatment target. Nat Rev Immunol *16*, 22-34.

Mizuno, K., Takahashi, H.K., Iwagaki, H., Katsuno, G., Kamurul, H.A., Ohtani, S., Mori, S., Yoshino, T., Nishibori, M., and Tanaka, N. (2005). Beta2-adrenergic receptor stimulation inhibits LPS-induced IL-18 and IL-12 production in monocytes. Immunol Lett *101*, 168-172.

Monastra, G., and Secchi, E.F. (1993). Beta-adrenergic receptors mediate in vivo the adrenaline inhibition of lipopolysaccharide-induced tumor necrosis factor release. Immunol Lett *38*, 127-130.

Moran, C.J., Walters, T.D., Guo, C.H., Kugathasan, S., Klein, C., Turner, D., Wolters, V.M., Bandsma, R.H., Mouzaki, M., Zachos, M.*, et al.* (2013). IL-10R polymorphisms are associated with very-early-onset ulcerative colitis. Inflamm Bowel Dis *19*, 115-123.

Moriyama, S., Brestoff, J.R., Flamar, A.L., Moeller, J.B., Klose, C.S.N., Rankin, L.C., Yudanin, N.A., Monticelli, L.A., Putzel, G.G., Rodewald, H.R.*, et al.* (2018). beta2-adrenergic receptor-mediated negative regulation of group 2 innate lymphoid cell responses. Science *359*, 1056-1061.

Mosser, D.M., and Edwards, J.P. (2008). Exploring the full spectrum of macrophage activation. Nat Rev Immunol *8*, 958-969.

Munoz, N.M., Vita, A.J., Neeley, S.P., McAllister, K., Spaethe, S.M., White, S.R., and Leff, A.R. (1994). Beta adrenergic modulation of formyl-methionine-leucine-phenylalanine-stimulated secretion of eosinophil peroxidase and leukotriene C4. J Pharmacol Exp Ther *268*, 139-143.

Muthu, K., Iyer, S., He, L.K., Szilagyi, A., Gamelli, R.L., Shankar, R., and Jones, S.B. (2007). Murine hematopoietic stem cells and progenitors express adrenergic receptors. J Neuroimmunol *186*, 27-36.

Myers, S.A., Eriksson, N., Burow, R., Wang, S.-C.M., and Muscat, G.E.O. (2009). Beta-adrenergic signaling regulates NR4A nuclear receptor and metabolic gene expression in multiple tissues. Mol Cell Endocrinol *309*, 101-108.

Nakada, T.-A., Russell, J.A., Boyd, J.H., Aguirre-Hernandez, R., Thain, K.R., Thair, S.A., Nakada, E., McConechy, M., and Walley, K.R. (2010). beta2-Adrenergic receptor gene polymorphism is associated with mortality in septic shock. Am J Respir Crit Care Med *181*, 143-149.

Nials, A.T., Ball, D.I., Butchers, P.R., Coleman, R.A., Humbles, A.A., Johnson, M., and Vardey, C.J. (1994). Formoterol on airway smooth muscle and human lung mast cells: a comparison with salbutamol and salmeterol. Eur J Pharmacol *251*, 127-135.

Nicod, L.P., el Habre, F., Dayer, J.M., and Boehringer, N. (1995). Interleukin-10 decreases tumor necrosis factor alpha and beta in alloreactions induced by human lung dendritic cells and macrophages. Am J Respir Cell Mol Biol *13*, 83-90.

Nielson, C.P. (1987). Beta-adrenergic modulation of the polymorphonuclear leukocyte respiratory burst is dependent upon the mechanism of cell activation. J Immunol *139*, 2392-2397.

Nijhuis, L.E., Olivier, B.J., Dhawan, S., Hilbers, F.W., Boon, L., Wolkers, M.C., Samsom, J.N., and de Jonge, W.J. (2014). Adrenergic β2 receptor activation stimulates anti-inflammatory properties of dendritic cells in vitro. PLoS One *9*, e85086.

Norris, S.L., Yen, P.Y., Dana, T.L., Care, B.R., and Burda, B.U. (2006). In Drug Class Review: Beta2-Agonists: Final Report (Portland (OR)).

Okabe, Y., and Medzhitov, R. (2016). Tissue biology perspective on macrophages. Nat Immunol *17*, 9-17.

Padgett, D.A., Sheridan, J.F., Dorne, J., Berntson, G.G., Candelora, J., and Glaser, R. (1998). Social stress and the reactivation of latent herpes simplex virus type 1. Proc Natl Acad Sci U S A *95*, 7231-7235.

Panina-Bordignon, P., Mazzeo, D., Lucia, P.D., D'Ambrosio, D., Lang, R., Fabbri, L., Self, C., and Sinigaglia, F. (1997). Beta2-agonists prevent Th1 development by selective inhibition of interleukin 12. J Clin Invest *100*, 1513-1519.

Panuncio, A.L., De La Peña, S., Gualco, G., and Reissenweber, N. (1999). Adrenergic innervation in reactive human lymph nodes. J Anat *194 (Pt 1)*, 143-146.

Papa, I., Saliba, D., Ponzoni, M., Bustamante, S., Canete, P.F., Gonzalez-Figueroa, P., McNamara, H.A., Valvo, S., Grimbaldeston, M., Sweet, R.A.*, et al.* (2017). TFH-derived dopamine accelerates productive synapses in germinal centres. Nature.

Pasare, C., and Medzhitov, R. (2004). Toll-like receptors: linking innate and adaptive immunity. Microbes Infect *6*, 1382-1387.

Pattison, M.J., Mitchell, O., Flynn, H.R., Chen, C.S., Yang, H.T., Ben-Addi, H., Boeing, S., Snijders, A.P., and Ley, S.C. (2016). TLR and TNF-R1 activation of the MKK3/MKK6-p38alpha axis in macrophages is mediated by TPL-2 kinase. Biochem J *473*, 2845-2861.

Pavlov, V.A., and Tracey, K.J. (2017). Neural regulation of immunity: molecular mechanisms and clinical translation. Nat Neurosci *20*, 156-166.

Pearen, M.A., Myers, S.A., Raichur, S., Ryall, J.G., Lynch, G.S., and Muscat, G.E.O. (2008). The orphan nuclear receptor, NOR-1, a target of beta-adrenergic signaling, regulates gene expression that controls oxidative metabolism in skeletal muscle. Endocrinology *149*, 2853-2865.

Pei, L., Castrillo, A., Chen, M., Hoffmann, A., and Tontonoz, P. (2005). Induction of NR4A orphan nuclear receptor expression in macrophages in response to inflammatory stimuli. J Biol Chem *280*, 29256-29262.

Philipp, M., and Hein, L. (2004). Adrenergic receptor knockout mice: distinct functions of 9 receptor subtypes. Pharmacol Ther *101*, 65-74.

Podojil, J.R., and Sanders, V.M. (2003). Selective regulation of mature IgG1 transcription by CD86 and beta 2-adrenergic receptor stimulation. J Immunol *170*, 5143-5151.

Pope, C., Kim, S.K., Marzo, A., Masopust, D., Williams, K., Jiang, J., Shen, H., and Lefrancois, L. (2001). Organ-specific regulation of the CD8 T cell response to Listeria monocytogenes infection. J Immunol *166*, 3402-3409.

Pourrajab, F., Yazdi, M.B., Zarch, M.B., Zarch, M.B., and Hekmatimoghaddam, S. (2015). Cross talk of the first-line defense TLRs with PI3K/Akt pathway, in preconditioning therapeutic approach. Mol Cell Ther *3*, 4.

Ram, C.V. (2010). Beta-blockers in hypertension. Am J Cardiol *106*, 1819-1825.

Rasmussen, S.G., DeVree, B.T., Zou, Y., Kruse, A.C., Chung, K.Y., Kobilka, T.S., Thian, F.S., Chae, P.S., Pardon, E., Calinski, D.*, et al.* (2011). Crystal structure of the beta2 adrenergic receptor-Gs protein complex. Nature *477*, 549-555.

Reilly, F.D., McCuskey, P.A., Miller, M.L., McCuskey, R.S., and Meineke, H.A. (1979). Innervation of the periarteriolar lymphatic sheath of the spleen. Tissue Cell *11*, 121-126.

Roewe, J., Higer, M., Riehl, D.R., Gericke, A., Radsak, M.P., and Bosmann, M. (2017). Neuroendocrine Modulation of IL-27 in Macrophages. J Immunol *199*, 2503-2514.

Rosas-Ballina, M., Olofsson, P.S., Ochani, M., Valdés-Ferrer, S.I., Levine, Y.A., Reardon, C., Tusche, M.W., Pavlov, V.A., Andersson, U., Chavan, S.*, et al.* (2011). Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. Science *334*, 98-101.

Rosenberg, J.C., Lillehei, R.C., Longerbeam, J., and Zimmermann, B. (1961). Studies on hemorrhagic and endotoxin shock in relation to vasomotor changes and endogenous circulating epinephrine, norepinephrine and serotonin. Ann Surg *154*, 611-628.

Sanders, V.M., Baker, R.A., Ramer-Quinn, D.S., Kasprowicz, D.J., Fuchs, B.A., and Street, N.E. (1997). Differential expression of the beta2-adrenergic receptor by Th1 and Th2 clones: implications for cytokine production and B cell help. J Immunol *158*, 4200-4210.

Saraiva, M., and O'Garra, A. (2010). The regulation of IL-10 production by immune cells. Nat Rev Immunol *10*, 170-181.

Sawada, M., Itoh, Y., Suzumura, A., and Marunouchi, T. (1993). Expression of cytokine receptors in cultured neuronal and glial cells. Neurosci Lett *160*, 131-134.

Schaljo, B., Kratochvill, F., Gratz, N., Sadzak, I., Sauer, I., Hammer, M., Vogl, C., Strobl, B., Müller, M., Blackshear, P.J.*, et al.* (2009). Tristetraprolin is required for full anti-inflammatory response of murine macrophages to IL-10. J Immunol *183*, 1197-1206.

Schroeppel, T.J., Fischer, P.E., Zarzaur, B.L., Magnotti, L.J., Clement, L.P., Fabian, T.C., and Croce, M.A. (2010). Beta-adrenergic blockade and traumatic brain injury: protective? J Trauma *69*, 776-782.

Seeley, E.J., Barry, S.S., Narala, S., Matthay, M.A., and Wolters, P.J. (2013). Noradrenergic neurons regulate monocyte trafficking and mortality during gram-negative peritonitis in mice. J Immunol *190*, 4717-4724.

Sekiya, T., Kashiwagi, I., Inoue, N., Morita, R., Hori, S., Waldmann, H., Rudensky, A.Y., Ichinose, H., Metzger, D., Chambon, P.*, et al.* (2011). The nuclear orphan receptor Nr4a2 induces Foxp3 and regulates differentiation of CD4+ T cells. Nat Commun *2*, 269.

Sekiya, T., Kashiwagi, I., Yoshida, R., Fukaya, T., Morita, R., Kimura, A., Ichinose, H., Metzger, D., Chambon, P., and Yoshimura, A. (2013). Nr4a receptors are essential for thymic regulatory T cell development and immune homeostasis. Nat Immunol *14*, 230-237.

Sekiya, T., Kondo, T., Shichita, T., Morita, R., Ichinose, H., and Yoshimura, A. (2015). Suppression of Th2 and Tfh immune reactions by Nr4a receptors in mature T reg cells. J Exp Med *212*, 1623-1640.

Sigola, L.B., and Zinyama, R.B. (2000). Adrenaline inhibits macrophage nitric oxide production through beta1 and beta2 adrenergic receptors. Immunology *100*, 359-363.

Snow, H.M. (1989). The pharmacology of xamoterol: a basis for modulation of the autonomic control of the heart. Br J Clin Pharmacol *28 Suppl 1*, 3S-13S.

Spencer, S.D., Di Marco, F., Hooley, J., Pitts-Meek, S., Bauer, M., Ryan, A.M., Sordat, B., Gibbs, V.C., and Aguet, M. (1998). The orphan receptor CRF2-4 is an essential subunit of the interleukin 10 receptor. J Exp Med *187*, 571-578.

Spengler, R.N., Chensue, S.W., Giacherio, D.A., Blenk, N., and Kunkel, S.L. (1994). Endogenous norepinephrine regulates tumor necrosis factor-alpha production from macrophages in vitro. J Immunol *152*, 3024-3031.

Spink, W.W., Reddin, J., Zak, S.J., Peterson, M., Starzecki, B., and Seljeskog, E. (1966). Correlation of plasma catecholamine levels with hemodynamic changes in canine endotoxin shock. J Clin Invest *45*, 78-85.

Steinman, L. (2004). Elaborate interactions between the immune and nervous systems. Nat Immunol *5*, 575-581.

Suzuki, K., Hayano, Y., Nakai, A., Furuta, F., and Noda, M. (2016). Adrenergic control of the adaptive immune response by diurnal lymphocyte recirculation through lymph nodes. J Exp Med *213*, 2567-2574.

Swanson, M.A., Lee, W.T., and Sanders, V.M. (2001). IFN-gamma production by Th1 cells generated from naive CD4+ T cells exposed to norepinephrine. J Immunol *166*, 232-240.

Takeda, K., and Akira, S. (2004). TLR signaling pathways. Semin Immunol *16*, 3-9.

Tan, K.S., Nackley, A.G., Satterfield, K., Maixner, W., Diatchenko, L., and Flood, P.M. (2007). Beta2 adrenergic receptor activation stimulates pro-inflammatory cytokine production in macrophages via PKA- and NF-kappaB-independent mechanisms. Cell Signal *19*, 251-260.

Thomas, B.J., and Smale, S.T. (2016). The Ly6C(lo)-Down: Targeting Developmental Enhancers for Cell Subset Depletion. Immunity *45*, 949-951.

Uematsu, S., and Akira, S. (2008). Toll-Like receptors (TLRs) and their ligands. Handb Exp Pharmacol, 1-20.

van der Poll, T., Barber, A.E., Coyle, S.M., and Lowry, S.F. (1996a). Hypercortisolemia increases plasma interleukin-10 concentrations during human endotoxemia--a clinical research center study. J Clin Endocrinol Metab *81*, 3604-3606.

van der Poll, T., Coyle, S.M., Barbosa, K., Braxton, C.C., and Lowry, S.F. (1996b). Epinephrine inhibits tumor necrosis factor-alpha and potentiates interleukin 10 production during human endotoxemia. J Clin Invest *97*, 713-719.

Veiga-Fernandes, H., and Mucida, D. (2016). Neuro-Immune Interactions at Barrier Surfaces. Cell *165*, 801-811.

Verhoeckx, K.C.M., Doornbos, R.P., van der Greef, J., Witkamp, R.F., and Rodenburg, R.J.T. (2005). Inhibitory effects of the beta-adrenergic receptor agonist zilpaterol on the LPS-induced production of TNF-alpha in vitro and in vivo. J Vet Pharmacol Ther *28*, 531-537.

Vida, G., Peña, G., Kanashiro, A., Thompson-Bonilla, M.d.R., Palange, D., Deitch, E.A., and Ulloa, L. (2011). β2-Adrenoreceptors of regulatory lymphocytes are essential for vagal neuromodulation of the innate immune system. FASEB J *25*, 4476-4485.

Wang, W., Xu, M., Zhang, Y.-Y., and He, B. (2009). Fenoterol, a beta(2)-adrenoceptor agonist, inhibits LPS-induced membrane-bound CD14, TLR4/CD14 complex, and

inflammatory cytokines production through beta-arrestin-2 in THP-1 cell line. Acta Pharmacol Sin *30*, 1522-1528.

Wang, Z., Benoit, G., Liu, J., Prasad, S., Aarnisalo, P., Liu, X., Xu, H., Walker, N.P., and Perlmann, T. (2003). Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. Nature *423*, 555-560.

Webster, J.I., Tonelli, L., and Sternberg, E.M. (2002). Neuroendocrine regulation of immunity. Annu Rev Immunol *20*, 125-163.

Weyer, C., Gautier, J.F., and Danforth, E., Jr. (1999). Development of beta 3-adrenoceptor agonists for the treatment of obesity and diabetes--an update. Diabetes Metab *25*, 11-21.

Willems, F., Marchant, A., Delville, J.P., Gerard, C., Delvaux, A., Velu, T., de Boer, M., and Goldman, M. (1994). Interleukin-10 inhibits B7 and intercellular adhesion molecule-1 expression on human monocytes. Eur J Immunol *24*, 1007-1009.

Wirtz, S., Popp, V., Kindermann, M., Gerlach, K., Weigmann, B., Fichtner-Feigl, S., and Neurath, M.F. (2017). Chemically induced mouse models of acute and chronic intestinal inflammation. Nat Protoc *12*, 1295-1309.

Wiysonge, C.S., Bradley, H.A., Volmink, J., Mayosi, B.M., and Opie, L.H. (2017). Beta-blockers for hypertension. Cochrane Database Syst Rev *1*, CD002003.

Wohleb, E.S., Franklin, T., Iwata, M., and Duman, R.S. (2016). Integrating neuroimmune systems in the neurobiology of depression. Nat Rev Neurosci *17*, 497-511.

Wynn, T.A., and Vannella, K.M. (2016). Macrophages in Tissue Repair, Regeneration, and Fibrosis. Immunity *44*, 450-462.

Xu, Z.-Z., Kim, Y.H., Bang, S., Zhang, Y., Berta, T., Wang, F., Oh, S.B., and Ji, R.-R. (2015). Inhibition of mechanical allodynia in neuropathic pain by TLR5-mediated A-fiber blockade. Nat Med *21*, 1326-1331.

Zigmond, E., Bernshtein, B., Friedlander, G., Walker, C.R., Yona, S., Kim, K.-W., Brenner, O., Krauthgamer, R., Varol, C., Müller, W.*, et al.* (2014). Macrophage-restricted interleukin-10 receptor deficiency, but not IL-10 deficiency, causes severe spontaneous colitis. Immunity *40*, 720-733.