Vitamin A in regulation of B cell functions

Agnete Bratsberg Eriksen

UNIVERSITY OF OSLO
Faculty of Medicine
Institute of Basic Medical Sciences
Department of Molecular Medicine

Thesis for the Degree of Philosophiae Doctor

June 2015
© Agnete Bratsberg Eriksen, 2015

*Series of dissertations submitted to the Faculty of Medicine, University of Oslo No. 2120*

ISBN 978-82-8333-130-1

All rights reserved. No part of this publication may be reproduced or transmitted, in any form or by any means, without permission.

Cover: Hanne Baadsgaard Utigard.
Print production: John Grieg AS, Bergen.

Produced in co-operation with Akademika Publishing.
The thesis is produced by Akademika Publishing merely in connection with the thesis defence. Kindly direct all inquiries regarding the thesis to the copyright holder or the unit which grants the doctorate.
TABLE OF CONTENTS

ACKNOWLEDGEMENTS ................................................................................................................. 1
LIST OF PUBLICATIONS ................................................................................................................. 2
ABBREVIATIONS ............................................................................................................................. 3
1 INTRODUCTION ............................................................................................................................ 7
  1.1 Vitamin A ................................................................................................................................... 7
    1.1.1 Vitamin A metabolism ........................................................................................................... 7
    1.1.2 Retinoic acid – mechanism of action .................................................................................... 9
  1.2 The immune system .................................................................................................................. 10
    1.2.1 Overview .............................................................................................................................. 10
    1.2.2 B cell development ............................................................................................................. 11
    1.2.3 Activation of B cells ............................................................................................................ 12
    1.2.4 Memory B cells ................................................................................................................... 13
    1.2.5 Plasma cells .......................................................................................................................... 13
  1.3 Toll-like receptors .................................................................................................................... 14
    1.3.1 TLR9 .................................................................................................................................... 14
    1.3.2 RP105 (CD180) .................................................................................................................... 16
  1.4 Vitamin A and the immune system .......................................................................................... 16
    1.4.1 Vitamin A and B cells .......................................................................................................... 18
  1.5 Autophagy .................................................................................................................................. 18
    1.5.1 The molecular mechanism of autophagy ............................................................................ 20
    1.5.2 Retinoic acid and autophagy ............................................................................................... 21
  1.6 Autophagy and the immune system ......................................................................................... 21
    1.6.1 Autophagy involved in direct elimination of pathogens ...................................................... 22
    1.6.2 Autophagy and lymphocytes ............................................................................................... 22
1.7 Multiple sclerosis ................................................................. 23
   1.7.1 Prevalence and etiology of MS ........................................... 24
   1.7.2 Diagnosis of MS ............................................................ 24
   1.7.3 Pathology of MS ............................................................ 24
   1.7.4 Role of B cells in MS disease activity ................................. 25
   1.7.5 Treatment of MS ............................................................ 25

2 AIMS OF THE STUDY .................................................................... 27

3 SUMMARY OF THE PAPERS .......................................................... 28

4 DISCUSSION .............................................................................. 30
   4.1 Methodological considerations ............................................. 30
      4.1.1 Methods for isolation of B cells ...................................... 30
      4.1.2 MS-derived B cells ....................................................... 31
      4.1.3 Activation of B cells ...................................................... 32
      4.1.4 Retinoic acid ............................................................... 32
      4.1.5 Proliferation assays ...................................................... 33
      4.1.6 Measurement of cytokines and immunoglobulins .............. 34
      4.1.7 Assays related to the analysis of autophagy ..................... 34
      4.1.8 Ethical considerations ................................................... 36
   4.2 General discussion .............................................................. 37
      4.2.1 RA-mediated proliferation and antibody production in human primary B cells .... 37
      4.2.2 The involvement of autophagy in RA-mediated antibody production ................ 39
      4.2.3 The impact of RA on MS-derived B cells ............................ 41

5 CONCLUSIONS ........................................................................... 44

6 FUTURE DIRECTIONS ................................................................. 45

7 REFERENCES ............................................................................ 46

8 APPENDIX: Paper I, II and III ...................................................... 81
ACKNOWLEDGEMENTS

The work presented in this thesis was performed at the Department of Molecular Medicine, Institute of Basic Medical Sciences, University of Oslo from October 2008 to June 2015, with financial support from the University of Oslo.

First, I would like to thank my supervisor Professor Heidi Kiil Blomhoff for excellent guidance and support. I greatly appreciate your enthusiasm and that you are always available for discussions and input. Your ability to find new approaches and strategies is very inspiring! I am very grateful for being a member of your group.

I thank Randi Larsen Indrevær and Kristine Lillebø Holm for interesting discussions on vitamin A and B cells, and for creating a friendly and positive work environment. I thank Virginie Follin-Arbelet for all the years we shared office, frustrations and laughs! I also thank Janicke Holmseth Bukve and Camilla Solberg for their support and enthusiasm. I am grateful to all past and current members of our group; Nina Richartz, Seham Skah, Elin Hallan Naderi, Hege Ugland, Martine Müller Kloster, Britt Fux-Nilsen and Soheil Naderi.

I would also like to thank all my collaborators on the autophagy project. I thank Maria Lyngås Torgersen and Professor Anne Simonsen for sharing their knowledge about autophagy and for excellent technical guidance. I thank Greger Abrahamsen and Professor Anne Spurkland for support regarding Image Stream analysis.

All the researchers involved in the MS project deserve a big thank you. I thank Professor Hanne Flinstad Harbo and Tone Berge for extensive help in recruiting MS patients and for fruitful discussions. I also thank Marte Wendel Gustavsen, Stefan Daniel Bos and Ingvild Sørum Leikfoss for their assistance and time spent isolating B cells.

Finally, I would like to thank my family. I thank my parents for always supporting and believing in me. I thank my husband Andreas Eriksen for providing humorous and philosophical perspectives to our life as PhD students. Special thanks to our children; Ludvig, Mathea and Selma, for distractions filled with laughter, hugs and joy!

Oslo, June 2015
Agnete Bratsberg Eriksen
LIST OF PUBLICATIONS

Paper I

Eriksen AB, Indrevaer RL, Holm KL, Landskron J, Blomhoff HK.
**TLR9-signaling is required for turning retinoic acid into a potent stimulator of RP105 (CD180)-mediated proliferation and IgG synthesis in human memory B cells.**
*Cell Immunol* 2012 Sep 19;279(1):87-95

Paper II

**Retinoic acid-induced IgG production in TLR-activated human primary B cells involves ULK1-mediated autophagy.**
*Autophagy* 2015 March 4;11(3):460-71

Paper III

Eriksen AB, Berge T, Gustavsen MW, Leikfoss IS, Bos SD, Spurkland A, Harbo HF, Blomhoff HK.
**Retinoic acid enhances the levels of IL-10 in TLR-stimulated B cells from patients with relapsing-remitting multiple sclerosis.**
*J Neuroimmunol* 2015 Jan 15;278:11-8

Reprints were made with permission.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>AMP</td>
<td>adenosine monophosphate</td>
</tr>
<tr>
<td>APL</td>
<td>acute promyelocytic leukemia</td>
</tr>
<tr>
<td>ATG</td>
<td>autophagy-related</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BBB</td>
<td>blood-brain barrier</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BDS</td>
<td>bright detail similarity</td>
</tr>
<tr>
<td>Blimp1</td>
<td>B-lymphocyte-induced maturation protein 1</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CFSE</td>
<td>5- (and -6)-carboxyfluorescein diacetat succinimidyl ester</td>
</tr>
<tr>
<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CMA</td>
<td>chaperone-mediated autophagy</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CpG</td>
<td>cytosine-phosphate-guanine</td>
</tr>
<tr>
<td>CRBP-II</td>
<td>cellular retinol-binding protein II</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>CSR</td>
<td>class switch recombination</td>
</tr>
<tr>
<td>CVID</td>
<td>common variable immunodeficiency</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DR</td>
<td>direct repeat</td>
</tr>
<tr>
<td>EDSS</td>
<td>expanded disability status scale</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
</tbody>
</table>
ER
ERK
GA
GABARAP
GALT
GATE16
GC
\(^3\)H
HDAC
HSC
IFN
Ig
IL
ILC
LAP
LC3B/MAP1LC3B
LPS
MAPK
MD-1
MHC
MMP
MRI
mRNA
MS
mTOR
MyD88

doplasmic reticulum
extracellular signal-regulated kinase
glatiramer acetate
GABA(A)-receptor associated protein
gut-associated lymphoid tissue
golgi-associated ATPase enhancer of 16 kDa
germinal center
tritium
histone deacetylase
hematopoietic stem cell
interferon
immunoglobulin
interleukin
innate lymphoid cell
LC3-associated phagocytosis
microtubule-associated protein 1 light chain 3 \(\beta\)
lipopolysaccharide
mitogen-activated protein kinase
myeloid differentiation protein-1
major histocompatibility complex
metalloproteinase
magnetic resonance imaging
messenger ribonucleic acid
multiple sclerosis
mechanistic target of rapamycin (serine/threonine kinase)
myeloid differentiation primary response 88
NF-κB  nuclear factor kappa B
OCB  oligoclonal bands
ODN  oligodeoxynucleotide
PAMP  pathogen-associated molecular pattern
PBMC  peripheral blood mononuclear cell
PC  plasma cell
PE  phosphatidylethanolamin
PI  propidium iodide
PI3K  phosphoinositide 3-kinase
PKCα  protein kinase C α
PML/RARA  promyelocytic leukemia/retinoic acid receptor α
PPMS  primary progressive multiple sclerosis
PRR  pattern recognition receptor
RA  all-trans retinoic acid
RALDH  retinaldehyde dehydrogenase
RAR  retinoic acid receptor
RARE  retinoic acid response element
RB1CC1/FIP200  RB1-inducible coiled coil 1
RBP  retinol binding protein
RNA  ribonucleic acid
RP105  radioprotective 105 kDa
RRMS  relapsing-remitting multiple sclerosis
RXR  retinoic X receptor
SAC  staphylococcus aureus Cowan I
SHM  somatic hypermutation
siRNA  small interfering RNA
SLR  sequestosome 1-like receptor
SMRT silencing mediator for retinoid and thyroid hormone receptor
SPMS secondary progressive multiple sclerosis
STRA6 stimulated by retinoic acid 6
SQSTM1/p62 sequestosome 1
Th T helper
TI T cell-indepent
TIR toll/IL-1 receptor
TLR toll-like receptor
TNF tumor necrosis factor
TRAF6 TNF receptor associated factor 6
Treg regulatory T cell
TTNPB tetrahydrotetramethylnaphtalenylpropenylbenzoic acid
ULK1 unc-51 like autophagy activating kinase 1
UPR unfolded protein response
VAD vitamin A deficiency
XBP1 X-box binding protein 1
1 INTRODUCTION

1.1 Vitamin A

Vitamin A was discovered in 1913 as a fat soluble factor that was essential for mammalian growth and survival (1,2) and has later been ascribed crucial roles in vital processes in the body, such as in eyesight, metabolism, organogenesis, immunological fitness and in the nervous system (3-6). The important contribution of vitamin A to health has been demonstrated by the dramatically curbed degree of childhood mortality in endemic regions of malnutrition obtained by supplementation of vitamin A (7,8). It is estimated that 100-200 million children worldwide suffer from vitamin A deficiency, and that approximately 10 % of these children will die from infectious diseases (9). Hence, the reduced mortality obtained by vitamin A supplementation has primarily been linked to reduced extent and severity of infectious diseases (10-12). On the other hand, it has been reported that excess intake of vitamin A may, due to its fat-solubility, result in accumulation of vitamin A in the liver and adipose tissues and have negative consequences such as embryonic malformation (13) and reduced bone mineral density (14). Signs of hypervitaminosis A can be seen after excess dietary intake of vitamin A and are related to the skin, circulation, internal organs, nervous system and the musculoskeletal system (15,16).

1.1.1 Vitamin A metabolism

The term vitamin A includes all compounds that possess the biological activity of all-trans retinol. Vitamin A is an essential vitamin that cannot be synthesized de novo by animals. The major sources of vitamin A in the human diet are the provitamin A carotenoids (mainly β-carotene) in vegetables and fruits, as well as the retinyl esters (mainly retinyl palmitate) in animal-based foods such as cod liver oil, fatty fish and egg (4,17). β-carotene is converted to retinol (via retinal) in the intestinal mucosa (18), while the retinyl esters are hydrolyzed to free retinol and fatty acids prior to intestinal absorption (19). Enterocytes absorb the free retinol (20) where it is bound to the binding protein CRBP-II (Cellular Retinol-Binding Protein II) (21), re-esterified with long-chain saturated fatty acids, packed into chylomicrons and secreted into the intestinal lymph (22). The released chylomicrons move into the circulation and form chylomicron remnants, that are taken up by parenchymal liver cells (hepatocytes) (23). Extrahepatic uptake of chylomicron remnants occurs also in the bone marrow, peripheral blood cells and the spleen, which is of great importance for the immune
system (24). The hepatocytes secrete retinol bound to RBP (Retinol Binding Protein) into plasma, but also to perisinusoidal stellate cells in the liver for storage (23). The released retinol-RBP in plasma is kept at a concentration of approximately 2 μM depending on the daily intake of vitamin A (23), and the complexes are subsequently taken up by target cells via the RBP-binding receptor STRA6 (25). Once inside the cell, alcohol dehydrogenases (ADH) oxidize retinol into retinal, which can be further oxidized into retinoic acid via retinaldehyde dehydrogenases (RALDH). Retinoic acid can exist in multiple isoforms (26), with all-trans retinoic acid (RA) being the most metabolically active and predominant isoform in most tissues (27). Trace amounts (approximately 10 nM) of RA bound to albumin is normally also present in blood (28,29). See Figure 1 for the structure of selected retinoids.

![Structure of selected retinoids](image)
1.1.2 Retinoic acid – mechanism of action

The intracellular activity of RA is mediated via receptors termed retinoic acid receptors (RAR) and retinoic X receptors (RXR), with variants such as α, β and γ. These belong to the superfamily of nuclear receptors that are transcription factors, and they function as heterodimers or homodimers. RAR is a ligand-dependent transcription factor that requires the association with all-trans retinoic acid, while 9-cis retinoic acid can bind to both RARs and RXRs (30-33). The binding of the RAR/RXR heterodimer to the retinoic acid response element (RARE) sequence in the promoter region of target genes results in the transcription of these genes (Figure 2). In the absence of ligands the RAR/RXR heterodimer recruits co-repressors such as the silencing mediator for retinoid and thyroid hormone receptor (SMRT) and the nuclear receptor corepressor (NCoR) - both of them binding to and blocking the ligand-binding domains of RAR and RXR. In turn, histone deacetylases (HDAC) are recruited to the complexes, leading to deacetylation of histones and thereby to chromatin that is less available for transcription factors to bind to transcription start sites (34). Binding of the ligands will promote the replacement of HDAC with co-activators leading to transcription of the target genes. The RARE sequences consists of two core hexamer motifs, (A/G)G(G/T)TCA, separated by 1, 2 or 5 base pairs, DR1, DR2 and DR5, respectively. The RAR/RXR heterodimer preferentially binds to the DR2 and DR5 motifs, whereas DR1 most often binds RXR homodimers (33,35,36). Also transcription-independent effects of RARs such as phosphorylation of the RARα ligand binding domain and RAR-independent effects of RA such as direct binding and inhibition of PKCα have been reported (37-40); however these are not as common as the classical RA-mediated transcriptional regulation via RARs.

Figure 2. RA-mediated gene transcription via RAR/RXR binding to RARE.
1.2 The immune system

1.2.1 Overview

The immune system is the collection of cells, tissues and molecules that protects the body against pathogenic microbes and toxins. The first line of defense encountered by pathogens consists of physical and biochemical barriers, such as the skin, mucosal linings, cilia of the respiratory tract and lysozymes in tears. The pathogens that are able to pass these barriers will encounter the body’s next line of defense, the immune cells. Within a few hours a nonspecific immune reaction is activated by cells belonging to the innate immune system, such as dendritic cells, monocytes, macrophages, granulocytes and innate lymphoid cells. The innate immune system is the most ancient and responds to structures common to several pathogens, such as pathogen-associated molecular patterns (PAMPS) recognized by toll-like receptors (TLRs) (41-43). After a few days, the specific (adaptive) immune system is activated and it collaborates with the innate immune system to fight the pathogens. The adaptive immune response consists of a humoral and a cellular response mediated by B- and T lymphocytes respectively, and it is highly specific by recognizing structures that are exclusively present on the pathogens. The lymphocytes continuously circulate the blood and lymphatic system and are also present in lymphoid organs such as the lymph nodes, thymus, spleen and appendix (see Figure 3).

Figure 3. Primary (yellow) and secondary (blue) lymphoid organs of the immune system. Adapted from (44).
1.2.2 B cell development

Both B cells and T cells originate from a common lymphoid progenitor in the bone marrow. Whereas T cells complete their maturation in the thymus, the B cells develop from B progenitor cells (pro-B cells) to the immature B cell state in the bone marrow and complete maturation in the periphery (Figure 4) (45). During development in the bone marrow the immunoglobulin genes are rearranged, leading to the generation of cytoplasmic expression of the IgM heavy chain (µ-chain) together with the so-called surrogate light chain defining the pre-B cell stage (46). The pre-B cells further develop into immature B cells expressing surface IgM, and potentially autoreactive cells are at this point eliminated. The translational B cells expressing both IgM and IgD are released into the periphery with a unique surface B-cell receptor (BCR) (47). The BCR is required for further B cell development and survival (48). When migrating to secondary lymphoid organs, such as the spleen or lymph nodes, the B cells may encounter antigens through interactions with immune cells like dendritic cells or macrophages. The B cell can then either differentiate into a short lived plasma cell or form germinal centers (GCs) (see Figure 4). GCs are sites within the secondary lymphoid organs where the B cell can undergo somatic hypermutation (SHM), class switch recombination (CSR) and clonal expansion. This process leads to the production of memory B cells (see section 1.2.4) and high affinity antibody-producing plasma cells (see section 1.2.5) (49). The memory B cells are long-lived and responsible for maintaining immunity against a certain pathogen. Also long-lived plasma cells can be generated from the GC reaction, and these cells migrate to and can reside in the bone marrow for several years (50,51). CD19 is a cell surface marker that is present on B cells from the B cell progenitor stage, whereas CD20 is expressed from the pre-B cell stage. Both CD19 and CD20 are lost at the plasma cell stage and are therefore used as markers to isolate, deplete or distinguish B cells from plasma cells (52,53).
**1.2.3 Activation of B cells**

Activation of B cells can be induced by two different classes of antigens, T cell-dependent- and T cell-independent antigens. T cell-dependent antigens are typically pathogen-derived proteins, and T cell-dependent activation occurs in a two-step process. The first step involves binding of the antigen to the BCR, followed by internalization and degradation of the antigen into peptides that are presented on MHC class II complexes on the cell surface of the B cell. The second step involves T cells that recognize the antigen bound to MHC class II complexes, and the ligation of the CD40 receptor with the CD40-ligand on the surface of T cells (54-56), resulting in proliferation, CSR, SHM, and generation of memory B cells and plasma cells (57,58). T cells are also able to provide B cells with co-stimulatory molecules such as IL-2, IL-4, and IL-10 that enhance BCR-mediated stimulation of B cells. T cell-independent activation of B cells involves activation of the BCR by antigens characterized by repeated structures such as polysaccharides typically found at the surface of pathogens (55,59). Several antigen-bound BCRs will then cluster together and become extensively cross-linked, resulting in activation of the B cell. T cell-independent activation of B cells can also include activation via the toll-like receptors (TLRs) (see chapter 3).
1.2.4 Memory B cells

A mature B cell that has not yet encountered any antigen is termed a naïve B cell. When a naïve B cell encounters an antigen, it will either differentiate into a plasma cell (see section 1.2.5) or into a long-lived memory B cell (60). Upon the second encounter with the antigen, the memory B cells will experience a proliferative burst and differentiate at a faster pace than the naïve B cells (61-64). Naïve B cells can be distinguished from memory B cells based on their expression of CD27, IgM and IgD (65-67). Whereas naïve B cells are CD27-IgM⁺IgD⁺, memory B cells are CD27⁺ (68) and can further be divided into two subgroups. One subgroup has not yet undergone isotype switching and still express IgM and various degrees of IgD on the cell surface (CD27+IgM⁺IgD sildenafil) (65,69,70), and upon activation these cells differentiate into IgM-secreting plasma cells. The other is the ‘classic’ memory B cell subgroup that has undergone class switching in the GC in response to T cell-dependent antigens. Here they undergo hypermutation of the variable (V) regions of the immunoglobulin (Ig) genes, express surface CD27 and switch to any other class of Ig than IgD and IgM, resulting in the IgM⁺IgD⁻ phenotype (71,72). Upon activation, the IgM⁺IgD⁻ memory B cells differentiate into IgG-, IgA- or IgE-secreting plasma cells. The memory B cells formed in the GC reaction require lower concentrations of antigen and T cell help for their activation (50). Recently, generation of memory B cells independent of GC reactions have also been identified (73,74).

1.2.5 Plasma cells

Plasma cells lack the surface molecules CD19 and CD20 (52,53), but are characterized by the expression of the cell surface markers CD38 and CD138 (75,76). The plasma cells are responsible for the total antibody production in the body and can secrete up to 10 000 molecules per second (77). Differentiation of B cells into plasma cells involves expansion of the endoplasmic reticulum (ER) required for secretion of such large amounts of antibodies (78,79). Plasma cells that reside in the bone marrow are considered as long-lived. These cells produce antibodies for several years (50,51), which is important given the short half-life of antibodies in the circulation (80).

1.2.5.1 Antibodies

Antibodies are large Y-shaped proteins that can bind and neutralize antigens and thereby help the immune system to eliminate pathogens. The general structure of an antibody consists of two heavy peptide chains and two light peptide chains. Each heavy and light chain consists of a constant and a variable region. The variable regions determine the specificity of the
antibody; whereas the constant regions of the heavy chain determine the class of antibody. There are five different classes; IgA, IgD, IgE, IgM and IgG, each with several subclasses. IgAs are mainly found in the mucosal areas of the body and pass into breast milk, whereas IgDs are primarily expressed as antigen receptors on the surface of B cells. IgEs are involved in immune reactions to parasitic worms and are also involved in allergic reactions. IgMs are expressed as monomers on the surface of B cells, but can also be secreted as pentamers and circulate as part of the initial immune reaction. IgG is the most abundant antibody in blood and it also passes through the placenta (81).

1.3 Toll-like receptors

TLRs are pattern recognition receptors (PRRs) that recognize signature patterns of pathogens (PAMPs) (41-43). TLRs are type 1 integral membrane glycoproteins and have a trimodular structure. The extracellular N-terminal domain consists of 16-28 leucine-rich repeats, whereas the intracellular C-terminal end forms the Toll/IL-1 receptor (TIR) domain. The general pattern-recognizing ability of TLRs classifies them as part of the innate immune system. However, TLRs also play important roles in development of pathogen-specific adaptive immunity mediated by the B and T cells (82), and as such they can be considered as bridges between innate and adaptive immune systems (83). The TLR family comprises 10 members (TLR1-10) in humans and 12 in mice (TLR1-9, TLR11-13). The TLRs located on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11) recognize membrane components; whereas the TLRs expressed in intracellular vesicles such as the endosomes and ER (TLR3, TLR7, TLR8 and TLR9) recognize nucleic acids (41-43). The various receptors are differently distributed among the cells of the immune system (84).

1.3.1 TLR9

TLR9 is located in the endosomes of monocytes, natural killer cells, dendritic cells and B cells. Memory human B cells express TLR9 at substantially higher levels than naïve human B cells (85). TLR9 recognizes genomic DNA rich in unmethylated CpG motifs – typically found in bacteria and viruses (86,87). Pathogen-mediated stimulation of TLR9 can be mimicked in vitro by CpG oligodeoxynucleotides (CpG-ODNs). CpG-ODNs are short single stranded unmethylated DNA sequences containing a cytosine triphosphate deoxynucleotide connected with a phosphodiester bond to a guanine triphosphate deoxynucleotide. Stimulation of TLR9 activates MyD88, TRAF6 and other signaling pathways ultimately leading to the
nuclear translocation of NF-κB and AP-1. This will in turn lead to transcriptional activation of pro-inflammatory genes, resulting in enhanced proliferation and differentiation (see Figure 5) (88,89). *In vivo* CpG-ODNs can stimulate human memory B cells inducing the differentiation into plasma cells, and hence function as a way to maintain long-term memory in the absence of further exposure to the antigen (90). TLR9-activation has recently been linked to BCR signaling via autophagy. Activation of BCR leads to mobilization of TLR9-containing endosomes and their fusion with autophagosomes. After the antigen-triggered BCR internalization, BCR-containing vesicles will fuse with the TLR9-containing autophagosomes, and TLR9 will be activated if the BCR-cargo contains CpG ODNs (91,92).

*Figure 5. TLR9 signaling. Modified from (84).*
1.3.2 RP105 (CD180)

RP105 (CD180) was originally discovered as a radioprotective B cell surface protein with a size of 150 kDa (93). The physiological ligand of RP105 has not yet been identified, but the receptor is activated in vitro by cross-linking with anti-RP105 antibodies. It has been shown that activation of the receptor both in vitro (93,94) and in vivo (95) enhances proliferation and Ig production in human and murine B cells, and it was recently shown that activation of RP105 also enhances TLR9-mediated viability and proliferation of naïve peripheral B cells (96). RP105 is structurally regarded as a TLR4 homolog (97), but RP105 lacks the intracellular TIR domain (93,98). The surface expression of RP105 is dependent on MD-1 (99,100), and although the protein is primarily expressed on mature B cells (94,101), it can also be detected on dendritic cells and macrophages (102,103). Dysregulation of RP105 has been linked to atherosclerosis (104), cardiac dysfunction (105) and systemic lupus erythematosus (SLE) (106-108).

1.4 Vitamin A and the immune system

The first discovery of vitamin A as a regulator of the immune system was made in the 1920s, with the recognition of its anti-infective effects in animal models (109). Vitamin A deficiency (VAD) is a major health problem in developing countries, and can arise both from a vitamin A deficient diet, but also from infectious diseases which deplete the body of vitamin A (10). Studies on preschool children in developing countries have shown that vitamin A supplementation reduces the incidences of diarrhea and measles, and importantly also the mortality rates (110). Recently a link between a well-functioning immune system and diet of pregnant mice was found; it was seen that dietary vitamin A of the mother was crucial for the proper development of lymph nodes in the fetus and for the immunity in the adult offspring (111). The beneficial effect of vitamin A on the immune system has over the years been firmly established (10,110-113), and involves immune cells such as dendritic cells, macrophages, T cells, innate lymphoid cells (iLCs) and B cells (section 1.4.1).

In dendritic cells RA has been demonstrated to regulate survival and antigen-presentation (114), promote differentiation (115,116) and migration by enhancing the production of metalloproteinases (MMP) (117). Interestingly, also the ability of dendritic cells to induce the expression of mucosal homing receptors on B and T cells depends on RA (118,119), and this effect of RA is activated via RAR-mediated signaling and transcription (120). Dendritic cells
are one of the types of immune cells that are able to metabolize RA intracellularly, via RALDH2 enzyme activity that converts retinal to retinoic acid (118,121,122). The subsequent release of RA makes it available also for other immune cells that do not possess RALDH2, such as B cells. Actually synthesis of RA by dendritic cells in the gastrointestinal tract and associated lymphoid tissue (GALT) is crucial for the generation of IgA+ B cells, and hence the presence of mucosal IgA (119,123).

In macrophages and monocytes RA mediates cytokine production that favors the generation of CD4+ Th2 cells (124-126), which are immune cells important for the clearance of helminths and extracellular pathogens. Also RA in itself has been shown to promote the Th2 cell response at the expense of Th1 responses in vivo (127). Our group and others have previously shown that also T cell proliferation and IL-2 secretion is induced by RA (128-130). RA is important for self-tolerance and prevention of autoimmune diseases by promoting the conversion of naïve CD4+ T cells into iTregs (induced FoxP3+ T cells) (131-134). At the same time as RA promotes iTreg-formation it inhibits the formation of the proinflammatory Th17 cells from CD4+ T cells, and plays therefore an important role in regulation of the balance between Tregs and Th17 cells (135-138).

Recently, a role for RA in modulating the behavior of ILCs at the interface of innate and adaptive immunity was discovered. ILCs are newly identified cell types that communicate with a variety of cells to orchestrate immunity, inflammation and homeostasis in tissues throughout the body (139-141). The family of ILCs is divided into three groups based on their cytokine expression profile; ILC1, which protects against bacteria, ILC2, which protects against parasitic worms and ILC3, which also protects against bacteria (142-144). Recent research shows that vitamin A deficiency (VAD) results in decreased frequencies of ILC3s resulting in increased risk of bacterial infections. Interestingly however, VAD also resulted in increased frequencies of ILC2 in mice (145). Being linked to protection against nutrient-consuming parasitic worms, the increased level of ILC2 resulted in increased protection towards the nematode *Trichuris muris*. The implication of this study is that VAD does not lead to a universal immune suppression, but that it in fact may result in activation of distinct parts of the immune system. Hence, the VAD-mediated increase in ILC2s might be beneficial for avoiding competition from nutrient-consuming parasitic worms in endemic areas.
1.4.1 Vitamin A and B cells

RA is implicated in a variety of B cell processes. Hence, VAD in mice has been shown to reduce both fetal and adult B cell lymphopoiesis, and the presence of RA is found to accelerate B cell development in the adult bone marrow (146). In VAD animals also reduced levels of antibody-secretion in response to T cell dependent- and T cell independent type 2 (TI-2) B cell activation has been observed (147).

The role of RA in regulating B cell proliferation is complex. Given the early recognition of the important role of vitamin A for a functional immune system (148), it came as a surprise when our group showed that physiological levels of RA inhibits BCR-mediated proliferation in vitro (149,150). Our results were in line with others demonstrating that RA also inhibits LPS-, CD38- and CD40-mediated proliferation (151,152). Later we could demonstrate that the ability of RA to regulate B cell proliferation was highly dependent on the context whereby the B cells were stimulated and also the subgroup of B cell in question. Hence, whereas proliferation of both memory and naïve B cells stimulated via the BCR was reduced by RA, the proliferation of naïve and memory B cells stimulated via TLR9 was greatly enhanced (153). In the same paper we demonstrated that RA also promoted Ig production in the presence of TLR9 stimulation, and several reports have demonstrated an important role of RA in B cell differentiation and in particular for CSR to IgA- and IgE-producing plasma cells (151,154,155). RA is also involved in the GC formation, by increasing the CD40 expression on dendritic cells that enhances the activation of B cells (156). Furthermore, RA has been shown to increase the expression of homing receptors that promote B cell migration to the gut (119).

1.5 Autophagy

Autophagy is an evolutionary conserved process that ubiquitously occurs in all eukaryotic cells (157). It is part of a lysosomal pathway for degradation of cytoplasmic constituents such as damaged organelles, microbes and long-lived proteins. There are three main types of autophagy; chaperone-mediated autophagy (CMA), microautophagy and macroautophagy. The process of CMA utilizes a chaperone to recognize pentapeptide motifs of cytosolic proteins to directly transfer the protein into the lysosome (158). Microautophagy is the term used to for the process where invagination of the lysosomal membrane directly engulfs cytoplasmic components (159), whereas macroautophagy (hereafter referred to as autophagy),
describes the sequestration of cytoplasmic material into *de novo*-formed double-membrane autophagosomes that eventually fuses with lysosomes (Figure 6) (160,161). Both micro- and macroautophagy have the capacity for sequestration of large structures, such as entire organelles. Autophagy has many physiological roles; it is responsible for the basal turn-over of damaged organelles and long-lived proteins, it is involved in the cellular stress responses to low levels of nutrients (starvation), heat and oxidative stress, it is important in cellular differentiation, and it is involved in both innate and adaptive immunity (see section 1.6) (162-166).

*Figure 6. Autophagy. Modified from (167).*
1.5.1 The molecular mechanism of autophagy

More than 30 autophagy-related (ATG) genes have been identified, that all contribute to the molecular process of autophagy (168). The initiation of autophagy is characterized by the formation of a phagophore/isolation membrane. The origin of the membrane is still debated, but it has recently been suggested that it either is formed from the membrane regions where the ER meets the Golgi (Golgi-ER intermediate compartment) (169) or where the ER meets the mitochondria (ER-mitochondria contact sites) (170). Two important protein complexes are required for the initiation of the phagophore; the ATG1/unc-51-like autophagy activating kinase 1 (ULK1) complex (171,172) and the autophagy-specific class III PI3K complex (173). The ULK complex and the PI3K complex phosphorylate various target proteins that leads to the nucleation of the phagophore and recruitment of other ATG proteins such as the transmembrane protein mAtg9 and its cycling machinery (174). The phagophore expands in a process requiring two ubiquitin-like conjugation systems. One of the conjugating systems contains ATG12, ATG5 and ATG16L1, with ATG7 being responsible for the ligation of ATG12 to ATG5 (175), whereas the other system contains LC3 (ATG8) (176). The ATG12-ATG5-ATG16L1 complex mediates the lipidation (conjugation to phosphatidylethanolamine (PE)) of LC3 and its family members GATE16 and GABARAP, enabling them to associate with the phagophore membrane as it expands. The function of LC3 is to aid in the expansion and the closure of the membrane (177,178). During the formation of the autophagosome, the membrane engulfs material destined for degradation. p62/SQSTM1 is a polyubiquitin-binding autophagy receptor that interacts with LC3 and recognizes polyubiquitinated protein aggregates (179). Before closure, the ATG proteins bound to the membrane dissociate while LC3 and its family members remain attached (180). The presence of lipidated LC3 (also referred to as LC3-PE or LC3-II) on the inner side of the autophagosomal membrane, makes it a suitable marker for analyzing autophagy (181).

The autophagosomes can be formed throughout the cytoplasm. However, as the autophagosome matures, it moves inwards in the cell while fusing with multivesicular bodies and endosomes (182). Finally the autophagosome fuses with the lysosome forming an autolysosome, where the internal components are degraded by hydrolases at low pH, and the content released into the cytoplasm for re-use (163). p62/SQSTM1 is also degraded, and the reduction of cellular levels of p62/SQSTM1 can be used to monitor the autophagic process (183).
1.5.1.1 ULK1

ULK1 is a serine/threonine kinase that is located in complex with ATG13 and FIP200 (RB1CC1). This complex is considered as an initiator of the autphagic cascade, and it plays an important role in linking the sensing of nutrient deprivation to the induction of autophagy (171,172). The activation status of this complex relies on upstream signals such as mechanistic target of rapamycin (mTOR) and AMP kinase (AMPK). Hence, the nutrient sensor mTOR will under nutrient rich conditions inactivate the ULK1 complex by phosphorylating ULK1 and ATG13 (184-186). In contrast, AMPK, which is activated upon nutrient deprivation, will phosphorylate and activate ULK1 (187,188). Moreover, activation of ULK1 can also be mediated by ubiquitylation by TRAF6 (189). It is not yet established how the ULK1 complex further orchestrates the signals that lead to autophagy. It was recently shown that ULK1 can phosphorylate Beclin-1 in response to amino acid withdrawal (190), important for both autophagosome formation and maturation. It should, however be emphasized that LC3-conversion has been reported also in cells where ULK1 has been knocked down (191), suggesting that ULK1-independent initiation of autophagy may occur.

1.5.2 Retinoic acid and autophagy

The role of micronutrients in the regulation of autophagy has not been thoroughly studied. However, there are a few studies linking RA to the induction of autophagy. It has been reported that RA can induce autophagy both in an mTOR-dependent (192-194) and mTOR-independent (195) manner. The studies on mTOR-dependent autophagy all explored the induction of autphagic degradation of the PML-RARα oncoprotein in APL (acute promyelocytic leukemia)-derived cell lines, and found that the RA-mediated autophagy was either dependent on ULK1 (192), p62/SQSTM1 (193), or independent of Beclin1 (194). The mTOR-independent effect of RA was shown to involve the redistribution of the cation-independent mannose-6-phosphate receptor from the trans-Golgi region to the acidified autophagosomes in a process that resulted in autophagosome maturation (195).

1.6 Autophagy and the immune system

There has been an emerging awareness of the role of autophagy in the immune system. Hence, it is now established that autophagy is important in processes such as direct elimination of pathogens, in antigen presentation, and in lymphocyte homeostasis (for reviews, see 162-166).
1.6.1 Autophagy involved in direct elimination of pathogens

Intracellular microorganisms can be recognized by PRRs such as TLRs, and these receptors can in turn activate signaling pathways involved in the induction of autophagy (196). One of the mechanisms whereby autophagy eliminates intracellular microorganisms is called xenophagy. Xenophagy involves the direct uptake of the microorganism into a double-membrane autophagosome which subsequently fuses with a lysosome and degrades the microorganism (162,197). Also, a process called LC3-associated phagocytosis (LAP) can occur, and this process engages the autophagic machinery when the microorganism is already inside a phagosome, leading to the degradation of the pathogen (198,199). If the pathogen escapes the autophagy barriers controlled by the PRRs, autophagic adaptors called the sequestosome 1-like receptors (SLRs) can be involved in the elimination (200).

1.6.2 Autophagy and lymphocytes

Autophagy is involved in several aspects of lymphocyte development and function. In fact, it has been shown that autophagy is important already at the stem cell stage, being required for the maintenance of the hematopoietic stem cell that gives rise to both myeloid and lymphoid progenitor cells (201).

1.6.2.1 T cells and autophagy

Autophagy has been linked to a variety of T cell functions, from the survival of resting naïve T cells (202-204), to the maturation of the naïve T cells after exiting the thymus (205). T cell activation depends on the presentation of antigens by antigen presenting cells (APCs) such as dendritic cells and B cells, and it has been shown that autophagosomes are required for this process by transporting the antigen fragments from the cytoplasm into the lumen of antigen-processing compartments (206-210). Furthermore, it has been shown that autophagy is required for sustained calcium homeostasis in T cells by maintaining the ER compartment (211).

1.6.2.2 B cells and autophagy

It was initially assumed that autophagy was only required for the earliest stages of B cell development, such as the transition from the pro- to the pre-B cell state (212). However, it is now acknowledged that autophagy is important also for later stages of B cell development. Activation of mature B cells via the BCR leads to autophagy (92). On the other hand, autophagy has also been shown to bridge the innate and adaptive parts of the immune system by promoting the fusion of TLR9-containing endosomes with internalized BCRs in
autophagosomes in a process that enhances BCR-signaling (213). The early events leading to the induction of autophagy can include activation of TLRs by PAMPs (196,214,215), and a direct link between the TLR-mediated TRAF6 activity and ULK1 stability and function was recently discovered (189).

Autophagy has also proven its vital role in plasma cell formation and homeostasis. The massive production of immunoglobulins in plasma cells results in pronounced ER stress (216), and it has been shown that autophagy is required for both keeping up sustained levels of ATP and for providing the necessary building blocks (217,218). Recently, autophagy was also suggested to be involved in the long-term survival of memory B cells (219). Hence, whereas no autophagy-related genes were induced at early stages of memory B cell formation, the levels of such gene products increased over time. The numbers of autophagosomes and transcription factors required for the transcription of autophagy-related genes were also gradually increased, all in support of an important role of autophagy in maintaining long-term memory.

1.7 Multiple sclerosis

Multiple sclerosis (MS) is a chronic autoimmune demyelinating disease that involves neurodegeneration, caused by a response to self-antigens such as fragments of myelin protein (220). The heterogeneity of MS has led to grouping of the patients into three categories (221). The most common disease course (85 % of the patients) is relapsing-remitting MS (RRMS) (222). RRMS is characterized by recurrent attacks and often reversible neurological deficits. An attack is defined by the presence of a new symptom that lasts for more than 24 hours, such as changes in sensation, muscle weakness, chronic pain and fatigue. Within a decade, the disease activity of 50 % of the RRMS patients will develop into secondary progressive MS (SPMS). SPMS is characterized by the initial relapse-course (RRMS diagnosis) followed by irreversible neurological decline without any relapses (223,224). 10-15 % of the MS patients are diagnosed as primary-progressive MS (PPMS), which is recognized by a gradual neurological decline from the onset and can occur both with or without separate attacks (225).
1.7.1 Prevalence and etiology of MS

Approximately 2.5 million people worldwide are diagnosed with MS (226). The median age of onset for RRMS is 30 years, whereas the median onset of PPMS is 40 years (225). The highest incidence of MS is noted in Europe and North-America (227,228), with a prevalence rate of 83 per 100,000 individuals in Europe (229). The etiology of MS is not well understood, but it has been linked to both genetic and environmental factors (230). The general view is that a person who has a genetic predisposition to develop MS is more likely to develop MS if he/she is exposed to the Epstein-Barr virus, cigarette smoking and/or reduced sunlight exposure/vitamin D levels. Interestingly, MS disease activity has also been linked to low serum levels of vitamin A (231,232), and it is therefore a growing interest in Vitamin A supplementation in the treatment of MS (233-235).

1.7.2 Diagnosis of MS

MS diagnosis involves the exclusion of alternative diagnoses, and follows the McDonald criteria (236-238). The McDonald criteria includes magnetic resonance imaging (MRI) to detect lesions in the white matter of the brain; T2 hyperintense lesions and/or gadolinium-enhancing T1 lesions disseminated both in time and space. Gadolinium is a dye that serves as a marker of focal inflammation by leaking through vessels due to the permeability and breakdown of the blood-brain barrier (BBB). The McDonald criteria also include analysis of the cerebrospinal fluid (CSF) to detect elevated immunoglobulin G (IgG) levels and oligoclonal bands (OCB) and finally the criteria include the presence of either neurological decline or at least one attack. MS is a heterogenic disease, with a wide range of symptoms. Hence, not all of the criteria need to be fulfilled to achieve an MS diagnosis. The level of disability in a patient is measured by the Expanded Disability Status Scale (EDSS) (239). The course of MS is highly unpredictable in individual patients, which makes the prognosis difficult to predict (220).

1.7.3 Pathology of MS

The main hallmark of MS is the focal demyelinated plaques within the central nervous system (CNS) (240-242), with variable degrees of inflammation, gliosis and neurodegeneration. The destruction of myelin sheets that surround the axons impairs the neuronal signaling, which gives rise to a variety of symptoms such as visual and sensory disturbances, limb weakness, fatigue, spasticity and cognitive impairment (220). CD4+ T cells have long been thought to be the most important immune cell subset involved in the pathogenesis of MS, mainly due to the
fact that MS can be induced in animal models upon transfer of myelin-reactive T cells (243,244). The autoreactive T cells are activated in lymphoid tissues such as lymph nodes (245,246), and these T cells migrate through the BBB, which has become more susceptible for cell infiltration as a part of the systemic inflammatory response (247-249). The migration of the T cells across the BBB increases its permeability, which favors the infiltration of other immune cells (250). Together this initiates a strong immune response inside the CNS, leading to demyelination and tissue damage. During the last years, various T cell subsets and other immune cells such as dendritic cells, natural killer cells and B cells (section 1.7.4) have gained novel interest and been implicated in the disease activity of MS (251).

1.7.4 Role of B cells in MS disease activity
The predominant evidence for a role of B cells in MS is the presence of intrathecal antibodies (OCB) in the CSF of MS patients (252). Myelin-specific antibodies bound to myelin have been found in macrophages at the sites of demyelinating lesions (248,253). Clonally expanded B cells have been detected in the brain parenchyma, and these cells are responsible for the production of the OCB (254-256). In the perivascular spaces within subacute and chronic MS plaques, plasma cells have been observed in large numbers, and it is likely that antigen processing and presentation occur at these sites (257,258). Also, an important indicator of the role of B cells in MS is the reduction of new gadolinium enhancing-lesions (259) and the proportion of patients experiencing relapses (260) when B cells are depleted. B cell depletion occurs via treatment of patients with Rituximab that targets all B cells via the CD20-receptor. MS-derived B cells have been shown to have a reduced production of the anti-inflammatory cytokine IL-10 (261-265), which has been known to modulate autoimmunity (266).

1.7.5 Treatment of MS
Since the etiology of MS is not well established, there are no available cures. Generally, current therapies involve modulation or suppression of the immune system to reduce MRI measures of inflammation and relapse rates. 20 years ago the two first disease-modifying drugs were approved for treating MS patients. These were interferon-β-1b (IFN-β-1b) (267) and glatiramer acetate (GA) (268), which both are still widely used. IFN-β reduces MS disease-activity by increasing anti-inflammatory Th2 cytokines such as IL-10, decreasing the pro-inflammatory Th1 and Th17 cytokines, and by reducing cell trafficking across the BBB (269). The effect of GA involves shifting the immune cytokine balance from pro-inflammatory to anti-inflammatory. This is achieved by the interaction of GA with the CD4+ and the CD8+ T cells and antigen-presenting cells. GA also has neuroprotective effects
(270). The most widely used drugs containing IFN β-1b are called Beta-feron and Extavia, while the most common GA-containing drug is Copaxone. There are, however, several other established disease-modifying agents approved for treatment of MS patients, and these include natalizumab, fingolimod, teriflunomide and dimethyl fumarate. As previously mentioned, also monoclonal antibodies such as Rituximab (271) that targets and depletes all CD20+ cells (all B cells except pre-B cells and plasma cells) are also used in the treatment of MS. However, a recent Cochrane report (272) did not support use of Rituximab in treatment of MS, due to lack of convincing evidence.
2 AIMS OF THE STUDY

The overall aim of the present project is to elucidate the mechanisms whereby vitamin A stimulates the immune system. The specific aims of the thesis have been to:

1) Elucidate the effect of RA on TLR9/RP105-mediated B cell proliferation and antibody production

2) Reveal the involvement of autophagy in RA-mediated stimulation of antibody production

3) Explore a possible beneficial impact of RA on cytokine production in B cells from patients with multiple sclerosis
3 SUMMARY OF THE PAPERS

PAPER I:

**TLR9-signaling is required for turning retinoic acid into a potent stimulator of RP105 (CD180)-mediated proliferation and IgG synthesis in human memory B cells.**

In this paper we demonstrated the impact of physiological and pharmacological concentrations of retinoic acid on activation of human peripheral blood B cells stimulated via TLR9 and RP105. Interestingly, whereas RA alone inhibited RP105-mediated proliferation and differentiation, the co-stimulation via TLR9 significantly turned the effect of RA from being inhibitory to strongly stimulatory. Concomitant with the enhanced proliferation and antibody production induced by RA was the secretion of the anti-inflammatory cytokine IL-10. By using antibodies to block the IL-10 receptor, the effect of RA on proliferation and differentiation was abrogated.

PAPER II:

**Retinoic acid-induced IgG production in TLR-activated human primary B cells involves ULK1-mediated autophagy.**

To further explore the mechanisms whereby RA induces antibody production from human B cells co-stimulated via TLR9 and RP105, we here focused on the role of autophagy in the process. We used several different techniques to show that RA increased autophagy in these cells. Hence, we demonstrated that the level of LC3-II (LC3 bound to autophagosomal membranes) and the ratio of membrane-bound and cytosolic LC3 (LC3-II/LC3-I) increased. We also found enhanced numbers of LC3-positive puncta representing autophagosomes in the cytoplasm of RA-stimulated B cells. Furthermore, we detected reduced expression of p62/SQSTM1, which is a cargo-receptor that is degraded during autophagy, and finally we demonstrated enhanced co-localization between autophagosomes and lysosomes. Together these techniques revealed that RA induced autophagosome formation and enhanced autophagic degradation. We also showed that RA induced the expression of ULK1 at the transcriptional level via RARs, and that blocking ULK1 both abolished the RA-induced autophagy and reduced the IgG-secretion. The impact of RA on IgG production was also significantly reduced when autophagic degradation was blocked by lysosomal inhibitors.
Taken together, the results presented in paper II show that ULK1-mediated autophagy is crucial for the ability of RA to potentiate TLR9/RP105-mediated IgG secretion.

PAPER III:

Retinoic acid enhances the levels of IL-10 in TLR-stimulated B cells from patients with relapsing-remitting multiple sclerosis.

Multiple sclerosis (MS) is an autoimmune disorder associated with reduced secretion of IL-10 and reduced IL-10/TNF-α ratio. In paper III we explored the ability of RA to skew the cytokine production in TLR9/RP105-stimulated B cells from MS patients in favor of IL-10 production. We showed that MS-derived B cells had a diminished capacity to secrete IL-10 upon TLR9/RP105 stimulation, and that this defect could be restored in the presence of RA. Importantly, the beneficial effect of RA could also be noted in B cells from patients that were treated with interferon β or glatiramer acetate. Taken together, the results presented in paper III encourage future trials with vitamin A in the treatment of MS.
4 DISCUSSION

4.1 Methodological considerations

4.1.1 Methods for isolation of B cells

As a source of normal peripheral blood B cells in papers I and II, we isolated CD19+ B cells from buffy coats from healthy blood donors. The buffy coat is the thin layer of cells between plasma and red blood cells after density grade centrifugation of whole blood. We obtained the buffy coats from the Blood Bank of Oslo University Hospital, Ullevål, and we isolated the B cells by using anti-CD19-coated magnetic beads according to the procedure developed by Funderud and colleagues (273). In short, the anti-CD19-coated magnetic beads are mixed with the buffy coat, leading to the binding of magnetic beads to the CD19 surface marker on the B cells. The flask with the suspension is placed next to a samarium cobalt magnet, resulting in the separation of the CD19+ B cells from the rest of the cells. The beads with cells are thoroughly washed and left for overnight incubation in order for the cells to detach from the beads. This detachment is the result of the CD19 cell surface protein being internalized upon binding of the cells to the anti-CD19 antibodies on the beads. The yield of CD19+ B cells from one buffy coat varied between 5 and 50 million, and in several of the experiments in papers I and II it was necessary to pool cells from different donors. The purity of the B cells was >99 %, which was assessed by flow cytometry of cells stained for the pan B cell marker CD20 (274). In order to verify that the results obtained on positively selected CD19+ B cells was not influenced by the binding of anti-CD19 to its receptor, negative selection of B cells was occasionally performed – by isolating the peripheral blood mononuclear cells (PBMCs) by Ficoll-Hypaque (Lymphoprep), and then using the Dynabeads Untouched Human B cell kit from Life Technologies.

In paper I we wanted to address the effects of RA, CpG and anti-RP105 on the memory and naïve B cells. Hence, the CD19+ B cells were further subfractionated into CD27+ memory B cells and CD27- naïve B cells, respectively, by using anti-CD27 magnetic beads. The subpopulation of CD27+ B cells ranged from 10 % to 40 % of the total CD19+ B cell population, and the purities of both the CD27+ and CD27- B cells were >95 %.

There are both advantages and challenges attached to studies on primary human B cells. As compared to B cell lines, the results obtained on primary B cells might be considered as more
physiologically relevant, due to the normal karyotypes of the cells. Primary B cells are also naturally synchronized in the resting G0 state, which makes activation of the cells more physiological compared to continuously dividing B cell lines. The advantage of using B cells from peripheral blood as compared to cells from other lymphoid organs is that the peripheral blood B cells are generally less pre-activated in vivo prior to isolation. The disadvantage of studying primary human B cells is first of all that the procedure is expensive and time consuming. However, another major disadvantage is the extensive variation between the results from different blood donors. Hence, in order to be able to draw statistically significant conclusions on primary human B cells, experiments on cells from many donors are usually required.

4.1.2 MS-derived B cells

In paper III, all experiments were performed on CD19+ B cells isolated from whole blood samples (35 ml) obtained from MS patients and healthy controls in collaboration with clinicians at Oslo University Hospital, Ullevål. The CD19+ B cells were isolated by an autoMACS cell separator using anti-CD19 magnetic beads on a population of PBMCs. The purity of these cells was >98%. In a few experiments in paper III, CD19+ B cells were manually isolated from whole blood samples using the anti-CD19-coated beads. The yield of CD19+ B cells obtained by either isolation method was typically between 1 and 4 million cells, and the yield did not statistically differ between MS patients and healthy controls.

In the major parts of paper III, our aim was to explore the effects of vitamin A on B cells derived from untreated MS patients. The reason for choosing untreated patients was to avoid the interference of the treatment protocols on our results. On the other hand, this could theoretically lead to a selection bias, since patients that do not require treatment may experience a lower disease activity than other MS patients. Even though the median EDSS is low, they do represent an average MS population with modest disease activity. However, to further exclude the possibility of selection bias, we also included results from MS patients on treatment for 6 to 18 months by Copaxone (glatiramer acetate), Betaferon or Extavia (both IFN β-1b). These patients had higher EDSS scores and more extensive MS disease activity. Still, as shown in paper III we observed the same stimulating effects of vitamin A on these B cells as on B cells from the untreated MS patients.
4.1.3 Activation of B cells

B cells can be activated *in vitro* via different cell surface - and intracellular receptors (see section 1.2.3). In all three papers included in the thesis, the B cells were activated via the toll-like receptors TLR9 and/or RP105, and we used modified CpG-2006 oligodeoxynucleotide (ODN) phosphorothionate and/or anti-RP105 antibodies, respectively. ODNs mimic the CpG-rich motifs of bacterial and viral DNA that activate TLR9 *in vivo*. Due to the rapid degradation of bacterial and viral DNA in cell cultures, synthetic CpG-DNA used for *in vitro* activation of B cells contains phosphorothionate backbones to preserve its stability (275). The *in vivo* ligand of RP105 is still not known, so we used anti-RP105 to crosslink and activate RP105 *in vitro* (98). The B cells were recently also stimulated with *Staphylococcus aureus* Cowan I (SAC) to activate the BCR (data not shown). SAC is known to bind the BCR and stimulate the B cells into entering the S-phase of the cell cycle (276). We found that the potentiating effect of RA remains even when the B cells are stimulated with SAC in addition to CpG/anti-RP105.

4.1.4 Retinoic acid

All-*trans* retinoic acid is considered to be the most active vitamin A derivate in the immune system alongside with 9-*cis* retinoic acid (277), and it primarily acts as a ligand for the RAR/RXR heterodimer transcription factors (32). In contrast to for instance dendritic cells (278), B cells do not possess the necessary enzymes to convert retinol into all-*trans* retinoic acid and therefore rely on all-*trans* retinoic acid from neighboring cells or from the circulation. However, despite the fact that no retinol-metabolizing enzymes so far have been detected in B cells, our group previously showed that retinyl esters, retinol-RBP and retinol-loaded chylomicron remnants all had the same effect as RA in BCR-activated B cells (149). In the three papers included in the present thesis, we therefore used all-*trans* retinoic acid dissolved in ethanol to treat the B cells.

RA is present in blood at a strictly regulated concentration between 5 and 10 nM (28,29). However, when patients are treated with pharmacological doses of vitamin A, the serum concentration of RA can reach 0.5 – 3 μM (279,280). In paper I we examined the dose responses of RA ranging from 0.1 nM to 1000 nM, and we found 100 nM of RA to be the optimal dose in combination with TLR9 and RP105. This concentration of RA is also in accordance with a previous study by our group (153).
Although most of the effects of RA are mediated via RAR/RXR-induced transcription, both transcriptional-independent effects of RAR/RXR – and even RAR/RXR-independent effects of RA have been reported (37-39). To evaluate whether or not the effects of RA occurred via RAR/RXR-mediated transcription, we used the RAR-specific agonist (TTNBP) and antagonist (Ro 41-5253) in paper II. The RAR-specific agonist was used at the same concentration as RA, i.e. 100 nM. Complete inhibition of RAR requires a 200-1000 fold molar excess of the antagonist (281,282). To obtain this molar excess and at the same time minimize the toxic effects of the antagonist, we therefore used the antagonist at a concentration of 500 nM concomitant with RA at 1 nM.

4.1.5 Proliferation assays
Incorporation of radioactively labelled thymidine into DNA is a commonly used method for determination of proliferation in lymphocytes (283), and this method was used to analyze proliferation in papers I and III. The extent of DNA synthesis in the S-phase of the cell cycle is reflected by the amount of radioactivity incorporated into DNA, i.e. taken up by the cells (284). The method is easy to perform, and a large amount of samples can be assessed simultaneously. The limitation to this method is the reported lack of correlation between thymidine incorporation and cell proliferation in lectin-stimulated lymphocytes (285). Furthermore, the method does not give an exact measure of the number of cells that are actually present in the S-phase at a given time point.

In order to measure proliferation by an alternative method, we measured the number of cell divisions by flow cytometry analysis of cells stained with carboxyfluorescein succimidyl ester (CFSE). CFSE is added to the cell cultures prior to cell stimulation, and the dye is taken up by cells and bound to amines. After each cell division the two daughter cells will have 50 % of the CFSE of the mother cell (285,286). Hence, analysis by flow cytometry allows the determination of the number of cell divisions as well as the number/percentages of cells in each cell generation. We obtained similar effects of the various stimulants by the two different methods used for measuring cell proliferation in paper I.

Finally, a more direct method for measuring proliferation was used in paper I by counting the cells in the culture plates. Although being a simple and easy method to perform, direct counting of cell numbers is not highly suitable for analysis of primary lymphocytes. This is due to a relatively low percentage of cells actually entering into the cell cycle upon stimulation of B cells, and also due to the high extent of apoptosis in B cell cultures.
4.1.6 Measurement of cytokines and immunoglobulins

Analysis of secreted cytokines (IL-10 and TNF-α in papers I and III) and immunoglobulins (IgM and IgG in papers I, II and III) was performed by enzyme-linked immunosorbent assays (ELISA) on cell culture supernatants. ELISA is based on specific antibodies against the protein to be analyzed, and it is a convenient and easy method for analysis of proteins in supernatants (287). However, the method does not provide any information on whether the altered levels of secreted proteins are due to changes in the rates of transcription or translation, or whether it simply reflects differences in secretion as such. Furthermore, the ELISA method cannot determine if a given level of secreted protein is due to moderate levels being secreted from the majority of the cells in a culture, or whether it is due to high levels secreted from only a small fraction of the cells. Therefore, in order to estimate the number of cells secreting IL-10, we also used ELISPOT analysis in paper I. This is a method that enables the analysis of the cells on a single cell level. In short, the B cells are stimulated on an ELISPOT plate, and the spots that appear represent the levels of IL-10 secreted from the cells (one spot per cell). This makes it possible to distinguish between the amount of IL-10 secreted from one cell (reflected by an increase in the size of the spot) and the number of cells secreting IL-10 (reflected by an increase in the number of spots) (288). As seen in paper I, both methods showed that RA enhances TLR9/RP105-mediated IL-10 secretion. Furthermore, the ELISPOT assay indicated that it is not the number of IL-10-secreting cells that is enhanced, but rather the amount of IL-10 secreted from each cell.

4.1.7 Assays related to the analysis of autophagy.

In paper II we followed the guidelines suggested by Klionsky and coworkers (181) to monitor the levels of autophagy. In most of the experiments in paper II, inhibitors were added to the cell cultures to provide a block in the autophagic pathway leading to the accumulation of autophagosomes. In order to distinguish between enhanced autophagic flux and autophagic block, we therefore also performed control experiments in the absence of these inhibitors. There are several inhibitors available to block the autophagic pathway. Pepstatin A and e64D inhibit the lysosomal proteases (289), bafilomycin A1 inhibits the fusion of autophagosomes with lysosomes by blocking the H+ATPase (290), whereas 3-methyladenin (3-MA) blocks autophagosome formation by inhibiting PI3K (291). We initially tested all compounds, and based on cell viability and ability to increase the accumulation of autophagosomes, we found the combination of pepstatin A and e64D to be the preferred inhibitors. This combination of inhibitors was therefore used throughout paper II.
The advantage of using lysosomal inhibitors in assays aimed at monitoring autophagy, is that their effects are immediate. However, due to potential side-effects of lysosomal inhibitors, we also inhibited autophagy by siRNA against specific autophagy-related proteins. Hence, in paper II we studied the effect of vitamin A on IgG production both in the presence of lysosomal inhibitors and in the presence of cells transfected with siRNA against ULK1. In both cases the potentiating effect of RA on IgG-secretion was significantly reduced, underlining the importance of the induction of autophagy by RA in its promotion of IgG secretion.

A well-known marker of autophagosomes is the LC3 protein (181). As described in section 1.5.1 the cytosolic and unconjugated form of LC3 is termed LC3-I. However, when LC3 becomes bound to autophagosomal membranes, it also becomes conjugated to phosphatidylethanolamine (PE). This conjugated form of LC3 is termed LC3-II and is specific for the autophagic process (292). We analyzed the levels of LC3 by Western blot analysis. The advantage of using Western blot analysis is that LC3-II migrates faster than LC3-I in an SDS-PAGE gel, and it is therefore possible to distinguish LC3-II from LC3-I. The ratio of LC3-II compared to LC3-I estimates the amount of autophagosomes present, and Western blot analysis allows the comparison of the levels of autophagosomes in differently treated cell samples. In such Western blot analyses it is important to perform experiments also without the autophagic inhibitors, to verify that the treatment indeed induces autophagy and not simply blocks autophagy. The Western blot experiments clearly showed that RA enhanced the LC3-II/LC3-I ratio.

A limitation to Western blot analysis in general, is that it only reveals the level of a given protein and not its cellular localization. To visualize the presence of autophagosomes in the cytoplasm, confocal microscopy was performed by staining the cells with anti-LC3 to identify the punctual localization of LC3 when bound to autophagosomal membranes. The results from confocal microscopy were in line with the results obtained by Western blot analysis supporting the notion that RA enhances the levels of autophagosomes.

To verify that the enhanced formation of autophagosomes reflected the degree of autophagic degradation, we used Image Stream analysis for quantification of the co-localization between autophagosomes and lysosomes, by staining the cells with anti-LC3 antibodies and Lyso-ID. This method verified that the autophagosomes induced by RA actually fuse with the lysosomes, leading to enhanced autophagosomal degradation. The benefit of using the Image
Stream analysis is that it combines the advantages of flow cytometry with the advantage of microscopy by allowing the measurement of a large number of cells (30 000 – 40 000 in our experiments). Finally, we also used degradation of the cargo-receptor p62/SQSTM1 as a marker of autophagy (183), and this protein was analyzed by Western blot analysis. A reduction in the level of p62 suggests that the rate of autophagic degradation is enhanced. Hence, it is important that the autophagic inhibitors are not present when these experiments are performed. The results from analysis of p62/SQSTM1 levels were in accordance with the Image Stream analyses, and further supported the notion that RA enhances the formation of autophagosomes and autophagosomal degradation.

4.1.8 Ethical considerations

There are ethical concerns related to collecting blood samples from patients and healthy volunteers. Although collection of peripheral blood generally is considered as a gentle method with minimal amount of pain, there is always a risk of infections when the skin is punctured. However, the isolation of B cells from buffy coats (obtained from the Blood Bank, Oslo University Hospital – see papers I and II) or from whole blood from MS patients or healthy controls (see paper III) was always performed with consent from patients and volunteers, and the projects were approved by the Regional Committee for Medical and Health Research Ethics in South-Eastern Norway Regional Health Authority. The investigation adhered to the principles outlined in the Declaration of Helsinki.
4.2 General discussion

4.2.1 RA-mediated proliferation and antibody production in human primary B cells

Proliferation of B cells is not only important for expanding the B cell pool, but it is also a critical aspect of the differentiation process of B cells into antibody-secreting plasma cells (61-64). The effect of RA on proliferation of B cells seems to depend on the additional co-stimulatory factors the B cells are exposed to. Whereas RA has been shown to inhibit proliferation of B cells that are co-stimulated via BCR (149,150), LPS, CD38 and CD40 (151,152), RA potentiates proliferation of TLR9-stimulated B cells in the presence (paper I-III) or absence (153) of anti-RP105. However, the levels of proliferation in the presence of RA and TLR9 stimulation are significantly higher in the memory B cell population than in the naïve B cell population (paper I). The inhibitory effect of physiological levels of RA on BCR- and CD38-stimulated B cells appears to be in contrast with the general notion that RA strengthens the immune response (10,110-113). Intriguingly, it was shown that a smaller group of the B cells in these cultures in fact differentiate towards plasma cells (151,152). Altogether, these results suggest that the effect of RA is not only dependent on the type of co-stimulation of B cells but also on the subpopulations of B cells.

A previous study revealed a synergy between TLR9 and RP105 in activation of human B cells (96). We therefore aimed at exploring the effect of RA in combination with TLR9 and RP105 stimulation. Our results showed a strong potentiating effect of RA on TLR9/RP105-mediated proliferation of both naïve and memory B cells (paper I). However when B cells were stimulated via RP105 alone, an inhibitory effect of RA on proliferation was noted, underlining the view that effects of RA highly depend on the co-stimulatory factors. By adding TLR9 ligands to the B cell cultures stimulated with anti-RP105, the effects of RA on proliferation changed from being inhibitory to stimulatory. We recently also included SAC to stimulate the BCR in TLR9/RP105-stimulated B cells, and again RA induced a strong proliferative signal (Randi Larsen Indrevær, personal communication). Hence, our results support the idea recently presented by Ross and coworkers, that RA might be a “fourth signal” in B cell activation together with antigens, TLR-ligands and co-stimulatory signals (147).

Whereas Yamazaki et al. (96) reported that the synergy between TLR9 and RP105 stimulation was most prominent in naïve B cells, we found that the RA-mediated enhancement of TLR9/RP105-mediated proliferation was evident in both naïve and memory B cells (paper I). In fact, the effect of RA was even stronger in memory B cells. Most of the
memory B cells have already undergone SHM and CSR, and respond much faster to a given stimulus than the naïve B cells (63), and they also have higher levels of TLR9 (85). TLR9 activation has previously been suggested as a mechanism for maintaining the levels of memory B cells (90). Hence, our observation of a strong effect of RA on the memory B cell pool suggests that RA may be particularly important for keeping up the repertoire of polyclonally activated B cells in the circulation.

In paper I we found that RA not only enhanced the TLR9/RP105-mediated proliferation of B cells, but that also the levels of IL-10 secreted from the B cells was enhanced. IL-10 is an anti-inflammatory cytokine that regulates the behavior of B cells by binding to IL-10 receptors on the cell surface (293-295). We used neutralizing antibodies to block the effects of IL-10, and in line with a previous report from our group on TLR9-stimulated cells (153), we showed that the RA-induced proliferation of TLR9/RP105-stimulated cells involved induction of IL-10.

Having shown that RA was able to enhance proliferation of TLR9/RP105-stimulated B cells, we also examined the influence of RA on B cell differentiation and antibody production. RA enhances B cell differentiation in various settings (296-298), and our group has previously shown that RA enhances Ig production when cells are stimulated via TLR9 alone (153). In paper I we found similar effects of RA on antibody production as we did on proliferation. Hence, whereas RA inhibited the RP105-mediated IgG production from memory B cells, co-stimulation with TLR9 turned RA from an inhibitor to a stimulator of Ig production. A stimulatory effect of RA on TLR9/RP105-mediated IgM production was also noted. In paper I we used IgG production as a measure of B cell differentiation. Later, another project in our lab has confirmed that RA really turns TLR9/RP105-stimulated B cells into plasmablasts/plasma cells by demonstrating the upregulation of the plasma cell surface markers CD38 (299) as well as CD138 concomitant with reduced levels of CD20 (300).

There have been several kinases implicated in TLR9- and RP105-signaling, such as p38MAPK, Akt and ERK (86,96,301,302). In a previous study by our group, we showed that RA potentiated TLR9-mediated antibody secretion by enhancing the phosphorylation of p38MAPK (153). However, when stimulating the cells via both TLR9 and RP105, RA was no longer able to enhance the activation of p38MAPK (paper I). The lack of effects was ascribed to the extremely high activation of the kinase in the presence of anti-RP105 alone (data not included in the paper). The RP105-mediated activation of p38MAPK was surprising as
stimulation via RP105 by itself does not promote differentiation or proliferation of human primary B cells, nor does it contain the active intracellular TIR domain such as other TLRs (93,98). We also measured the effects of RA on other signaling events implicated in TLR-signaling, such as ERK, Akt and NFκB (86,301,302). Although all of these molecules were activated via TLR9 in combination with RP105, RA did not further enhance their activation (paper I).

In a later paper published by our group (299), we used a RAR agonist and a RAR antagonist to show that the RA-mediated induction of IgG involved RAR-mediated gene expression. In paper II we further analyzed the molecular mechanisms behind the RA-mediated IgG production of TLR9/RP105-stimulated cells. We showed that the process involved induced autophagy, and that RA promoted autophagy via its induction of ULK1. ULK1 is one of the early proteins in the autophagic cascade (171), and we showed that the effect of RA on ULK1 was via induction of RAR-mediated transcription (paper II). The role of autophagy in RA-mediated plasma cell formation will be further discussed in section 4.2.2.

It has been shown that RA may increase the level of TLR5 in human THp1 cells (303), and reduce the level of TLR2 in primary human monocytes (304). We therefore explored the possibility that the effects of RA could be mediated via increased levels of TLR9. However, we did not find that RA enhanced the levels of TLR9 (paper I). This result was supported by recent findings in our lab, showing that RA did not enhance the expression of TLR9 at the mRNA level in TLR9/RP105-stimulated cells (Randi Larsen Indreæ, unpublished results). Unfortunately, it was impossible to analyze the expression levels of the RP105 protein, since the antibody we used for cross-linking RP105 is the only commercial clone available (MHR73-11). Thus, any analysis of cell surface expression of RP105 with methods involving antibodies, such as Western blot analysis and flow cytometry is precluded. Interestingly however, recent experiments in our lab on gene expression profiling of B cells stimulated via TLR9 and RP105 have revealed that RA slightly enhances the mRNA expression of RP105 (Randi Larsen Indreæ, unpublished results).

4.2.2 The involvement of autophagy in RA-mediated antibody production
Until recently it was generally assumed that autophagy was only involved in the early B cell maturation stages (212) and for the survival of hematopoietic stem cells (201). In paper II we showed that TLR9/RP105-mediated Ig production and differentiation of B cells was associated with induced autophagy. Furthermore, we showed that the RA-mediated
Ig production was mediated via ULK1-induced autophagy. These results are in accordance with several recent studies that have shed light on the important implication of autophagy also in B cell survival and in the final stages of B cell differentiation (217-219,305). Hence, Conway and coworkers (218) showed a reduction in IgG secretion from autophagy-deficient (Atg5 knock out) murine B cells, while Pengo et al. (217) found a reduction in IgM and IgG3 secretion in vivo using the same mouse model. Interestingly, in vitro Pengo et al. showed that immunoglobulin synthesis was enhanced in autophagy deficient cells, simultaneously with enhanced ER-stress, less ATP and more cell death. Together these results indicate that plasma cell differentiation is maintained even in the absence of autophagy and that autophagy is involved in the cytoprotective trade-off between Ig synthesis and viability by providing the ATP levels required for long-term plasma cell survival. In line with this we found that the level of plasma cell differentiation remained the same in the absence of ULK1 when B cells were stimulated via TLR9/RP105, and that it was the specific effect of RA on IgG secretion that disappeared (paper II).

Chen and coworkers have recently shown that autophagy also may be linked to the survival and maintenance of memory B cells (219). Recent studies in our lab on B cell survival versus differentiation have shown that after 5 days of stimulation with RA in the presence of CpG and anti-RP105, only 30 % of the viable cells have differentiated into plasmablasts/plasma cells as demonstrated by the gain of CD38 and loss of the CD20 cell surface markers (300). We assume that all the viable cells are stimulated, since our experience is that unstimulated B cells otherwise undergo apoptosis in culture. It therefore seems that as much as 70 % of the viable cells are stimulated but undifferentiated. In light of the recent paper that connects autophagy to the survival and maintenance of memory B cells (219), one might speculate that these undifferentiated B cells actually have high levels of autophagy as well. In fact, it might be autophagy that keeps these cells alive. In order to further explore this possibility one may take advantage of the CD20 marker which is lost upon differentiation of B cells into plasma cells (306). Hence, LC3-levels and co-localization between LC3-puncta and lysosomes could be examined in CD20 positive versus negative B cells by Image Stream analysis or by cell sorting.

XBP1 and Blimp1 are transcription factors important for the enhanced protein production during plasma cell differentiation, and XBP1 has also been shown to mediate the cross-talk between autophagy and the unfolded protein response (UPR) (79,307). In autophagy-deficient (Atg5-knock out) murine B cells simulated with LPS, Conway and coworkers (218) found that
expression of XBP1 and Blimp1 were not induced; while in contrast, Pengo and coworkers (217) showed that the mRNA levels of XBP1 and Blimp1 were enhanced in these cells. Although inconclusive, the two papers suggest a link between autophagy, plasma cell generation and the expression levels of XBP1 and Blimp1. Interestingly, our lab recently showed that RA enhances the levels of XBP1 and Blimp1 via IRF4 in TLR9/RP105-stimulated B cells concomitant with the generation of plasma cells (300). It will be interesting to explore the effects of RA on XBP1- and Blimp1-expression when autophagy is inhibited. These studies will also further explore the role of IL-10 in the crossroad between autophagy and IgG production/plasma cell formation. In papers I and III we showed that RA enhanced the TLR9/RP105-mediated induction of IL-10. When autophagy was blocked by Pepstatin A and E64d or by ULK1 siRNA, we found that the RA-mediated induction of IgG was diminished (paper II). Interestingly, also the IL-10 production was reduced by blocking autophagy (data not shown). Hence, it will be interesting to determine the order of events in RA-stimulated B cells activated via TLR9 and RP105, including ULK1, IL-10, IRF4, XBP1, Blimp1 and IgG production. In light of the key roles of mTOR and AMPK in autophagy (171,172), we also explored the possibility that the RA-mediated induction of ULK1 would involve either inhibitory phosphorylation by mTOR or activating phosphorylation by AMPK. However, RA did not affect the phosphorylation of ULK1 (data not shown), supporting our conclusion that RA directly induces ULK1 at the transcriptional level via RAR.

4.2.3 The impact of RA on MS-derived B cells

4.2.3.1 RA enhances the IL-10/TNF-α ratio secreted by MS-derived B cells

MS has generally been regarded as a T cell-mediated disease due to studies demonstrating development of a MS-like disease in animal models upon transfer of myelin-reactive T cells (243,244). Recently however, there have been increasing focus on the involvement of B cells in MS (308). B cells have been found to cross the BBB (309), and antibodies specific for myelin have been detected in lesions of active myelin break down in the CNS (310-312). Furthermore, the presence of OCBs in the CSF is a typical finding in MS patients (252). We therefore aimed to study the possibility of using vitamin A as a tool for increasing anti-inflammatory behavior of MS-derived B cells.

IL-10 is an important anti-inflammatory cytokine secreted by activated B cells (293-295). Several studies have demonstrated that the levels of IL-10 secreted from B cells is lowered in MS-derived B cells (261-265), and a link between reduced levels of IL-10 and the severity of autoimmune diseases has also been established (266). In line with these reports, we showed a
statistical significant reduction in IL-10 secreted from TLR9/RP105-stimulated MS-derived B cells as compared to normal B cells (paper III). However, in contrast to what has been reported by Hirotani and coworkers (262), we did not find a statistical significant difference between IL-10 secreted from MS- and control B cells when the cells were stimulated via TLR9 alone. We believe that this discrepancy might be due to the relative low number of patients and controls included in our study. Importantly though, RA was able to restore the IL-10 levels secreted from MS-derived B cells stimulated via TLR9 alone or via the combination of TLR9 and RP105 (paper III). Even more important was the ability of RA to increase the IL-10/TNF-α ratio in the MS-derived B cells by increasing the levels of IL-10 and keeping constitutive levels of the anti-inflammatory cytokine TNF-α. TNF-α has been ascribed a disease-promoting role in MS (313), but recent control trials have also indicated that inhibition of TNF-α levels may enhance MS disease activity (314,315). That RA was able to keep the levels of TNF-α constant in MS-derived B cells while increasing the levels of IL-10, suggest that RA might have a beneficial effect in MS patients.

4.2.3.2 Clinical aspects of RA in treatment of MS
There has been increasing awareness of a possible beneficial role of vitamin A in MS (235). A recent clinical study found lower concentrations of serum retinol in the blood of MS patients compared to healthy controls, and the low serum retinol levels were associated with poor MRI outcomes (232). We believe that our results demonstrating a skewing of the cytokine production in MS-derived B cells in favor of anti-inflammatory IL-10 production (paper III) adds to the potential role of vitamin A in MS.

In paper III we used RA at the optimal concentration of 100 nM, but we have previously shown that concentrations of RA as low as 1 nM potentiates TLR9/RP105-stimulated B cells (paper I). Whereas the concentration of RA in plasma is around 10 nM (28,29), the pharmacological concentrations of RA can reach as high as 0.5 – 3 μM (279,280). This means that the 100 nM of RA used in the experimental settings in paper III might be of clinical relevance. However, any use of RA on MS patients will need careful optimization both as to type of retinoic acid to be used and the dose. RA exists in many isoforms (26), and it would be worthwhile to test both 13-cis RA, 9-cis RA and synthetic analogues of retinol - in addition to all-trans RA. 13-cis RA is a particular interesting candidate, due to its enhanced stability compared to all-trans RA, and that it is already being used clinically for treatment of acne vulgaris (316,317).
When considering RA in treatment of MS, it is important to relate its effect to conventional treatments of MS, such as IFN β-1b and glatiramer acetate. A recent paper showed that IFN β-1b-treatment enhances anti-inflammatory behavior in RRMS patients without increasing the serum levels of IL-10 (318). In paper III we were able to demonstrate that RA enhanced the IL-10 secretion also in TLR9/RP105-stimulated B cells isolated from IFN β-1b- or glatiramer acetate-treated RRMS patients. In a previous study combining IFN β-1b and vitamin A (319), a synergistic effect on T cell suppressor function was discovered - supporting the possible benefit of combining the two compounds.

Although the results presented in paper III encourage future trials of vitamin A supplementation in MS patients, it should be emphasized that the MS-derived B cells in our in vitro study were co-stimulated via TLR9 and RP105. We would not suggest that CpG and/or anti-RP105 should be added in any trial on RA in treatment of MS. Despite its promising role as an adjuvant in several clinical settings (320), there is an awareness of the possibility of CpG promoting autoimmunity (321,322). In light of MS being an autoimmune disease, one should of course be cautious not to include anything that might increase the risk of promoting disease activity. However, as the B cells in our body are constantly being exposed to pathogens that stimulate TLRs like TLR9 and RP105, we believe that RA by itself will be able to enhance the levels of IL-10 in vivo – even in the absence of administrating CpG and/or anti-RP105. In support for such a notion, a previous trial involving the administration of vitamin A to vitamin A-deficient CVID patients resulted in enhanced plasma ratio of IL-10/TNF-α (323). Although we cannot totally exclude the possibility that RA by itself might promote autoimmunity in MS patients, we believe that the anti-inflammatory impact and the stimulatory effect of RA on the immune system in general will be beneficial for these patients.
5 CONCLUSIONS

The results presented in this thesis support the notion that RA strengthens B cell functions. In addition, we have introduced autophagy as one of the main mechanisms whereby RA can induce antibody secretion. Finally, we discovered a potential role for vitamin A in the treatment of MS by identifying its ability to normalize the production of IL-10 from MS-derived B cells.

The specific conclusions are:

1) RA enhances TLR9/RP105-mediated proliferation and differentiation of human peripheral blood B cells into IgG- and IgM-producing cells in an IL-10-dependent manner.

2) RA induces the autophagy-inducing protein ULK1 at the transcriptional level via RARs. ULK1-mediated autophagy is crucial for the ability of RA to potentiate TLR9/RP105-mediated IgG secretion.

3) B cells from RRMS patients have a diminished capacity to secrete IL-10 when stimulated via TLR9 and RP105. RA is able to restore the levels of IL-10 both from untreated MS patients and patients that are undergoing treatment with IFN β-1b or glatiramer acetate.
6 FUTURE DIRECTIONS

Based on the results from the papers presented in this thesis, the points below indicate possible future directions of our research.

1) In this thesis we show that RA-induced differentiation of TLR9/RP105-stimulated B cells into Ig-producing cells involves ULK1-mediated autophagy (paper II). The transcription factors XBP1 and Blimp1 have also been implicated in autophagy in B cells (217,218), and Randi Larsen Indrevaer in our group has recently identified the transcription factor IRF4 as a critical RA target upstream of XBP1 and Blimp1 in TLR9/RP105-mediated Ig production (300). We will now study the interrelationship between these events. Thus, by using siRNA against IRF4, XBP1 and/or Blimp1 we will elucidate whether these factors are involved in ULK1-mediated autophagy leading to Ig production. Vice versa, we will use siRNA against ULK1 to reveal the possibility that RA-induced autophagy regulates the transcription factors driving B cell differentiation and Ig production.

2) In paper II we found that RA enhances the level of autophagy in TLR9/RP105-stimulated B cells, and that autophagy is important for antibody production in these cells. We have previously shown that B cells from patients with common variable immune deficiency (CVID) have a diminished capacity to secrete IgG when activated via TLR9 and RP105 (299), and that RA is able to partially restore the IgG levels in B cells from a few of these patients. We would now like to explore the possibility that the diminished capacity of CVID B cells to secrete IgG involves defects in the autophagic machinery, and whether the beneficial effects of RA on IgG production in certain CVID patients involves modulation of autophagy.

3) MS patients frequently experience both low levels of serum retinol and reduced secretion of the anti-inflammatory cytokine IL-10 from their peripheral blood B cells. In paper III we demonstrated the ability of RA to restore the IL-10 secretion in MS-derived B cells stimulated via TLR9 and RP105. It would now be interesting to elucidate the autophagic machinery in B cells from MS patients and to link reduced IL-10 production to defects in this machinery. Accordingly, it would also be interesting to explore a possible link between the ability of RA to restore the IL-10 production in MS-derived B cells and any effect of RA on the autophagy machinery in these cells.
7 REFERENCES


Errata

Page 2  Last sentence added: Reprints were made with permission.

Page 9  Figure 2, sequence of RARE: (A/G)G(G/T)TCAinnnnn(A/G)G(G/T)T to (A/G)G(G/T)TCAinnnnn(A/G)G(G/T)TCA

Page 21 Section 1.5.2 line 1: micro nutrients to micronutrients

Page 35 Section 4.17 third paragraph, line 8: estimate to estimates