The Pennsylvania State University
The Graduate School
The Huck Institutes of The Life Sciences

ALL-TRANS RETINOIC ACID COMBINED WITH
POLYRIBOINOSINIC:POLYRIBOCYTIDYLIC ACID PROMOTES
TETANUS TOXOID-INDUCED VACCINE RESPONSES

IN ADULT AND NEONATAL MICE

A Thesis in
Integrative Biosciences

by
Yifan Ma

© 2006 Yifan Ma

Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Doctor of Philosophy

May 2006
The thesis of Yifan Ma has been reviewed and approved* by the following:

A. Catharine Ross
Professor of Nutrition
Thesis Advisor
Chair of Committee

Margherita Cantorna
Associate Professor of Immunology and Nutrition

Andrea M. Mastro
Professor of Microbiology and Cell Biology

John E. Smith
Associate Professor of Nutrition

Richard J. Frisque
Professor of Molecular Virology
Co-director of Integrative Biosciences Graduate Program

* Signatures are on file in the graduate school
ABSTRACT

Vitamin A (VA) is characterized as an anti-infective vitamin that plays an essential role in both innate and adaptive immunity. Polyriboinosinic: polyriboctydilic acid (PIC), a synthetic double-stranded RNA (dsRNA), is well known as an inducer of IFNs and an adjuvant, which can induce anti-viral and anti-tumor reactions as well as a variety of immune responses. Previous studies have shown that retinoic acid (RA), a bioactive metabolite of VA, and PIC synergistically increased primary and secondary anti-tetanus toxoid (TT) antibody responses in VA-deficient animals. However, their immunoregulatory effects in VA-sufficient animals, particularly the regulation of IgG isotypes and memory responses, are not well elucidated.

Early-life vaccination is an important strategy to protect neonates and infants from infectious diseases. Unfortunately, the immaturity of immune system hampers the generation of protective vaccine response. Hence, new strategies are sought to improve vaccine efficiency in neonates and infants. Because RA and/or PIC successfully promoted TT-induced vaccine response in VA-deficient adult animals, it was interesting to determine if RA and PIC could benefit neonatal vaccine response. Hence, the overall hypothesis in the present study was that RA and PIC cooperatively enhance anti-TT IgG responses but differentially modulate IgG isotypes in both VA-sufficient adult and neonatal mice. The immunoregulatory function of RA and PIC may be attributed to their distinct effects on type 1/type 2 cytokine response, immune-cell populations and differentiation, as well as the GC response.
In the first part of study, the effects of RA and/or PIC on anti-TT vaccine response were evaluated in VA-sufficient adult mice. The results showed that co-administration of RA and/or PIC with TT priming cooperatively enhanced both primary and secondary anti-TT IgG responses, but differentially regulated IgG isotypes. RA alone selectively increased anti-TT IgG1, while PIC alone increased all IgG isotypes (IgG1, IgG2a, and IgG2b). Whereas RA+PIC synergistically enhanced IgG and IgG1, this combination attenuated IgG2a production as compared to PIC alone, thereby keeping the ratio of IgG1:IgG2a close to the control level. Multiple mechanisms might be involved in the regulation of anti-TT antibody response by RA and PIC treatments. First of all, RA and PIC differentially regulated type 1/type 2 cytokine mRNA levels. Whereas RA reduced type 1 cytokines (IFN-γ and IL-12), PIC enhanced both type 1 and type 2 cytokines (IL-4 and IL-10) and Th1-related transcription factors. The mRNA levels of type 1 and type 2 cytokines are strongly correlated with the titers of IgG2a and IgG1, respectively, suggesting that RA and PIC might regulate anti-TT antibody response by shaping type 1/type 2 cytokine responses. Secondly, RA and/or PIC significantly regulated lymphocyte populations and the expression of costimulatory molecules. RA and PIC differentially modulated NK/NKT-cell populations and the expression of CD80/CD86 costimulatory molecules within 3 days of primary immunization. Also, the NKT: NK and CD80: CD86 ratios were positively correlated with the IL-4: IFN-γ ratio, implying that RA and PIC treatments might regulate type 1/type 2 cytokines and anti-TT antibody response by modulating NK/NKT cell populations as well as the expression of CD80/CD86. Thirdly, RA and PIC increased the generation of TT-induced germinal center (GC) B cells and the expression of GC-related markers,
suggesting that these agents could promote the GC reaction, thereby enhancing anti-TT antibody response.

In the second part of study, the adjuvant effects of RA and/or PIC on TT-induced vaccine response were evaluated in neonatal mice. Early-life treatments with RA and/or PIC were well tolerated, and stimulated both primary anti-TT IgG production in infancy and the memory response in adulthood. TT-specific lymphocyte proliferation and type 1/ type 2 cytokine production were also significantly augmented. In addition, RA and PIC modulated the maturation and/or differentiation of neonatal B cells, natural killer (NK)/NKT cells, and antigen-presenting cells (APCs). Whereas RA alone increased neonatal anti-TT antibody response, it selectively increased anti-TT IgG1 and IL-5, resulting in a skewed type 2 response. PIC, a potent adjuvant in adult mice, elevated neonatal anti-TT IgG as well as all IgG isotypes (IgG1, IgG2a, IgG2b), and induced TT-specific IFN-γ, an important type 1 cytokine; however, PIC alone failed to benefit the memory response. The combination of RA+PIC was more potent than either agent alone in elevating primary and secondary anti-TT IgG responses as well as IgG isotypes. Moreover, RA+PIC increased TT-specific IFN-γ and IL-5, suggesting the combination effectively promoted both type 1 and type 2 responses in neonatal mice.

Overall, RA and/or PIC treatments significantly enhanced TT-induced vaccine response in both adult and neonatal mice. The combination of RA+PIC stimulated a robust, durable and balanced increase in all of the anti-TT IgG isotypes (IgG1, IgG2a, IgG2b), suggesting that this combination may be a promising adjuvant for both adult and neonatal vaccination.
# TABLE OF CONTENTS

List of Figures ........................................................................................................... x

List of tables ............................................................................................................. xiii

List of Abbreviation .................................................................................................. xiv

Acknowledgment ..................................................................................................... xvii

Chapter 1. Literature review ..................................................................................... 1

I. Introduction .......................................................................................................... 2

II. Overview of T helper (Th)-cell activation .......................................................... 4

   Th-cell activation ................................................................................................. 4

   Th1/Th2 response ............................................................................................... 5

Molecular regulation of Th1 response ..................................................................... 7

Molecular regulation of Th2 response ..................................................................... 11

Th1/Th2 cytokines and type 1/type 2 responses ................................................. 14

The role of antigen-presenting cells (APCs) in Th-cell response .................. 16

The role of NK cells and NKT cells in Th-cell activation ............................... 17

III. Overview of B-cell activation and antibody response .................................. 21

   TD antigen-induced B-cell activation ............................................................. 21

   Regulation of germinal center reaction ......................................................... 23

   Regulation of immunoglobulin (Ig) isotype switching .............................. 29
III. Immune response in early life .................................................. 32

Neonatal innate immunity ......................................................... 32

Neonatal adaptive immunity .................................................... 34

T-cell responses in neonates ................................................... 34

B-cell responses in neonates ................................................... 37

Functions of antigen-presenting cells in neonates ................. 39

Vaccination: a major strategy protecting neonates and infants from
infection .................................................................................. 40

Neonatal tetanus and tetanus toxoid ........................................ 41

IV. Vitamin A: metabolism and roles in immune functions .......... 44

VA absorption and metabolism ................................................. 44

VA status in children ............................................................. 46

VA deficiency increases the risk of infectious diseases in children ..... 47

VA supplementation effectively reduces the severity of infectious
diseases in VA-deficient children ........................................... 47

VA or RA stimulates the immune functions in VA-sufficient
corelations ............................................................................. 48

Possible mechanisms by which VA and RA regulate the immune
functions ................................................................................ 49

VA supplementation and vaccination ....................................... 54

V. PIC regulates immune functions ............................................. 57

Biological functions of PIC ..................................................... 57
TLR3 and IFNAR1 mediate the biological functions of PIC ............ 58
PIC and RA interactively regulate immune responses .................... 59
VI. Hypothesis and objectives .................................................. 60

Chapter 2. Retinoic acid and polyriboinosinic: polyribocytidylic acid stimulate robust anti-tetanus antibody production while differentially regulating type 1/type 2 cytokines and lymphocyte populations ......................................................... 65

Abstract ................................................................. 66
Introduction ............................................................... 67
Materials and methods ..................................................... 70
Results .................................................................. 74
Discussion ................................................................. 95

Chapter 3. The anti-tetanus vaccination response of neonatal mice is augmented by all-trans retinoic acid combined with polyriboinosinic acid: polyribocytidylic acid ................................................................. 101

Abstract ................................................................. 102
Introduction ............................................................... 103
Materials and methods ..................................................... 107
Results and Discussion ................................................... 111
Conclusions ................................................................. 139
Chapter 4. Retinoic acid and polyriboinosinic acid:polyriboctidylic acid regulate TT-induced germinal center response in adult mice ................. 141

Introduction ................................................................. 142
Materials and methods .................................................... 144
Results and discussion ..................................................... 146

Chapter 5. Discussion and future directions ................................. 160

RA and PIC co-operatively enhance anti-TT vaccine response in both adult and neonatal mice .................................................... 162

What is the potential mechanism? ............................................ 165

Conclusions ................................................................. 168

Future directions ............................................................. 168

References ................................................................. 174

Appendix A: List of primers .................................................. 198

Appendix B: List of antibodies ............................................... 199

Appendix C: Reprint permission .............................................. 201
LIST OF FIGURES

Fig. 1-1  Overview of TD antigen-induced Th-cell and B-cell activation  …  6

Fig. 1-2  Overview of Th1/Th2 response  ........................................... 15

Fig. 2-1  RA and PIC synergistically enhance primary anti-TT IgG but
differentially regulate IgG isotypes  ............................................ 75

Fig. 2-2  RA and PIC differentially regulate mRNA levels of type 1/type 2
cytokines  ................................................................. 80

Fig. 2-3  RA and PIC significantly regulate mRNA levels of IL-4 and IFN-γ 3
days after priming  ............................................................. 84

Fig. 2-4  The ratio of IL4/IFN-γ is significantly associated with the ratio of
NK/NKT cells, and the ratio of CD80/CD86 costimulatory molecules 90

Fig. 2-5  RA and/ or PIC treatments given with the primary immunization
enhance secondary anti-TT antibody IgG responses  ....................... 92

Fig. 3-1  RA and/ or PIC treatments do not affect the growth of neonatal mice 113

Fig. 3-2  RA and/ or PIC treatments significantly enhance primary anti-TT
antibody response in neonatal mice  ........................................... 114

Fig. 3-3  Co-administration of RA and/ or PIC with TT priming during the
neonatal period significantly enhance anti-TT memory response in
adulthood  .................................................................................. 116
Fig. 3-4  RA and/or PIC treatments significantly enhance TT-specific lymphocyte proliferation and production of IL-5 and IFN-γ  ........ 119

Fig. 3-5  RA and/or PIC treatments do not significantly affect anti-CD3 induced lymphocyte proliferation and production of IL-5 and IFN-γ .. 121

Fig. 3-6  Neonatal splenocyte population is more heterogeneous than adult cell population  ................................................................. 124

Fig. 3-7  RA and/or PIC significantly regulate cell proportion in the A and n1 gates  ................................................................. 126

Fig. 3-8  RA and/or PIC treatments regulate neonatal lymphocyte populations  129

Fig. 3-9  RA and/or PIC regulate NK and NKT cell populations  .......... 131

Fig. 3-10  RA and/or PIC treatments regulate populations and differentiation of neonatal macrophages and DCs  ........................................ 134

Fig. 4-1  RA and PIC treatments increase the percentage of B220⁺PNA⁺ cells  148

Fig. 4-2  RA and/or PIC treatments do not significantly affect IgM expression.  150

Fig. 4-3  RA and/or PIC treatments regulate the expression of IgG1 on B cells.  ................................................................. 151

Fig. 4-4  RA and/or PIC treatments regulate the expression of MHCII on B cells  ................................................................. 153

Fig. 4-5  RA and/or PIC treatments regulate the expression of Fas on B cells .. 156
Fig 4-6  The expression of IgG1 is significantly correlated with the expression of MHC II and Fas on B220<sup>+</sup>PNA<sup>Hi</sup> cells .......................... 157

Fig. 5-1  The potential mechanisms by which RA and PIC regulate T-induced vaccine response  ................................................................. 173
LIST OF TABLES

Table 2-1  Regulation of Th1/Th2-related genes by RA, (PIC), and RA/PIC in combination ...................................................... 82

Table 2-2  The correlation between anti-TT antibody isotypes and the mRNA levels of cytokines ................................................. 83

Table 2-3  Regulation of T-cell and NK/NKT-cell populations by RA, PIC, and RA/PIC in combination ........................................... 87

Table 2-4  Expression and distribution of CD80/CD86 molecules on splenic lymphocytes ......................................................... 89

Table 2-5  ELISPOT assay of splenic anti-TT antibody-secreting cells (ASC) .................................................................................. 94

Table 3-1  RA and/ or PIC treatments regulate lymphocyte populations in neonatal mice ............................................................. 136

Table 3-2  RA and/ or PIC treatments regulate the populations of antigen-presenting cells and expression of co-stimulatory molecules in neonatal mice ................................................................. 138

Table 4-1  RA and/ or PIC treatments regulate the expression of IgG1, MHC II, and Fas on B cells .................................................. 159
LIST OF ABBREVIATION

Ab: Antibody

ADCC: Antibody-dependent cell-mediated cytotoxicity

Ag: Antigen

α-GalCer: α-galactosylceramide

APC: Antigen-presenting cell

ARAT: Acyl-CoA: retinol transferase

ASC: Antibody-secreting cell

BCR: B-cell receptor

Bcl-6: B-cell lymphoma 6

BLIMP1: B-Lymphocyte-Induced Maturation protein 1

BM: Bone marrow

CSR: Class switch recombination

CTL: Cytotoxic T lymphocyte

CTLA-4: Cytotoxic T lymphocyte-associated molecule-4

DC: Dendritic cell

dsRNA: Double-stranded RNA

DT: Diphtheria and tetanus toxoids

DTP: Diphtheria, Tetanus, and Pertussis Vaccine

DISC: Death-inducing signaling complex

EAE: Experimental autoimmune encephalomyelitis

FADD: Fas-associated death domain

FDC: Follicular dendritic cells
GC: Germinal center
HLX: H2.0-like homebox1
IFN: Interferon
LC: Langerhans cell
L. major: Leishmania major
Ig: Immunoglobulin
LRAT: Lecithin:retinol acyltransferase
MMC: Mitomycin C
MTA3: Metastasis-Associated 1 family member 3
NK cell: Natural killer cell
NKT cell: Natural killer T cell
Pax5: Paired box protein 5
PIC: Polyriboinosinic: polyribocytidylic acid
PMBC: Peripheral blood mononuclear cell
PNA: Peanut agglutinin
RA: Retinoic acid
RE: Retinyl esters
STAT: Signal transducer and activator of transcription
T-bet: T-box expressed in T cells
Te cells: T cytotoxic cells
TCR: T-cell receptor
TD: Thymus-dependent
TI: Thymus-independent
**Th cell**: T Helper cell

**TLR**: Toll-like receptor

**TT**: Tetanus toxoid

**VA**: Vitamin A

**XBP1**: X-box-Binding Protein 1
ACKNOWLEDGMENTS

I would like to take this opportunity to extend my sincere gratitude to the special people who provided valuable assistance in my graduate study.

First and foremost, I wish to extend my sincere gratitude to my advisor, Dr. A. Catherine Ross, for the continuing support, guidance, patience, and encouragement in the past five years. I have learned from you not only about science but also about how to become a good scientist. Your professional and personal guidances are the most precious gift in my life.

My gratitude also goes to my committee members: Dr. Cantorna and Dr. Mastro for their generosity of sharing the knowledge and expertise in the immunology field; and Dr. Smith for his continuing encouragement and insightful suggestions in nutritional biochemistry and animal experiments.

I would like to thank the Department of Nutritional Sciences and the Huck Institutes of Life Sciences for the financial support of my graduate study.

I am also thankful to my colleagues and friends in the Penn State University: Dr. Kunze and Susan in Center of Cell Quantitative Analysis for their assistance in flow cytometry; Dr. Qiuyan Chen for her friendship, advice, and patience in training me in the first two years of my graduate study; Dr. Reza Zolfaghari for his encouragement and extensive knowledge in molecular biology; Dr. Nanqian Li for her valuable advice and continuing support in animal experiments; Xin Luo, Chris Cifelli, Yao Zhang, and Lili Wu, for their friendship, encouragement, and assistance in my graduate research; Madeline Stull for her sweet smile and very nice help in my research and study.
My deepest gratitude goes to my parents, Zhong-pu Ma and Bei-hui Zhang, my husband, Lintao Cai, and my son, Kevin. Thank you very much for endless love, unconditional support, and constant encouragement. I could not complete this thesis without all of you.
CHAPTER 1

LITERATURE REVIEW
I. Introduction

Vitamin A (VA) has been recognized as “the anti-infective vitamin” since the 1920s. In humans, vitamin A deficiency is strongly associated with increased mortality in children and pregnant women (1,2). Providing VA supplements to VA-deficient children aged 6-72 months, reduces all-cause mortality by 23%, measles-related mortality by 50%, and diarrheal disease mortality by 33% (3). Hence, periodic high-dose supplementation with VA is considered as a highly cost-effective approach to prevent VA deficiency and save children’s lives (4). In addition, VA supplementation also benefits women at childbearing age, which reduces the severity of diseases during pregnancy and lactation, and decreases the mortality related to pregnancy (5,6). The anti-infective effect of vitamin A could be partially attributable to the prevention of VA deficiency. More importantly, VA and its active metabolite, retinoic acid (RA), regulate a variety of immune responses, such as dendritic cell (DC) maturation, cytokine production, T- and B-cell activation, as well as mucosal immunity, which also contribute to the anti-infective effects (7-9).

Polyriboinosinic : polyribocytidylic acid (PIC), as a synthetic dsRNA, is well known for its ability to induce type I/ type II IFNs, and enhance anti-viral and anti-tumor reactions in several models (10-12). PIC is also a potent immune adjuvant, regulating both innate and adaptive immunity. Previous studies have demonstrated that PIC synergized with RA to enhance both primary and secondary anti-TT IgG responses in VA-deficient rats (13). However, the immunoregulatory role of RA and PIC in
VA-sufficient populations, particularly in neonates and infants, is not elucidated yet. The following literature review will focus on the roles of VA/RA and PIC in immune system. The general immune responses and neonatal immunity will also be discussed as well.
II. Overview of T helper (Th)-cell activation

The immune system in vertebrates comprises innate immunity and adaptive immunity. Innate immunity includes anatomic and physiological barriers as well as phagocytes, all of which provide non-specific but the first line of the host defense against infection. Unlike innate immunity, adaptive immunity is characterized by antigen (Ag) specificity, diversity, and immunologic memory, usually providing more effective protection than innate immunity. Adaptive immunity can be divided into two types: cell-mediated immunity and humoral immunity. The former is mediated by Ag-specific T lymphocytes, and responsible for eliminating intracellular pathogens, such as bacteria and virus. The latter is mediated by antibody derived from B lymphocytes, which can bind to extracellular pathogens and neutralize them or facilitate their elimination.

Mature T lymphocytes consist of two subpopulations: CD4+ T cells and CD8+ T cells, which function as T helper (Th) cells and T cytotoxic (Tc) cells, respectively. Upon activation, Th cells can secrete various cytokines and regulate the activation of many cell types, such as B cells, Tc cells, and macrophages. Therefore, Th cells play a critical role in the regulation of immune responses.

Th-cell activation

Th-cell activation requires two signals. The first signal is derived from interaction of the T-cell receptor (TCR)-CD3 complex with an Ag peptide presented by a MHC II molecule on an APC. The TCR-Ag interaction triggers multiple signaling
pathways and consequently induces Th-cell proliferation and differentiation. Moreover, full Th-cell activation requires a co-stimulatory signal that is provided by interactions between CD28 on the T cells and B7 molecules on the APCs (14) (see fig. 1-1). Once activated, Th cells proliferate and differentiate into either effector T cells or memory T cells. The effector Th cells secrete cytokines and initiate primary response; while the memory T cells will respond to a subsequent challenge with the same Ag, generating a secondary response.

**Th1/Th2 response**

Th1 cells and Th2 cells are two subsets of Th cells (Th), existing in human beings as well as mice. Upon activation, Th0 cells can differentiate into either Th1 or Th2 cells, which secrete different cytokines and regulate immune functions. Mature Th1 cells principally secrete IFN-γ, IL-2, and lymphotoxin, driving the cell-mediated response against intracellular pathogens, such as bacteria, parasites, viruses, as well as cancer cells. Th2 cells, on the other hand, produce IL-4, IL-5, and IL-10, promoting B-cell activation and humoral response to eliminate extracellular pathogens (15) (Fig. 1-1 and 1-2). Despite their critical roles in immune response, both Th1 and Th2 cells are also related to pathological impairments. Overreaction of Th1 cells is strongly associated with organ-specific autoimmune diseases, such as multiple sclerosis, type 1 diabetes, and arthritis. Overreaction of Th2 cells is considered as a key mechanism contributing to allergy and asthma (16). Therefore, it is essential to maintain the balance of Th1/Th2 response.
Fig. 1-1. Overview of TD antigen-induced Th-cell and B-cell activation. TD Ag-induce Th-cell activation is initiated by the TCR-Ag-MHC II interaction, and requires co-stimulatory signals derived from APCs, such as B7 molecules. Once activated, Th cells proliferate and differentiate into either Th1 or Th2 cells. The former secretes IFN-γ, IL-2, and lymphotoxin, driving the cell-mediated response against intracellular pathogens; and the latter produces IL-4, IL-5, and IL-10, promoting B-cell activation and humoral response. TD Ag-induced B-cell activation requires a direct contact with Th cells. Upon activation, most of B cells migrate into the secondary follicles and participate in the germinal center (GC) response. Within GCs, B cells undergo affinity maturation, isotype switching, and then differentiate into high-affinity plasma cells or long-lived memory B cells. B-cell proliferation and differentiation require the involvement of Th cytokines, such as IL-2, IL-4, and IL-5. Th1/Th2 cytokines are also key factors regulating Ig isotype switching. APC, antigen-presenting cell; DC, dendritic cell; Mø, macrophage; CD40L, CD40 ligand.
Molecular regulation of Th1 response

Cytokines are considered as key factors regulating Th1 response. The major cytokines promoting Th1 response include IL-12, IFN-$\gamma$, and IL-18 (Fig. 1-2). IL-12 is a pleiotropic cytokine, which is mostly produced by activated APCs, such as macrophages and DCs. It is composed of two subunits: p35 and p40. The former is constitutively expressed at a low level, and the latter is highly induced by pathogens in activated APCs. The major function of IL-12 is to induce IFN-$\gamma$ production in T cells and NK cells, directing Th1 differentiation. In addition, IL-12 can act as a growth factor, inducing T-cell and NK-cell proliferation. The functions of IL-12 are mediated by IL-12 receptor (IL-12R) complex, which consists of IL-12R$\beta$1 and IL-12R$\beta$2. Although both IL-12R$\beta$1 and IL-12R$\beta$2 are required for IL-12 binding, their functions are somewhat different. The IL-12R$\beta$2 subunit has conserved tyrosine residues in the cytoplasmic portion, suggesting that it can act as an important signal-transduction component (17). Furthermore, IL-12R$\beta$2 but not IL-12R$\beta$1 is selectively expressed on Th1 cells, and can be induced by IL-12 and type I IFNs (18). Maintenance of IL-12R$\beta$2 expression is required for normal Th1 differentiation. Hence, IL-12R$\beta$2 plays a critical role in the IL-12 signaling transduction and Th1 differentiation (19). The interaction of IL-12 and IL-12R activates the Signal transducer and activator of transcription (STAT)-4, driving Th1 polarization. Lacking IL-12, IL-12R$\beta$1, IL-12R$\beta$2, or STAT-4 significantly reduces Th1 response against infection (15,20-22).

Interestingly, despite of its importance, IL-12 may not be indispensable for Th1 response. Jankovic et al. (23) showed that IL-12 p40 deficient mice could produced
IFN-γ in response to *Toxoplasma gondii* or *Mycobacterium avium* infection, although the level of IFN-γ was much lower than that of wild type animals. These data suggest that IL-12 may not be essential for the initiation of Th1 responses. However, IL-12 is critical for optimizing IFN-γ production.

IFN-γ, as a Th1 signature cytokine, is also crucial for Th1 differentiation. IFN-γ is a type II interferon primarily produced by activated CD4 T cell, CD8 T cells, and NK cells. It can not only act as an antiviral agent, but also activate several cell types, such as macrophages, NK cells, and neutrophils, enhancing their ability to eliminate pathogens (24). Biological functions of IFN-γ are mediated by Jak/Stat1 signaling pathway. Once secreted, IFN-γ binds to IFN-γ receptor (IFN-γ R1 and R2), activating Jak1/Jak2, the receptor-associated Janus-family protein. The activated Jak2 and Jak2 can recruit Stat1, facilitating its phosphorylation and dimerization. The activated Stat1 then translocates into nuclear and regulates a variety of gene transcription. The role of IFN-γ in Th1 response has been demonstrated in many studies. In vitro, IFN-γ alone can elicit Th1 differentiation, and administration of anti-IFN-γ Ab inhibits the development of Th1 cells (25). In vivo, IFN-γ deletion significantly reduces the Th1 response against *Leishmania major* (*L. major*) but promotes Th2 response, resulting in a poor resistance to *L. major* infection (26). The effect of IFN-γ on Th1 response can be partially attributable to the induction of T-box expressed in T cells (T-bet), a Th-1 specific transcription factor, which will be reviewed later. IFN-γ, probably derived from pathogen-activated NK cells, initiates Th1 differentiation by inducing T-bet expression
in Th cells. The increased T-bet in turn promotes IFN-γ production and elevates IL-12Rβ2 expression, which further drives Th1 differentiation (20) (Fig. 1-2).

**IL-18** is another potent promoter for Th1 differentiation. IL-18 is a member of IL-1 family and produced by macrophages and DCs. Although IL-18 itself does not direct the development of Th1 cells, it synergizes with IL-12 to stimulate IFN-γ production (20). Moreover, IL-12 and IL-18 co-stimulation can elicit Th1 differentiation in the absence of TCR ligation (27,28). IL-18 deficiency significantly impairs BCG-specific Th1 response and LPS-induced IFN-γ production (29). Therefore, IL-18 is considered as an IL-12 cofactor directing Th1 response.

Recently, IL-23 and IL-27, two IL-12-family members, have been identified as novel factors promoting Th1 response. IL-23 is composed of IL-12p40 and a unique subunit, p19, which has homology with IL-12p35 (19). The major function of IL-23 is to induce IFN-γ production in T cells. Moreover, IL-23 can induce proliferation of memory T cells (30). Although the absence of IL-23 does not affect the development of Th1 response, administration of IL-23 in IL-12p40−/− mice significantly enhances their resistance against *Toxoplasma gondii* (31). Hence, IL-23 may be involved in Th1 commitment, especially when IL-12 is absent. The function of IL-27 is somewhat similar to IL-18. IL-27 itself can not drive IFN-γ production, however it synergizes with IL-12 to induce IFN-γ production in naive CD4+ T cells (32,33). Also, IL-27 stimulation during T-cell activation strongly induces the expression of T-bet and IL-12Rβ2, while suppressing expression of GATA-3, a key transcriptional factor of Th2 differentiation (33). Notably, although the absence of IL-27 significantly impairs Th1
response in the early stage of infection, the mice lacking IL-27 can eventually produce IFN-γ and control the parasite infection (34,35). Hence, IL-27 may play an important role in the early development of Th1 response.

Th1 response is also regulated by several Th1-specific transcription factors. T-bet, a member of T-box transcriptional factor family, has been identified as a key regulator for Th1 response (36,37). It is rapidly and selectively induced in Th1 but not Th2 cells, playing a critical role in Th1 differentiation. T-bet can initiate chromatin remodeling of the IFN-γ gene and induce IFN-γ production (20). T-bet also induces IL-12Rβ2, which consequently increases the responsiveness to IL-12 and optimize IFN-γ production. The importance of T-bet in Th1 differentiation is further confirmed in transgenic models. CD4 T cells isolated from T-bet−/− mice produce a significantly lower level of IFN-γ than wild type cells in response to CD3 stimulation. Under Th1 condition, IFN-γ secretion is also severely impaired in T-bet−/− CD4 T cells. In vivo, T-bet−/− mice fail to produce IFN-γ against TNP-KLH immunization or L. major, an intracellular protozoan (37). The induction of T-bet during T cell activation is strongly dependent on IFN-γ/STAT1 activation but independent on STAT-4 (38). These data suggest a positive feedback loop during Th1 differentiation that IFN-γ first induces T-bet expression in CD4+ T cells, and increased T-bet in turn promotes IFN-γ production and drives Th1 differentiation.

In addition to promoting Th1 differentiation, T-bet appears to suppress Th2 differentiation. Retroviral gene transduction of T-bet into polarized Th2 cells
significantly induces IFN-γ but reduces IL-4 and IL-5 production, suggesting T-bet can redirect Th2 cells into Th1 direction (36). On the other hand, T-bet deletion elevates IL-4 and IL-5 in response to CD3 stimulation or *L. major* infection in mice. Therefore, T-bet not only drives Th1 differentiation but also actively suppresses Th2 differentiation (Fig.1-2).

Recently, H2.0-like homebox1 (HLX) has been identified as another Th1-specific transcription factor involved in Th1 response. HLX is one of target genes of T-bet, selectively expressed in Th1 cells but not in naïve T cells and Th2 cells (39). In vivo, the HLX transgenic mice generate more Th1 cells than wild type mice in response to KLH immunization. In vitro, the HLX transgenic CD4 T cells can produce a high level of IFN-γ under Th2-polarizing conditions. More importantly, HLX and T-bet synergistically enhance IFN-γ production in Th1 cells (40). Thus, HLX may interact with T-bet and optimize IFN-γ production.

**Molecular regulation of Th2 response**

IL-4, a Th2 signature cytokine, is known as a key regulator directing Th2 differentiation (Fig. 1-2). Administration of IL-4 significantly induces the production of Th2 cytokines, such as, IL-4 and IL-5, but reduces IFNγ production in activated T cells (41,42). The biological functions of IL-4 are mediated by IL-4 receptor. Previous studies have identified two types of IL-4R complexes: type I and type II IL-4Rs. The former consists of IL-4Rα chain and the common γ chain (γc), and is exclusive for IL-4 binding. The latter consists of IL-4Rα and a low affinity binding receptor for IL-13,
IL-13Rα1, which is a high affinity receptor complex for both IL-13 and IL-4 (43). Ligation of IL-4 with IL-4Rs induces the activation of Jak family members, such as Jak1, Jak2, and Jak3, which in turn initiates tyrosine phosphorylation of IL-4Rα cytoplasmic domain, triggering Stat6 activation. The phosphorylated IL-4Rα can also recruit a group of Phosphotyrosine Binding-Domain (PTBD) adaptor proteins, such as Insulin Receptor Substrate (IRS)-1 and -2, and consequently activates PI3 kinase pathway. Mice lacking IL-4Rα or Stat6 show a significantly reduced Th2 response triggered by parasites and allergen sensitization (21,43). In vitro, Stat6-/- lymphocytes not only fail to develop into Th2 cells but also can not switch to IgG1 and IgE in response to LPS+IL-4 (44). Therefore, Stat6 plays a critical role in IL-4-induced Th2 response. In addition to Stat6, IRS-2 appears to be involved in IL-4 induced Th2 differentiation. Although IRS-2-/- T cells can differentiate into Th2 cells in vitro, the production of Th2 cytokines, such as IL-4 and IL-5, are reduced by 30-40% (45). Overall, IL-4 and its downstream signaling pathway play a central role in Th2 commitment.

Moreover, Th2 response is regulated by several Th2-specific transcription factors. GATA-3, as a zinc-finger transcriptional factor, has been identified as a key regulator for Th2 differentiation (Fig. 1-2). GATA-3 is rapidly induced in Th2 cells but declines in Th1 cells (46). Induction of GATA-3 in Th1 cells enhances IL-4 and IL-5 production, while inhibiting IFN-γ production. In contrast, antisense GATA-3 significantly reduces the production of IL-4, IL-5, and IL-13 in Th2 cells (21,47,48). Notably, overexpression of GATA-3 in Stat6-deficient T cells completely restores Th2
cell development, suggesting that GATA-3 can direct Th2 differentiation in a Stat6-independent manner. Moreover, GATA-3 protein seems to be able to self-activate GATA-3 transcription, indicating a autoregulated positive feedback driving Th2 commitment (49,50).

The mechanism by which GATA-3 directs Th2 differentiation could be due to its direct involvement in Th2 cytokine transcription. Previous studies have identified several GATA-3 binding sites that exist in IL-5 and IL-13 promoters. Ectopic expression of GATA-3 in Th1 cells activates the IL-5 or IL-13 promoter, while mutations of the GATA-3 binding site abrogate the promoter activation in Th2 cells (46,51,52). Therefore, GATA-3 can induce Th2 cytokine expression by transactivating the gene promoters. Moreover, GATA-3 may also be involved in chromatin remodeling. Introducing GATA-3 into Th1 cells induces chromatin remodeling at the flanking regions of the IL-4 and IL-13 genes and the IL-4/IL-13 intergenic regulatory region (52). These data suggest that GATA-3 may enhance Th2 cytokine gene transcription by remodeling chromatin into an accessible conformation.

In addition to GATA-3, c-Maf is another transcription factor involved in Th2 differentiation. c-Maf is a basic region/leucine zipper transcription factor that is selectively expressed in Th2 cells rather than Th1 cells. In vivo, overexpression of c-maf increases the production of Th2 cytokines and the Th2-related Ig, such as IgG1 and IgE, skewing the Th immune response towards a Th2 pathway (53). In vitro, introducing c-maf into Th1 cells significantly decreases IFN-γ production. Unlike GATA-3, c-Maf selectively induces IL-4 transcription rather than other Th2 cytokines. Deletion of c-Maf severely impairs IL-4 production, while the levels of IL-13 and IgE
are still normal (54). The induction of IL-4 by c-Maf is mediated by a c-Maf response
element (MARE) located in the proximal IL-4 promoter. Introduction of c-Maf in Th1
cells and B cells significantly transactivates the IL-4 promoter (55). Hence, c-Maf
directly activates IL-4 promoter and induces IL-4 expression, thereby promoting Th2
differentiation.

**Th1/Th2 cytokines and type 1/type 2 responses**

Although Th1/Th2 cytokines are initially defined and characterized in Th1/Th2
cell clones in vitro, many other leukocytes, such as monocytes/macrophages, B cells,
and NK/NKT cells, can also produce these cytokines. Therefore, it is very difficult to
isolate effects of Th1/Th2 cells from other cell types in an in vivo immune system. In
addition, several cytokines not produced by Th1/Th2 clones, such as IL-12, make
important contributions to the regulation of Th1/Th2 differentiation and immune
responses. Due to these reasons, Lucey et al. (56) suggested to use “type 1” instead of
“Th1” and “type 2” instead of “Th2” to characterize in vivo immune responses.
According to this nomenclature, type 1 cytokines are defined as IFN-γ, IL-2,
lymphotoxin, and IL-12; and type 2 cytokines are defined as IL-4, IL-5, IL-6, IL-10,
and IL-13. A type 1 response refers to a strong cellular immune response with increased
level of IL-2, IFN-γ, lymphotoxin, and IL-12; while a type 2 response refers to a strong
humoral response with increased level of IL-4, IL-5, IL-6, IL-10, and IL-13. In the
present study, most of experiments were conducted in vivo, so it is better to use type
1/type 2 cytokines and type 1/type 2 responses instead of Th1/Th2 responses to describe
our study.
**Fig. 1-2. Overview of Th1/Th2 response.** Th1 response is initiated by NK cell-derived IFN-γ, which induces T-bet expression in CD4⁺ T cells. The increased T-bet in turn promotes the production of IFN-γ and expression of IL-12Rβ2, directing Th1 differentiation. IL-12 and IL-18, produced by pathogen-activated macrophages or DCs, can amplify the production of IFN-γ and further drive Th1 commitment. Th2 response is initiated by IL-4, a cytokine produced by the activated T cells or by several cell types, such as NKT cells. IL-4 induces the expression of GATA-3 in T cells, which in turn leads to Th2 differentiation. T-bet and GATA-3 are the key transcription factors, driving Th1 and Th2 differentiation, respectively. On the other hand, T-bet and GATA-3 may also act as antagonists suppressing the development of the opposite subset. DC, dendritic cell; Mø, macrophage.
The role of antigen-presenting cells (APCs) in Th-cell response

As described above, APCs play an indispensable role in Th-cell response. Professional APCs include dendritic cells (DCs), macrophages, and B cells, all of which can ingest Ag and present a part of Ag by MHC II molecules to Th cells. The Ag presentation by APCs consequently initiates Th-cell activation. APCs also deliver costimulatory signals, facilitating full activation of Th cell. The most important co-stimulatory molecules expressed on APCs are CD80 (B7.1) and CD86 (B7.2), the members of the B7 protein family. Interaction of B7 molecules and their receptor on T cells, CD28, activates multiple signaling pathways as well as transcription factors, finally resulting in T-cell proliferation, differentiation, cytokine production, and cell survival (14,57) (Fig. 1-1). In addition to CD28, CD80 and CD86 also bind to the Cytotoxic T lymphocyte-associated molecule-4 (CTLA-4), which is a surface molecule expressed on activated T cells. The interaction of CTLA-4 and B7 molecules provides a negative signal to T-cell activation, limiting immune responses (57).

CD80 and CD86 are widely involved in T-cell activation and differentiation. Activation of naïve T cells strongly depends on the CD28-B7 interaction. Although memory T cells can be activated in absence of CD80/CD86 molecules, CD80/CD86 co-stimulation enhances their activation (58). CD80/CD86 co-stimulation also plays an important role in the Th1/Th2 differentiation. Increasing CD28 ligation during primary culture of CD4+ T cells enhances the production of IL-4 and IL-5 (59). However, blocking CD80/CD86 co-stimulation by using CTLA4-Ig significantly inhibits Th2 response (60). These results suggest that CD80 and CD86 are more involved in Th2
response than Th1 response. Furthermore, CD80 and CD86 play an important role in TD Ag-induced humoral response. Blocking CD80/CD86 co-stimulation with CTLA4-Ig dose-dependently inhibits primary antibody response against TD antigens, such as SRBCs and KLH (58). Also, the absence of CD28 significantly reduces Ig isotype switching against vesicular stomatitis virus infection (61). Hence, CD80 and CD86 signals are essential for TD Ag-induced humoral response.

In addition to the direct involvement in Th-cell activation, APCs can regulate Th1/Th2 response by secreting a variety of cytokines. IL-12, a key cytokine for Th1 differentiation, is produced primarily by activated macrophages and DCs (62). Activate APCs also produce other Th1-inducing factors, including IL-18 and IL-23, inducing Th1 response. Recent studies have indicated that a specific subpopulation of DCs called DC type 2 (DC2) may induce Th2 response, particularly in parasitism and allergic disease. Although the underlying mechanism is unclear, DCs might potentially promote Th2 response by secreting IL-10, a Th2-inducing cytokine (63,64). Hence, APCs act as a key factor participating in both initiation and regulation of Th-cell response.

The role of NK cells and NKT cells in Th-cell activation

Natural killer (NK) cells are a small population of lymphocytes, contributing to innate defense against viral infections and tumor cells (65). NK cells can recognize those abnormal cells lacking MHC I molecules, such as tumor cells and virus-infected cells, and mediate cytotoxicity to eliminate them. More importantly, NK cells can rapidly produce IFN-γ, which in turn activates phagocytosis of macrophages and
increases resistance to virus, thereby enhancing anti-tumor and anti-viral effects of NK cells (65). Recently, it has been shown that NK cells are also involved in the regulation of adaptive immunity, especially Th1/Th2 differentiation. As described previously, IFN-γ is a critical factor that directs Th1 differentiation. NK cells, as an early source of IFN-γ, play an important role in the initiation of Th1 response (Fig. 1-2). Inhibiting NK-cell activity significantly reduced production of IFN-γ by CD4+ T cells, but enhanced the production of Th2 cytokines (66). Thus, NK cells are involved in the regulation of Th1/Th2 differentiation.

Natural killer T (NKT) cells represent a subset of mature T lymphocytes expressing a restricted TCR as well as NK cell markers, such as NK1.1 and CD122 (IL-2Rβ). In mice, most of NKT cells express a restricted αβTCR repertoire that consists of an invariant Vα14Jα281 chain and certain Vβ chains (Vβ8.2, Vβ7, and Vβ2). In humans, NKT cells primarily express an invariant Vα24JαQ chain paired with Vβ11, the human homolog of mouse Vβ8.2 (67). The invariant TCR specifically recognize glycolipid antigens presented by monomorphic MHC I-like molecule CD1d, which consequently triggers NKT-cell activation. Although natural ligand of NKT cells has not been identified yet, α-galactosylceramide (α-GalCer) is proved as a synthetic NKT-cell ligand, which can be presented by CD1d and selectively stimulates both human and mouse NKT cells (67,68).

NKT cells play an important role in the regulation of innate and adaptive immune responses. NKT cells are shown to be essential for preventing autoimmune tissue destruction. Both human and mouse studies have indicated that depletion of NKT
cells is associated with severity of certain autoimmune diseases, such as Type 1 diabetes, multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), systemic lupus erythematosus (SLE), and rheumatoid arthritis. Increasing NKT-cell number and activity could help to prevent these diseases (68). Also, NKT cells participate in protective immune responses against several pathogens, such as mycobacteria, plasmodia, listeria, cryptococci, and trypanosomes (68). More importantly, NKT cells play an important role in the regulation of Th1/Th2 differentiation (Fig. 1-2). In vivo, a single injection of $\alpha$-GalCer rapidly triggers NKT-cell activation and induces the production of IL-4 and IFN-$\gamma$. However at later time points, particularly with repeated doses, $\alpha$-GalCer induces NKT cells to secrete IL-4 and IL-10 but dramatically reduces IFN-$\gamma$ production (69,70). Moreover, $\nu\alpha14$TCR transgenic mice showed elevated serum IgE and about 10-fold increase in IL-4 production (71). These data implied that NKT cells could promote Th2 responses. Interestingly, NKT cells may contribute to Th1 responses as well. Cui et al. (72) reported that activated NKT cells produced a large amount of IFN-$\gamma$, which in turn inhibited IL-4 production and reduced serum IgE. Moreover, IFN-$\gamma$ produced by NKT cells also stimulated NK cells, thereby further enhancing IFN-$\gamma$ production and promoting Th1 immunity (73). Hence, NKT cells can produce both Th1 and Th2 cytokines, involved in the regulation of Th1/Th2 responses and their balance.

Overall, Th-cell activation depends on the TCR-Ag-MHC II interaction and co-stimulatory signals derived from APCs. Moreover, signals from innate immunity,
such as cytokines secreted by macrophages, DCs, NK cells, and NKT cells, are also involved in the regulation of Th-cell activation (Fig.1-1 and 1-2).
III. Overview of B-cell activation and antibody response

Antigen triggered B-cell activation and antibody production mediate humoral immunity, a major branch of the adaptive immune response. Depending on different antigens, B cells can be activated in different ways. Thymus-independent (TI) antigens, such as some bacterial cell-wall components, can activate B cells without direct contact with Th cells. In contrast, the B-cell response to thymus-dependent (TD) antigens, including most of soluble protein antigens (e.g. TT), requires the direct contact with Th cells. TI Ag-induced antibody response is generally weak and has a limited isotype switching but no memory response. However, TD Ag-induced antibody response is strong and involves memory response, isotype switching as well as affinity maturation.

TD antigen-induced B-cell activation

TD Ag-induced B-cell activation is initiated by the interaction of B-cell receptor (BCR) with TD Ag. The subsequent interaction between CD40 on B cells and CD40 ligand (CD40L) on T cells provides another essential signal to stimulate B-cell activation. Additionally, various cytokines from Th cells, such as IL-2, IL-4, and IL-5, are also required for supporting B-cell proliferation and differentiation, including the generation of plasma cells and memory cells, affinity maturation, and immunoglobulin (Ig) class switching (see fig.1-1).

TD Ag-induced B-cell responses can be divided into 4 phases (74). Phase I refers to Ag-specific Th-cell activation and differentiation, which mainly occurs at 3-5 days after initial exposure to antigens. In this stage, Ag-activated APCs (mostly DCs)
migrate to the T-cell zone of secondary lymphoid tissues, presenting Ag peptides to naïve Th cells and initiating their activation. In addition, cytokines produced by APCs, such as IL-12 and IL-6, and B7 molecules expressed on APCs regulate Th-cell activation and differentiation. Phase II refers to Th cell-dependent B-cell activation, which mainly occurs at 5-7 days after initial exposure to antigens. In this stage, B cells first recognize antigens and present it with MHC II molecules. The activated Th cells then bind to B cells via the TCR-Ag-MHC II interaction and initiate B-cell activation. Moreover, the interaction between CD40 on B cells and CD40L on T cells is also critically involved in Ag-induced B cell activation. After activation, the Ag-specific B cells can undergo isotype switching and differentiate into low-affinity plasma cells. Alternatively, the Ag-activated B cells migrate to the secondary follicles and participate in the germinal center (GC) response, which is defined as Phase III. About 7-10 days after Ag priming, the secondary follicle polarizes into the dark zone and the light zone, the former contains rapidly dividing B cells (centroblasts), and the latter contains non-cycling B cells (centrocytes). This polarized structure is called a GC. Within GCs, B cells undergo affinity maturation, positive/negative selection as well as isotype switching, and eventually differentiate into high-affinity plasma cells or long-lived memory B cells. In this stage, the active Th cells can be recruited to GC and involved in B-cell selection. Also, cytokines derived from Th cells, such as IL-2, IL-4, and IL-10, regulate the generation of plasma cells and memory B cells (75). Phase IV refers to the memory response, which may occur weeks, months, or years after the initial priming. In this stage, interactions between memory B cells and memory T cells contribute to a
rapid memory response against Ag re-challenge (74). Overall, Th cells play a central role in TD Ag-induced B-cell activation and differentiation.

**Regulation of the germinal center reaction**

GCs are Ag-inducible microenvironments and principal anatomic sites for Ag-specific B-cell clone expansion, somatic hypermutation, affinity maturation, Ig isotype switching, and generation of plasma cells and memory B cells. Within GCs, the Ag-specific B cells first enter into the dark zone and differentiate into centroblasts. In the dark zone, centroblasts proliferate very fast and undergo somatic mutation, a serial of mutations occurring in Ig V-region genes. The result of somatic mutation is to increase the diversity of BCR repertoires. Centroblasts then move into the light zone and differentiate into centrocytes. In the light zone, B cells are subject to Ag-mediated positive selections based on their capability of binding Ag-Ab immune complexes on follicular dendritic cells (FDC). B cells with high-affinity BCR receptors are more likely selected and survive. However, those low-affinity B cells will not be selected and undergo apoptosis. In addition to positive selections, B cells are also subject to negative selections to eliminate self-reactive clones that are randomly generated by somatic hypermutation. After passing selection, the selected B cells need to process and present the Ag-derived peptides through MHC II to GC Th cells. The cognate T-B cell interaction not only provides an indispensable survival signals to B cells, but also drive B cells to switch isotype and differentiate into either memory or plasma cells. The B cells that fail to present Ag to T cells can not survive and will undergo apoptosis (75,76). Therefore, both cell survival and cell death are essential for the GC reaction.
The equilibrium between survival and death signals determines GC homeostasis (77). GC B-cell apoptosis is mediated by Fas (CD95), a member of the tumor necrosis factor receptor family, which binds to Fas ligand (FasL) and triggers apoptosis in many types of cells. Stimulation and clustering of Fas leads to the recruitment of Fas-associated death domain (FADD) protein to the intracellular death domain of fas, which in turn recruits procaspase-8/FLICE, resulting in the formation of a death-inducing signaling complex (DISC). The formation of DISC in turn causes the activation of caspase 8, initiating the apoptotic signaling (77). Fas-mediated GC B-cell apoptosis is required for both positive and negative selection to eliminate the low-affinity B cells as well as the self-reactive B cells. Fas mutation not only impairs selection of high-affinity B cells within GCs, but also leads to an extensive generation of self-reactive B cells (78,79). Interestingly, GC B cells are characterized as apoptosis-sensitive cells. Compared with other B cells, GC B cells lack Bcl-2, an anti-apoptotic proteins, but express a significantly higher level of apoptosis-inducing genes, such as Fas, Bax, c-myc (80). GC B cells also contain a preformed DISC, suggesting that GC B cells are programmed to cell death (81). Interestingly, the preformed DISC in GC B cells is originally in an inactive form, which is bound to cellular FLIP (c-FLIP), a major anti-apoptotic protein that can inhibit Fas-induced apoptosis by blocking the activation of caspase-8. Without exogenous stimuli, c-FLIP dissociates from DISC very quickly, thereby leading to GC B-cell apoptosis. However, in the presence of survival signals, such as Ag binding, the CD40/CD40L signaling, and FDC-derived anti-apoptotic signals, the binding of c-FLIP with DISC could be maintained, and GC B cells can survive (81,82).
GC B-cell survival and death are mostly control by two cell types: Follicular dendritic cells (FDCs) and GC Th cells. FDCs are the stromal cells in the GC, and play a crucial role in GC response. The primary function of FDCs is to drive positive selection and affinity maturation of B cells. FDCs present intact Ag on cell surface by Fc receptors (CD32 and CD23), or by complement receptors (CD21 and CD35), which select the B cells with high-affinity Ig, promoting affinity maturation. Moreover, FDCs express a group of adhesion molecules, such as ICAM-1, VCAM-1, VLA-4, and CD23, which bind to their respective ligands on GC B cells, providing strong survival signals to GC B cells (75,83). In addition, FDCs can produce the anti-apoptotic molecules, such as Baff/Blys, and a number of growth factors, such as 8D6, IL-15 and IL-6, which prevent GC B-cell apoptosis and promote proliferation (83).

Ag-specific Th cells also play a critical role in the GC reaction. The cognate T-B cell interaction, primarily mediated by the MHCII/Ag/TCR complex and the CD40/CD40L interactions, plays an essential role in the GC formation. Particularly, the CD40/CD40L interaction is required for B-cell proliferation and survival (84). CD40 is an integral membrane protein of the tumor necrosis factor receptor (TNF-R) super family, which is constitutively expressed on almost all mature B cells. CD40L is expressed on activated T cells but not resting T cells (85). CD40L prevents GC B-cell apoptosis by maintaining c-FLIP with DISC and keeps DISC in a nonfunctional form (81). Also, the CD40/CD40L signaling is required for Ig isotype switching and memory B-cell generation (81,86). Administration of anti-CD40 antagonist significantly reduces GC formation (87). In addition to the CD40/CD40L interaction, cytokines
secreted by T cells also regulate GC B-cell differentiation. For example, IL-2 and IL-4 can promote proliferation of GC B cells and direct GC B cells to differentiate into memory B cells, whereas IL-10 is required for plasma cell generation (88). Th1/Th2 cytokines are also key factors regulating Ig isotype switching, which will be described later. Recently, Kim et al. (89) showed that upon TCR stimulation, GC Th cells produced a significantly higher level of CXCL13 than other CD4+ T cells. Since CXCL13 is a critical chemokine for B-cell migration to lymphoid follicles, the up-regulation of CXCL13 on GC Th cells suggests their essential role in the GC formation.

The GC reaction is modulated by several transcription factors. Paired box protein 5 (Pax-5) and Bcl-6 are major transcription factors essential for the GC reaction. Pax-5 was originally known as B-cell-lineage-specific activator protein (BASP), and required for B-cell lineage commitment and development as well as B-cell function through the GC stage (90). Pax-5 can activate many B-cell-related genes, such as CD19, CD79A, B-cell linker (BLNK), and Activation-Induced cytidine Deaminase (AID), an enzyme essential for B-cell identity, B-cell activation, and the GC reaction (91). On the other hand, Pax-5 represses the genes that are related to antibody secretion, such as X-box-Binding Protein 1 (XBP1), IgH, IgL, and the J chain, thereby blocking the development of plasma cells (92). Inhibition of Pax-5 down-regulates IL-4/LPS-induced Ig class switching (93); in contrast, overexpression of Pax-5 stimulates B-cell proliferation but suppresses Ig synthesis in both late B cell lines and plasma cell lines (94). Hence, Pax-5 promotes the GC reaction but suppresses plasma cell differentiation.
B-cell lymphoma 6 (Bcl-6) is another transcription factor involved in the GC formation. Although Bcl-6 mRNA in resting B cells and GC B cells are identical, Bcl-6 protein was expressed about 3 to 34-fold higher in GC B cells than in resting B cells (95). The major function of Bcl-6 is to inhibit the expression of Blimp-1, a transcription factor inducing plasma-cell differentiation, which allows the GC reaction to continue but prevents premature plasma cell differentiation (91). Absence of Bcl-6 leads to significantly impaired GC formation but increased generation of plasma cells (96). Recently, Fujita et al. (97) reported Metastasis-Associated 1 family member 3 (MTA3) as a cofactor for Bcl-6 signaling. MTA3 is a cell type-specific subunit of the corepressor complex Mi-2/NuRD, and can be physically bound to Bcl-6 and mediated the repressive activity of Bcl-6. Although MTA3 depletion does not affect the expression of Bcl-6, Bcl-6 induced gene repression is significantly impaired as shown with increased Blimp-1. Introduction of Bcl-6 together with MTA3 in a plasma cell line significantly inhibits the plasma cell-related transcripts, but restores the B-cell transcriptional program (97). Thus, Bcl-6 and MTA3 play crucial roles in maintaining the GC reaction and preventing the plasma-cell differentiation.

The regulation of plasma-cell differentiation also requires the involvement of transcription factors, including B-Lymphocyte-Induced Maturation protein 1 (Blimp-1), X-box-Binding Protein 1 (XBP1), and IRF-4. Blimp-1 is a 98-kDa transcriptional repressor, expressed in all plasma cells and a subset of GC B cells. It plays an essential role in plasma-cell formation and Ig secretion. Introduction of Blimp-1 into B cells directly repressed genes involved in mature B cell functions, including the genes for B
cell identity, BCR signaling components, and genes required for Ig class switching. Moreover, Blimp-1 directly inhibited Pax-5 and Bcl-6, two major transcription factor genes essential for the GC formation. In contrast, Blimp-1 induced several genes related to plasma-cell differentiation and Ig secretion, such as XBP1 and J chain. Hence, Blimp-1 terminates the GC reaction but promotes plasmacytic differentiation by initiating and regulating a cascade of gene expression (98).

X-box-Binding Protein 1 (XBP1) is a basic-region leucine zipper protein and a member of the CREB/ATF family of transcription factors, which is crucial for plasma-cell differentiation and Ig secretion. Compared with other immature and mature B cells, the level of XBP-1 is much higher in plasma cell lines. XBP-1 deficiency significantly impairs plasma cell differentiation and severely reduced serum Antibody levels. Oppositely, introduction of XBP-1 into B-lineage cells initiates plasma cell differentiation (99).

IRF4, a member of the interferon regulatory factor family, may also be involved in the generation of plasma cells. IRF4 is primarily expressed in lymphocytes. In B lineage, IRF4 is highly expressed on a subset of cells in the light zone of GCs that also express Blimp-1 and Syndecan-1 (CD138, a surface marker of plasma cells). IRF4--/--mice showed severe defects in both T-cell and B-cell functions as well as the antibody response (100).

Overall, the GC reaction involves several cell types (such as FDCs and GC Th cells), and is positively and negatively regulated by a group of transcription factors. The
outcome of GC reaction is to produce long-lived plasma cells and memory B cells with high-affinity BCR repertoires.

**Regulation of immunoglobulin (Ig) isotype switching**

Immunoglobulin (Ig) isotype switching or class switching refers to a process by which B cells shift from producing IgM to one of IgG, IgE or IgA classes. It is very critical for generating functional diversity in humoral responses. Ig isotype switching is mediated by class switch recombination (CSR), a deletional DNA recombination that occurs between two switch (S) regions located upstream of each heavy-chain constant region (C_H). CSR results in replacement of the C_µ gene by one of the downstream C_H genes (C_γ, C_α, or C_ε), which consequently leads to the production of IgG, IgA, and IgE (101). IgG is the most dominant class in serum that constitutes about 80% of the total serum Ig. Based on the C_H genes, the IgG class is further divided into four subclasses: IgG1, IgG2, IgG3, and IgG4 in human; and IgG1, IgG2a, IgG2b, and IgG3 in mice.

Although the primary function of antibody is to binding Ag, antibody also participates in a variety of biological activities in order to effectively remove Ag. The antibody-mediated effector responses mostly include opsonization (Ig can bind to the Fc receptors on phagocyte to promote Ag phagocytosis), complement activation, and antibody-dependent cell-mediated cytotoxicity (ADCC), all of which are related to the C_H region. Therefore, Ig isotype switching can significantly affect antibody functional properties. For example, in humans, only IgM, IgG1, IgG2, and IgG3 can activate the classical pathway of complement. Also, only IgG and IgA but not other Ig isotypes can bind to Fc receptor on neutrophils and induce opsonization (102). Whereas IgG1 and
IgG3 appear to be the major Ig isotypes mediating the response to protein Ags, IgG2 mostly mediates the response against polysaccharide in human (103). The functions of mouse Ig isotypes are different from that of human Ig. Mouse Ig2a can bind to high-affinity FcR (FcγI) on macrophages and neutrophils, inducing the greatest level of ADCC. However, mouse IgG2b and IgG1 can only bind to low-affinity FcRs (FcγII and FcγIII). Hence, mouse IgG2b mediates a intermediate level of ADCC, and IgG1 is least effective to mediate ADCC (104,105). Moreover, mouse IgM and IgG2a are much more effective than other mouse IgG isotypes in inducing C3-mediated bactericidal and opsonophagocytic activity (106). Thus, Ig isotype switching directly impacts host response against Ags.

Previous studies have shown that many factors are involved in the regulation of Ig isotype switching. Among them, Th1/Th2 cytokines serve as crucial regulators, driving activated B cells to switch to specific isotypes. For example, Th2 cytokines, such as IL-4, direct murine B cells to produce IgG1 and IgE, and human B cells to produce IgE and IgG4 (107,108). Th1 cytokines, such as IFN-γ, induce switching to IgG2a and IgG3 (108). In addition, interaction of CD40 and CD40 ligand (CD40L) provides another signal essential for Ig isotype switching. In vitro, treatment with anti-CD40 or CD40L induced B cells to undergo isotype switching to IgG1 and IgE. Human or animal with mutant CD40L could not undergo Ig isotype switch in response to TD Ag (85,109). The CD40L-mediated Ig isotype switch occurs at the germline transcriptional level, the initial step of Ig class switch recombination, and does not
depend on IL-4 and other cytokines induced by CD40L stimulation. However, CD40L
and IL-4 synergistically enhanced γ1 germline transcription (109).

Overall, TD Ag-induced B-cell activation and differentiation requires direct
contact with activated Th cells and Th1/Th2 cytokines. Moreover, signals from innate
immunity, such as APCs, NK cells, and NKT cells, are also directly or indirectly
involved in the regulation of B-cell activation (see fig. 1-1).
III. Immune response in early life

Infants and neonates are the population with a high risk of infectious diseases. According to estimates by the World Health Organization (WHO), about 2.5 million infants between 1 and 12 months of age die each year of infectious diseases (110). The high susceptibility of infants and neonates to infections is mostly attributed to the relative immaturity of innate and adaptive immune system.

Neonatal innate immunity

Innate immunity provides the first line of defense against microbial invasion. These early defense mechanisms are particularly important during the neonatal stage when adaptive immune system is not fully developed. Unfortunately, neonatal innate immunity is relatively immature as compared with adults.

Several studies have shown that the function of neonatal neutrophils is defective. Neutrophils are one of key cellular effectors of innate immunity that function as active phagocytic cells. Neutrophils contain a variety of lytic enzymes and bactericidal substance in cytoplasmic granules, which can kill and digest microorganisms. Compared with the older children and adults, neonates have a similar level of neutrophils in blood; however, the function of neonatal neutrophils is immature. Kallman et al. (111) reported that phagocytosis and opsonization by neutrophils was significantly impaired in neonates, particularly in preterm neonates. The capacity of phagocytosis and opsonization was correlated with maturation of the newborn children (111,112). Moreover, chemotaxis of neutrophils is significantly reduced in newborns as
compared with adults (113). Term infants do not achieve adult level of chemotaxis until 2 weeks of age. Therefore, the immaturity of neonatal neutrophils may partially contribute to the high mortality of infectious diseases.

Macrophages, as another key cellular effector of innate immunity, have also been shown to be immature during the neonatal stage. The functions of macrophages include Ag uptake and catabolism, cytotoxicity, and the production of cytokines (such as IL-1 and IL-12). In addition, activated macrophages serve as APCs, facilitating Th-cell activation. In neonates, the ability of Ag up-taking and digestion by macrophages has been shown to be identical to adult macrophages. However, neonatal macrophages show impaired cytotoxicity, reduced cytokine secretion, such as IL-1, IL-12, and TNF-α, and immature Ag presentation (114,115). The immaturity of neonatal macrophages could be partially due to the absence of Ia expression on macrophages in the neonatal spleen and peritoneal cavity after birth (116). Also, neonatal macrophages show a significantly decrease in the upregulation of CD80 and/or CD86 by stimulation with IFN-γ, cAMP, CD40L, and anti-CD3 (117). CD80 and CD86 are costimulatory molecules expressed on APCs and essential for T cell activation. Therefore, the suppressed induction of CD80/CD86 molecules on neonatal macrophage can directly reduce their Ag-present capability, thereby impairing T-cell activation. In addition, impaired macrophage function in neonates may also be partially due to an excess production of IL-10, since blocking IL-10 production restored the secretion of IL-1 and TNF-α in neonatal macrophages (115). Hence, neonatal macrophages are
immature in both phenotype and function, which could in turn diminish T cell-dependent immunity against pathogens, increasing the risk of infection in neonates.

**Neonatal adaptive immunity**

**T-cell responses in neonates**

Neonatal T cells are both quantitatively and functionally distinct from adult cells. Compared with adults, neonates have fewer immune cells in the peripheral lymphoid organs (118,119). Also, neonatal T cell expresses lower density of the TCR-CD3 complex, CD4, and CD8 than adult cells. In addition, some adhesion molecules, such as CD2, leukocyte functional Ag (LFA)-1, and LFA-3 are expressed at lower level on neonatal T cells than on adult T cells (120).

The function of T cells in neonates is also immature. First of all, The TCR signaling appears to be defective in neonates. Adkins et al. (121) investigated neonatal T-cell activation in vitro. The results showed that although TCR-independent stimulation, such as PMA, was able to induce adult-like T-cell proliferation and IL-2 production, anti-CD3 induced T-cell proliferation and IL-2 production were significantly diminished in neonates. However, increasing the dose of costimulation, such as anti-CD28, significantly elevated neonatal T-cell proliferation and IL-2 production (122). The increased requirement of costimulation suggests that the threshold of T-cell response may be changed in neonates (123).

Secondly, neonatal Th-cell responses tend to polarize towards a Th2 pattern. In human neonates, serum IL-4 is not different from adult level, while IFN-γ is much less
than that of adult, suggesting a Th2 biased cytokine profile (124). In mice, neonatal splenocytes secrete significantly higher levels of Th2 cytokines, such as IL-4 and IL-5, but lower level of IFN-γ than adults (125,126). The quality of primary Th-cytokine response in neonatal spleen appears to be different from that in lymph nodes. Neonatal lymph node T cells can develop a mature and balanced Th1/Th2 primary response within the first week of life. In contrast, neonatal spleen T cells develop a Th2-biased primary response (127). Interestingly, although neonatal mice produce a mixed Th1/Th2 primary response in lymph nodes, memory response in neonates is still Th2 dominant (128).

There are several possible mechanisms contributing to the Th2-biased cytokine response: 1) The neonatal primary Th2 response is prolonged. In adult mice, the primary Ag-specific Th2 response disappears at 1-2 weeks postimmunization. However, Th2 response in neonates can persist till 5 weeks in both spleen and lymph nodes (125,128). 2) The generation of Th1 memory response is poor. Adult mice usually show a rapid increase in both IL-4 and IFN-γ productions, which peaks at 2-3 days after re-immunization. In contrast, the neonatal mice show a rapid increase in IL-4 but not in IFN-γ production (128). Therefore, the generation of Th1 memory response in neonatal mice is selectively impaired. 3) Primary neonatal Th1 cells undergo apoptosis upon Ag restimulation. Li et al. (129) used a T-cell transfer system to track neonatal T cells. The result showed that although neonatal mice developed both Th1 and Th2 cells during primary response, re-exposure to Ag significantly induced apoptosis in the Th1 population. The Ag-induced Th1-cell apoptosis was IL-4-dependent, since
neutralization of IL-4 or blockage of IL-4R/IL-13Rα1 rescued Th1 cells and restored Th1 memory response. 4) The fetal-derived T cells may be a potential source of Th2 cytokines in neonates. During the neonatal stage, some of T cells are derived from fetal thymic precursors and functionally distinct form adult-derived cells. Atkins et al. (130) showed that fetal-derived T cells produced 5 to 10 folds more of Th1 and Th2 cytokines than adult-derived T cells of, and evoked a Th2-dominant response. Therefore, the existence of fetal-derived T cells may contribute to the skewed Th2 response in neonates.

In addition to a Th2-skewed Th-cell response, cytotoxic T lymphocyte (CTL) response in neonate is also immature. CTL response plays an important role in reducing viral load and preventing viral infection. In murine neonates, Ag-specific CTL response is limited as compared with adults (126,131). In humans, cord blood cells generat less alloreactive CTLs than adult cells in vitro. Upon allostimulation, cord blood-derived CD8+ allospecific clones secrete less IFN-γ than adult cells (132). In HIV-infected population, infants usually show a reduced HIV-specific CTL response (133). Although the underlying mechanism is under investigation, previous studies showed that administration of anti-IL-4 Ab or IL-12 significantly enhanced CTL responses in neonatal mice, suggesting the dominant Th2 response is an important factor contributing to defective CTL in neonatal stage (134,135). Also, the dose of Ag seems to be critical for CTL response. Sarzotti et al. (136) showed that only low doses but not high doses of virus were able to induce protective CTL response in neonatal mice. A recent study done by Adkins et al. (137) confirmed this phenomenon that mouse
neonates were able to develop a fully mature alloreactive CTL response when exposed to low doses of cells. These results suggest that neonates may achieve an adult-like CTL response under certain circumstance despite the immaturity of T cells.

In summary, neonatal T-cell function is relatively immature as compared with adult level, which is shown as impaired TCR signaling transduction, Th2-biased cytokine response, and limited CTL response. The immaturity of T-cell response is considered as a major factor related to the high susceptibility of neonates to infectious diseases.

**B-cell responses in neonates**

Although young children can produce IgM and IgG antibodies upon Ag challenge, the antibody response against TD and TI type-2 Ag is especially low in infants and children <2 y of age, as well as in young animals, such as mice and rats. Compared with adult antibody response, neonatal antibody response to TD Ags generally shows the following characters: 1) Delayed generation and low titers: Schallert et al. (138) showed that primary anti-TTP30 IgG in neonatal mice was generated about 1 wk later than that of adult. Also the titer of IgG in neonates was significantly lower than that of adults. 2) Imbalanced Ig isotype switching: Although B cells from both human and murine neonates can go through the isotype switching from IgM to IgG, the switching is usually dominant with IgG1 but lacking IgG2 in human or IgG2a in mice (110). 3) Poor Ig affinity maturation: In human, infants immunized with conjugated polysaccharide vaccines, such as Haemophilus influenzae type b (Hib) and meningococcal vaccines, produced Ag-specific antibodies with lower avidity than that
of older children and adults (139,140). In murine models, standard human vaccine, such as TT and PT formulated in an aluminum-based adjuvant, can induce antibody response with adult-like avidity in neonatal mice. However, TT-P30, an immunodominant peptide, only induces low-avidity antibodies in neonates (138).

The defective TD antibody responses in neonates can be due to the immaturity of B cells and BCR signaling. Previous studies and our data have shown that neonatal spleen contains a significant number of immature B cells (IgM+IgD-). These immature B cells usually fail to become active and to proliferate upon BCR ligation. Instead, the BCR ligation induces negative signals and triggers B-cell apoptosis. Furthermore, the anatomical microstructure of the lymphoid organs is far from mature. In murine spleen, B-cell follicle and GC formation are not recognizable until 2-3 weeks of age. The capacity to trap immune complexes on FDCs occurs at 1-2 weeks, and mature FDC network only appears at 2 wk old (141,142). Since GCs are important sites for Ig isotype switching, affinity maturation, and memory responses, the immaturity of GC formation and FDC network could hamper TD antibody response in neonates. Moreover, a Th2-dominant cytokine profile in neonates also partially contributes to the imbalanced Ig isotype switching. In addition, neonates and infants up to 2 months of age show very few mutations of Ig V(H)6 sequence. Somatic mutation of Ig V-region genes is a part of selection of high-affinity variants and affinity maturation. Ridings et al. (143) showed that although the somatic hypermutation of Ig gene occurred in the human neonate, mutated neonatal V(H)6 genes contained relatively fewer mutations as compared with adult genes. Lacking somatic mutation may lead to a poor affinity maturation of
neonatal antibody response. Recently, Pihlgren et al. (144) showed that the migration of neonatal plasma B cell to bone marrow was significantly delayed and reduced compared with adult level. Since bone marrow is an important site for establishing a long-lived antibody secreting cell pool, the ineffective bone marrow homing of neonatal plasma cells might limit the persistence of TD antibody response in neonates.

In addition to TD antibody response, neonatal response to TI-2 Ags is reduced. Rijkers et al. (145) reported that newborns and infants up to the age of 1.5-2 years of age are not able to produce antibodies against bacterial capsular polysaccharides, a major type of TI-2 Ag. CD21, the C3d receptor and a BCR coreceptor, has been shown to play an essential role in TI-2 antibody response. Peset Llopis et al. (146) reported that pneumococcal polysaccharide (PPS), was mostly co-localized with C3 on CD21+ B cells, suggesting that complement C3 fragments (such as C3d) could bind to PPS and facilitate Ag localization to B-cell through CD21. However, the expression of CD21-(C3d/EBV-R) is significantly reduced on cord blood B cells and neonatal B cells (145). Also spleen marginal zone, the major site of TI-2 antibody response, is immature in infants (< 2 years of age), which is characterized with absence of CD21 but increased IgM+IgD+ cells (147). Reduced CD21 expression and immature marginal zone may be major factors hampering TI-2 antibody response in neonates.

**Functions of neonatal antigen-presenting cells (APCs)**

APCs, such as DCs, B cells, and macrophages, play an essential role in Th-cell activation and TD antibody response. However, neonatal APCs are functionally immature. Muthukkumar et al. (148) showed that B cells and DCs from neonatal mice
express very low levels of MHC II, CD80, and CD86. Moreover, neonatal B cells and DCs were severely defective in presenting Ag to Ag-specific T cell clones. The Ag-presenting ability of APCs was parallel to the antibody titer in neonatal mice. In human, the percentage of DC in cord blood is significantly less that adult peripheral blood. Also, compared with adult DCs, cord-blood DCs express lower levels of the IL-2 receptor $\gamma$ (CD132) and CD86, and secrete less IL-1beta and IL-6 (149). These data suggest the immaturity of neonatal APC function, which could be another factor contributing to defective immune response in neonate.

**Vaccination: a major strategy protecting neonates and infants from infection**

Immunization is one of most important strategies to protect neonates and infants from infectious diseases. In general, conventional vaccines protect the host from infections by inducing the production of specific protective antibodies, which can neutralize pathogens (or their toxins) at mucosal surface or right after invasion. For most viral pathogens, both antibody neutralization and cell-mediated immunity (cytotoxicity and Th1 cytokines) are essential for viral clearance (150). Unfortunately, the immaturity of the neonatal immune system significantly hampers the generation of a protective vaccine response (110). Hence, strategies to enhance vaccination efficiency in early life are highly sought.

Recent studies have reported promising results with strategies using certain adjuvants, such as IL-12 and CpG oligonucleotides, can induce type-1 cytokine production, elicit CTL responses, and augment antibody production in neonatal mice (151-153). IL-12 is a cytokine produced by DCs and macrophages, which is essential
for Th1 differentiation. IL-12 treatment during the neonatal stage significantly enhances both primary and secondary IgG responses to vaccine. IgG isotype analysis indicates that IL-12 mostly induces IgG2a and IgG2b subtypes (151,154). Moreover, IL-12 treatment during the neonatal stage significantly increases the production of IFN-γ and elicits Th1 response against vaccine antigens (155). CpG oligonucleotides are bacterial oligonucleotides containing unmethylated CpG dinucleotides in particular base contexts (CpG motifs). Kovarik et al. (152) showed that CpG oligonucleotides significantly enhanced vaccine-induced antibody response. Moreover, neonatal mice treated with CpG oligonucleotides produced adult-levels of IgG2a and IFN-γ production against TT and measles vaccines. Despite the promising results in animal models, the application of these adjuvants in human neonates is still limited because of the potential adverse effects, such as weight loss, fever, adjuvant arthritis, septic shock, neurotoxicity, etc (155-157). Hence, it is necessary to find an effective and safe adjuvant to promote neonatal vaccination.

**Neonatal tetanus and tetanus toxoid**

Tetanus is a serious disease typically caused by infection of a skin injury with an anaerobic bacterium *Clostridium tetani*. The bacteria can produce tetanus toxin, a potent neurotoxin, which binds to neuronal tissue, causing nerve and muscle damage (158). In developing countries, neonatal tetanus is the most common form of tetanus and usually occurs through infection of the unhealed umbilical stump, particularly when the stump is cut with an unsterile instrument. According to WHO estimate, neonatal tetanus caused about 200,000 of deaths in 2000, which contributed over 5% of global neonatal
mortality. Hence, neonatal tetanus is considered as a major public health problem in the world (159).

Fortunately, tetanus can be prevented by immunization with tetanus toxoid (TT). TT is derived from tetanus toxin, which is inactivated by formaldehyde and then absorbed onto aluminum salt. The function of TT is to induce specific Abs that can neutralize tetanus toxin. In human beings, TT mostly induces IgG1, which counts over 90% of total anti-TT antibodies (160). In mice, TT also evokes an IgG1-dominant antibody response. In addition, TT induces a small amount of IgG2a and IgG2b, contributing about 20% of anti-TT antibodies in mice (161). To prevent neonatal tetanus, TT is usually administrated with diphtheria toxoid and pertussis vaccine as a triple vaccine (DTP), providing basic protection to infants. Moreover, pregnant women are required to receive TT immunization. Maternal anti-TT Abs, such as IgG1, can pass to the fetus across the placenta and provide a passive protection against tetanus. Also, maternal TT immunization can protect the mothers from tetanus (159).

The efficiency of TT immunization can be determined by measuring anti-TT antibodies. The toxin neutralization test, an in vivo test, is regarded as a gold standard that directly measures the biological activity of anti-TT antibodies. However, this test is expensive and requires a lot of animals. The ELISA test is a simple, inexpensive, and rapid method that is widely used to quantify anti-TT antibodies (162). Despite some discrepancies between these two assays, the level of anti-TT antibodies determined by ELISA is highly correlated with anti-toxin potency determined by the toxin neutralization test (163). Hence, the anti-TT antibody measured by ELISA is considered
as a valid predictor for the efficiency of TT immunization and for the immune status against tetanus infection.
IV. Vitamin A: metabolism and role in immune functions

Vitamin A absorption and metabolism

Vitamin A (VA) is a fat-soluble vitamin required for vision, growth and development, cell differentiation, as well as maintenance of immune functions. Dietary VA usually exists as retinyl esters (RE) in foods of animal origin (e.g. liver, yolks, dairy products, etc), and as provitamin A in some vegetables (e.g. carrots, dark green leafy vegetables, yams, tomatoes, etc). The overall absorption efficiency of preformed VA is about 60%-90%. However, the absorption efficiency of provitamin A is only about 30-50%. Once uptake, REs is hydrolyzed to retinol in lumen and then enters into the enterocyte. In the enterocyte, retinol is esterified to RE by Lecithin:retinol acyltransferase (LRAT) or Acyl-CoA: retinol transferase (ARAT) for further absorption. For provitamin A, such as β-carotene, it has to subject to enzyme-mediated cleavage in the enterocyte, which mostly produces retinal. Retinal is reduced to retinol and then esterified to form RE (164). The newly absorbed REs are packed into chylomicrons and delivered to other tissues. Approximately 75% of chylomicron REs are transferred to the liver, the principal site for VA metabolism and storage; and the rest 25% are taken up by extrahepatic tissues, such as skeletal muscle, adipose tissues, heart, spleen, and kidney (165). When VA status is adequate, over 90% of VA in liver is stored as REs in stellate cells. On the other hand, REs can be hydrolyzed to form retinol, which will then bind to the RBP-TTR complex and be released into blood circulation.

Plasma retinol bound with RBP-TTR can be taken by many tissues in the body. In target tissues, retinol is metabolized into two kinds of active retinoids: 11-cis-retinoid
and acidic retinoids. The former participates in the transduction of light energy into neural signals in the retina; and the latter, particularly all-trans RA and 9-cis RA, are involved in many biological processes, such as growth, development, and maintenance of a functional immune response. The plasma level of RA is fairly low as compared with the plasma retinol level. In health human adults, plasma retinol concentration is about 1.5-3 μmol/L (43-86 μg/dL). However, the fasting plasma concentration of all-trans RA is in the range of 4-14 nmol/L, which is about 0.2-0.7% of plasma retinol level (166,167). The low level of RA in the circulation may be partially due to the low biosynthesis of RA. In vitro studies indicated that when retinol was added into cultured mammalian cells (e.g., human epidermal keratinocytes and pig kidney cell line), less than 5% of retinol was converted into RA (168,169). Moreover, RA concentration is tightly regulated by several members of cytochrome P450, such as CYP26, which oxidize RA to polar metabolites (e.g. hydroxyl-RA and oxo-RA), facilitating its secretion from body (170). Dietary VA or RA supplementation can significantly induce CYP26 mRNA and accelerate RA metabolism (171). Therefore, the homeostasis of RA is tightly controlled by RA biosynthesis and metabolism.

The biological functions of RA are mediated by two families of nuclear receptors: the RA receptor family (RARα, β, and γ) and the retinoid X receptor family (RXRα, β, and γ). The former are activated by both all-trans RA and 9-cis RA, and the latter are selectively activated by 9-cis RA. Upon activation, RAR and RXR form a dimer of either RAR/RXR or RXR/RXR, which can directly bind to a RA-response element (RARE or RXRE) and regulate gene expression (170).
Vitamin A status in children

Young children are considered as the population vulnerable to VA deficiency (172). Dahro et al. (173) measured VA concentration in the livers of 33 well-nourished children from 2 weeks to 9 years of age. In infants under 1 month of age, the median VA concentration was very low (~4 ug/g), suggesting that their VA storage was deficient. The liver VA concentration increased rapidly during the first 6 months of life, and reached to the adult-like level at one year of age (173). In rats fed with a high-vitamin A diet (15 ug/g diet), the retinol concentration in pup liver is 15 ug/g, which is only about 1/40 of the maternal level (172). Hence, despite maternal VA status, humans and animals are usually born with a low VA storage.

During the lactation, breast milk is a major source of VA for infants. Both human and animal studies have shown that maternal VA status directly affects the VA concentration in breast milk and the VA status in infants. Maternal VA deficiency during lactation results in a severe VA deficiency in their nursing babies (174). Increasing VA in maternal diet can not only elevate the VA concentration in breast milk during lactation, but also increase the liver VA level in rat pups (175).

After weaning, dietary VA is the most important factor that determines the VA status in children (172). However, even in developed countries, such as the United States, serum retinol concentration of children (<14 years of age) is still about 40% lower than the adult level. The plasma retinol level in children increases with age, and reaches to the adult level at about 14-18 years of age (176). Hence, VA storage and
serum VA level in children, particularly in neonates and infants, are significantly lower than that of adults.

**Vitamin A deficiency increases the risk of infectious diseases in children**

Vitamin A deficiency is a public health problem in more than 118 countries and affects about 140-250 million preschool children worldwide (177). VA deficiency during childhood may lead to xerophthalmia (dry eyes), a leading cause of childhood blindness in developing countries (7). Furthermore, VA deficiency increases the risk of infection in children. In 1968, Scrimshaw et al. (178) first suggested the association between VA deficiency and prolonged infection. Since then, many clinical studies have shown that VA deficiency is closely related to decreased resistance to infections and increased children’s mortality. Sommer et al. (179) reported that the total mortality of VA-deficient children with was on average 3 times higher than that of children without clinical VA deficiency. Also, VA-deficient children showed a 2 or 3-fold increase in the risk of developing respiratory disease and diarrhea than VA-sufficient children (180).

**Vitamin A supplementation effectively reduces the severity of infectious diseases in VA-deficient children**

Since VA deficiency is tightly associated with decreased resistance to infection during childhood, it is necessary to determine whether supplemental VA can reduce the risk of infectious diseases. Currently, VA supplementation has been proved as an effective treatment for some infectious diseases in children, such as measles and diarrhea. Sommer et al. (4) reported that VA administered at doses of 200,000 IU (60
mg) every 6 months reduced total mortality by 34% in preschool children. Hussey et al. (181) showed that co-treatment with VA (400,000 IU) not only decreased measles mortality but also reduced the duration of major complications, such as pneumonia and diarrhea, in children with severe measles. In addition, VA supplementation was shown to decrease the severity of diarrhea among the VA-deficient children (182). Due to the protective role of VA, WHO/UNICEF recommends that in those countries where the measles fatality rate is 1% or greater, all the children diagnosed as measles should receive 30-60 mg of VA immediately (183).

**Vitamin A or retinoic acid can stimulate the immune functions in vitamin A-sufficient population**

In addition to the protective role in VA-deficient populations, VA and its active metabolite, RA, may enhance immune response in VA-sufficient animals. Cui et al. (184) found that the mice treated with a high-vitamin A diet (250,000 IU/kg diet) had greater salivary IgA titers to influenza A virus than the control group. DeCicco et al. (185) reported that VA-sufficient mice, when receiving all-trans RA supplementation, showed a three-fold increase in both primary and secondary antibody responses. These data suggest that VA and RA may stimulate immune functions even in VA-sufficient populations.
Possible mechanisms by which vitamin A and retinoic acid regulate the immune functions

Previous studies have shown that VA and its active metabolite, RA, not only enhanced the immune responses in VA-deficient populations, but also potentially stimulated the immune functions in VA-sufficient populations. However, the underlying mechanism by which VA modulates immune responses is not clear. It has been indicated that VA is essential for maintaining integrity of the lymphoid organs. More importantly, VA regulates both adaptive and innate immunity, thereby strengthening the host’s immune defense (8).

VA is essential for maintaining integrity of lymphoid organs

VA plays an important role in maintenance of integrity of lymphoid organs. VA-deficient rats showed diminished splenic GCs, decreased spleen cells, and probably reduced weight of spleen and thymus (178). Furthermore, VA deficiency affected major lymphocyte populations. Semba et al. (186) reported that children with xerophthalmia had a lower proportion of CD4+ T cells and a lower ratio of CD4/CD8 cells, but higher proportions of CD8+ and CD45RO+ T cells than children without xerophthalmia. VA supplementation (60 mg RE), however, significantly increased the proportion of CD4+ T cells and the ratio of CD4/CD8, but reduced the proportions of CD8+ and CD45RO+ T cells. Dawson et al. (187) showed that marginal VA deficiency was associated with an increased percentage of CD8+ T cells and a decreased ratio of CD4/CD8 in old rats.
Hence, normal VA status is required for maintaining the integrity of lymphoid organs and the distribution of major lymphocyte populations.

**VA regulates antibody production and Ig isotype switching**

VA plays an important role in Ag-specific antibody responses. VA deficiency impairs the antibody responses against TD antigens, such as TT and diphtheria toxoid (DT) (178). DeCicco et al. (13) reported that VA-deficient rats produced lower levels of primary and secondary anti-TT IgG than VA-sufficient rats. Also, VA deficiency significantly decreased all isotypes of anti-TT IgG, including IgG1, IgG2a, and IgG2b. Repletion with oral RA effectively recovered both primary and secondary anti-TT IgG responses. In addition to the TD antigens, VA deficiency also significantly diminishes the antibody responses against TI type 2 antigens, such as bacteria polysaccharides (178). Pasatiempo et al. (188) found that VA-deficient rats a significantly lower antibody response specific to pneumococcal polysaccharide (SSS-III) than controls. Repletion with VA, however, effectively restored the anti-SSS-III antibody responses. Hence, VA status is crucial for both TD Ag and type 2 TI Ag-induced antibody responses.

Moreover, VA may modulate Ig isotype switching. Tokuyama et al. (189) reported that whereas RA alone did not affect Ig isotype switching, it enhanced LPS-induced IgA production by splenic B cells when combined with IL-5. Furthermore, RA strongly inhibited IL-4-dependent IgG1 production and S μ -S γ 1 switch rearrangement (189,190). A recent study showed that although RA inhibited BCR and/or CD38 triggered IgG1 germline transcription, it increased the percentage of
CD19$^+$sIgG1$^+$ cells (191). These data suggest that Ig germline transcriptional level may not necessarily be equivalent to Ig isotype switching. The role of RA in Ig isotype switching needs to be further confirmed.

**VA modulates Th1/Th2 response**

Although the effect of VA on Th1/Th2 differentiation is still an open question, some studies have reported that VA deficiency disrupted the balance of Th1/Th2 response. Cantorna et al. (192) reported that mesenteric lymph node cells (MLNC) from VA-deficient mice secreted more IFN-$\gamma$ but less IL-5 and IL-10 than those from VA-sufficient mice. Repletion with all-trans RA in vitro dramatically reduced IFN-$\gamma$ production and enhanced IL-5 secretion. DeCicco et al. (13) reported that VA-deficient rats showed an elevated mRNA level of IL-12 in spleen, while oral RA repletion significantly down-regulated it. Recently, Nozaki et al. (193) reported that a single administration of RA could help to treat lupus nephritis partially by reducing Th1 cytokine production, such as IL-2, IL-12 and IFN-$\gamma$. These results suggest that VA deficiency inhibited the Th2 response and skewed the immune response into a Th1 direction. The imbalanced Th1/Th2 response may be an important mechanism contributing to poor antibody responses in VA-deficient populations. RA repletion or supplementation, however, inhibited the Th1 response but promoted the Th2 response, thereby helping to rebalance the ratio of Th1/Th2 response.

The mechanism by which VA regulates the Th1/Th2 response is still under investigation. It has been shown that VA can regulate the Th1/Th2 response by directly affecting Th-cell differentiation. Cantorna et al. (192) showed that VA deficiency did
not affect the frequency of IFN-γ-secreting T cells; however, the IFN-γ-secretion rate (shown as pg/h/100 secretors) in VA-deficient mice was 6-fold faster than that of VA-sufficient mice. Treatment with RA in vitro decreased this rate by 50%. In addition, VA deficiency significantly reduced the IL-5-secreting cell frequency, and RA repletion significantly increased this number (192). Iwata et al. (194) investigated effects of RA on the differentiation of naïve CD4+ T cells into Th1 and Th2 cells in vitro. RA treatment consistently suppressed IFN-γ production but enhanced IL-4 production in vitro. Moreover, RA treatment reduced the expression of Th1-related genes, such as T-bet and IL-12Rβ2, while inducing the expression of Th2-related genes, such as GATA-3, c-Maf, and IL-4R (194). Thus, RA treatment directly inhibited Th1 differentiation and promoted Th2 differentiation both in vivo and in vitro.

Furthermore, APCs play an important role in VA-regulated Th1/Th2 response. Spleen APCs derived from VA-deficient mice stimulated more IFN-γ than those from VA-sufficient mice. Pretreatment of RA in VA-deficient APCs, however, significantly down-regulated their ability to stimulate IFN-γ production (192). Hoag et al. (195) reported that RA treatment enhanced Th2 development through APCs rather than T cells. In addition, 9-cis RA and LG69, a RXR agonist, were shown to inhibit LPS-induced IL-12 production by mouse macrophages. These data suggested that VA might influence Th1/Th2 differentiation through the regulation of cytokine production by APCs (196). Overall, VA can regulate Th1/Th2 differentiation by directly affecting Th-cell differentiation as well as APC functions.
VA regulates the differentiation and maturation of dendritic cells

Dendritic cells (DCs) are professional Ag-presenting cells that play an important role in both innate and adaptive immunity. Although VA has been shown to be widely involved in immune response, the effect of VA on DCs has not been well elucidated. Several recent studies demonstrated that VA and its active metabolite, RA, could regulate the generation and maturation of DCs. Hengesbach et al. (9) investigated the effect of VA on the generation of myeloid DCs. When bone marrow (BM) cells were cultured in low-VA medium, GM-CSF induced DCs differentiation was significantly reduced. Instead, GM-CSF induced BM cells to differentiate into granulocytes. Repletion with RA significantly induced DCs, while inhibiting granulocyte development. These data suggested that VA deficiency might impair the differentiation of immature myeloid DC from myeloid progenitors, and adequate VA or RA repletion could promote the development of myeloid DCs. Furthermore, VA may enhance DC maturation. Geissmann et al. (197) reported that RA does-dependently increased the expression of MHC II and CD86 on immature Langerhans cell (LC)-type DCs induced by TNF-α, suggesting RA enhanced TNF-α induced DC maturation. RA also enhanced Ag-presenting capability of LC-type DCs. The effects of RA on DC maturation were mediated by both an RXR-dependent pathway and an RARα/RXR pathways. Hence, VA and RA may promote DC development and activation, thereby regulating immune functions.
**VA regulates NK/NKT-cell populations and their functions**

NK cells, as a part of the innate immune system, play an important role in host defense against tumors and viral infections. NKT cells, a subset of TCRαβ⁺ T lymphocytes sharing some characteristics with NK cells, can produce a large amount of cytokines, regulating antibody production and inflammatory responses. Previous studies have shown that VA can regulate NK/NKT cell populations and their functions. Dawson et al. (187) showed that marginal VA deficiency differentially regulated NK and NKT cell populations. Marginal VA deficiency resulted in a significant reduction of NK-cell number in peripheral blood mononuclear cells (PMBCs) from young, middle-aged, and old rats. In contrast, marginal VA deficiency was associated with increased percentage and number of NKT cells in PMBCs from middle-aged and old rats. Ross et al. (198) reported that both NK-cell number and NK-cell cytotoxicity were significantly reduced in spleen cells from VA-deficient rats. Hence, VA status is critical for both NK/NKT cell populations and their functions.

**Vitamin A supplementation and vaccination**

In the past decades, a series of clinical trials have indicated that providing VA supplements to VA-deficient children aged 6-72 months reduces all-cause mortality by 23%, measles-related mortality by 50%, and diarrheal disease mortality by 33% (3,4). Hence, periodic high-dose supplementation with VA is considered as a highly cost-effective approach to prevent VA deficiency in infants and preschool children and save children’s lives. To facilitate delivery of VA supplements to young children, WHO
has recommended integrating VA supplementation as a part of the Expanded Program of Immunization (EPI) in countries where VA deficiency is still prevalent. For the 6- to 12-month old infants, 100,000 IU of VA (equivalent to 30 mg of retinol) is recommended to be given along with measles immunization (3,199), and for infants under 6 months of age, 25,000 IU of VA to be given along with diphtheria-pertussis-tetanus (DPT) vaccines (200).

The integration of VA administration into early-life vaccination programs has been shown as a safe and effective way to improve the VA status of infants (200). Moreover, co-administration of VA with measles or DPT vaccination significantly elevates vaccine-induced antibody responses in infants, suggesting the potential benefit of VA on the vaccine response in early life (199,201,202). Benn et al. (201) reported that giving children 100,000 IU of VA supplementation with measles vaccine at age of 9 months significantly enhanced titers of measles-specific antibody at age of 18 months. In addition, VA supplementation tended to increase antibody titers when these children reached age 6-8 years, which indicated its long-term benefit on measles-specific antibody responses. Rahman et al. (202) evaluated the effect of simultaneous VA supplementation on DPT vaccination in India. Infants aged 6-17 wk were randomly given 15 mg oral VA or placebo at the time of their DPT immunization. Although VA administrated with DPT vaccination did not affect antibody response against pertussis and tetanus, it significantly increased titers of anti-diphtheria IgG compared with placebo group. These studies suggested that VA supplementation with vaccination could be a promising way to enhance vaccine-induced antibody responses in those areas...
with high prevalence of VA deficiency. However, the effect of VA supplementation on vaccine responses in VA-sufficient populations is still controversial. Cherian et al. (203) reported that 100,000 IU of VA supplemented with measles vaccination did not affect titers of measles antibody in healthy VA-sufficient infants. Therefore, the potential benefit of VA supplementation on vaccine responses in VA-sufficient populations, particularly in neonates and infants, needs to be further investigated.
V. Polyriboinosinic:polyribocytidylic acid (PIC) regulates immune functions

Biological functions of PIC

PIC is a synthetic double-stranded polynucleotide that can mimic a viral infection and induce a large amount of type I interferon (IFNs). In vivo, PIC injection rapidly induces a high level of IFN in both animal and human models (11,204,205). In vitro, PIC induces the production of IFN in a variety of cells, including macrophage and human PBMC (206). Also, PIC can serve as an antiviral agent that effectively inhibits virus replication and increases the resistance to virus infection in vivo and in vitro (11,204). In addition, PIC can act as an anti-tumor agent, which not only inhibits the growth of transplanted tumors but also prevents the occurrence of malignancy (11). Furthermore, PIC regulates a series of immune responses, such as Th1/Th2 response, DCs maturation, and NK cell activity. In vivo, PIC induces the production of IFN-γ, IL-12, and TNF-α, and enhances Ag-specific IgG production (207). In vitro, PIC treatment suppresses the production of Th2 cytokines and skews Th-cell differentiation to a Th1 direction. The effects of PIC on Th1 development is partially mediated by its ability to induce production of IFN-α and IL-12 by monocytes (206). PIC also potentially promotes the maturation of DCs. Verdijk et al. (208) reported that PIC treatment increased the expression of co-stimulatory molecules, such as CD80 and CD86, as well as a maturation marker, CD83, on human DCs. The phenotypic maturation was accompanied by enhanced Ag-presenting activity and IL-12 production but down-regulated pinocytic activity. In addition, PIC significantly augments NK-cell cytotoxicity both in vitro and in vivo, and increases the production of IFN-γ and TNF-α.
Overall, PIC not only serves as an effective antiviral and anti-tumor agent but also acts as an adjuvant regulating a variety of immune responses.

**TLR3 and IFNAR1 mediate the biological functions of PIC**

Toll-like receptors (TLRs) are a family of innate immune-recognition receptors that recognize molecular patterns associated with microbial pathogens and subsequently induce antimicrobial immune responses. So far, ten TLRs have been identified in humans and mice, and each of them recognizes different microbial products. For example, TLR2 mostly recognizes bacteria peptidoglycan (PGN), lipoproteins, and LPS; TLR4 recognizes LPS; TLR9 recognizes unmethylated CpG motifs in the genome of bacteria and viruses. Recently, TLR3 has been identified as a receptor responsible for the recognition of dsRNA, such as PIC. TLR3 is a type 1 integral membrane glycoprotein located in intercellular endosomal membranes (211). It has been shown to be expressed in macrophages, DCs, as well as NK cells (209,212). In vitro, PIC triggers NF-κB activation in the 293T cells transfected with human TLR3. However, the cells expressing other TLRs do not respond to the PIC treatment. Furthermore, cotransfection with a dominant negative version of TLR3, but not other TLRs, completely blocks the effect of PIC, suggesting that TLR3 is responsible for the recognition of PIC. To further confirm the role of TLR3 in the PIC recognition, Alexopoulou et al. (213) generated a TLR3-knockout (TLR3<sup>−/−</sup>) model. Although TLR3<sup>−/−</sup> mice had normal B-cell and T-cell populations, they were resistant to the lethal effect of PIC (10-50 μg/mouse). In addition, TLR3 deletion significantly impaired the response of macrophages and DCs to PIC treatment and decreased PIC-induced cytokine production, such as IL-6, IL-12.
and TNF. Therefore, TLR3 plays a crucial role in the biological functions of PIC. In addition to TLR3, IFN-α/β receptor (IFNAR1) is also involved in the functions of PIC. Honda et al. (214) showed that IFNAR1 deletion not only blocked PIC-induced DC phenotype maturation and but also suppressed PIC-triggered cytokine secretion in DCs. Moreover, IFNAR1−/− DCs failed to stimulate the activation of CD4+ T cells and CD8+ T cells. Hence, both TLR3 and IFNAR1 are essential for the biological functions of PIC.

**RA and PIC interactively regulate immune responses**

Previous studies in our lab indicated a strong interaction between RA and PIC on TD Ag-induced antibody response. VA-deficient rats produced lower levels of primary and secondary anti-TT IgG than VA-sufficient rats. PIC alone effectively increased titers of primary anti-TT IgG but failed to improve secondary anti-TT IgG. However, PIC combined with RA synergistically induced both primary and secondary anti-TT IgG in VA-deficient rats (13). Also, this combination significantly induced mRNA levels of IL-2Rβ, IRF-1, and STAT-1 in VA-deficient spleens (215). In VA-sufficient rats, the combination of PIC and RA additively enhanced the titer of primary anti-TT IgG. Moreover, this combination up-regulated the percentage of B cells, the expression of MHC II, and the NK-cell population in spleen and lymph nodes. In addition, treatment with RA+PC in vivo significantly enhanced T-cell proliferation to anti-CD3/phorbol myristyl acetate+IFN in vitro (185). All these data suggest that the combination of RA and PIC might be a promising strategy to stimulate TD Ag-specific antibody response and cell-mediated immunity.
**VI. Hypothesis and objectives**

Previous studies have demonstrated that PIC combined with RA synergistically enhanced both primary and secondary anti-TT IgG responses in VA-deficient rats (13). However, the immunoregulatory role of RA and PIC in VA-sufficient animals has not been well elucidated. Although RA and PIC were shown to enhance primary anti-TT antibody response in VA-sufficient animals (185), whether RA and PIC could benefit memory response and Ig isotype switching are unknown. Also, the underlying mechanisms by which RA and PIC promote anti-TT antibody response in VA-sufficient animals are not clear.

Early-life vaccination is an important strategy to protect neonates and infants from infectious diseases. Unfortunately, the immaturity of neonatal immune system hampers the generation of protective vaccine responses. Hence, a safe and effective way is highly desired to improve vaccine efficiency in neonates and infants. Currently, VA supplementation has been integrated with immunization programs in many developing countries where VA deficiency is still prevalent (3). The combination of VA with vaccination not only improves VA status but also enhances vaccine response in VA-deficient children, suggesting its potential adjuvant effect (199,202). Since RA, an active metabolite of VA, and PIC can synergistically enhance anti-TT antibody response in VA-deficient animals, it is interesting to determine if RA combined with PIC can benefit neonatal vaccine response.

Therefore, the goal of the present study is to determine the effects of RA and PIC treatments on TT-induced vaccine response in VA-sufficient adult and neonatal
mice, and to explore potential mechanisms contributing to their immunoregulatory effects.

**Hypothesis:**

RA and PIC cooperatively enhance anti-TT IgG responses but differentially modulate IgG isotypes in both VA-sufficient adult and neonatal mice. The immunoregulatory function of RA and PIC may be attributed to their distinct effects on type 1/type 2 cytokine response, immune-cell populations and differentiation, as well as the GC response.

**Objectives**

1. **To evaluate the effects of RA and/or PIC treatments on primary and secondary anti-TT antibody responses in VA-sufficient adult mice.**

   The effect of RA and/or PIC treatments on primary and secondary anti-TT antibody responses as well as IgG isotypes (IgG1, IgG2a, and IgG2b) was evaluated in VA-sufficient adult mice. The results showed that RA and PIC cooperatively enhanced primary and secondary anti-TT antibody responses, while differentially regulating the IgG isotypes. ELISPOT assay indicated that the enhancement of anti-TT antibody response by RA and PIC might be partially due to the induction of B cells differentiation into effector Ag-secreting cells (ASCs). These data will be described and discussed in Chapter 2.
2. To explore the potential mechanisms by which RA and PIC regulate the anti-TT antibody responses in VA-sufficient adult mice.

(a): To evaluate the effect of RA and PIC on type 1/type 2 cytokine gene expression

(b): To determine how RA and/ or PIC treatments regulate the populations of lymphocytes (such as T cells, B cells and NK/NKT cells), and the expression of co-stimulatory molecules (CD80 and CD86) in VA-sufficient mice

(c): To explore the effect of RA and PIC on TT-induced GC response

Type 1/type 2 cytokines play an important role in the regulation of Ag-specific antibody response and Ig isotype switching. Therefore, it is necessary to determine if RA and PIC could affect anti-TT antibody response by modulating type 1/type 2 cytokines. Here, two independent studies were conducted, which evaluated the effect of RA and PIC on type 1/type 2 cytokine gene expression in the early stage (3 days after priming) and the late stage (11 days after priming) of immune response, respectively. The result showed that RA and PIC differentially regulated type 1 /type 2 cytokine mRNA expression. Moreover, the mRNA levels of type 1 /type 2 cytokines were significantly correlated with the titers of IgG isotypes. These data will be described in Chapter 2.

TT-induced antibody response involves several types of immune cells, including T cells, B cells, macrophages, DCs, as well as NK/NKT cells. Therefore, the effect of RA and/ or PIC treatments on the populations and differentiation of immune cells was also evaluated. The result showed that RA and/or PIC differentially regulated NK/NKT-cell populations and the expression of the costimulatory molecules
The correlation between the ratio of IL4/IFN-γ and the ratio of NKT/NK cells as well as the ratio of CD80/CD86 suggested that RA and/or PIC might promote TT-induced vaccine responses by regulating immune-cell populations and differentiation. These data will be described in Chapter 2.

GCs are principal anatomic sites for Ag-specific B-cell clone expansion, affinity maturation, Ig isotype switching, and generation of plasma cells and memory B cells. Therefore, the effect of RA and PIC on TT-induced GC reaction was also assessed. The result showed that RA and PIC not only increased the proportion of GC B cells but also up-regulated the expression of GC-related markers. These data will be described in Chapter 4.

3. To evaluate the adjuvant effect of RA and/or PIC treatments on the TT-induced vaccine response in neonatal mice.

(a): To determine if early-life treatments with RA and/or PIC could enhance primary anti-TT antibody response in infancy and memory response in adulthood.

(b): To evaluate the effect of RA and/or PIC treatments during the neonatal stage on lymphocyte proliferation and type 1/type 2 cytokine production.

(c): To assess the effect of RA and/or PIC treatments on the maturation and differentiation of neonatal immune cells.

Stimulating a strong humoral response and providing a good memory are required properties of an adjuvant. Hence, to determine if RA and PIC could be a
possible adjuvant for neonatal vaccination, I first evaluated their effect on neonatal antibody response. The result showed that co-administration of RA and/or PIC with TT priming during the neonatal period stimulated both primary anti-TT IgG production in infancy and the memory response in adulthood. These data will be described in Chapter 3.

A poor antibody response in neonates can be partially due to impaired lymphocyte activation and imbalanced type 1/type 2 cytokine response. Therefore, the effect of RA and/or PIC treatments on TT-induced lymphocyte proliferation and type 1/type 2 cytokines in neonatal mice were assessed using in vitro cell culture. The result showed that RA and PIC both enhanced TT-induced lymphocyte proliferation but differentially regulated type 1 (IFN-γ) and type 2 cytokines (IL-4). Remarkably, the combination of RA+PIC significantly enhanced both IFN-γ and IL-4, suggesting its capability of enhancing both type 1 and type 2 responses. These data will be described in Chapter 3.

At last, the effect of RA and/or PIC treatments on the maturation and differentiation of immune cells was determined in neonatal mice. Lacking mature immune cells, such as CD4 and CD8 T cells, and NK cells, in neonatal peripheral lymphoid organs is considered as a major factor contributing to weak immune responses in neonates. My results showed that RA and PIC not only promoted the generation of immune cells (such as B cells, NK/NKT cells, and DCs) in neonatal mice, but also induced expression of CD80, a major costimulatory molecule expressed on APCs. These data will be described in Chapter 3.
CHAPTER 2

Retinoic acid and polyriboinosinic: polyribocytidylic acid stimulate robust anti-tetanus antibody production while differentially regulating type 1/type 2 cytokines and lymphocyte populations
ABSTRACT

Retinoic acid (RA), a bioactive retinoid, and polyriboinosinic:polyribocytidylic acid (PIC), a inducer of IFNs, are known to promote immunity in vitamin A-deficient animals. We hypothesized herein that RA, PIC and the combination can provide significant immunoadjuvant activity even in the vitamin A-adequate state. Six-week old C57/Bl6 mice were immunized with tetanus toxoid (TT) and treated with RA and/or PIC at priming in three independent studies of short and long duration. The results showed that RA and PIC differentially regulated both primary and secondary anti-TT IgG isotypes, whereas the combination of RA+PIC stimulated the highest level of anti-TT IgG production and, concomitantly, a ratio of IgG1 to IgG2a similar to that of the control group. The regulation of antibody response was strongly associated with type 1/type 2 cytokine gene expression. Whereas RA reduced type 1 cytokines (IFN-γ and IL-12), PIC enhanced both type 1 and type 2 cytokines (IL-4 and IL-10) and cytokine-related transcription factors. Despite the presence of PIC, the IL-4:IFN-γ ratio was significantly elevated by RA. In addition, RA and/or PIC modulated NK/NKT-cell populations and the level of expression of the costimulatory molecules CD80/CD86, evident three days after priming. Notably, the NKT:NK and CD80:CD86 ratios were correlated with the IL-4:IFN-γ ratio, indicative of multiple converging modes of regulation. Overall, RA, PIC and RA+PIC rapidly and differentially shaped the anti-tetanus Ig response. The robust, durable and proportionate increase in all anti-TT IgG isotypes induced by RA+PIC suggests that this combination is promising as a means to enhance the antibody response to tetanus toxoid and similar vaccines.
INTRODUCTION

Soon after its discovery, vitamin A was characterized as “the anti-infective vitamin” due to the observation that vitamin A-deficient animals succumbed to infections which vitamin A-adequate animals survived (7). In humans, vitamin A deficiency is now recognized as a highly significant risk factor associated with increased mortality in children and pregnant women (1,2). Intervention studies in at-risk populations have clearly demonstrated that providing vitamin A to children, ranging from newborn to 5 years of age, decreases child mortality rates by an average of 23%, with a 50% reduction observed in some studies (216,217). Furthermore, vitamin A supplementation has reduced measles-related mortality and the severity of several infectious diseases including measles, diarrhea, malaria, and HIV infection (181,218-220). These encouraging results have prompted the distribution of vitamin A to children in at-risk populations, sometimes in concert with immunization programs, including vaccinations against measles and tetanus (177,201,202).

The anti-infective effect of vitamin A is thought to be attributable to immune stimulation and/or regulation. Vitamin A and its transcriptionally active metabolite, retinoic acid (RA)³ have been shown to modulate several indices of innate and adaptive immunity, such as dendritic cell (DC) maturation, cytokine production, T- and B-cell activation and antibody responses, as well as mucosal immunity (7-9). Whereas most immunological research has addressed the effects of vitamin A and RA in remediating vitamin A deficiency, it is also important to evaluate vitamin A and/or RA in the normal state not only because these nutrients may potentially be useful in strategies to improve vaccine efficiency, but because many of the recipients of current vitamin A
distribution-immunization programs are not themselves vitamin A deficient (177). Therefore, understanding the consequences of vitamin A and its metabolite RA on the development of antigen-specific antibody responses can aid in their appropriate use in both clinical and public health settings.

Polyriboinosinic:polyriboctidylic acid (PIC), a synthetic double-stranded polyribo-nucleotide and a mimetic of double-stranded RNA viruses, is known for its ability to induce type I/ type II IFNs, increase anti-viral and anti-tumor reactions in several models (11,12,221), and activate both innate and adaptive immunity (10,11,207,208). Multiple mechanisms appear likely in that PIC has promoted DC maturation, stimulated NK cell cytotoxicity (207,208,222), and also increased antigen-specific total IgG and IgG isotypes (207). Besides immune effects of PIC alone, it is of interest that combinations of IFNs and retinoids have shown promise in cancer therapy, which may in part be due to their regulatory effects on the immune system (223).

Tetanus toxoid (TT) is a classical T-cell dependent antigen and a clinically important vaccine especially in young children and women of child-bearing age (224). Previously, we demonstrated that the combination of RA and PIC synergistically increased anti-TT antibody production in vitamin A-deficient rats and mice (13), and elevated primary anti-TT IgG response in vitamin A-adequate rats (185). However, little is yet known of the independent and combined effects of these agents on the production of IgG subtypes, memory responses to TT, and on cellular and molecular markers of type 1/type 2 immunity. The aim of the present study was to evaluate RA
and PIC, alone and in combination, as modulators of molecular and cellular aspects of immunity in non-immunocompromised vitamin A-sufficient mice.
MATERIALS AND METHODS

Animals and experimental design

Animal protocols were approved by the Institutional Animal Use and Care Committee of the Pennsylvania State University. Six-week old C57BL/6 female mice (Charles River Laboratories, Wilmington, MA) were divided into 4 groups: control, RA, PIC, and RA+PIC, and fed a nutritionally complete diet (LabDiet 5001 which contains vitamin A 22 IU/g, Purina Mills, St. Louis, MO) throughout the experimental period. Three independent experiments were conducted. For the first study (11-day study), mice were immunized with TT (Connaught Laboratories, Swiftwater, PA), 10 μg, by intraperitoneal injection (i.p.), and cotreated with all-trans RA (Sigma, St. Louis, MO) 50 μg, orally in 25 μl canola oil and/or PIC (stabilized with poly-L-lysine and carboxymethycellulose, provided by the late Dr. H. Levy, NIH), at a dose of 2 μg i.p. on the first day of the experiment (day 0). From days 1–10 after immunization, the mice were fed the same dose of RA or oil daily. Blood was collected 7 and 10 days after priming for antibody quantification. On day 11, the mice were re-immunized with TT and co-treated with same doses of RA and/or PIC as on day 0. After 24 h, spleens were collected for later analysis. For the second study (3-day study), mice were immunized with TT and co-treated with RA and/or PIC as described for the first study, except that blood and spleen were collected 3 days after priming. For the third study (secondary anti-TT antibody response), mice were immunized with TT ± RA (37.5 μg, orally) and/or PIC (2 μg, i.p.) on day 0, followed by an additional 6-day treatment with RA or oil. Five weeks after priming, without further treatment with either RA or PIC, mice
were re-immunized with TT and blood and spleen were collected 7 days later. Throughout the studies, the treatment groups did not differ in body weight, and liver and spleen weights did not differ at the end of study.

**Serum anti-TT antibody analysis**

Serum anti-TT IgM, total anti-TT IgG, and anti-TT IgG isotypes were quantified by ELISA as previously described (225). Serum was serially diluted to assure measurements were in a linear dose-response range. A standard of serially-diluted pooled immune serum was included on every plate and titers of antibody were calculated based on this standard curve, in which 1 unit was defined as the dilution fold that produced 50% of maximal optical density for the standard sample.

**Spleen cell isolation**

Spleen cells were isolated as reported previously (25). Briefly, spleen tissues were gently mashed with sterile screen wire mesh, and the resulting cell suspensions were layered over Ficoll-Hypaque (1.083 g/ml, Sigma, St. Louis, MO) followed by centrifuged at 2500 rpm for 20 min at 20°C. The mononuclear cells were carefully collected, washed twice and suspended in RPMI-1640 medium (Invitrogen, Carlsbad, CA) with 10% FBS.

**Enumeration of anti-TT antibody-secreting cells (ASCs)**

TT-specific ASCs were assessed by ELISPOT assay as previously described (226). Spleen cell suspensions (10^6 cells/ml) from individual mice were serially diluted and incubated in TT-precoated filtration plates (Multiscreen HA, Millipore Corporation,
Bedford MA) at 37°C for 4-5 h. The plates were then washed and incubated with alkaline phosphatase (AP)-conjugated goat anti-mouse antibodies (Binding Site, San Diego CA) at 4°C, overnight, followed by reaction with AP-substrate solution (Bio-Rad, Hercules CA). The developed spots were counted under a dissection microscope and calculated as number of spots per 10⁶ cells.

**Flow cytometry**

Splenic mononuclear cells were suspended in RPMI-1640 medium (Invitrogen, Carlsbad, CA) containing 1% FBS and incubated with combinations of appropriately diluted fluorochrome-conjugated monoclonal antibodies (BD PharMingen, San Diego, CA) at room temperature for 40 min. For T-cell population, the cells were double stained with PE-CD4 (H129.19) and FITC-CD8 (53-6.7). For B-cell population, the cells were stained with PE-B220 (RA3.6B2). For NK/NKT-cell populations, the cells were double stained with FITC-CD3 (17A2) and PE-NK1.1 (PK136). For APCs, the cells were double stained with PE-CD11b (M1/70) and FITC-CD80 (16-10A1) or FITC-CD86 (GL1). The cells were then washed, fixed with 1% paraformaldehyde in PBS, and analyzed on a Coulter XL-MLC flow cytometer (185).

**Reverse transcription-polymerase chain reaction (RT-PCR)**

One μg of total RNA were subjected to reverse transcriptase reaction (Promega, Madison, WI), followed by ³³P-labeled PCR as described previously (227). Sense and anti-sense primers were as reported previously (185,215) or were as follows: IL-4 (NM 021283) 5’-AGAGCTATTGATGGTGCTCA, 3’-GGCTTTCAAGGAAGTCTTTC, 401bp; T-bet (AF241242) 5’-GATCGTCCTGCAGTCTTCC, TCTCC,
The PCR products were separated on a non-denaturing polysacrylamide gel and then exposed to X-ray film. For quantification, individual bands were excised and counted in liquid scintillation fluid (SciintiVerse, Fisher, Pittsburgh, PA), and normalized with 18S or GAPDH mRNA that was similarly quantified.

**Statistical analysis**

Data are reported as mean ± SE. The main effects of RA, PIC, and the interaction of RA and PIC were evaluated by two-way ANOVA. Differences among groups were determined using Fisher’s protected least significant difference (LSD) test (SuperAnova, Abacus Software, Berkeley CA). When group variances were unequal, data were subjected to log₁₀ or square-root transformation before statistical analysis. Simple linear regression was determined using the same software. A value of $P < 0.05$ was considered statistically significant.
RESULTS

Primary anti-TT antibody production is enhanced by RA and PIC

Primary anti-TT IgM and IgG production was determined 7 and 10 days after priming, respectively. IgM was elevated only by PIC alone and RA+PIC, each about four-fold (Fig. 2-1a). Whereas RA and PIC alone significantly increased IgG, the combination of RA+PIC increased anti-TT IgG levels >80-fold compared to the response of normal control mice (Fig. 2-1b). Surprisingly, RA and PIC differentially regulated anti-TT IgG isotypes (Fig. 2-1c to e). RA alone selectively increased IgG1 and IgG2b, and elevated the IgG1/IgG2a ratio, an indicator of type 1/type 2 balance. PIC alone strongly increased all anti-TT IgG isotypes (IgG1, IgG2a, and IgG2b), while the IgG1/IgG2a ratio was not different from control. Compared with PIC alone, RA+PIC more potently increased ant-TT IgG1, but attenuated the induction of IgG2a, thereby maintaining the IgG1/IgG2a ratio similar to control group (Fig. 2-1f). Two-way ANOVA confirmed that RA was a positive regulator for IgG1 and IgG2b but a negative regulator for IgG2a, whereas PIC was a positive regulator for all of the IgG isotypes (Fig. 2-1c to e). Hence, RA and PIC cooperatively promoted a robust primary anti-TT IgG response but differentially regulated IgG isotypes in vitamin A-sufficient mice.
**A**

RA: NS  
PIC: $P = 0.001$  
RA x PIC: NS

**B**

RA: $P = 0.001$  
PIC: $P < 0.001$  
RA x PIC: $P = 0.05$

**C**

RA: $P = 0.0001$  
PIC: $P = 0.0001$  
RA x PIC: $P < 0.01$

**D**

RA: $P = 0.05$  
PIC: $P = 0.01$  
RA x PIC: $P < 0.05$

**E**

RA: $< 0.01$  
PIC: $P = 0.001$  
RA x PIC: $P < 0.01$

**F**

RA: $< 0.001$  
PIC: $P < 0.05$  
RA x PIC: $P < 0.05$
FIGURE 2-1. RA and PIC synergistically enhance primary anti-TT IgG but differentially regulate IgG isotypes. Six-week old mice were immunized with TT ± RA and/or PIC, followed by an additional 10-d treatment with RA or oil (control). Plasma anti-TT IgM (a) and IgG (b) including IgG isotypes (c,d,e) were measured by ELISA on day 7 (IgM) and day 10 (IgGs) after priming. The antibody titers were calculated based on a standard curve, in which one unit was defined as the dilution fold that gave 50% of OD_{max}. Bars show mean ± SE, n = 12-16 mice/group. Different letters above bars within panels indicate significant differences (P < 0.05, a < b < c). Results of two-way ANOVA for each factor (RA, PIC, and interaction) are also shown in each panel.
Type 1/ type 2 cytokines and Th1/Th2-related gene expression are regulated by RA and PIC

Having observed that RA and PIC differentially regulated anti-TT IgG isotypes, we next asked whether type 1/ type 2 cytokines, the key regulators of Ig isotype switching, were also regulated. The effects of RA and PIC on type 1/type 2 cytokine mRNA levels were evaluated in two independent studies: an 11-day study with re-immunization of TT with and without RA, PIC or RA+PIC 24 hr before spleens were collected, and a 3-day study to examine effects in the early stage of priming. On day 11, RA alone selectively reduced the mRNA expression of type 1 cytokine genes (IFN-γ and IL-12, Fig. 2-2a and b), and elevated the ratio of IL-4 to IFN-γ (IL4/IFN-γ), a commonly used index of the balance of type 1/type 2 responses (Fig. 2-2a, b and e). PIC alone significantly enhanced both type 1 cytokines (IFN-γ and IL-12) and type 2 cytokines (IL-4 and IL-10, Fig. 2-2c and d) without altering the ratio of IL4 to IFN-γ compared to the ratio in the control group. The combination of RA+PIC abrogated the induction of IFN-γ and IL-12 by PIC (RA+PIC <PIC), thereby elevating the ratio of IL-4 to IFN-γ (Fig. 2-2e). Moreover, RA and PIC also regulated several transcriptional factors and receptors involved in type 1/type 2 (Th1/Th2) responses. T-bet and GATA-3 are two major transcriptional factors essential for Th1 and Th2 differentiation, respectively (20). IRF-1 is such a factor involved in Th1 differentiation. IL-12Rβ2, a subunit of IL-12 receptor, is required for IL-12-induced Th1 differentiation (15,20). Overall, RA slightly suppressed Th1-related gene expression, while PIC alone enhanced both Th1 and Th2-related gene expression especially IRF-1, which was significantly
induced by PIC. The combination of RA+PIC tended to attenuate the induction of Th1-related genes by PIC (Table 2-1). Despite weaker regulation of Th1/Th2-related transcription factors than cytokines, RA was still a negative regulator for each of these Th1-related genes, as shown by two-say ANOVA, whereas PIC was a positive regulator for both Th1-related and Th2-related genes, patterns consistent with the regulation of type 1/type 2 cytokines.

Regression analysis indicated that anti-TT IgG1 titers were positively correlated with IL-4 \((r = 0.47, P < 0.01)\) and IL-10 \((r = 0.39, P < 0.05)\), while anti-TT IgG2a titers were highly correlated with the level of IL-12 \((r = 0.60, P < 0.01)\). Interestingly, IgG2b titer was correlated with both IL-4 \((r = 0.43, P < 0.05)\) and IL-12 \((r = 0.58, P < 0.01)\). These data imply that RA and/or PIC in vivo differentially modulated type 1/type 2 responses, which in turn contributed to the regulation of anti-TT antibody response and IgG isotypes (Table 2-2).

To further determine if RA and PIC could regulate type 1/ type 2 cytokines in the early stage of response to immunization (prior to primary antibody response and before or during Ig isotype switching), we also measured IL-4 and IFN-\(\gamma\) mRNA levels on day 3 after priming (Fig. 2-3). PIC alone selectively induced IFN-\(\gamma\) mRNA (Fig. 2-3a), whereas RA alone only slightly enhanced IL-4 mRNA (Fig. 2-3b). The combination of RA+PIC significantly increased IL-4 while it abrogated the induction of IFN-\(\gamma\). In consequence, the ratio of IL-4: IFN-\(\gamma\) was significantly increased in mice that received RA+PIC compared to PIC alone (Fig. 2-3c). Two-way ANOVA confirmed that PIC was a positive regulator for IFN-\(\gamma\), and RA was a positive regulator for IL-4.
but a negative regulator for IFN-γ. Thus, RA and PIC were able to manipulate the expression of type 1/type 2 cytokine genes in the early stage of the response to immunization.
FIGURE 2-2. RA and PIC differentially regulate mRNA levels of type 1/type 2 cytokines. Six-week old mice were immunized TT ± RA and/or PIC, followed by additional 10-day treatment with RA or oil. On day 11 after priming, the mice were re-challenged with TT ± RA and/or PIC. Spleens were collected 24 hr later, and RNA was extracted. The mRNA levels of IL-4 (a), IL-10 (b), IL-12 (c), and IFN-γ (d) were quantified by $^{33}$P-labeled PCR, normalized, and then shown as fold induction relative to control. Bars shown are means ± SE, $n = 12-16/\text{group}$. Bands from two representative mice per group are shown for illustration below the group means. Different letters above bars within panels indicate significant differences ($P < 0.05, a < b < c$). Results of two-way ANOVA for each factor (RA, PIC, and interaction) are also shown in each panel.
TABLE 2-1. Regulation of Th1/Th2-related genes by RA, PIC, and RA/PIC in combination

<table>
<thead>
<tr>
<th></th>
<th>T-bet (mRNA fold induction)</th>
<th>IRF-1 (mRNA fold induction)</th>
<th>IL-12Rbeta-2 (mRNA fold induction)</th>
<th>GATA-3 (mRNA fold induction)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>1.00 ± 0.30&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.00 ± 0.33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.00 ± 0.38&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.00 ± 0.26&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>RA</strong></td>
<td>0.67 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.66 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.45 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.87 ± 0.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>PIC</strong></td>
<td>1.20 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.14 ± 1.24&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.43 ± 0.65&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.20 ± 0.25&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>RA/PIC</strong></td>
<td>0.97 ± 0.46&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>1.34 ± 0.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.06 ± 0.69&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.24 ± 0.43&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

**Two-way ANOVA<sup>2</sup>:**

<table>
<thead>
<tr>
<th></th>
<th><strong>RA</strong></th>
<th><strong>PIC</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P</em> &lt; 0.05 (-)</td>
<td><em>P</em> &lt; 0.01 (-)</td>
</tr>
<tr>
<td><strong>RA</strong></td>
<td><em>P</em> = 0.06 (+)</td>
<td><em>P</em> &lt; 0.05 (+)</td>
</tr>
</tbody>
</table>

1 Mice were treated as described in Fig. 2-2. The mRNA levels of genes in spleen tissues were quantified by <sup>33</sup>P-labeled PCR. Data were normalized with 18S rRNA and shown as fold induction relative to the control group. Values are mean ± SE, *n* = 8/ group. Different superscript letters in a column indicate significant differences (*P* < 0.05, a < b).

2 The effect of each factor (RA, PIC, and interaction) was evaluated by two-way ANOVA. (+) and (-) indicate positive and negative regulation, respectively. There were no significant interactions between RA and PIC.
TABLE 2-2. The correlation between anti-TT antibody isotypes and the mRNA levels of cytokines as well as Th1/Th2-specific transcription factors

<table>
<thead>
<tr>
<th></th>
<th>IgG1</th>
<th>IgG2a</th>
<th>IgG2b</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>0.469**</td>
<td>0.272</td>
<td>0.427*</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.393*</td>
<td>0.069</td>
<td>0.159</td>
</tr>
<tr>
<td>IFN</td>
<td>0.321</td>
<td>0.237</td>
<td>0.239</td>
</tr>
<tr>
<td>IL-12</td>
<td>0.049</td>
<td>0.599**</td>
<td>0.582**</td>
</tr>
<tr>
<td>T-bet</td>
<td>0.232</td>
<td>0.125</td>
<td>0.185</td>
</tr>
<tr>
<td>GATA-3</td>
<td>0.480**</td>
<td>0.09</td>
<td>0.116</td>
</tr>
</tbody>
</table>

1 Mice were treated as described in Table 2-1. The correlation between the titers of anti-TT antibody isotypes and the mRNA levels of cytokines as well as Th1/Th2-specific transcription factors was assessed by simple regression analysis. *, P< 0.05; **, P< 0.01.
FIGURE 2-3. RA and PIC significantly regulate mRNA levels of IL-4 and IFN-γ 3 days after priming. Six-week old mice were immunized TT ± RA and/or PIC, followed by additional 2-day treatment of RA or oil. Spleens were collected on day 3 after priming, and RNA was extracted. The mRNA levels of IL-4 (a) and IFN-γ (b) were quantified by $^{33}$P-labeled PCR, normalized, and then shown as fold induction relative to control. Bars shown are means ± SEM, $n = 12-16$ / group. Bands from two representative mice per group are shown for illustration. Different letters above bars within panels indicate significant differences ($P < 0.05$, a < b < c). Results of two-way ANOVA for each factor (RA, PIC, and interaction) are also shown in each panel.
Lymphocyte populations and antigen-presenting cell differentiation are regulated by RA and PIC

T cells, B cells, antigen-presenting cells (APCs), and NK/NKT cells are directly or indirectly involved in the regulation of thymus-dependent antibody production. Therefore, we wanted to determine if RA and PIC modulate these cell types. The 3-day study showed that T- and B-cell populations were not affected by the treatments except for CD8\(^+\) T cells, which were significantly reduced by PIC (Table 2-3). RA and PIC differentially regulated NK and NKT-cell populations. The proportion of NK cells (CD3\(^-\)NK1.1\(^+\)) was increased by PIC alone, whereas the proportion of NKT cells (CD3\(^+\)NK1.1\(^+\)) was increased by RA. Two-way ANOVA confirmed that RA was a positive regulator for the NKT-cell population, and PIC was a positive regulator for the NK-cell population (Table 2-3).

Moreover, RA and PIC regulated populations and differentiation of APCs. We evaluated markers associated with three types of professional APC: dendritic cells (DC, CD11c\(^+\)), macrophages (CD11b\(^+\)), and B cells (B220\(^+\)). Treatment with RA and/or PIC for 3 days did not significantly affect B-cell and DC populations (Table 2-3 and data not shown); however, RA and RA+PIC up-regulated CD11b, a major marker of macrophages (Table 2-4). Regarding costimulatory molecules, the treatments did not affect MHC II and CD40 (data not shown). However, RA and RA+PIC induced the expression of the costimulatory molecule CD80 (B7-1), whereas PIC alone induced the expression of CD86 (B7-2). Two-way ANOVA indicated that RA was a positive
regulator for CD80 but a negative regulator for CD86; while PIC was a positive regulator for CD86. Interestingly, RA and RA+PIC together up-regulated CD80 expression on CD11b^{+} cells, whereas PIC induced CD86 on CD11b^{-} cells (Table 2-4). These data indicated that RA and PIC affected different cell types and regulated the expression of CD80/CD86 differentially.

Simple regression showed that the ratio of IL4 to IFN-\gamma, described above, was significantly correlated with both the ratio of NKT to NK cells (Fig. 2-4a) and the ratio of CD80 to CD86 (Fig. 2-4b). These results imply a possible functional association between the balance of NK/NKT cells, CD80/CD86 molecules, and type 1/ type 2 cytokine responses.
TABLE 2-3. Regulation of T-cell and NK/NKT-cell populations by retinoic acid (RA), polyriboinosinic:polyribocytidylic acid (PIC) and RA/PIC in combination

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RA</th>
<th>PIC</th>
<th>RA+PIC</th>
<th>Two-way ANOVA²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RA (P)</td>
</tr>
<tr>
<td><strong>T-cell population</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4⁺ (%)</td>
<td>14.21 ± 1.40</td>
<td>14.82 ± 1.68</td>
<td>11.14 ± 1.30</td>
<td>13.03 ± 1.06</td>
<td>NS</td>
</tr>
<tr>
<td>CD8⁺ (%)</td>
<td>8.28 ± 1.27b</td>
<td>7.58 ± 1.24ab</td>
<td>5.02 ± 0.81a</td>
<td>5.30 ± 0.75a</td>
<td>NS</td>
</tr>
<tr>
<td>CD4⁺ to CD8⁺ ratio</td>
<td>1.85 ± 0.14a</td>
<td>2.11 ± 0.20ab</td>
<td>2.40 ± 0.21ab</td>
<td>2.72 ± 0.34b</td>
<td>NS</td>
</tr>
<tr>
<td><strong>B-cell population</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220⁺ (%)</td>
<td>74.20 ± 1.98</td>
<td>71.92 ± 2.97</td>
<td>77.61 ± 2.38</td>
<td>75.58 ± 1.92</td>
<td>NS</td>
</tr>
<tr>
<td><strong>NK/NKT-cell populations</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NK1.1⁺ (%)</td>
<td>5.27 ± 0.62a</td>
<td>6.37 ± 0.59ab</td>
<td>6.54 ± 0.75ab</td>
<td>7.40 ± 0.59b</td>
<td>NS</td>
</tr>
<tr>
<td>CD3⁻NK⁺ (%)</td>
<td>3.52 ± 0.45a</td>
<td>3.98 ± 0.46ab</td>
<td>5.06 ± 0.60b</td>
<td>4.75 ± 0.43ab</td>
<td>NS</td>
</tr>
<tr>
<td>CD3⁺NK⁺ (%)</td>
<td>1.20 ± 0.17ab</td>
<td>1.71 ± 0.12c</td>
<td>0.99 ± 0.15a</td>
<td>1.54 ± 0.11b</td>
<td>&lt; 0.01 (+)</td>
</tr>
<tr>
<td>NKT to NK ratio</td>
<td>0.34 ± 0.03bc</td>
<td>0.43 ± 0.03c</td>
<td>0.20 ± 0.03a</td>
<td>0.34 ± 0.03b</td>
<td>&lt; 0.01 (+)</td>
</tr>
</tbody>
</table>
Six-week old mice were immunized with TT, and co-treated with RA and/or PIC. On day 3 after priming, spleen cells were isolated and stained with fluochrome-conjugated antibodies as described in Materials and Methods. Values are mean ± SE, n = 8/group. Different superscript letters within a row indicate significant differences (P < 0.05, a < b < c).

The effect of each factor (RA, PIC, and interaction) was evaluated by two-way ANOVA. (+) and (-) indicate positive and negative regulation, respectively. There were no significant interactions between RA and PIC.
**TABLE 2-4. Expression and distribution of CD80/CD86 molecules on splenic lymphocytes**

1 Mice were treated as described in Table 2-3. Values are mean ± SE, \( n = 8/\) group. Different superscript letters in a row indicate significant differences (\( P < 0.05, a < b < c \)).

2 The effect of each factor (RA, PIC, and interaction) was evaluated by two-way ANOVA. (+) and (-) indicate positive and negative regulation, respectively. There were no significant interactions between RA and PIC.

3 FI (total fluorescence intensity) = % of positive cells \( \times \) median fluorescence intensity.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>RA</th>
<th>PIC</th>
<th>RA+PIC</th>
<th>Two-way ANOVA²</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RA (P)</td>
<td>PIC (P)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD11b⁺ (FI)³</td>
<td>639.93 ± 58.39⁻</td>
<td>927.53 ± 80.56⁻</td>
<td>636.90 ± 64.65⁻</td>
<td>915.62 ± 91.02⁻</td>
<td>0.01 (+)</td>
</tr>
<tr>
<td>CD80⁺ (%)</td>
<td>10.49 ± 0.84⁻ab</td>
<td>12.75 ± 0.91⁻c</td>
<td>9.93 ± 0.42⁻a</td>
<td>12.91 ± 0.71⁻c</td>
<td>&lt; 0.01 (+)</td>
</tr>
<tr>
<td>CD80⁺CD11b⁻ (%)</td>
<td>2.79 ± 0.27</td>
<td>3.19 ± 0.13</td>
<td>3.09 ± 0.17</td>
<td>3.01 ± 0.16</td>
<td>NS</td>
</tr>
<tr>
<td>CD80⁺CD11b⁺ (%)</td>
<td>3.65 ± 0.39⁻a</td>
<td>4.53 ± 0.45⁻ab</td>
<td>3.75 ± 0.20⁻ab</td>
<td>4.80 ± 0.41⁻b</td>
<td>&lt; 0.05 (+)</td>
</tr>
<tr>
<td>CD86⁺ (%)</td>
<td>6.25 ± 0.32⁻a</td>
<td>5.62 ± 0.24⁻a</td>
<td>8.30 ± 0.59⁻b</td>
<td>6.86 ± 0.53⁻a</td>
<td>&lt; 0.05 (-)</td>
</tr>
<tr>
<td>CD86⁺CD11b⁻ (%)</td>
<td>2.05 ± 0.17⁻ab</td>
<td>1.19 ± 0.14⁻a</td>
<td>4.59 ± 0.77⁻c</td>
<td>2.62 ± 0.44⁻b</td>
<td>&lt; 0.01 (-)</td>
</tr>
<tr>
<td>CD86⁺CD11b⁺ (%)</td>
<td>1.54 ± 0.23</td>
<td>1.52 ± 0.10</td>
<td>1.61 ± 0.13</td>
<td>1.54 ± 0.15</td>
<td>NS</td>
</tr>
<tr>
<td>CD80 to CD86 ratio</td>
<td>1.67 ± 0.08⁻b</td>
<td>2.26 ± 0.11⁻c</td>
<td>1.25 ± 0.10⁻a</td>
<td>1.89 ± 0.14⁻b</td>
<td>0.0001 (+)</td>
</tr>
</tbody>
</table>
FIGURE 2-4. The ratio of IL4/IFN-γ is significantly associated with the ratio of NK/NKT cells, and the ratio of CD80/CD86 costimulatory molecules. Mice were treated as described in Table 2-3. The relationship between ratio of IL4/IFN-γ and (a) ratio of NK/NKT cells and (b) ratio of CD80/CD86 was assessed by simple regression analysis.
Secondary anti-TT antibody production is enhanced by RA and/ or PIC

Next, we determined if the strong effects of RA and PIC on anti-TT antibody response observed in the primary response were durable, as would be crucial for a vaccine adjuvant. As for the primary response, RA and/or PIC given only with priming significantly enhanced secondary anti-TT IgG production (Fig. 2-5). Regarding anti-TT IgG isotypes, RA selectively enhanced IgG1 and IgG2b. Although PIC robustly increased all anti-TT IgG isotypes, it significantly reduced the IgG1 to IgG2a ratio as compared to the control group. The combination of RA+PIC stimulated the highest levels of IgG and IgG1, but suppressed induction of IgG2a by PIC, which in turn partially restored the ratio of IgG1 to IgG2a towards the control level (Fig. 2-5e).

Finally, the effects of RA and PIC on the secondary antibody response were further determined by measuring anti-TT antibody-secreting cells (ASCs). Consistent with the plasma antibody response, RA and PIC also differentially regulated splenic ASCs (Table 2-5). The number of ASCs was highly correlated with the corresponding plasma anti-TT IgG isotypes (IgG1: $r = 0.727$, $P < 0.0001$; IgG2a: $r = 0.804$, $P < 0.0001$; IgG2b: $r = 0.73$, $P < 0.0001$).
FIGURE 2-5. RA and/or PIC treatments given with the primary immunization enhance secondary anti-TT antibody IgG responses. Six-week old mice were immunized TT ± RA and/or PIC, followed by additional 6-day treatment of RA or oil. Five weeks later, the mice were reimmunized with TT only. Secondary anti-TT IgG (a) and IgG isotypes (b-e) in plasma were measured by ELISA day 7 days after reimmunization. Different letters above bars within panels indicate significant differences ($P < 0.05$, a < b < c). Results of two-way ANOVA for each factor (RA, PIC, and interaction) are also shown in each panel.
Table 2-5. ELISPOT assay of splenic anti-TT antibody-secreting cells (ASC)\(^1\)

<table>
<thead>
<tr>
<th></th>
<th>IgG1 (ASC/10(^6) cells)</th>
<th>IgG2a (ASC/10(^6) cells)</th>
<th>IgG2b (ASC/10(^6) cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51.8 ± 15.2(^a)</td>
<td>0.4 ± 0.4(^a)</td>
<td>2.8 ± 1.9(^a)</td>
</tr>
<tr>
<td>RA</td>
<td>79.1 ± 9.1(^{ab})</td>
<td>1.3 ± 0.6(^a)</td>
<td>6.3 ± 1.8(^a)</td>
</tr>
<tr>
<td>PIC</td>
<td>247.4 ± 84.4(^{bc})</td>
<td>30.6 ± 9.9(^c)</td>
<td>32.4 ± 7.2(^b)</td>
</tr>
<tr>
<td>RA/PIC</td>
<td>396.8 ± 116.7(^c)</td>
<td>12.5 ± 4.4(^b)</td>
<td>26.1 ± 7.1(^b)</td>
</tr>
</tbody>
</table>

1 Mice were treated as described in Figure 5. On day 7 after reimmunization, spleen cells were isolated and anti-TT ASCs were detected by ELISPOT assay. Values are mean ± SE, n = 8/group. Different superscript letters in a column indicate significant differences (\(P < 0.05\), \(a < b < c\)).
DISCUSSION

The combination of RA and IFNs has been demonstrated to be an effective strategy for cancer chemoprevention and chemotherapy (228). However, the immunoregulatory effect of RA and IFNs combined has not yet been well characterized. DeCicco et al. (13,215) reported that RA and PIC synergistically enhanced both primary and secondary anti-TT antibody responses in vitamin A-deficient rats, suggesting that RA and IFNs can interactively promote immune functions. However, whether RA and IFNs, especially in combination, can effectively modulate primary immune responses and promote long-term immunity in the healthy state had not been studied. Therefore we evaluated the immunoregulatory effects of RA, PIC, and their combination in a model of normal immunocompetent mice. Several new findings resulted from these studies.

Firstly, RA and/ or PIC, which were given at priming, modulated important immune regulators within a few days of immunization. On day 3 after priming, RA and PIC differentially regulated mRNA levels of IFN-γ and IL-4, the “signature” cytokines of type 1 and type 2 immune responses, respectively. Previous studies showed that PIC selectively induced type 1 cytokines, such as IFN-γ and IL-12, while suppressing type 2 cytokines, such as IL-4 and IL-5 (206,207). In contrast, RA suppressed Th1 cytokines but enhanced Th2 cytokines in both vitamin A-deficient animals and in vitro culture (194,229). In the present study PIC significantly induced IFN-γ mRNA 3 days after priming. RA, on the other hand, was a positive, albeit modest, regulator for IL-4 but a
negative regulator for IFN-γ. In consequence, RA was a strong positive regulator of the ratio of IL-4 to IFN-γ gene expression. Therefore, RA, PIC and their combination had already shaped the developing type 1/type 2 response within 3 days of immunization and treatment.

Retinoic acid and PIC also differentially regulated NK/NKT-cell populations by 3 days after treatment and priming. NK cells are considered as an early source of IFN-γ (230), while NKT cells have been shown to secrete IL-4 and IL-10 and promote a Th2 (type 2) response (67,70). In the present study, RA was a positive regulator for NKT cells, whereas PIC was a positive regulator for NK cells. The ratio of NKT cells to NK cells was positively correlated with the ratio of IL-4 to IFN-γ mRNAs, implying that RA and PIC treatments might regulate type 1/type 2 cytokines and anti-TT antibody response through modulating NK/NKT cell populations.

Furthermore, RA and PIC significantly affected APC characteristics within 3 days of immunization. PIC has been shown to induce dendritic cell maturation and expression of CD80 and CD86 (208). Several studies suggested that RA could regulate immune response by targeting APCs (195,196). In the present study, RA and RA+PIC significantly induced expression of CD11b, consistent with our previous results in the human monocytic cell line THP-1 (227). Moreover, RA and PIC differentially regulated the expression of CD80 and CD86, the major co-stimulatory molecules for T-cell activation. The regulation of CD80 and CD86 molecules were associated mostly with CD11b+ cells and CD11b- cells, respectively, suggesting the interesting and unexpected finding that these co-stimulatory molecules can be modulated individually. Although
APC function was not assessed in the present study, the differences observed in cell markers imply that RA and PIC can modulate APC function, thereby affecting T-cell activation as well as downstream antibody responses. Notably, the ratio of CD80/CD86 was positively correlated with the ratio of IL-4/IFN-γ. The differential regulation of CD80 and CD86 by RA and PIC is not readily explained at this time because the possibly distinct functions of CD80 and CD86 in type 1/type 2 responses have not yet been clarified. Kuchroo et al. (231) suggested that CD80 and CD86 are involved in the generation of Th1 and Th2 responses, respectively. Lang et al. (232) observed that CD86 was essential for both Th1 and Th2 responses, while CD80 provided a negative signal for Th1 response. Despite the present uncertainty about the individual roles of CD80 and CD86, the significant positive correlation observed between the ratio of IL-4 to IFN-γ and the ratio of CD80 to CD86 suggests that the early regulation of CD80 and CD86 molecules by RA/PIC might make a significant contribution to the ability of these treatments to rapidly modulate type 1/type2 cytokines.

Later, by day 10-12 after priming, the effects of RA and PIC on immune function became more dramatic as it was evident that RA and/or PIC robustly promoted the primary anti-TT IgG response. Compared to the control level of anti-TT IgG produced by normal mice, treated mice produced levels that were up to 80-fold higher. Moreover, RA and PIC differentially regulated anti-TT IgG isotypes. RA alone shifted anti-TT IgG production towards IgG1 and therefore elevated the ratio of IgG1/IgG2a. On the other hand, PIC strongly boosted all of the IgG isotypes, without changing the ratio of IgG1 to IgG2a titers. Surprisingly, whereas RA+PIC
synergistically enhanced IgG1, this combination attenuated IgG2a production as compared to PIC alone. Therefore, RA+PIC not only potently stimulated total anti-TT IgG production, but also kept the balance of IgG1/ IgG2a antibodies similar to that in control mice.

At the same time, the regulation of type 1/type 2 cytokines by RA and PIC was also very significant. PIC, which was expected to induce type 1 cytokines, significantly induced both type 1 and type 2 cytokines as well as Th1/Th2-related genes. The enhancement of type 2 cytokines by PIC was probably due to its ability to induce IFN-β (10), which has been shown to reduce type 1 cytokines (e.g., IL-12) but increase type 2/regulatory cytokines (e.g., IL-10) as observed in treatment of multiple sclerosis (233). Thus, PIC appears to be a relatively indiscriminant but potent inducer of immune responses, with very broad inducing effects on both type 1 and type 2 immunity. The enhancement of type 1/type 2 cytokines by PIC was well correlated with the increased production of all anti-TT IgG isotypes. Oppositely, RA inhibited type 1 cytokines and Th1-related genes, confirming previous reports (194,229). Interestingly, this inhibition occurred despite presence of PIC and was strongly correlated with the attenuation of IgG2a, suggesting that RA could abolish part of PIC-induced IgG2a production by suppressing type 1 cytokine expression. Although RA did not significantly induce type 2 cytokines, it consistently suppressed type1 cytokines and therefore skewed the balance in the type 2 direction, which likely enhanced the production of anti-TT IgG1. Nevertheless, RA combined with PIC manipulated type 1/type 2 cytokine expression,
which in turn contributed to the enhancement of anti-TT antibody response and directed Ig isotype switching towards a nearly normal balance.

Because a strong memory response is a hallmark of successful vaccination, it was important to determine if the immunoregulatory effects of RA and PIC were durable. Indeed, providing RA and PIC (only at the time of priming) greatly enhanced the secondary anti-TT IgG response. Consistent with increased plasma Ig isotypes, RA and PIC also up-regulated the number of splenic anti-TT ASCs. These data provided insight that RA and PIC enhanced secondary anti-TT IgG responses by regulating the clonal expansion of memory B cells and the differentiation of B cells into effector ASCs.

Formulation of vaccines with potent adjuvants is an important approach for improving vaccine efficiency. When incorporated into vaccines, adjuvants can accelerate, prolong, or enhance the quality of specific immune response to vaccine antigens. In the past decades, many adjuvants have been developed and tested, however, few of them are used for human vaccines because of potential toxicity and adverse effects (234). Therefore, developing effective adjuvants for human vaccines remains a challenge for the vaccine industry. The present study has demonstrated that RA and/or PIC treatments can robustly and durably enhance anti-TT antibody response in vitamin A-sufficient mice, suggesting that a simple nutritional intervention, RA, coupled with PIC can effectively improve vaccine performance. This outcome appears to involve multiple mechanisms, including early regulation of NK/NKT-cell and APC populations and shaping of type 1/type 2 cytokine gene expression. Compared with RA or PIC alone,
RA+PIC not only was more potent in increasing the TT-specific IgG response but also maintained the balance of IgG1/IgG2a. Thus, RA+PIC may serve as a promising strategy for increasing vaccine efficiency in not only vitamin A deficient but also in healthy populations.
CHAPTER 3

The anti-tetanus vaccination response of neonatal mice is augmented by all-trans retinoic acid combined with polyriboinosinic: polyribocytidylic acid
ABSTRACT

Neonates are highly susceptible to infectious diseases and, in general, respond poorly to conventional vaccines due to immaturity of the immune system. In the present study, we hypothesized that the anti-tetanus toxoid (TT) vaccine response of neonatal mice to could be enhanced by retinoic acid (RA), a bioactive retinoid, and polyriboinosinic acid:polyribocytidylic acid (PIC), an inducer of interferons (IFN). Early-life treatments with RA and/or PIC were well tolerated, and stimulated both primary anti-TT IgG production in infancy and the memory response in adulthood. TT-specific lymphocyte proliferation and type 1/ type 2 cytokine production were also significantly augmented. In addition, RA and PIC modulated the maturation and/or differentiation of neonatal B cells, natural killer (NK)/NKT cells, and antigen-presenting cells. Whereas RA alone increased neonatal anti-TT antibody response, it selectively increased anti-TT IgG1 and IL-5, resulting in a skewed type 2 response. PIC, a potent adjuvant in adult mice, elevated neonatal anti-TT IgG as well as all IgG isotypes (IgG1, IgG2a, IgG2b), and induced TT-specific IFN-γ, an important type 1 cytokine; however, PIC alone failed to benefit the memory response. The combination of RA+PIC was more potent than either agent alone in elevating primary and secondary anti-TT IgG responses as well as IgG isotypes. Moreover, RA+PIC increased TT-specific IFN-γ and IL-5, suggesting the combination effectively promoted both type 1 and type 2 responses in neonatal mice. Thus, RA combined with PIC, a nutritional-immunological intervention, appears promising as an adjuvant for early-life vaccination.
INTRODUCTION

Infants and neonates are well known to be at high risk of infectious diseases. According to estimates by the World Health Organization (WHO), about 2.5 million infants between 1 and 12 months of age die each year of infectious diseases (110). The high susceptibility of infants and neonates to infections is mostly attributed to the relative immaturity of immune system, which may involve several aspects: First, compared with adults, the cellularity of the peripheral lymphoid organs is lower in neonates, and the microarchitecture of secondary lymphoid organs is not well developed (118,119). Secondly, neonatal immune cells are functionally immature as compared to similar cells from adults. The antibody response of B-cells to T-cell dependent (TD) and TI type-2 antigens is especially low in infants and children <2 y of age and in the young of comparable animals used as experimental models. The neonatal antibody response to TD antigens is characterized by weak Ig isotype switching (lack IgG2a), poor Ig affinity maturation, and restricted heterogeneity due to limited use of V region Ig genes (235). Moreover, T-helper (Th)-1 cell and cytotoxic T lymphocyte (CTL) responses are low in neonates, which are considered to be responsible for the increased susceptibility of neonates to intracellular pathogens (119). In addition, the limited immune response in neonates may also be due to an immaturity of antigen-presenting cells (APCs), as shown by a low expression of costimulatory molecules, impaired antigen-presenting ability, and reduced interleukin (IL)-12 production following infection or vaccination (119,236,237).
Currently, early-life vaccination is an important strategy for protecting neonates and infants from infectious diseases. In children >2 y and adults, conventional vaccines can generally induce sufficient production of specific protective antibodies to neutralize pathogens (or their toxins) and thereby protect the host from infections. However, the immaturity of the neonatal immune system significantly hampers the generation of a protective vaccine response (110). Hence, strategies to enhance vaccination efficiency in early life are highly sought. Several recent studies have reported promising results with strategies using certain adjuvants, such as IL-12 and CpG-containing oligonucleotides, can induce type-1 cytokine production as well as CTL responses and successfully increase antibody production in neonatal mice (151-153). However, in some of these strategies weight loss was observed (155), and the potential adverse effects of these treatments would appear to be limitations to their application in human neonates.

Vitamin A (VA) is known to play an important role in both innate and adaptive immunity. Vitamin A-deficient children suffer a significant increase mortality and infection diseases, particularly measles and diarrhea, are more severe (182,216). By providing VA supplements to VA-deficient children aged 6-72 months, all-cause mortality has been reduced by 23%, measles-related mortality by 50%, and diarrheal disease mortality by 33% (3). Therefore, periodic high-dose supplementation with VA is considered as a highly cost-effective approach to prevent VA deficiency and save children’s lives (4). To facilitate delivery of VA supplements to young children, WHO has recommended integrating VA supplementation as a part of the Expanded Program
of Immunization (EPI) in countries where VA deficiency is prevalent. For the 6- to 12-month old infants, 100,000 IU of VA (equivalent to 30 mg of retinol) is recommended to be given along with measles immunization (199), and for infants under 6 months of age, 25,000 IU of VA to be given along with diphtheria-pertussis-tetanus (DPT) vaccines (200). The integration of VA administration into early-life vaccination programs has been shown to be as a safe and effective way to improve the VA status of infants (200). Moreover, co-administration of VA with measles or DPT vaccination significantly elevated vaccine-induced antibody responses in infants, suggesting the potential benefit of VA on the vaccine response in early life (199,201,202).

Polyriboinosinic acid: polyribocytidylic acid (PIC) is a synthetic dsRNA, which triggers TLR3 and its downstream signaling molecules (213), activating both innate and adaptive immunity. PIC is well known for its ability to induce type I/ type II IFNs and enhance anti-viral and anti-tumor reactions in several models (10-12). Furthermore, PIC is an potent immune adjuvant, which can promote dendritic cell (DC) maturation, increase NK-mediated cytotoxicity, and enhance CD8 T cell response as well as Th1-cytokine production (206,208).

Previously, we reported that co-administration of RA and PIC with tetanus toxoid (TT) immunization cooperatively enhanced the anti-TT antibody response in vitamin A-adequate adult mice (13,238). Moreover, the combination of RA+PIC stimulated a robust, durable and balanced increase in all of the anti-TT IgG isotypes (IgG1, IgG2a, IgG2b). These results suggested to us that this combination might act as a promising strategy for enhancing the response to TT and similar vaccines in healthy
populations. However, whether RA and PIC, alone or in combination, can effectively
enhance antibody production in a neonatal vaccination model is still unknown.
Therefore, in the present study we sought to evaluate the adjuvant effects of RA, PIC,
and their combination on anti-TT vaccination response in neonatal mice.
MATERIALS AND METHODS

Animals

Animal protocols were approved by the Institutional Animal Use and Care Committee of the Pennsylvania State University. Adult C57BL/6 mice purchased from Charles River Laboratories (Wilmington, MA) were bred under specific pathogen-free conditions, and the date of birth was recorded. Pups remained with their mothers until weaning at the age of 4 weeks. During the experimental period, all the mice were fed with a nutritionally complete diet (LabDiet 5001 which contains vitamin A 22 IU/g, Purina Mills, St. Louis, MO).

Vaccine, adjuvants, and immunization procedures

Tetanus Toxoid (TT) was purchased from Connaught Laboratories (Swiftwater, PA). All-trans-RA (Sigma, St. Louis, MO) was dissolved in canola oil at 4 mg/ml. PIC, stabilized with poly-L-lysine and carboxymethylcellulose, was kindly provided by Dr. H. Levy in NIH. The doses of TT, all-trans RA, and PIC for neonatal mice were calculated by adjusting the doses of adult mice with metabolic body weight \( (BW^{0.75}) \) (238), which were approximately 3.5 µg, 12 µg, and 0.7 µg per pup, respectively. One-wk old mice were randomly divided into 4 groups: control, RA, PIC, RA+PIC. One day before primary immunization (day –1), the pups were fed with RA or canola oil only. On the next day (day 0), each pup was immunized with TT by intraperitoneal (i.p.) injection, and co-treated with RA orally and/or PIC by i.p. injection. From day 1–5 after priming, the pups were fed the same dose of RA or oil daily. To evaluate the
effects of RA and/or PIC treatments on neonatal lymphocyte populations, some spleens were collected 3 days after priming for flow cytometry. The rest of the pups remained with their mothers until weaning, and were re-immunized with TT (10 µg/mouse) at 6-wk old without further treatment with RA/PIC. Blood was collected 14 days after priming and 7 days after re-immunization for determination of primary and secondary antibody responses, respectively. Two weeks after re-immunization, spleens were collected for in vitro proliferation assay and cytokine response.

**Serum anti-TT antibody analysis**

Serum anti-TT IgG and anti-TT IgG isotypes were quantified by ELISA using serially diluted serum samples as previously described (225). Measurements in a linear dose-response range were compared to a standard of serially-diluted pooled immune serum, included on every ELISA plate, to calculate the titers of anti-TT IgG; 1 unit was defined as the dilution fold that produced 50% of the maximal optical density for the standard sample.

**Lymphocyte proliferation**

Spleen mononuclear cells were isolated as reported previously (185), and suspended at $5 \times 10^6$ cells/ml in RPMI-1640 with 10% FBS. To assess TT-induced cell proliferation, 96-well plates were coated with TT (2.5µg/ml) at 4°C overnight, followed by washing. Then $5 \times 10^5$ cells/well were added in triplicate and incubated in the presence of soluble TT (2.5 µg/ml) at 37°C for 96 hours. For comparison, cells were also incubated with plate-bound anti-mouse CD3 (145-2C11, BD PharMingen, San
Diego, CA) in 96-well plates at 37°C for 72 hours. Cell proliferation was determined by the incorporation of [methyl-3H]-thymidine (Amersham Biosciences, Piscataway, NJ) as described previously (185). The stimulation index (SI) was defined as the ratio of experimental cpm/ control cpm (without stimulation).

**Quantification of cytokine production**

To assess TT-specific cytokine production, spleen mononuclear cells (5×10^6 cells/ml) were cultured in TT-coated 24-well plates, and incubated with soluble TT (2.5 µg/ml) at 37°C for 96 hours. For anti-CD3-induced cytokine production, cells were also incubated in anti-CD3-bound 24-well plates at 37°C for 72 hours. Cytokines (IL-4, IL-5 and IFN-γ) in culture supernatants were detected by a sandwich ELISA according to the protocol from BD Biosciences. Purified anti-mouse IL-4 (11b11), IL-5 (TRFK4) and IFN-γ (R4–6A2) mAbs, as well as biotinylated anti-mouse IL-4 (BVD6-24G2), IL-5 (TRFK4) and IFN-γ (XMG1.2) mAbs were obtained from BD PharMingen (San Diego, CA). The values of cytokines were expressed by reference to a standard curve, which was established by assaying serial dilutions of the respective mouse cytokine standards (155).

**Flow cytometry**

Splenic mononuclear cells in RPMI-1640 medium containing 1% FBS were incubated with combinations of appropriately diluted fluorochrome-conjugated monoclonal antibodies (BD PharMingen) at room temperature for 40 min. For T-cell population analysis, the cells were double stained with PE-anti-CD4 (H129.19) and
FITC- anti-CD8 (53-6.7). B cells were stained with PE- anti-B220 (RA3.6B2) or FITC- anti-CD19 (1D3). For NK/NKT-cell populations, the cells were double stained with FITC- anti-CD3 (17A2) and PE- anti-NK1.1 (PK136). For APCs, the cells were double stained with PE- anti-CD11b (M1/70) and FITC- anti-CD80 (16-10A1) or FITC- anti-CD86 (GL1). The cells were then washed, fixed with 1% paraformaldehyde in PBS, and live-gated cells were detected on a Coulter XL-MLC flow cytometer. The results were analyzed with Flow-Jo software (Tree Star Inc., Ashland, OR).

**Statistical analysis**

Data are reported as mean ± SE. The main effects of RA, PIC, and the interaction of RA and PIC were evaluated by two-way ANOVA. Differences among groups were determined using Fisher’s protected least significant difference (LSD) test (SuperAnova, Abacus Software, Berkeley CA). When group variances were unequal, data were subjected to log$_{10}$ or square-root transformation before statistical analysis; a $P$-value less than 0.05 was considered statistically significant.
RESULTS AND DISCUSSION

Because we previously observed that the combination of RA, an active metabolite of VA, and PIC was well tolerated by adult mice and led to a robust and durably enhanced anti-TT antibody response (238), we considered it possible that this combination of nutritional-immunological intervention would also function as an adjuvant for neonatal vaccination.

Early-life treatments with RA and/or PIC augment both primary and secondary anti-TT IgG responses.

Early-life treatments with RA and/or PIC were well tolerated and did not affect the growth of neonates (Fig. 3-1). Although the primary anti-TT antibody response in neonatal mice was very weak, both RA and PIC alone significantly increased anti-TT IgG titers, and the combination of RA+PIC further increased it by 80% (Fig. 3-2A). Furthermore, RA and/ PIC enhanced IgG isotype switching in neonatal mice. RA alone selectively increased the titers of IgG1 (Fig. 3-2B), and elevated the ratio of IgG1/IgG2a (Fig. 3-2E), an index reflecting the balance of type 2 to type 1 immune response. PIC significantly enhanced all of the IgG isotypes (IgG1, IgG2a, and IgG2b) without affecting the IgG1/IgG2a ratio. Compared with PIC alone, the combination of RA+PIC further increased IgG1 and IgG2b, while still maintaining the IgG1/IgG2a ratio similar to the control level (Fig. 3-2B to E). Two-way ANOVA confirmed that RA was a positive regulator for IgG1, and PIC was a positive regulator for all of the IgG isotypes.
Hence, RA and PIC treatments significantly promoted the primary anti-TT antibody response and differentially regulated IgG isotypes in neonatal mice.

Of particular interest with respect to vaccination is the recall response to antigen. The co-administration of RA and/or PIC with TT immunization during the neonatal stage also significantly enhanced the secondary (recall) antibody response when the pups grew up till 6-7 weeks old. The secondary anti-TT IgG level was slightly enhanced by PIC alone but significantly up-regulated by RA and by RA+PIC (Fig. 3-3A). Similarly to the primary response, RA alone selectively induced secondary anti-TT IgG1 and elevated the ratio of IgG1/IgG2a (Fig. 3-3B and E). PIC alone selectively induced IgG2a and IgG2b without affecting the IgG1/IgG2a ratio (Fig. 3-3C and D). Notably, RA+PIC significantly increased all the IgG isotypes, while the ratio of IgG1/IgG2a was equivalent to that in the control group (Fig. 3-3). Overall, early treatment with RA and/or PIC successfully enhanced both the primary antibody response in neonates and their memory responses as young adults.
**FIGURE 3-1. RA and/or PIC treatments do not affect the growth of neonatal mice.**

One-wk old C57BL/6 mice were pretreated with RA or oil for one day, followed by TT immunization ± RA and/or PIC as described in *Material and Methods*. The body weight of neonatal mice was measured on the day of immunization, and 1 or 2 weeks after priming. Curves shown are means ± SE, \( n = 10-12 \) group. The neonatal body weight was not significantly different among all of the groups at each time point.
FIGURE 3-2. RA and/ or PIC treatments significantly enhance primary anti-TT antibody response in neonatal mice. One-wk old C57BL/6 mice were pretreated with RA or oil for one day, followed by TT immunization ± RA and/ or PIC as described in Material and Methods. Blood samples were collected at 2 weeks after priming. Anti-TT IgG and IgG isotypes were measured using ELISA. A, total IgG; B, IgG1, C, IgG2a; D, IgG2b; E, ratio of titers of IgG1 to IgG2a as indicator of type 2 to type 1 balance. Bars shown are means ± SE, \( n = 10-12/ \) group. Different letters above bars within panels indicate significant differences \( (P < 0.05, \ a < b < c) \). Results of two-way ANOVA for each factor (RA, PIC, and interaction) are also shown in each panel.
FIGURE 3-3. Co-administration of RA and/ or PIC with TT priming during the neonatal period significantly enhances anti-TT memory response in adulthood. One-wk old C57BL/6 mice were pretreated with RA or oil for one day, followed by TT immunization ± RA and/ or PIC as described in Material and Methods. Five weeks after priming, the mice were re-immunized with 10 μg of TT only. Blood samples were collected at 1 week after re-immunization. Anti-TT IgG and IgG isotypes were measured using ELISA. A, total IgG; B, IgG1; C, IgG2a; D, IgG2b; E, ratio of titers of IgG1 to IgG2a as indicator of type 2 to type 1 balance. Bars shown are means ± SE, n = 10-12/ group. Different letters above bars within panels indicate significant differences (P < 0.05, a < b < c). Results of two-way ANOVA for each factor (RA, PIC, and interaction) are also shown in each panel.
Early-life treatments with RA and/or PIC increase TT-specific lymphocyte proliferation and cytokine production.

Because TT-induced antibody production requires the involvement of active T helper cells and type 1/type 2 cytokines, we evaluated the effect of RA and PIC on lymphocyte proliferation and cytokine production. RA, PIC, and RA+PIC significantly doubled or tripled TT-induced lymphocyte proliferation (Fig. 3-4A). Moreover, RA and PIC significantly regulated TT-specific type 1/type 2 cytokines. Type 1/type 2 cytokines are the key regulators of Ig isotype switching. In the present study, we measured IFN-γ, a major “signature” of type 1 cytokine responses, and IL-4 and IL-5, two type 2 cytokines. Since TT-induced IL-4 was not significantly detectable, we focused on the regulation of IFN-γ and IL-5. TT-induced IL-5 was significantly increased by RA alone and by RA+PIC (Fig. 3-4B). TT-induced IFN-γ, on the other hand, was markedly elevated by PIC alone. Although the combination of RA+PIC slightly attenuated PIC-induced IFN-γ production, it still induced a significantly higher level of IFN-γ compared to the control group (Fig. 3-4C). Consequently, the ratio of IL-5/IFN-γ, an index of the balance of type 2 relative to type 1 response, was elevated about 2-fold by RA, while RA+PIC together rebalanced this ratio close to the control level (Fig. 3-4D). Therefore, RA and PIC promoted TT-specific type 1 and type 2 responses, respectively; while the combination of RA+PIC promoted both type 1 and type 2 responses, resulting in a high-level, balanced response.
Fig. 3-4. RA and/or PIC treatments significantly enhance TT-specific lymphocyte proliferation and production of IL-5 and IFN-γ. One-wk old C57BL/6 mice were pretreated with RA or oil for one day, followed by TT± RA and/or PIC as described in Material and Methods. Five weeks after priming, the mice were re-immunized with 10 μg of TT only. Two weeks after re-immunization, spleen cells were isolated and stimulated with TT for 96 hours in vitro. A, Lymphocyte proliferation determined by [methyl-3H]-thymidine incorporation. B and C, cytokines in supernatant detected by ELISA. D, ratio of IL5:IFN-γ as an indicator of type 2 to type 1 cytokine balance. Bars shown are means ± SE, n = 10/group. Different letters above bars within panels indicate significant differences (P < 0.05, a < b < c). Results of two-way ANOVA for each factor (RA, PIC, and interaction) are also shown in each panel.
It is interesting that anti-CD3 induced T-cell proliferation and cytokine production was similar regardless of prior RA/PIC treatments during the neonatal stage (Fig. 3-5). To confirm this result, spleen cells were also collected 14 days after neonatal priming, and stimulated with anti-CD3. Similarly, RA and/or PIC did not significantly affect anti-CD-3 induced T-cell proliferation and cytokine production (data not shown). These data implied that the immunoregulatory effects of RA/PIC were apparently antigen-specific, without affecting the general T-cell response to TCR/CD3 stimulation. These TT-responding cells showing regulation by RA and/or PIC represented a memory pool, which might be increased in number or antigen-responsiveness by RA and/or PIC treatments in the neonatal period, survived, and were than capable of responding to TT upon later re-stimulation. Anti-CD3, on the other hand, is expected to stimulate both naïve and memory CD3+ T cells, and apparently these cells or their progenitors were unaffected by treating neonates with RA and PIC. Currently, several agents, such as IL-12, CpG-containing oligonucleotides, and complete Freud’s adjuvant, have been shown to effectively boost vaccine responses in neonates (119,154,226). However, a major concern is that these agents might potentially lead to a state of heightened inflammation later on, increasing the risk of inflammation and autoimmune diseases (119). Although further studies are necessary, our current results suggest that the effects of RA and PIC could be focused on antigen-specific T cells, without having a long-lasting effect on the general population of T cells.
Fig. 3-5. RA and/ or PIC treatments do not significantly affect anti-CD3 induced lymphocyte proliferation and production of IL-5 and IFN-γ. One-wk old C57BL/6 mice were pretreated with RA or oil for one day, followed by TT± RA and/ or PIC as described in Material and Methods. Five weeks after priming, the mice were re-immunized with 10 μg of TT only. Two weeks after re-immunization, spleen cells were isolated and stimulated with plate-bound anti-CD3 for 72 hours in vitro. A, Lymphocyte proliferation was determined by [methyl-3H]-thymidine incorporation. B and C, the cytokines in supernatant were detected with sandwich ELISA. D, ratio of IL5:IFN-γ as an indicator of type 2 to type 1 cytokine balance. Bars shown are means ± SE, n = 10/ group. Different letters above bars within panel indicate significant differences (P < 0.05, a < b).
RA and/or PIC treatments promote neonatal lymphocyte maturation and differentiation

A lack of mature lymphocytes in peripheral lymphoid organs is believed to be an important factor in the weak immune response of neonates (119). Therefore, we determined if RA and/or PIC treatments could directly affect the maturation and development of neonatal immune cells. Forward scatter analysis using forward angle light scatter (FALS) and side scatter (SS) showed that more than 20% of neonatal spleen cells were larger (increased FALS) and/or appeared to be more granular (increased SS) than the great majority of adult spleen cells (fig. 3-6A). Thus, in addition to analyzing the effects of RA and PIC on the total spleen cell population, we separately quantified cells using the following three gates: an “A” (adult) gate set according to the distribution of live cells in adult mouse spleen; an “n1” (neonatal 1) gate, and an “n2” (neonatal 2) gate (Fig. 3-6A). In the “A” gate of neonatal mice, the expression of CD3+ (a T-cell marker), NK1.1+, CD11b+ (a marker of macrophages), and CD11c+ (a DC marker) were all much lower than the adult levels (Fig.3-6B). Whereas the percentage of B220+ cells in the A gate was comparable to the adult level, the expression of CD19, a B-cell receptor (BCR) coreceptor, was much less than that of adults (Fig. 3-6B and 3-8D). In addition, the percentage of IgM+ IgD− cells (immature B cells) was over 25%, suggesting the cell population in the “A” gate of neonates were relatively immature. The distribution of cells in the n1 gate was similar to that in the A gate, except that the CD11b+ cells were notably enriched in the n1 gate as compared to the A gate population. Also, the n1 gate contained 6% of IgM+ IgD− cells, and most of
B220-positive cells expressed CD19 (Fig. 3-6B). By these criteria, the n1 gate contained more mature cells than the “A” gate. Although the n2 gate covered only 6.5% of splenic mononuclear cells, nearly all of these cells were CD11b positive (Fig. 3-6B and 3-10B). Moreover, the medium fluorescence intensity (MdFI) of CD80 and CD86 of cells in the n2 gate were about 1 or 2-fold higher than those of cells in the A and n1 gates (Fig. 3-6B). Hence, the n2 gate mostly comprised of relatively mature macrophages. Although RA and/or PIC treatments did not produce a significant change in the number of total spleen cells, RA and RA+PIC significantly reduced cell proportion in the A gate but increased cell proportion in the n1 gate, suggesting that these treatments promoted lymphocyte maturation in neonatal mice (Fig.3-7).
### A.

![Flow cytometry plots](image)

T: total  
A: adult gate  
n1: neonatal gate 1  
n2: neonatal gate 2

### B.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Adults (%)</th>
<th>Neonates</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Total</td>
<td>A</td>
<td>n1</td>
<td>n2</td>
</tr>
<tr>
<td>CD3+</td>
<td>17.3 ± 2.2</td>
<td>7.2 ± 0.6</td>
<td>7.6 ± 0.7</td>
<td>7.9 ± 0.6</td>
<td>2.4 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>CD4+</td>
<td>13.3 ± 1.3</td>
<td>4.0 ± 1.1</td>
<td>4.8 ± 1.3</td>
<td>2.0 ± 0.4</td>
<td>0.5 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>CD8+</td>
<td>6.5 ± 1.0</td>
<td>2.2 ± 0.8</td>
<td>1.8 ± 0.8</td>
<td>2.6 ± 0.9</td>
<td>2.0 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>B220+</td>
<td>74.2 ± 2.3</td>
<td>63.0 ± 1.3</td>
<td>70.4 ± 1.4</td>
<td>56.6 ± 3.1</td>
<td>6.1 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>CD19+</td>
<td>73.4 ± 4.1</td>
<td>20.6 ± 4.7</td>
<td>32.4 ± 5.5</td>
<td>53.7 ± 7.1</td>
<td>8.7 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>IgM+IgD-</td>
<td>4.8 ± 0.2</td>
<td>20.2 ± 0.9</td>
<td>25.8 ± 0.8</td>
<td>6.3 ± 0.3</td>
<td>1.9 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>NK1.1+</td>
<td>5.3 ± 0.6</td>
<td>2.3 ± 0.2</td>
<td>2.3 ± 0.2</td>
<td>1.3 ± 0.1</td>
<td>1.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>NK</td>
<td>4.3 ± 0.4</td>
<td>2.1 ± 0.3</td>
<td>2.2 ± 0.3</td>
<td>1.7 ± 0.4</td>
<td>1.2 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>NKT</td>
<td>1.4 ± 0.1</td>
<td>0.7 ± 0.2</td>
<td>0.6 ± 0.2</td>
<td>1.1 ± 0.3</td>
<td>1.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>CD11b+</td>
<td>9.5 ± 0.7</td>
<td>16.1 ± 0.7</td>
<td>6.8 ± 0.3</td>
<td>32.9 ± 2.3</td>
<td>90.7 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>CD11b-Mdx</td>
<td>45.6 ± 3.5</td>
<td>104.1 ± 12.3</td>
<td>41.8 ± 20.2</td>
<td>132.5 ± 13.1</td>
<td>108.1 ± 11.6</td>
<td></td>
</tr>
<tr>
<td>CD11c+</td>
<td>6.5 ± 0.4</td>
<td>2.8 ± 0.1</td>
<td>2.2 ± 0.1</td>
<td>5.1 ± 0.2</td>
<td>2.2 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>CD80</td>
<td>9.9 ± 0.7</td>
<td>7.3 ± 0.6</td>
<td>7.6 ± 0.6</td>
<td>9.4 ± 0.7</td>
<td>9.4 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>CD80-Mdx</td>
<td>8.7 ± 0.4</td>
<td>12.2 ± 0.1</td>
<td>8.5 ± 0.2</td>
<td>14.8 ± 0.9</td>
<td>20.3 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>CD86</td>
<td>6.1 ± 0.2</td>
<td>4.3 ± 0.2</td>
<td>4.3 ± 0.2</td>
<td>4.5 ± 0.4</td>
<td>4.4 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>CD86-Mdx</td>
<td>8.7 ± 0.3</td>
<td>8.4 ± 0.1</td>
<td>5.5 ± 0.1</td>
<td>9.3 ± 0.2</td>
<td>17.1 ± 1.4</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 3-6. Neonatal splenocyte population is more heterogeneous than adult cell population. One-wk (neonate) and 6-wk (adult) old C57BL/6 mice were immunized with TT. Three days later, spleen cells were isolated and stained with fluorochrome-conjugated mAbs. A, Based on the light-scatter plots, the neonatal splenocyte population was gated into 3 gates: the A (adult-like) gate, the n1 (neonatal 1) gate, and the n2 (neonatal 2) gate. Values shown are the average cell proportions in each gate. B, The percentages of different cell types in each gate, mean ± SE (n = 4).
Fig. 3-7. RA and/or PIC significantly regulate cell proportion in the A and n1 gates. One-wk old C57BL/6 mice were immunized with TT, and spleen cells were isolated 3 days later. According to the gating shown in Fig. 3-6A, cell proportions for each treatment group (Control, RA, PIC and RA+PIC) were determined using light-scatter analysis. Means ± SE, n = 5/group. *, P < 0.05 versus control group.
Retinoic acid and/or PIC treatments at the neonatal stage also regulated neonatal lymphocyte populations. The percentage of CD3⁺, CD4⁺, and CD8⁺ T cells in neonatal mice was about 60% less than those of adult mice (Fig. 3-6B). RA and/or PIC treatments did not significantly affect neonatal CD4⁺ cells, however the percentage of CD8⁺ cells were significantly reduced by RA and RA+PIC in the total as well as in the A and the n1 gates (Fig. 3-8B). Consequently, the ratio of CD4⁺ cells to CD8⁺ cells in neonatal mice was significantly decreased by RA and RA+PIC (Fig. 3-8C). Interestingly, the number of CD8⁺ cells was not significantly changed by the treatments (Fig.3-8B), suggesting that the treatments in our study did not directly reduce CD8⁺ T cells. Instead, RA and RA+PIC might promote the expansion of other cell types (such as B cells and NK/NKT cells, which will be described later), thereby indirectly reducing the percentage of CD8⁺ cells.

The B-cell population (B220⁺ cells) in neonatal spleen, compared to adults, was only slightly reduced; however, neonatal B cells expressed much less CD19 (Fig. 3-8D). CD19 is a 95-kDa transmembrane protein expressed from the early stage of B-cell development up to the stage of plasma cell differentiation. It is known as an essential downstream element of BCR signaling required for B-cell maturation and activation, TD antigen-specific antibody responses, and germinal center formation (239,240). Notably, RA and RA+PIC, but not PIC alone, up-regulated the percentage and number of CD19⁺ cells (Fig. 3-8D), which might in turn enhance antigen-triggered
BCR signaling transduction, thereby promoting anti-TT antibody response in neonatal mice.

Furthermore, RA and PIC also regulated NK/NKT-cell populations in neonatal mice. The proportions of NK cells (NK1.1^+CD3^-) and NKT cells (NK1.1^+CD3^+) were much lower in neonates than adults (NK: 2.08% versus 4.33%; NKT: 0.7% versus 1.36%). However, RA+PIC significantly increased the percentage of NK cells and number of NK and NKT cells (Fig.3-9A and B). NK cells are known as an early source of IFN-γ, while NKT cells have been shown to secrete IL-4 and IL-10 (as well as IFN-γ) and, in general, to promote a type 2 response (67,230). Hence, the increase in NK and NKT cells could be responsible in part for the increased type-1/type-2 cytokine production by RA/PIC treated neonatal mice.
Fig. 3-8. **RA and/or PIC treatments regulate neonatal lymphocyte populations.** One-week old C57BL/6 mice were pretreated with RA or oil for one day, followed by TT ± RA and/or PIC. Three days after priming, spleen cells were isolated and stained with fluorochrome-conjugated mAbs as described in *Materials and Methods*. *A*, Number of total spleen cells; *B*, the percentage of CD8⁺ T cells; *C*, the ratio of CD4 to CD8 cells; *D*, histogram of the percentage of CD19⁺ B cells (control group is illustrated; the gray areas represent the isotype control) and the percentage of CD19⁺ B cells. Cells were quantified as the total cells and as cells residing in the A, n1 and n2 gates. The number of each cell type is calculated based on the number of total spleen cells. Means ± SE, *n* = 6/ group. *, *P < 0.05 versus control group.
Fig. 3-9. RA and/or PIC regulate NK and NKT cell populations. A, Proportion of NK cells in the adult (A) gate and neonatal gates n1 and n2 (see Fig. 3A for gating). B, number of NK and NKT cells in control, RA, PIC and RA/PIC-treated neonates 3 days after initial treatment. Means ± SE, n = 5/group. *, P < 0.05 versus control group.
In addition to lymphocyte population, RA and/or PIC regulated the generation and differentiation of DCs (CD11c\(^+\)) and macrophages (CD11b\(^+\)). The proportion of CD11c\(^+\) cells in neonatal mice was less than 50% of the adult level (Fig. 3-6B). In contrast, the proportion and MdFI of CD11b\(^+\) cells in neonatal mice were about twice the adult level (Fig. 3-6B and 3-10B). As macrophages are a major part of innate immunity, a relatively high proportion of macrophages implies the importance of innate immunity in controlling infections during the neonatal period, when the ability to mount adaptive immune responses is not yet well developed. Whereas the percentage of CD11b\(^+\) cells were not significantly affected, the percentage of CD11c\(^+\) cells was significantly increased by PIC in total and the n1 gate, and by RA+PIC in both the A and n2 gates (Fig. 3-10A). Therefore, RA and/or PIC treatments enhanced the generation of DCs in neonatal mice. Since macrophages and DC also function as professional APCs, we therefore measured CD80 and CD86, the co-stimulatory molecules expressed on APCs. The percentage of CD80 and CD86 was reduced about 30% in neonatal mice as compared with adult mice (Fig.3-6B). RA and/or PIC treatments slightly induced the percentage of CD80, but did not affect CD86 (data not shown). Notably, RA and PIC alone increased the percentage of CD11b\(^+\)CD80\(^+\) cells in the n2 gate, and RA+PIC combined significantly increased CD11b\(^+\)CD80\(^+\) cells in all four gates. Besides, the number of CD11b+CD80+ spleen cells was significantly elevated in RA- and RA+PIC-treated neonates (Fig. 3-10C). Hence, RA and/or PIC treatments induced CD80 expression on neonatal macrophages. Muthukkumar et al.
(237) reported that the defective antigen-presenting ability of neonatal APCs was strongly associated with the absence of costimulatory molecules, such as CD80 and CD86. The up-regulation of CD80 on macrophages implies that RA and/ or PIC may enhance the antigen presenting capacity of neonatal macrophages, thereby contributing to augmentation of anti-TT lymphocyte responses and antibody production.

Two-way ANOVA showed that RA was a positive regulator for CD19⁺ cells, NK cells and NKT cell, CD11b⁺CD80⁺ cells (Table 3-1 and 3-2), indicating RA treatment significantly increased a wide spectrum of immune cells in neonates. PIC, on the other hand, was a positive regulator for NK cells and for CD11c⁺ cells in total (Table 3-1 and 3-2), suggesting that PIC enhanced the generation of NK cells and DCs. The regulation of immune cell populations by RA and/ or PIC might directly affect functional outcomes in neonates.
Fig. 3-10. RA and/or PIC treatments regulate populations and differentiation of neonatal macrophages and DCs. One-week old C57BL/6 mice were pretreated with RA or oil for one day, followed by TT ± RA and/or PIC. Three days after priming, spleen cells were isolated and stained with fluorochrome-conjugated mAbs. A, The percentage of CD11c+ cells in the total gate and each subgate is shown. B, CD11b+ cells in total, A, n1 and n2 sub-gates; the gray areas represent the isotype control (control group illustrated). C, CD11b+CD80+ cells. Bars show means ± SE, n = 6/group. *, P < 0.05 versus control group.
Table 4-1. RA and/ or PIC treatments regulate lymphocyte populations in neonatal mice

<table>
<thead>
<tr>
<th></th>
<th>RA (P value)</th>
<th>PIC (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Total No.</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CD8+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.01 (-)</td>
<td>N/A</td>
</tr>
<tr>
<td>A</td>
<td>0.01 (-)</td>
<td>N/A</td>
</tr>
<tr>
<td>n1</td>
<td>0.05 (-)</td>
<td>N/A</td>
</tr>
<tr>
<td>n2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Total No.</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>A</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>n1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Total No.</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>B220+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Total No.</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>CD19+ (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.001 (+)</td>
<td>0.04 (-)</td>
</tr>
<tr>
<td>A</td>
<td>0.001 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>n1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Total No.</td>
<td>0.01 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>NK1.1+</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.05 (+)</td>
<td>0.05 (+)</td>
</tr>
<tr>
<td>A</td>
<td>N/A</td>
<td>0.01 (+)</td>
</tr>
<tr>
<td>n1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Total No.</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>NK cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.05 (+)</td>
<td>0.001 (+)</td>
</tr>
<tr>
<td>A</td>
<td>N/A</td>
<td>0.01 (+)</td>
</tr>
<tr>
<td>n1</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>n2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Total No.</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>NKT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Total No.</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
</tbody>
</table>
One-week old C57BL/6 mice were pretreated with RA or oil for one day, followed by TT ± RA and/ or PIC. Three days after priming, spleen cells were isolated and stained with fluorochrome-conjugated mAbs. The effects of RA and PIC on the number and the percentage of lymphocytes in each subgate were analyzed by a Two-way ANOVA. The data shown are P values of each factor, and a P value < 0.05 is considered statistically significant. There is no significant interaction between RA and PIC. “(+),” a positive regulation; “(-),” a negative regulation; N/A, not significant.
Table 4-2. RA and/or PIC treatments regulate the population of antigen-presenting cells and expression of co-stimulatory molecules in neonatal mice

<table>
<thead>
<tr>
<th></th>
<th>RA (P value)</th>
<th>PIC (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD11b+</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Total No.</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>CD80+</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n2</td>
<td>N/A</td>
<td>0.05 (+)</td>
</tr>
<tr>
<td>Total No.</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>CD80+CD11b+</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.01 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>A</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>n1</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>n2</td>
<td>0.01 (+)</td>
<td>0.001 (+)</td>
</tr>
<tr>
<td>Total No.</td>
<td>0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>CD80/CD86</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>0.01 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>A</td>
<td>0.001 (+)</td>
<td>0.05 (-)</td>
</tr>
<tr>
<td>n1</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>n2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>CD11c+</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T</td>
<td>N/A</td>
<td>0.05 (+)</td>
</tr>
<tr>
<td>A</td>
<td>N/A</td>
<td>0.01 (+)</td>
</tr>
<tr>
<td>n1</td>
<td>0.01 (-)</td>
<td>0.05 (+)</td>
</tr>
<tr>
<td>n2</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Total No.</td>
<td>0.07 (+)</td>
<td>N/A</td>
</tr>
</tbody>
</table>

1 One-week old C57BL/6 mice were pretreated with RA or oil for one day, followed by TT ± RA and/or PIC. Three days after priming, spleen cells were isolated and stained with fluorochrome-conjugated mAbs. The effects of RA and PIC on the number and the percentage of cells in each subgate were analyzed by a Two-way ANOVA. The data shown represent P values of each factor, and a P value < 0.05 is considered statistically significant. There is no significant interaction between RA and PIC. “(+)”, a positive regulation; “(-)”, a negative regulation; N/A, not significant.
CONCLUSIONS

In this study, early-life treatments with RA and/or PIC enhanced the TT-induced vaccine response in neonatal mice. RA alone given at the neonatal stage significantly enhanced both primary and secondary anti-TT IgG responses as well as TT-specific lymphocyte proliferation. However, it selectively increased type 2 responses as indicated by the induction of TT-specific IL-5 and IgG1 production, therefore further skewing the neonatal immune response towards a type 2 direction. Because the lack or immaturity type 1 responses is a major contributor to the increased susceptibility of neonates to intracellular pathogens (119), a selective induction of type 2 immunity by RA could be disadvantageous for the development of type 1 responses and cell-mediated immunity in neonates. On the other hand, PIC, shown to be a potent adjuvant in adult mice (238), strongly increased primary anti-TT IgG and all of the IgG isotypes (IgG1, IgG2a, and IgG2b) in neonatal mice. PIC alone also significantly enhanced TT-specific lymphocyte proliferation and IFN-γ production but, unfortunately, PIC did not significantly benefit the secondary anti-TT IgG and IgG1 response, suggesting PIC alone did not provoke a good memory response. Therefore PIC alone may not be sufficient as an adjuvant for neonatal vaccination. Compared with either RA or PIC alone, the combination of RA+PIC was more potent in augmenting both primary and secondary anti-TT IgG responses as well as all IgG isotypes. Moreover, the balance of anti-TT IgG1/IgG2a was maintained close to that of vehicle-treated neonates. Furthermore, RA+PIC significantly increased the production of TT-specific IFN-γ and IL-5, thereby effectively promoting both type 1 and type 2 cytokine responses. Somewhat to our surprise, RA given at the neonatal stage did not significantly attenuate TT-specific type 1 responses (e.g., anti-TT IgG2a and IFN-γ production), whereas we have reported that RA attenuated PIC-induced levels of type 1
cytokine mRNAs and IgG2a production in healthy adult mice (238). Therefore it appears that neonatal mice as compared to adults do not respond identically to RA in terms of the balance of IgG isotypes and cytokines production. This difference could be partly due to the significant effect of RA on neonatal immune-cell maturation and differentiation, such as by promoting the differentiation of NK/NKT cells which are sources of IFN-γ and IL-4 (65,241), and macrophages as well. Nevertheless, RA+PIC at the neonatal stage was more effective and more durable than either of these agents alone in promoting anti-TT antibody production in infancy, stimulating both type 1 and type 2 cytokines, and providing long-term immunity (heightened recall response) in the young adult stage. Overall, the combination of RA+PIC, a novel nutritional-immunological intervention, can act as a powerful adjuvant for neonatal vaccination.
CHAPTER 4

Retinoic acid combined with polyriboinosinic: polyribocytidylic acid
regulates TT-induced germinal center response in adult mice
INTRODUCTION

Germinal centers (GCs) are antigen-induced lymphoid microenvironments that play a critical role in TD Ag-induced humoral responses. Upon activated by TD Ags (e.g., TT), some B cells directly go through isotype-switching and differentiate into low-affinity plasma cells. However, many activated B cells migrate into primary follicles, and then rapidly expand to form secondary follicles. About one week after Ag priming, the secondary follicle polarizes into the dark zone and the light zone, forming a dynamic structure call a GC. The GC is primarily comprised of antigen-specific B cells and Th cells, follicular dendritic cells (FDC), as well as macrophages. Within GCs, the activated B cells undergo B-cell clone expansion, somatic hypermutation, affinity maturation, and Ig isotype switching, and then differentiate into high-affinity plasma cells or memory B cells. Hence, GCs are essential for generating high-affinity antibody response as well as memory response (74).

GC B cells can be identified using two surface markers: B220 and peanut agglutinin (PNA). PNA is a plant lectin that specifically binds to lymphocyte glycoprotein on terminal galactosyl residues (242). PNA was first reported as a surface marker for immature (cortical) thymocytes, which could bind over 90% of thymus cells (243,244). Later studies showed that PNA also selectively bound to GC cells in peripheral lymphoid organs. Compared with other B cells and T cells, GC B cells bind about 10-30 times more of PNA. Based on these results, PNA is used as a major marker of GC B cells, and GC B cells are defined as B220^+PNA^{Hi} cells. GC B cells have been
shown to uniformly express the B-cell lineage markers, such as CD19, CD21/35, CD22, and CD40, indicating these cells are mature B cells (245). Moreover, GC B cells can be characterized by several unique surface markers, which discriminate GC B cells from non-GC B cells. Compared with non-GC B cells, GC B cells express higher levels of MHC II and CD86, suggesting their potent antigen-present capability (245,246). Also, as a principal site for Ig isotype switching, GC B cells express a higher level of IgG isotypes, such as IgG1, than non-GC B cells. In addition, the expression of Fas is significantly elevated on GC B cells as compared with non-GC B cells (80,247). Fas is a member of the tumor necrosis factor receptor family, which binds to Fas ligand (FasL) and triggers apoptosis in many types of cells. Although the role of Fas in the GC reaction has not been well elucidated, Fas-mediated B-cell apoptosis is known to be required for both positive and negative B-cell selection in GCs. However, increased expression of Fas on GC B cells does not necessarily cause cell death. In the presence of survival signals, such as Ag stimulation and the CD40/CD40L interaction, GC B cell can be rescued from apoptosis (88). Hence, a relatively high expression of Fas is a unique characteristic of GC B cells.

In Chapter 2 and 3, RA and/or PIC treatments were shown to significantly enhanced primary and secondary anti-TT IgG production in both VA-sufficient adult and neonatal mice. Also RA and PIC differentially regulated anti-TT IgG isotype switching. However, the effect of RA and PIC on TT-induced GC reaction is unknown. Therefore, the present study tested hypothesis that RA and PIC could enhance anti-TT antibody response by regulating the GC response.
MATERIALS AND METHODS

Animals and experimental design

Animal protocols were approved by the Institutional Animal Use and Care Committee of The Pennsylvania State University. Six-week old C57BL/6 female mice (Charles River Laboratories) were divided into 4 groups: control, RA, PIC, and RA+PIC, and maintained on a nutritionally completed diet (#5001, Labdiet) during the experimental period. The mice were immunized with TT (10 μg/mouse, i.p.; Connaught Laboratories) and treated at the same time with all-trans RA (37.5 μg/mouse, oral; Sigma) and/or PIC (2 μg/mouse) on the first day of the experiment (day 0). From day 1 to day 6 after immunization, the mice were fed the same dose of RA or oil daily. Blood and spleens were collected on day 10 post-immunization for later analysis.

Spleen cell isolation

Spleen cells were isolated as reported previously (25). Briefly, spleen tissues were gently dispersed with a sterile wire screen mesh, and the resulting cell suspensions were layered over Ficoll-Hypaque (1.083 g/ml, Sigma, St. Louis, MO) followed by centrifugation at 2500 rpm for 20 min at 20°C. The mononuclear cells were carefully collected, washed twice and suspended in RPMI-1640 medium (Invitrogen) with 10% FBS.

Flow cytometry

The phenotype of spleen GC B cells was determined using sequential triple-color staining. Spleen mononuclear cells were suspended in flow wash buffer
(PBS-1% FBS) and incubated with anti-CD16 (2.4G2, 0.5 ug/10^6 cells; BD Pharmingen) on ice for 15 min in order to block the Fc receptors. After washing, the cells were individually incubated with appropriately diluted PE-labeled antibodies, including PE-anti-IgM (R6-60.2, BD Pharmingen), PE-goat-anti-mouse IgG1 (Molecular Probes), PE-anti-CD19 (1D3, BD Pharmingen), PE-anti-CD40 (1C10, Southern Biotech), PE-anti-MHC II (AF6-120.1, BD Pharmingen), and PE-hamster-anti-mouse Fas (Jo2, BD Pharmingen) at 4°C for 30 min. After washing twice, the cells were incubated with a mixture of Cy5-anti-B220 (RA3-6B2, BD Pharmingen) and FITC-PNA (Vector Laboratories) at 4°C for 30 min. To exclude non-specific binding of PNA, cells were incubated with FITC-PNA in the presence of D-galactose (0.2 M), a competitive substrate for PNA, which then served as a negative control for PNA staining (243). After incubation, the cells were fixed with 1% paraformaldehyde in PBS, and live-gated cells were detected on a Coulter XL-MLC flow cytometer. The results were analyzed with Flow-Jo software (Tree Star Inc.).

**Statistical analysis**

Data are reported as mean ± SE. The main effects of RA, PIC, and the interaction of RA and PIC were evaluated by two-way ANOVA. Differences among groups were determined using Fisher’s protected least significant difference (LSD) test (SuperAnova, Abacus Software, Berkeley CA). The correlation between the expression of IgG1 and MHC II or Fas on each B-cell subset was assessed using simple regression analysis. A value of $P < 0.05$ was considered statistically significant.
RESULTS AND DISCUSSION

The present study evaluated the effects of RA and/or PIC treatments on TT-induced GC reaction in VA-sufficient adult mice. The GC reaction generally peaks at 1 to 2 weeks after priming and then declines rapidly (75). However, depending on different Ags, the peak time of GCs is variable. Pihlgren et al. (141) reported that the number of GCs induced by alum-absorbed TT peaked at 10-12 days in adult mice. In my pilot study, naive mice contained about 2-3% of GC B cells (B220⁺PNA⁺) in spleen (data not shown). GC B cells induced by TT immunization were evident on day 8 after priming, and then slightly decreased on day 12. Therefore, in the present study, I evaluated the GC reaction on day 10 after TT priming.

RA and PIC treatments increase the proportion of germinal center B cells

To identify GC B cells, spleen cells were double-labeled with Cy5-anti-B220 and FITC-PNA. Previous studies showed that immunization with SRBC or NP induced a discrete population of B220⁺PNA⁺ cells in mouse spleens (245,247). However, in the present study, TT immunization only induced a shift from PNA Lo cells to PNA Hi cells instead of a relatively discrete B220⁺PNA⁺ population. Therefore, according to the intensity of PNA, B220⁺ cells were divided into 3 subsets: B220⁺PNA Lo cells, B220⁺PNA Int cells, and B220⁺PNA Hi cells (Fig.4-1A). B220⁺PNA Int cells are the largest population, comprising about 70% of B220⁺ cells; while B220⁺PNA Lo cells and B220⁺PNA Hi cells made up about 16% and 10% of B220⁺ cells, respectively. Over 98%
of B220<sup>+</sup>PNA<sup>Lo</sup> cells, B220<sup>+</sup>PNA<sup>Int</sup> cells, as well as B220<sup>+</sup>PNA<sup>Hi</sup> cells coexpressed CD19<sup>+</sup>, confirming that all three cell populations were B cells (data not shown). Whereas the proportions of B220<sup>+</sup>PNA<sup>Lo</sup> and B220<sup>+</sup>PNA<sup>Int</sup> cells were not affected by the treatments, RA increased the percentage of B220<sup>+</sup>PNA<sup>Hi</sup> cells about 40%, and PIC significantly increased it about one-fold (Fig.4-1B). Since B220<sup>+</sup>PNA<sup>Hi</sup> cells represent GC B cells, the upregulation of B220<sup>+</sup>PNA<sup>Hi</sup> cells suggests that RA or PIC alone induces the generation of GC B cells. Interestingly, although the combination of RA+PIC synergistically promoted anti-TT antibody response in VA-sufficient mice, it did not significantly increase the percentage of B220<sup>+</sup>PNA<sup>Hi</sup> cells. The discrepancy between antibody production and the generation of GC B cells may be due to an earlier peak time of GC formation induced by RA+PIC. Also, RA+PIC may accelerate the differentiation of plasma cells and memory B cells, thereby diminishing GCs. Hence, the effect of RA+PIC on GC B-cell population needs to be further investigated.
Fig. 4-1. RA and PIC treatments increase the percentage of B220^{+}PNA^{\text{Hi}} cells. Six-wk old C57BL/6 mice were immunized with TT ± RA and/ or PIC as described in Material and Methods. Ten days after priming, spleen cells were isolated and double-stained with FITC-PNA and Cy5-anti-B220. (A) B220^{+} cells were divided into 3 subsets based on the intensity of PNA: B220^{+}PNA^{\text{Lo}} cells, B220^{+}PNA^{\text{Int}} cells, and B220^{+}PNA^{\text{Hi}} cells. Data shown in the figure are the average percentage of each B-cell subset. (B) The effect of RA and/or PIC on the percentage of B-cell subsets. Bars shown are means ± SE, n = 4/ group. Different letters above bars within panel indicate significant differences (P < 0.05, a < b).
**RA and/or PIC treatments regulate the expression of IgG1, MHCII, and Fas on germinal center B cells**

Next, the effect of RA and PIC on the expression of GC-related markers was evaluated. IgM and IgG1 are two surface markers related to Ig isotype switching, a major outcome of the GC reaction. Compared with B220⁺PNA² cells, B220⁺PNA² cells expressed a higher level of IgM. However, neither the percentage of IgM on total spleen cells nor the expression of IgM on each B-cell subset was significantly affected by RA and/or PIC treatments (Fig.4-2). IgG1, a major isotype induced by TT, was expressed at a fairly low level on B220⁺PNA² cells and slightly increased on B220⁺PNA² cells. On B220⁺PNA² cells, the total fluorescence intensity (FI) of IgG1 was remarkably elevated about 3 to 5-fold as compared with B220⁺PNA² cells, confirming that GCs are important site for Ig isotype switching. Although the percentage of IgG1 on total spleen cells was not significantly affected by the treatments, RA and RA+PIC increased the FI of IgG1 about 30% on B220⁺PNA² cells and on B220⁺PNA² cells. On B220⁺PNA² cells, RA alone increased FI of the IgG1 about 50%, and the combination of RA+PIC significantly increased the FI of IgG1 about 75% (Fig. 4-3). Two-way ANOVA analysis indicated that RA was a positive factor for IgG1 expression on B220⁺PNA² cells, while PIC did not have any effect (Table 4-1). Therefore, RA rather than PIC positively regulated IgG1 isotype switching in GCs. Compared with either RA or PIC alone, the combination of RA+PIC was more potent in promoting GC Ig isotype switching.
Fig. 4-2. RA and /or PIC treatments do not significantly affect IgM expression. Six-wk old C57BL/6 mice were immunized with TT ± RA and/ or PIC as described in Material and Methods. Ten days after priming, spleen cells were isolated and sequentially triple-labeled with FITC-PNA, Cy5-anti-B220, and PE-anti-IgM. The expression of IgM on total spleen cells (A) and on each B-cell subset (B) were detected on a Coulter XL-MLC flow cytometer and then analyzed with Flow-Jo software. Bars shown are means ± SE, n = 4/ group. FI (total fluorescence intensity) = % of positive cells × median fluorescence intensity.
Fig. 4-3. RA and/or PIC treatments regulate the expression of IgG1 on B cells.

Six-wk old C57BL/6 mice were immunized with TT ± RA and/or PIC as described in Material and Methods. Ten days after priming, spleen cells were isolated and sequentially triple-labeled with FITC-PNA, Cy5-anti-B220, and PE-anti-IgG1. The expression of IgG1 on total spleen cells (A) and on each B-cell subset (B) were detected on a Coulter XL-MLC flow cytometer and then analyzed with Flow-Jo software. Bars shown are means ± SE, n = 4/group. Different letters above bars within panel indicate significant differences (P < 0.05, a < b). FI (total fluorescence intensity) = % of positive cells × median fluorescence intensity.
MHC II is another important molecule related to the GC reaction. Within GC, B cells process and present the antigen-derived peptides through MHC class II complexes to memory GC Th cells. The GC Th cells in turn provide survival signals to the B cells, promoting Ig switch isotype as well as the generation of plasma cells and memory B cells (248). Therefore, MHC II plays a critical role in the GC reaction. A previous study showed that GC B cells expressed a higher level of MHC II than follicular B cells (245). In the present study, the FI of MHC II on B220<sup>+</sup>PNA<sup>Hi</sup> cells was about 3 to 5-fold higher than that on B220<sup>+</sup>PNA<sup>Lo</sup> cells, confirming previous result (Fig.4-4B). Although the percentage of MHC II on total spleen cells was not affected by the treatments, RA and RA+PIC significantly increased the FI of MHC II on both B220<sup>+</sup>PNA<sup>Lo</sup> and B220<sup>+</sup>PNA<sup>Int</sup> cells. On B220<sup>+</sup>PNA<sup>Hi</sup> cells, the FI of MHC II was induced about 25% by RA and RA+PIC (Fig. 4-4). Two-way ANOVA analysis indicated that RA was a positive regulator for MHC II expression on B220<sup>+</sup>PNA<sup>Lo</sup> cells, B220<sup>+</sup>PNA<sup>Int</sup> cells, and B220<sup>+</sup>PNA<sup>Hi</sup> cells (Table 4-1). These data indicate that RA promoted MHC II expression on the whole B-cell population, including GC B cells. Notably, the expression of MHC II was correlated with the expression of IgG1 on B220<sup>+</sup>PNA<sup>Hi</sup> cells and B220<sup>+</sup>PNA<sup>Int</sup> cells (Fig.4-6B and C). Particularly, the strong correlation between the expression of MHC II and IgG1 on B220<sup>+</sup>PNA<sup>Hi</sup> cells (R<sup>2</sup> = 0.63) suggests that the up-regulation of MHC II on GC B cells by RA may enhance the formation of cognate T-B cell interaction in GC center, thereby facilitating the GC reaction.
Fig. 4-4. RA and/or PIC treatments regulate the expression of MHCII on B cells.

Six-wk old C57BL/6 mice were immunized with TT ± RA and/or PIC as described in Material and Methods. Ten days after priming, spleen cells were isolated and sequentially triple-labeled with FITC-PNA, Cy5-anti-B220, and PE-anti-MHCII. The expressions of MHC II on total spleen cells (A) and on each B-cell subset (B) were detected on a Coulter XL-MLC flow cytometer and then analyzed with Flow-Jo software. Bars shown are means ± SE, n = 4/group. Different letters above bars within panel indicate significant differences (P < 0.05, a < b). FI (total fluorescence intensity) = % of positive cells × median fluorescence intensity.
Furthermore, the expression of Fas on GC B cells was evaluated in the present study. Fas is another surface marker related to GC B cells. Compared with naïve and memory B cells, GC B cells (centroblasts and centrocytes) express a significantly higher level of Fas mRNA and protein (80). GC cells also contain a preformed death-inducing signaling complex (DISC), which is involved in Fas-induced apoptosis (81). The heightened expression of Fas and the preformed DISC complex in GC B cells suggest these cells are programmed to die. However, in the presence of survival signals, such as the B cell-FDC interaction and the CD40/CD40L interaction, B cells can be rescued from Fas-triggered apoptosis (82). Therefore, a relatively high expression of Fas on GC B cells does not necessarily lead to cell death. In contrast, Fas plays a critical role in the GC reaction. Fas mutation not only impairs selection of high-affinity B cells within GCs, but also results in an extensive generation of self-reactive B cells (78,79). Furthermore, Fas appears to be critical for generating memory B cells (78). Therefore, increased Fas expression is one of major characteristics of GC B cells, which may be related to the GC reaction.

The present study showed that Fas was almost undetectable on B220⁺PNA<sub>Lo</sub> cells and slightly increased on B220⁺PNA<sub>Int</sub> cells, confirming previous studies (80). However, on B220⁺ PNA<sub>Hi</sub> cells, the FI of Fas was dramatically up-regulated about 50 to 60-fold, supporting that the B220⁺PNA<sub>Hi</sub> cells are GC B cells. Whereas the percentage of Fas on total spleen cells was not regulated by the treatments, the combination of RA+PIC significantly increased the FI of Fas on B220⁺PNA<sub>Hi</sub> cells about 4 folds (Fig. 4-5). Two-way ANOVA analysis indicated that both RA and PIC
both are positive factors for Fas expression on B220^{+}PNA^{Hi} cells (Table 4-1). Interestingly, the expression of Fas was strongly correlated with the expression of IgG1 only on B220^{+}PNA^{Hi} cells but not on the other B-cell subsets. These data suggest that RA/PIC-induced Fas expression on GC B cells might be associated with TT-induced GC reaction (Fig.4-6). The role of Fas in the GC reaction will be investigated in future study.

Overall, RA and PIC differentially regulated TT-induced GC reaction. Although RA alone slightly induced generation of B220^{+}PNA^{Hi} cells, which represent GC B cells, it was a positive regulator for the expression of IgG1, MHC II, and Fas on B220^{+}PNA^{Hi} cells (Table 4-1). Therefore, RA may function as a differentiator modulating the differentiation and activation of GC B cells. In contrast, whereas PIC significantly induced GC B cells, it did not significantly affect expression of GC-related markers on B220^{+}PNA^{Hi} cells except for Fas, which was positively regulated by PIC (Table 4-1). Hence, instead of regulating GC B-cell differentiation, PIC may primarily act as an amplifier of the GC reaction. Although the combination of RA+PIC did not significantly increase the generation of GC B cells, it resulted in the highest level of IgG1 and Fas on B220^{+}PNA^{Hi} cells. Hence, the combination of RA+PIC was more potent than either agent alone in regulating GC B-cell differentiation and promoting the GC reaction. In summary, RA and PIC differentially regulated the generation and differentiation of GC B cells, which could be an important mechanism contributing to RA/PIC-enhanced anti-TT antibody response.
Fig. 4-5. RA and/or PIC treatments regulate the expression of Fas on B cells. Six-wk old C57BL/6 mice were immunized with TT ± RA and/or PIC as described in Material and Methods. Ten days after priming, spleen cells were isolated and sequentially triple-labeled with FITC-PNA, Cy5-anti-B220, plus PE-anti-Fas. The expressions of Fas on total spleen cells (A) and on each B-cell subset (B) were detected on a Coulter XL-MLC flow cytometer and then analyzed with Flow-Jo software. Bars shown are means ± SE, n = 4/group. Different letters above bars within panel indicate significant differences (P < 0.05, a < b). FI (total fluorescence intensity) = % of positive cells × median fluorescence intensity.
Fig. 4-6. The expression of IgG1 is significantly correlated with the expression of MHC II and Fas on B220⁺PNA⁺⁺+ cells. Six-wk old C57BL/6 mice were immunized with TT ± RA and/or PIC as described in Material and Methods. Ten days after priming, spleen cells were isolated and sequentially triple-labeled with FITC-PNA, Cy5-anti-B220, plus PE-anti-IgG1, PE-anti-MHC II, or PE-anti-Fas. The expressions of IgG1, MHC II, and Fas were detected on a Coulter XL-MLC flow cytometer and then analyzed with Flow-Jo software. The correlation between the total fluorescence intensity (FI) of IgG1 and the FI of IgM (A, B, and C) or the FI of Fas (D, E, and F) on each B-cell subset was assessed by simple regression analysis. FI = % of positive cells × median fluorescence intensity.
Table 4-1. RA and/or PIC treatments regulate the expression of IgG1, MHC II, and Fas on B cells

<table>
<thead>
<tr>
<th></th>
<th>RA (P value)</th>
<th>PIC (P value)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IgG1 (Fl)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220⁺PNA(^{Lo})</td>
<td>0.09 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>B220⁺PNA(^{Int})</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>B220⁺PNA(^{Hi})</td>
<td>&lt; 0.01 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>MHC II (Fl)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220⁺PNA(^{Lo})</td>
<td>&lt; 0.01 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>B220⁺PNA(^{Int})</td>
<td>&lt; 0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td>B220⁺PNA(^{Hi})</td>
<td>&lt; 0.05 (+)</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Fas (Fl)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B220⁺PNA(^{Lo})</td>
<td>N/A</td>
<td>&lt; 0.01 (-)</td>
</tr>
<tr>
<td>B220⁺PNA(^{Int})</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>B220⁺PNA(^{Hi})</td>
<td>&lt; 0.05 (+)</td>
<td>&lt; 0.01 (+)</td>
</tr>
</tbody>
</table>

1 Six-wk old C57BL/6 mice were immunized with TT ± RA and/or PIC as described in Material and Methods. Ten days after priming, spleen cells were isolated and sequentially triple-labeled with FITC-PNA, Cy5-anti-B220, plus PE-anti-IgG1, PE-anti-MHCII, or PE-anti-Fas. The expressions of IgG1, MHC II, and Fas were detected on a Coulter XL-MLC flow cytometer and then analyzed with Flow-Jo software. The effects of RA and PIC on the total fluorescence intensity (Fl) of IgG1, MHC II, and Fas on each B-cell subset were analyzed by a Two-way ANOVA. The data shown represent P values of each factor, and a P value < 0.05 is considered statistically significant. There is no significant interaction between RA and PIC. “(+),” a positive regulation; “(-),” a negative regulation; N/A, not significant.

2 Fl (total fluorescence intensity) = % of positive cells × median fluorescence intensity
CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS
Vaccination is a cost-effective strategy to protect human beings from infectious diseases. Conventional vaccines, such as inactivated whole pathogens and purified microbial macromolecules, are designed essentially to induce neutralizing antibodies against viruses or bacteria invasion. However, for the vaccine targeting either chronic infectious diseases or intracellular pathogens, the capability of inducing type 1 (Th1) response (e.g., type 1 cytokines, IgG2/IgG2b production, and CTL response) is also highly desired (150). Vaccination is particularly important for neonates and infants, the population with a high risk of infectious diseases. Unfortunately, due to the immaturity of immune system, conventional vaccines usually cannot induce sufficient protective response, especially type 1 response, in neonates and infants. Hence, strategies to enhance vaccination efficiency and to induce type 1 response are highly sought. Currently, the combination of vaccine with certain Th1 adjuvants is considered as a promising strategy to induce Th1 response. Although a group of Th1 adjuvants, such as IL-12 and CpG oligonucleotides, have been shown to increase antibody titers and induce type 1 response in animal models (249), their application in human beings is hampered due to potential toxicity (234).

Previous studies have shown that RA and PIC synergistically or additively enhanced anti-TT antibody responses in VA-deficient animals, suggesting the combination of RA+PIC can promote vaccine-induced immune responses (13). However, the effect of RA and PIC on vaccine response in VA-sufficient population, especially in neonates and infants, has not been well elucidated. Therefore, the goal of
the present study was to evaluate the adjuvant effects of RA and PIC on TT-induced vaccine response VA-sufficient adult and neonatal mice.

**RA and PIC co-operatively enhance anti-TT vaccine response in both adult and neonatal mice**

Overall, RA and/or PIC treatments were well tolerated in both adult and neonatal mice, and did not affect the growth of animals. More importantly, RA and/or PIC treatments significantly increased primary anti-TT Ig response as well as IgG isotypes in both well-nourished adult and neonatal mice. In addition, the co-administration of RA and/or PIC with the primary TT immunization remarkably benefited anti-TT memory responses. Compared with either RA or PIC alone, the combination of RA+PIC was more potent and more durable in increasing anti-TT IgG response in both adult and neonatal mice. Also, this combination induced all IgG isotypes (IgG1, IgG2a, and IgG2b), while maintaining the balance of IgG1/IgG2a. In neonatal mice, the combination of RA+PIC increased both type 1 and type 2 cytokines, and led to an elevated but balanced type 1/type 2 responses. Therefore, RA and/or PIC treatments significantly promoted TT-induced vaccine response in both adult and neonatal mice.

Notably, adult and neonatal mice did not respond identically to RA and PIC treatments, even though the doses of TT, RA, and PIC in neonatal mice were comparable with the doses used for adult mice. First of all, RA and/or PIC treatments enhanced anti-TT antibody response more effectively in adult mice than in neonatal mice. In adults, RA alone increased IgG and IgG1 about 10-fold, and PIC or RA+PIC
dramatically increased IgG and IgG isotypes (IgG1, IgG2a, and IgG2b) about 50 to 120-fold. However RA alone increased IgG and IgG1 about 5-fold, and PIC or RA+PIC increased IgG and IgG isotypes (IgG1, IgG2a, and IgG2b) only about 4 to 16-fold. Therefore, despite their ability to promote the anti-TT antibody response in both neonatal and adult mice, RA and PIC were less effective in neonates than in adults. Particularly, the efficiency of PIC was reduced about 10-fold in neonates as compared with adults. The mechanism resulting in low efficiency of PIC in neonatal mice is unknown. It may be due to defective TLR3 signaling in neonates. As described previously, PIC is a synthetic dsRNA that can induce a large amount of type I IFNs, regulate Th1/Th2 response, and promote DC maturation as well as NK cell activity. The function of PIC is mediated by TLR3 expressed on macrophages, DCs, and NK cells (209,212,213) (Fig. 5-1). Once bound to TLR3, PIC can trigger the activation of MyD88-dependent and MyD88-independent signaling pathways, inducing cytokine production and DC maturation. Absence of TLR3 significantly impairs responses to PIC and reduces production of inflammatory cytokines in macrophages and DCs (213). In human newborns, TLR-mediated immune responses have been shown to be defective. Levy et al.(250) evaluated TLRs and TLR signaling in monocytes of human cord blood cells. Whereas the basal mRNA level of TLRs (TLR1 to TLR10) and TLR-related signaling molecules (MD-2, MyD88, TIRAP, IRAK-4, and CD14) in cord-blood monocytes were similar to the adult level, TNF-α production induced by TLR ligands, such as bacterial lipopeptides (a TLR1/2 ligand), LPS (a TLR4 ligand) and imiquimod (a TLR7 ligand) was significantly reduced. Moreover, p38 phosphorylation induced by LPS and R-848 (a TLR7/8 ligand) was also decreased in cord-blood monocytes. These
data suggest that the immune responses mediated by TLRs, including TLR1, 2, 4, 7, and 8, were impaired in newborn. Although TLR3 triggered signal transduction in neonates has not been reported yet, the production of IL-12p70 and IFN-α induced by PIC is significantly suppressed in human cord-blood cells (251). These data suggest that the TLR3 signaling pathway is defective in neonates, which consequently results in a reduced response to PIC.

Second, RA differentially regulated TT-specific type 1 responses (e.g., anti-TT IgG2a and IFN-γ production) in adult and neonatal models. In adult mice, RA treatment attenuated PIC-induced IFN-γ mRNA expression and anti-TT IgG2a production. However, RA did not significantly affect titers of anti-TT IgG2a and TT-induced IFNγ production in neonatal mice. This difference could be partly due to the significant effect of RA on neonatal immune-cell maturation and differentiation, such as by increasing the generation of NK cells and DCs, which can be early sources of IFN-γ and IL-12, driving the development of type 1 response in neonatal mice (15,65,241).

Third, the effect of PIC on neonatal memory response was somewhat different from that in adults. RA treatment significantly increased secondary anti-TT antibody response in both adult and neonatal mice, indicating the effect of RA was durable. PIC, on the other hand, dramatically enhanced memory response in adult mice; however it failed to benefit secondary IgG and IgG1 production in neonatal mice. The transient effect of PIC on neonatal IgG response suggest that although PIC itself could amplify immune response, it might not enhance the generation of memory effector cells in neonatal mice.
What is the potential mechanism?

The underlying mechanisms by which RA and PIC enhanced vaccine responses were investigated in the present study. The possible mechanisms include at least four aspects: 1) Regulation of lymphocyte activation, such as lymphocyte proliferation and type 1/type 2 cytokine response; 2) Regulation of lymphocyte populations; 3) Regulation of macrophage and DC populations; 4) Regulation of the GC reaction (Fig.5-1). RA and PIC both augmented TT-induced lymphocyte proliferation in neonatal mice. RA and PIC also differentially regulated type 1/type 2 cytokines in adult and neonatal mice. Although the mechanism by which PIC induced type 2 cytokine is unknown, the up-regulation of type 1 cytokines by PIC could be partially attributable to its direct effect on macrophages and DCs. PIC can trigger the TLR3 signaling pathway in macrophages and DCs, and induce the activation of NF-κB (206,208,252). The activated NF-κB then binds to the κB binding site in IL-12 p40 promoter, increasing the production of IL-12. In contrast, RA has been shown to inhibit the DNA binding of NF-κB and suppress its transcriptional activity through the RXR-NFκB interaction, thereby reducing IL-12 production in macrophages (196). RA also reduced the mRNA level of IL-12Rβ2, which could decrease the IL-12 responsiveness and further inhibit IL-12-elicited the development of Th1 cells. Moreover, RA negatively regulated the mRNA level of IRF-1, an IFN-γ downstream molecule. Previous studies have identified the IRF-1 binding sites in both IL-12 p40 and IL-4 promoters (196,253). IRF-1 can directly induce IL-12 p40 gene transcription but suppress IL-4 (253,254). Hence, the downregulation of IRF-1 by RA could be a major molecular mechanism contributing to the differential regulation of type 1/type 2 cytokine responses. The mRNA levels of
type 1 and type 2 cytokines were strongly correlated with the titers of IgG isotypes, implying that RA and PIC enhanced anti-TT IgG and IgG isotypes through modulating type 1/type 2 cytokine response.

RA and PIC also regulated the populations and differentiation of lymphocytes. Whereas RA and/or PIC did not affect the proportion of T and B cells in adult mice, RA and RA+PIC significantly induced expression of CD19 on neonatal B cells, suggesting that these treatments enhanced B-cell maturation in neonatal mice. Since neonates lack mature lymphocytes, promoting B-cell maturation may help to generate protective immune response in neonatal mice. In addition, RA and PIC also regulated NK/NKT cell populations in both adult and neonatal mice. In adult mice, RA and PIC were positive regulators for NKT and NK cells, respectively. Also, RA and RA+PIC increased the percentage of neonatal NK cells. As described before, NK cells are early sources of IFN-\(\gamma\) and participate in the initiation of Th1 (type 1) response (230), while NKT cells have been shown to secrete IL-4 and IL-10 and promote a Th2 (type 2) response (67,70). Therefore, RA and PIC might regulate anti-TT antibody production and type 1/type 2 cytokine responses by modulating NK/NKT-cell populations in adult and neonatal mice.

Moreover, RA and PIC significantly regulated the populations and differentiation of macrophages (CD11b\(^+\)) and DCs (CD11c\(^+\)). Macrophages and DCs are not only an important part of the innate immunity but also professional APCs participating in TD Ag-induced immune response. Whereas RA and RA+PIC significantly increased the percentage of macrophages in adult mice, PIC significantly
increased the percentage of DCs in neonatal mice. Moreover, RA and PIC regulated the expression of CD80 and CD86, the major co-stimulatory molecules expressed on APCs (255). Notably, the expression of CD80 on macrophages (CD80⁺CD11b⁺) was significantly induced by RA and RA+PIC in both adult and neonatal mice. On the other hand, CD86 was induced by PIC alone on non-macrophages (CD86⁺CD11b⁻), which presumably include DCs and activated B cells. The induction of CD80/CD86 costimulatory molecules implies that RA and PIC may enhance the antigen-presenting capability of APCs, thereby affecting T-cell activation as well as downstream antibody responses.

In addition, RA and/ or PIC modulated TT-induced GC reaction, which might be another important mechanism leading to the enhanced antibody response. GC is a principal site for generating high-affinity and long-lived plasma cells and memory B cells (76). Previous studies have shown that RA and PIC cooperatively enhanced primary and secondary anti-TT antibody responses in both VA-deficient and VA-sufficient animals (13). However, no report has yet addressed the effects of RA and PIC on the GC reaction. The present study showed that RA not only increased the percentage of GC B cells (PNA⁺B220⁺) but also positively regulated the expressions of GC-related markers, such as IgG1, MHCII, and Fas. Hence, RA promoted the generation and activation of GC B cells. Whereas PIC significantly increased the percentage of GC B cells, it only induced expression of Fas on GC B cells without affecting other GC-related markers. Hence, PIC more likely increased number of GC B cells but did not significantly affect activation of GC B cells. Despite no significant
effect on the percentage of GC B cells, RA+PIC resulted in the highest expressions of IgG1, MHCII, and Fas on the GC B cells, suggesting this combination significantly increased GC B-cell activation. Although the effect of RA and PIC on the GC reaction needs to be further confirmed, the present data indicate that RA and PIC can increase anti-TT antibody response by promoting the GC reaction.

**Conclusions**

In summary, co-administration of RA and/or PIC with TT priming significantly enhanced anti-TT vaccine response in both VA-sufficient adult and neonatal mice. The combination of RA+PIC was more effective than either agent alone in promoting primary anti-TT antibody response, stimulating both type 1 and type 2 cytokines, and providing long-term immunity (recall response). Hence, the combination of RA+PIC, a nutritional-immunological intervention, can act as a powerful adjuvant for adult and neonatal vaccination.

**Future directions**

Although the present study has demonstrated the adjuvant effects of RA and PIC on TT-induced vaccine response in both adult and neonatal mice, there are still many unresolved questions. One of the major questions is how RA and PIC regulate the GC reaction. The present study has shown that RA and PIC promoted GC B-cell generation and activation in TT-immunized adult mice. However, the effect of RA and PIC on TT-induced GC reaction in neonatal mice has not been tested yet. Also, whether RA and PIC can change the microstructure of GCs, such as the FDC network, is still an unknown question. Moreover, no report has yet addressed if RA and PIC could regulate
GC B- and T-cell recruitment. GC B- and T-cell recruitment is mediated by B-lymphocyte chemoattractant (BLC), which is produced by follicular stromal cells and FDCs. It attracts CXCR5+ B cells and T cells to lymphoid follicles, participating in the GC reaction (256). Recently, Iwata et al. (257) has shown that RA plays an essential role in directing T cells to the gut. However, the effect of RA on B- and T-cell homing to lymphoid follicles is unknown. Therefore, it will be interesting to determine if RA and PIC regulate GC B- and T-cell recruitment. In addition, the effect of RA and PIC on cell survival and cell death within GCs is unknown. To address these questions, the following experiments will be conducted in the future: 1) To determine if RA and PIC can regulate neonatal GC B cells using flow cytometry; 2) To evaluate the effect of RA and PIC on follicular dendritic cell (FDC) network as well as B-and T-cell recruitment in GCs using immunohistochemical staining; 3) To determine if RA and PIC can affect GC B-cell survival or apoptosis using immunohistochemical staining; 4) To assess the effect of RA and PIC on the expression of GC-related transcription factors using RT-PCR and Western blot analysis. Although Ag-induced GC reaction, FDC network as well as GC cell apoptosis have been described in both human and murine lymphoid tissues (141,258), the future studies will further help us to understand the immunoregulatory effect of RA and PIC treatments.

In the present study, all the animal experiments were established in C57BL/6 mice, a Th1-prone mouse strain (259). However, the adjuvant effects of RA and PIC have not been confirmed in other mouse strains. Although DeCicco et al. (13) reported that RA and PIC significantly increased anti-TT IgG response in VA-sufficient Balb/c
mice, the efficiency of PIC was about 20-fold less as compared with that in C57BL/6 mice, suggesting different genetic background could affect host responsiveness to the adjuvants. The difference in PIC sensitivity might be due to the variable TLR3 expression in different mouse strains. Also, the TLR ligands, such as lipoprotein (TLR2), LPS (TLR4), and CpG (TLR9), induce a higher level of IL-12 in DCs derived from C57BL/6 mice than that in Balb/c mice (260). In addition, the activation of NK/NKT cells, the important regulator for type 1/type 2 response, is also different in C57BL/6 and Balb/c mice (259). Therefore, it is necessary to confirm the adjuvant effects of RA and PIC in other mouse strains.

Since the present study has reported that RA combined with PIC promoted TT-induced vaccine response in both adult and neonatal mice, the following question will be how to apply to human vaccination. Currently, VA supplementation has been integrated with vaccination in many developing countries where VA deficiency is prevalent (3). Although the primary goal of this policy is to reduce VA deficiency in children, providing VA supplementation also potentially benefits vaccine-induced immune response (202,261). The present study showed that RA, the bioactive metabolite of VA, significantly enhanced anti-TT IgG production in VA-sufficient adult and neonatal mice. These data suggest that VA can promote TT- or similar vaccine-induced immune response through RA. Therefore, VA supplementation may be applied as an adjuvant to enhance vaccine efficiency in neonates and adults. Unfortunately, several studies conducted in VA-sufficient infants seem to disapprove this hypothesis. Kutukculer et al. (262) showed that co-administration of VA with DPT
vaccination in VA-sufficient infants increased primary anti-TT IgG about 30% without statistic significance. Cherian et al. (203) reported that VA supplementation did not enhance measles vaccine-induced antibody production in healthy infants. The discrepancy between human intervention and animal study implies that VA supplementation may not be as effective as RA in stimulating immune response, particularly in VA-sufficient populations. As described previously, the natural synthesis of RA is quite low. Also, dietary VA as well as RA itself can accelerate RA metabolism by inducing RA-metabolizing enzyme CYP26. Hence, a periodic supplementation of VA may not be able to build up a high enough level of RA to stimulate immune system in VA-sufficient population. If that is the case, what should we do? The recent data in our lab reported that oral administration of retinyl palmitate (RP) combined with a small amount of RA (1:10 molar of RP) synergistically increased lung VA concentration about 5 to 10-fold in rat pups, and this potency was maintained even though the dose of RP+RA was reduced to half or one fourth (Ross et al, unpublished data). These data suggest a novel supplementation that VA combined with a small amount of RA can not only reduce the dosage of VA but also increase the capacity of improving VA status. More importantly, the combination of VA and RA may be more effective than VA alone to stimulate immune system in VA-sufficient population. Therefore, in the future study, we will test the immunoregulatory effect of VA+RA in adult and neonatal mice.

Moreover, we notice that RA has to be combined with an appropriate adjuvant to achieve a satisfying adjuvant effect. In the present study, whereas RA enhanced anti-TT antibody response, it resulted in a type 2 (Th2) skewed response. For the
conventional vaccines, such as TT or similar vaccines, RA alone may improve vaccine efficiency by increasing Ag-specific antibody production. However, for the vaccines designed to induce cell-mediated immunity, such as live attenuated vaccines, RA alone may suppresses type 1 cytokines and CTL response, thereby diminishing the vaccine efficiency. In neonates and infants, the immaturity of type 1 response is considered as a major factor contributing to their high susceptibility to pathogens, particularly intracellular pathogens. Therefore, giving RA alone will further skew neonatal immune response towards a type 2 direction, decreasing their resistance to infection. The present study reported that the combination of RA with PIC, an IFN inducer and a powerful adjuvant, was more potent than either agent alone in increasing anti-TT antibody production, promoting memory response, and stimulating a balanced type 1/type 2 response. These data suggest that RA should be combined with an appropriate adjuvant, such as PIC, to promote vaccine-induced immune response. In addition to PIC, RA may also be combined with some other Th1 adjuvants, such as CpG oligonucleotide, to increase vaccine efficiency (263). The adjuvant effect of RA combined with other Th1 adjuvants is worth to be investigated in the future.

Overall, the present study suggests that RA combined with PIC, a nutritional-immunological intervention, may be a promising strategy to enhance the efficiency of vaccine in both adults and neonates.
Fig. 5-1. The potential mechanisms by which RA and PIC can regulate TT-induced vaccine response. Co-administration of RA and/or PIC with TT priming significantly enhances both primary and secondary anti-TT antibody response as well as IgG isotypes. RA and/or PIC also induce the generation and differentiation of immune cell, promote the germinal center reaction, and differentially regulate type 1/type 2 cytokine response. APC, antigen-presenting cell; DC, dendritic cell; Mø, macrophage; CD40L, CD40 ligand.
REFERENCES


23. Jankovic, D., Kullberg, M. C., Hieny, S., Caspar, P., Collazo, C. M. & Sher, A. (2002) In the absence of IL-12, CD4(+) T cell responses to intracellular pathogens fail to default to a Th2 pattern and are host protective in an IL-10(-/-) setting. Immunity 16: 429-439.


differentiation but are dispensable for IL-4-dependent rescue from apoptosis. Mol Cell Biol 22: 117-126.


## Appendix A. LIST OF PRIMERS

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-4</td>
<td>5'-AGAGCTATTGATGGGTCTCA 3'-GGCTTTCAAGGAAGTCTTC</td>
<td>401 bp</td>
</tr>
<tr>
<td>(NM 21283)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-10</td>
<td>5'-AATAACTGCACCCACCTTCCC 3'-CATGGGCTTTGTAGACACCTT</td>
<td>260 bp</td>
</tr>
<tr>
<td>(NM 010548)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12</td>
<td>5'-AGACCCTGCCATTTGAAGCTG 3'-TTCTCTACGAGGAACGCACC</td>
<td>288 bp</td>
</tr>
<tr>
<td>(NM 008352)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-AAAGACAACCAGGCCCATTGAAGCTG 3'-CGACTCCTTTTCCGCTTCCT</td>
<td>220 bp</td>
</tr>
<tr>
<td>(NM 008337)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IRF-1</td>
<td>5'-TGAGACCCTGCCTGAGGATG 3'-TACGCTGCTGCCGATGGC</td>
<td>173 bp</td>
</tr>
<tr>
<td>(NM 008390)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T-bet</td>
<td>5'-GATCGGTCTCTGCTAGGATG 3'-AATCAGGTGCTGAGGTCG</td>
<td>413 bp</td>
</tr>
<tr>
<td>(AF241242)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GATA-3</td>
<td>5'-CTTATCAAGGCCCCAGGAAG 3'-CAGGGATGACATGTGTCTGG</td>
<td>311 bp</td>
</tr>
<tr>
<td>(NM008091)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-12Rβ2</td>
<td>5'-TGACAGCTGCTGAGGCTAGGCTAGGTCCTTCC</td>
<td>269 bp</td>
</tr>
<tr>
<td>(NM008354)</td>
<td>3'-ATGATCAGGGGCTCAGGCTCTTCC</td>
<td></td>
</tr>
<tr>
<td>18S rRNA</td>
<td>5'-AATGGGTGCTACCCGGTCATTCC 3'-ACCTCTCTTACCGCTTCC</td>
<td>193 bp</td>
</tr>
<tr>
<td>(M35283)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-TGAAGGGTCGAGTCAACCGGATTGTGTTGTCAGTCAAGGGAAG 3'-CATGTGGGCCCATGAGGTCACCAC</td>
<td>982 bp</td>
</tr>
</tbody>
</table>
## Appendix B. LIST OF ANTIBODIES

<table>
<thead>
<tr>
<th>Ab</th>
<th>clone</th>
<th>Producer</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC-CD3</td>
<td>17A2</td>
<td>BD PharMingen</td>
<td>0.125 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-CD4</td>
<td>H129.19</td>
<td>BD PharMingen</td>
<td>0.2 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>FITC-CD8</td>
<td>53-6.7</td>
<td>BD PharMingen</td>
<td>0.125 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-B220</td>
<td>RA3.6B2</td>
<td>BD PharMingen</td>
<td>0.0625 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>Cy5-B220</td>
<td>RA3.6B2</td>
<td>BD PharMingen</td>
<td>0.01 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>FITC-CD19</td>
<td>1D3</td>
<td>BD PharMingen</td>
<td>0.25 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-CD19</td>
<td>1D3</td>
<td>BD PharMingen</td>
<td>0.05 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-CD21</td>
<td>7G6</td>
<td>BD PharMingen</td>
<td>0.2 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-CD38</td>
<td>NIMR-5</td>
<td>Southern Biotech</td>
<td>0.05 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-CD40</td>
<td>1C10</td>
<td>Southern Biotech</td>
<td>0.2 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-IgM</td>
<td>R6-60.2</td>
<td>BD PharMingen</td>
<td>0.1 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>FITC-IgD</td>
<td>11-26</td>
<td>BD Biosciences</td>
<td>0.0625 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-goat-anti-IgG1</td>
<td>Molecular probes</td>
<td>0.25 μg/5×10⁶ cells</td>
<td></td>
</tr>
<tr>
<td>Alxa488-IgG1</td>
<td></td>
<td>Molecular probes</td>
<td>0.2 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-Fas</td>
<td>Jo2</td>
<td>BD PharMingen</td>
<td>0.2 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-CD138</td>
<td>281-2</td>
<td>BD PharMingen</td>
<td>0.2 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-MHC II</td>
<td>AF6-120</td>
<td>BD PharMingen</td>
<td>0.03 μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-NK1.1</td>
<td>PK136</td>
<td>BD PharMingen</td>
<td>0.25μg/5×10⁶ cells</td>
</tr>
<tr>
<td>PE-CD111b</td>
<td>M1/70</td>
<td>BD PharMingen</td>
<td>0.25μg/5×10⁶ cells</td>
</tr>
<tr>
<td>FITC-CD80</td>
<td>16-10A1</td>
<td>BD PharMingen</td>
<td>0.125μg/5×10⁶ cells</td>
</tr>
<tr>
<td>Ab</td>
<td>clone</td>
<td>Producer</td>
<td>Concentration</td>
</tr>
<tr>
<td>---------------------</td>
<td>-------</td>
<td>------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>FITC-CD86</td>
<td>GL1</td>
<td>BD PharMingen</td>
<td>0.125 μg/5×10^6 cells</td>
</tr>
<tr>
<td>PE-CD86</td>
<td>GL1</td>
<td>Southern Biotech</td>
<td>0.1 μg/5×10^6 cells</td>
</tr>
<tr>
<td>FITC-PNA</td>
<td></td>
<td>Vector Laboratory</td>
<td>0.25 μg/5×10^6 cells</td>
</tr>
<tr>
<td>Purified anti-mouse CD3</td>
<td>145-2C11</td>
<td>BD PharMingen</td>
<td>2.5 μg/ml</td>
</tr>
<tr>
<td>Purified anti-mouse IL-4</td>
<td>11b11</td>
<td>BD PharMingen</td>
<td>2 μg/ml</td>
</tr>
<tr>
<td>Purified anti-mouse IL-5</td>
<td>TRFK4</td>
<td>BD PharMingen</td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>Purified anti-mouse IFN-γ</td>
<td>R4–6A2</td>
<td>BD PharMingen</td>
<td>4 μg/ml</td>
</tr>
<tr>
<td>Biotinylated anti-mouse IL-4</td>
<td>BVD6-24G2</td>
<td>BD PharMingen</td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>Biotinylated anti-mouse IL-5</td>
<td>(TRFK4)</td>
<td>BD PharMingen</td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>Biotinylated anti-mouse IFN-γ</td>
<td>(XMG1.2)</td>
<td>BD PharMingen</td>
<td>1 μg/ml</td>
</tr>
<tr>
<td>AP-anti-mouse IgG</td>
<td></td>
<td>The Binding Site</td>
<td>1:5000</td>
</tr>
<tr>
<td>AP-anti-mouse IgG1</td>
<td></td>
<td>The Binding Site</td>
<td>1:5000</td>
</tr>
<tr>
<td>AP-anti-mouse IgG2a</td>
<td></td>
<td>The Binding Site</td>
<td>1:2000</td>
</tr>
<tr>
<td>AP-anti-mouse IgG2b</td>
<td></td>
<td>The Binding Site</td>
<td>1:4000</td>
</tr>
</tbody>
</table>
Appendix C. REPRINT PERMISSION FROM JI

October 24, 2005

Yifan Ma
The Penn State University
The Department of Nutritional Sciences s-126 Henderson South
State college, PA 16801
USA
Phone: 814-237-8286
Fax:
Email: yum100@psu.edu

Dear Dr. Ma:

The Journal of Immunology grants permission to reproduce article found in Volume 174, p. 7961-7969, 2005, in your thesis contingent upon the following conditions:

1. That you give proper credit to the authors and to The Journal of Immunology, including in your citation the volume, date, and page numbers.

2. That you include the statement:
   Copyright 2005. The American Association of Immunologists, Inc.

Please understand that permission is granted for one-time use only for print and electronic format. Permission must be requested separately for future editions, revisions, translations, derivative works, and promotional pieces.

Thank you for your interest in The Journal of Immunology.

Very truly yours,

Regina B Prince
Web Content Coordinator
Yifan Ma

**Education**
The Pennsylvania State University, University Park, PA
Ph.D. in Integrative Biosciences  (2006)
Nanjing Medical University, Nanjing, P. R. China

**Professional Experience**
The Pennsylvania State University, University Park, PA
Graduate research assistant (2000-2005)
- The effects of retinoic acid and polyriboinosinic: polyribocytidylic acid (PIC) on Tetanus Toxoid-induced vaccine responses in both adult and neonatal mice
Jiangsu Provincial Center of Maternal and Child Health Care, Nanjing, P. R. China
Pediatrician (1997- 1999)
Nanjing Medical University, Nanjing, P. R. China
Graduate research assistant (1994 – 1997)
- The effects of vitamin A deficiency /excess on cytokine production and T-cell differentiation in rats

**Teaching Experience**
The Pennsylvania State University, University Park, PA
Teaching assistant (2001– 2002)
- Nutr 251 (Introductory principles of nutrition) & Nutr 451 (Diet and diseases)

**Awards and Honors**
- Life Science Consortium Fellowship (2000)
- Life Science Consortium Scholarship (2000)
- Distinguished Graduate Student Scholarship, Nanjing Medical University (1995)
- Madam Bu-Wei Yang Scholarship, Nanjing Medical University (1994)
- Distinguished Student Scholarship, Nanjing Medical University (1989-1993)

**Selected Publications**