A Complement to T Cell Immunity

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ACADEMIC DISSERTATION

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1 ABSTRACT

The human immune system consists of the innate and the adaptive immunity that together protect the body from pathogens. To complete this task, the immune system must be able to recognize and destroy the dangerous foreign structures but also not to react to host structures or innocuous foreign structures, such as proteins of food or the commensal microbes residing in the gut.

The innate immunity includes the phagocytes, such as the macrophages and neutrophils, and multiple molecular defensive systems, most importantly the complement system. The innate immunity reacts quickly to pathogens but its functions remain unchanged with repeated encounters with the intruder. The adaptive immunity is slower in its response, but it is more specific and it has memory; upon repeated exposure to a given pathogen, the adaptive immunity is activated more rapidly. Immunological tolerance, the unresponsiveness to self antigens, is a feature of the adaptive immunity. The adaptive immunity includes T and B lymphocytes and the antibodies produced by the B lymphocytes.

In spite of their interdependency, the innate and adaptive immune systems have often been studied separately. This thesis focuses on their interface by investigating the role of the innate complement system in the regulation of the adaptive immunity and of the T lymphocyte function in particular. We followed the immune response and the establishment of oral tolerance in a C3 deficient mouse model, where the function of the complement system is blocked. We also studied vaccination responses and mucosal immune homeostasis in C3 deficient human subjects.

The mice were immunized with ovalbumin in Complete Freund's adjuvant. In order to induce oral tolerance, some of the mice were given ovalbumin to the gastrointestinal tract prior to the immunizations. The ensuing immune response was monitored by assessing the lymphocyte fractions by flow cytometry and by stimulating splenocytes with ovalbumin and monoclonal antibodies *in vitro*, and measuring the proliferative response with a radioactive thymidine incorporation assay. The expression of cytokines and transcription factors in isolated cells and tissue samples was analyzed with quantitative real-time PCR. Serum antibody levels were determined by ELISA.

We isolated leukocytes from peripheral blood samples collected from the patients and healthy control subjects and analyzed the lymphocyte population with flow cytometry. Serum antibodies specific for intestinal commensal microbes and the vaccine antigens tetanus toxoid and diphtheria toxoid were measured with ELISA. Serum samples were also analyzed for the presence of a set of key cytokines.

The results indicate that complement plays a crucial role in the regulation of the functional differentiation of the T helper lymphocytes central to the adaptive immunity. Immunization with ovalbumin produced a weaker T cell proliferative response in the C3 deficient mouse model compared to the wild-type controls. The response of the T lymphocytes was also qualitative different, since the development of a T_H1 response was particularly impaired in the absence of a functional complement system, whereas the T_H2 response showed no difference between the

mouse strains. This was also reflected on the B lymphocyte response: The IgG2a and IgG3 response to the immunization was reduced in the C3 deficient mice but the IgE response was normal.

In addition to the general attenuation of the adaptive immunity, the C3 deficiency resulted in a disturbance of the intestinal immune tolerance in both mice and men. The administration of a foreign protein into the gastrointestinal tract of the C3 deficient mice failed to prevent the systemic immune response to the subsequent immunization with the same protein, i.e. the establishment of oral tolerance failed. The C3 deficient human subjects had more mucosally homing activated T lymphocytes in the peripheral blood and higher levels of serum IgG specific for intestinal commensal microbes. A further sign of the deficient immune tolerance in the C3 deficient human system was the lack of IgG4 response to the vaccine antigens. IgG3 antibodies specific for vaccine antigens were present at higher concentrations in the patient sera and the levels of the inflammatory cytokines IL-12 and IL-21 were also elevated. In contrast to the mouse, the profile of serum cytokines and antibody subclasses in the C3 deficient human subjects pointed at a pronounced T_H1 response.

The work presented in this thesis defines the complement system as a versatile regulator of the adaptive immunity and helper T lymphocytes. The normal functional differentiation of the T lymphocytes requires signals from the complement system and the establishment of immune tolerance both in the mucosal and systemic immune systems is particularly dependent on complement. The results present novel information on the interplay of the innate and adaptive immune systems and will probably affect the treatment strategies for food allergies and inflammatory bowel diseases.

2 TIIVISTELMÄ

Ihmisen immuunijärjestelmä jakautuu luontaiseen ja hankittuun immuniteettiin, jotka yhdessä suojelevat kehoa taudinaiheuttajilta. Onnistuakseen tässä tehtävässä immuunijärjestelmän on kyettävä tunnistamaan ja tuhoamaan vaaralliset vieraat rakenteet ja oltava reagoimatta kehon omiin rakenteisiin ja harmittomiin vieraisiin rakenteisiin, kuten ruuan proteiineihin ja suoliston normaaliflooran mikrobeihin.

Luontaiseen immuniteettiin kuuluvat fagosyytit, kuten makrofagit ja neutrofiilit, sekä erilaiset puolustusmolekyylit, tärkeimpänä näistä komplementtijärjestelmä. Luontainen immuniteetti reagoi nopeasti taudinaiheuttajiin, mutta sen toiminta ei kehity toistuvien kohtaamisten myötä. Hankittu immuniteetti reagoi hitaammin, mutta sen tunnistuskyky on tarkempi ja siihen liittyy immunologinen muisti; kun elimistö kohtaa saman taudinaiheuttajan uudelleen, aktivaatio tapahtuu nopeammin. Myös toleranssi, eli reagoimattomuus omiin rakenteisiin, on hankitun immuniteetin ominaisuus. Hankittuun immuniteettiin kuuluvat T- ja B-lymfosyytit, sekä jälkimmäisten tuottamat vasta-aineet.

Luontainen ja hankittu immuniteetti ovat riippuvaisia toisistaan, mutta niitä on usein tutkittu erillään. Tässä väitöskirjassa paneudutaan niiden rajapintaan selvittämällä luontaiseen immuniteettiin kuuluvan komplementtijärjestelmän toiminnan vaikutuksia hankitun immuniteetin säätelyyn, sekä erityisesti T-lymfosyyttien toimintaan. Tutkimus on toteutettu seuraamalla immunisaatiovastetta ja oraalisen toleranssin kehittymistä hiirikannassa, jonka komplementtijärjestelmä ei toimi C3-tekijän puutteen vuoksi. Lisäksi tutkimme rokotusvasteita ja kartoitimme suoliston immuunijärjestelmän tasapainoa C3-puutteisilla potilailla.

Koejärjestelyssä hiiret immunisoitiin ovalbumiinilla ja adjuvanttina käytettiin Complete Freund’s adjuvanttia. Oraalisen toleranssin synnyttämiseksi osalle hiiristä annosteltiin ovalbumiinia mahasuolikanavaan ennen immunisaatiota. Kehittynyttä immuunivastetta tutkittiin analysoimalla lymfosyyttipopulaatioiden koostumusta virtaussytometrialla ja stimuloimalla lymfosyyttejä soluviljelmässä ovalbumiinilla ja monoklonaalisilla vasta-aineilla, seuraten jakautumisvastetta radioaktiivisen tymidiinin sitoutumiseen perustuvalla koejärjestelyllä. Sytokiinien ja transkriptiotekijöiden ilmentymistä soluissa ja kudosnäytteissä tutkittiin reaaliaikaisella PCR:llä. Seerumin vasta-aineiden määrittämiseen käytettiin ELISA-menetelmää.

Potilaiden ja terveiden verrokkien verinäytteistä eristimme valkosolut ja analysoimme lymfosyyttipopulaatioiden koostumusta virtaussytometrialla. Suoliston normaaliflooraa, tetanustoksoidia ja difteriatoksoidia vastaan kehittyneitä seerumin vasta-aineita tutkimme ELISA-menetelmällä. Seeruminäytteistä määritettiin myös immuunivasteessa keskeisten sytokiinien pitoisuudet.

Tutkimuksen tulokset osoittavat, että komplementti vaikuttaa hankitun immuniteetin keskeisten solujen, auttaja T-lymfosyyttien, toiminnalliseen erilaistumiseen ratkaisevasti. C3-puutteisessa hiirikannassa immunisaatio ovalbumiinilla tuotti heikomman T-lymfosyyttien jakautumisvasteen kuin villityypin hiirissä. T-lymfosyyttien vaste immunisaatioon oli myös laadullisesti erilainen, sillä T_H1-

tyyppisten solujen kehitys oli erityisesti heikentynyttä komplementin puuttuessa, mutta T_H2 vaste oli normaali. Tämä heijastui myös B-lymfosyyttien tuottamiin vasta-aineisiin: IgG2a ja IgG3 vaste immunisaatioon oli heikentynyt C3-puutteisissa hiirissä, mutta IgE vaste oli normaali.

Hankitun immuniteetin yleisen heikentymisen lisäksi C3-puutos aiheutti sekä hiirissä että ihmisissä suoliston immunologisen toleranssin häiriön. C3-puutteisille hiirille ei kehittynyt oraalista toleranssia, eli mahasuolikanavaan annosteltu vieras proteiini ei kyennyt estämään immuunivastetta myöhemmässä immunisaatiossa. C3-puutteisilla potilailla puolestaan oli veressään enemmän suolistoon matkalla olevia aktivoituneita T-lymfosyyttejä, sekä enemmän suoliston normaaliflooraan kohdistuvia IgG-luokan vasta-aineita. Toleranssin häiriöön viittasi myös C3-puutteisten potilaiden puuttuva IgG4-vaste rokotuksille. Rokoteproteiineja vastaan tuotettuja IgG3 vasta-aineita potilaiden seerumissa oli merkitsevästi enemmän kuin terveillä verrokeilla. Potilaiden seerumissa oli myös enemmän tulehduksellisia IL-12 ja IL-21 sytokiineja. Ihmisellä C3-puutos vaikuttaisi siis johtaneen T_H1-vasteen voimistumiseen, toisin kuin hiirellä.

Väitöskirjani tulokset osoittavat, että komplementtijärjestelmä säätelee hankitun immuniteetin ja etenkin auttaja-T-lymfosyyttien toimintaa laaja-alaisesti. T-lymfosyyttien normaali toiminnallinen erilaistuminen tarvitsee komplementin aktivaation tuottamia viestejä ja etenkin toleranssin kehittyminen sekä suoliston alueella että immuunijärjestelmässä laajemmin häiriintyy komplementin puuttuessa. Löydökset tuovat uutta tietoa luontaisen ja hankitun immuniteetin yhteistoiminnasta ja asettavat etenkin allergioiden ja tulehduksellisten suolistosairauksien hoitomenetelmät uuteen valoon.

3 LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications. The publications are referred to in the text by Roman numerals (I-III).

I Pekkarinen PT, Vaali K, Junnikkala S, Rossi LH, Tuovinen H, Meri S, Vaarala O, Arstila TP. A functional complement system is required for normal T helper cell differentiation. *Immunobiology* 2011; 216:737-43.

II Pekkarinen PT, Vaali K, Jarva H, Kekäläinen E, Hetemäki I, Junnikkala S, Helminen M, Vaarala O, Meri S, Arstila TP. Impaired intestinal tolerance in the absence of a functional complement system. *The Journal of Allergy and Clinical Immunology* 2013; 131:1167-75.

III Pekkarinen PT, Heikkilä N, Kisand K, Peterson P, Botto M, Daha MR, Drouet C, Isaac L, Helminen M, Haahtela T, Seppälä I, Meri S, Jarva H, Arstila TP. Dysregulation of adaptive immune responses in complement C3-deficient patients. *Submitted*.

4 ABBREVIATIONS

APC	Antigen presenting cell (professional)
B7	Costimultatory signal molecule, multiple subtypes
Bcl-6	B-cell lymphoma 6
BCR	B cell (antigen) receptor
C	Complement
C3	Complement component 3
C3a	Complement component 3a; soluble anaphylatoxin
C3b	Complement component 3b; membrane-associated
$C3_{ctrl}$	C3-KO mice fed with saline, immunized with OVA
C3dg	Complement component 3dg; membrane-associated
$C3_{OVA}$	C3-KO mice fed with OVA, immunized with OVA
CD	Cluster of differentiation
CD19	Coreceptor of the BCR, present on all B cells
CD3	Coreceptor of the TCR, present on all T cells
CD4	Binds MHC II, present on T helper cells
CD45RO	Adhesion molecule expressed by activated/memory T cells
CD69	Early activation marker expressed by T cells
CD8	Binds MHC I, present on cytotoxic T cells
CFSE	Carboxyfluorescein diacetate succinimidyl ester
CR	Complement receptor
CRP	C-reactive protein; one of acute phase proteins
CTLA-4	Cytotoxic lymphocyte antigen 4
DAF	Decay accelerating factor

DC	Dendritic cell
DP	Double positive ($CD4^+CD8^+$ thymocyte)
DT	Diphtheria toxoid
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FDC	Follicular dendritic cell
FoxP3	Forkhead box P3
GATA-3	GATA-family transcription factor 3
HLA	Human leukocyte antigen
iC3b	Inactivated C3b; membrane-associated
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
KO	Knock-out
LP	Lamina propria
mAb	Monoclonal antibody
MAC	Membrane attack complex
MASP-2	Mannan-binding lectin associated serine protease 2
MCP	Membrane cofactor protein
MHC	Major histocompatibility complex
mRNA	Messenger RNA
MΦ	Macrophage
NK	Natural killer
OVA	Ovalbumin

PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PDT	Pertussis-Diphtheria-Tetanus vaccine
PRR	Pattern recognition receptor
pTα	pre-T cell receptor α-chain
qPCR	Quantitative PCR
RORγt	Retinoic acid receptor-related orphan receptor γt
SP	Single positive (either $CD4^+$ or $CD8^+$ thymocyte)
T-bet	T_H1-specific T box transcription factor
TAP	Transporter associated with antigen processing
TCR	T cell (antigen) receptor
T_{FH}	T follicular helper
TGF-β	Transforming growth factor β
T_H1	T helper 1
T_H17	T helper 17
T_H2	T helper 2
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor α
T_R1	T regulatory type 1
T_{reg}	Regulatory T cell
TT	Tetanus toxoid
WT	Wild-type
WT_{ctrl}	WT mice fed with saline, immunized with OVA
WT_{OVA}	WT mice fed with OVA, immunized with OVA

5 INTRODUCTION

Immunology, the study of the human defense system, is associated with all other branches of medicine. Immunological mechanisms are involved in biological processes ranging from the healing of minor wounds to the protection of the organism from cancer or invasive pathogens. However, our knowledge of the immune system remains incomplete and immunological reactions are often considered unpredictable and impossible to control. Misguided or excessive immune reactions can lead to detrimental conditions such as allergy, reperfusion injury following stroke or myocardial infarction and even multiple organ failure following uncontrolled inflammatory responses to systemic infections. These conditions are difficult to prevent or cure by the means of the contemporary medicine, emphasizing the need for further study of the underlying immunological mechanisms.

The beginning of the new millennium has brought about major leaps of knowledge in immunology: we have started to understand the interrelations between the innate and adaptive immune systems, and the paramount importance of the mucosal immune system to the human health has emerged to the attention of the scientific community. In addition, the application of the methods of molecular genetics in immunology has changed our conception of the functional differentiation of T helper cells. The classical view of stable T helper cell lineages has evolved to a more nuanced model emphasizing the plasticity of the lineages and the simultaneous and synergistic activity of different effector cell types. Due to the central role of the T helper cells in the adaptive immunity, this change of perspective has far-reaching consequences to the interpretation of the function of the immune system as a whole.

The work presented in this thesis concentrates on the role of the complement system of the innate immunity in regulating the adaptive immune responses and T helper cell responses in particular. Experiments performed with both human and murine samples demonstrate that complement is required for normal T cell responses and especially for the induction of tolerance, both in the mucosal and systemic immune systems. Important differences between the murine and the human system were also observed.

6 REVIEW OF THE LITERATURE

6.1 Innate immunity and the complement system

6.1.1 First lines of defense

The human body is protected against pathogens by a layered defense system. The first line of defense is formed by a continuous layer of epithelial cells joined by tight junctions covering all surfaces of the body. Breaching this barrier is the essential first step that a pathogen must take in order to establish a focus of infection and to cause disease.

The thick, stratified epithelium of the skin is impenetrable to most microorganisms, and, therefore, many pathogens prefer the mucosal surfaces of the respiratory and gastrointestinal tract as a route of entry. These epithelia are thinner than the skin to allow diffusion of respiratory gases in the lungs and absorption of nutrients in the gut. A protective layer of mucus partly compensates for the epithelial thickness, and the constant flow of intestinal contents towards the end of the gastrointestinal tract and the continuous transport of mucus by cilia of the respiratory epithelium serve to mechanically remove harmful agents from the mucosal surfaces. Additional protective measures in the mucosal surfaces include the acidity of the stomach and secreted bactericidal molecules, such as defensins and lysozyme. The abundant commensal flora colonizing the mucosa and actively competing for space and nutrients with the pathogenic organisms is also an important protective factor.

If a pathogen overcomes these initial obstacles and succeeds in penetrating the epithelium, the immune system will be activated to repel the invader. Activation of innate immunity leads to inflammation and is followed by an adaptive immune response, if the innate response fails to remove the threat by itself. As adaptive immunity is recruited, the innate system passes on crucial information concerning the type of pathogen and the infected tissue to the adaptive immunity, largely dictating the type of the ensuing adaptive response.

The extent of the interdependency between innate and adaptive immunity has only started to emerge during the past decade. This thesis focuses on one interesting piece in this puzzle, namely, the role of the complement system in regulating the adaptive immune response.

6.1.2 Complement activation

The complement system consists of over 30 proteins, which include soluble factors present in serum and other bodily fluids, and membrane-bound molecules on host cells (Ricklin et al., 2010). Complement factors are produced as inactive proenzymes and remain so until they are activated. A common feature of complement activation is the cleavage of the inactive proenzyme into two fragments: a large fragment, which binds to membranes and labels the target for phagocytosis, and a small soluble fragment, which often has signaling potential. The large, membrane bound fragment also contributes to activating the next factor in the cascade.

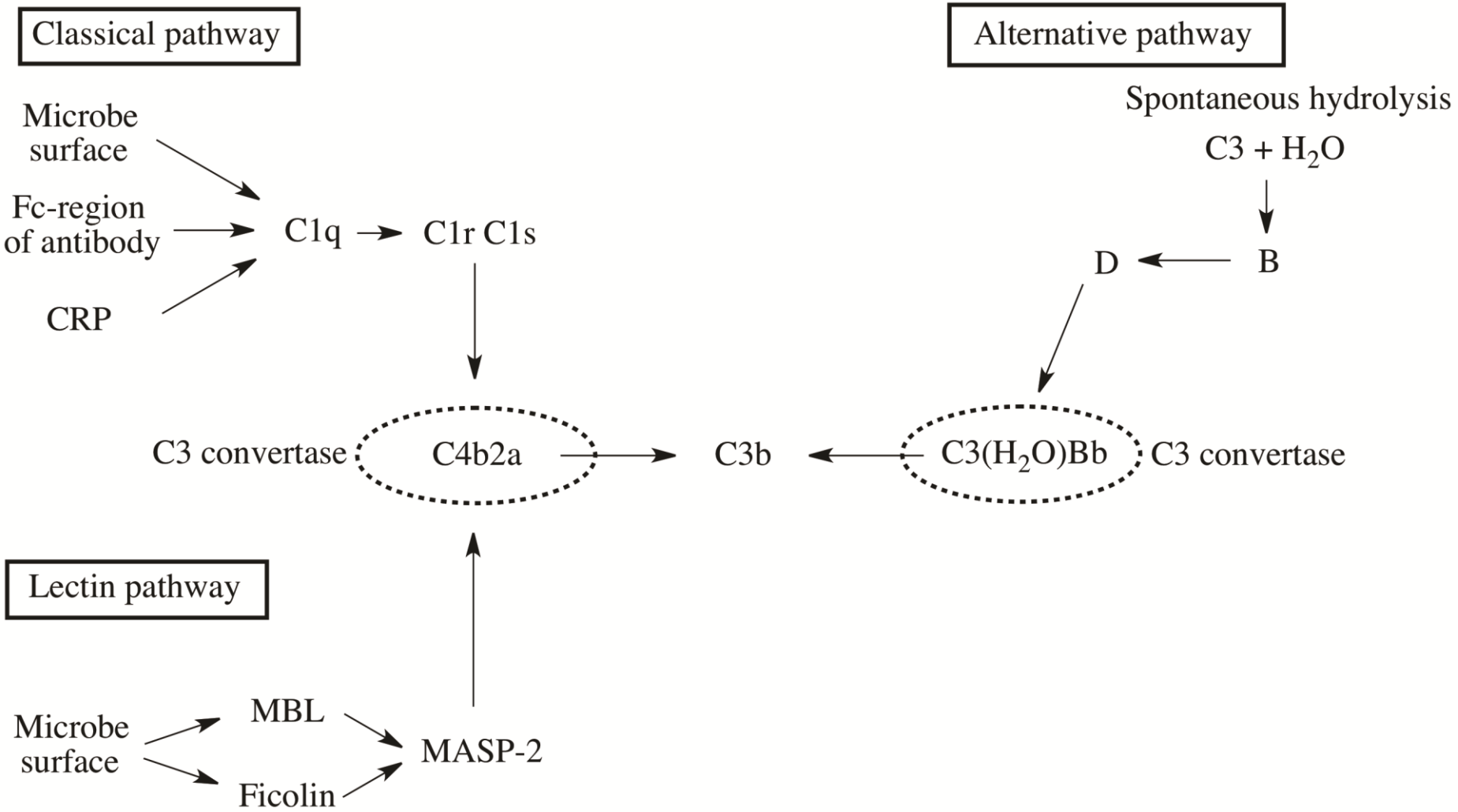

Classical pathway
Microbe surface
Fc-region of antibody
CRP
C1q
C1r C1s
Alternative pathway
Spontaneous hydrolysis
C3 + H2O
B
D
C3 convertase
C4b2a
C3b
C3(H2O)Bb
C3 convertase
Lectin pathway
Microbe surface
MBL
Ficolin
MASP-2

Figure 1. Complement activation pathways. The complement system has three activation pathways, which all converge at the production of membrane-bound C3b. The classical pathway is initiated by C1q binding directly to microbe surface or an antibody or CRP molecule associated with the surface. C1q forms a complex with C1r and C1s and the latter cleaves C4 to C4a and C4b, which binds to the surface. C2 is then cleaved by C1s and the larger fragment C2a forms a C3 convertase with C4b. The lectin pathway is initiated in a similar manner with MBL or ficolin as the pattern-recognition molecule and MASP-2 as the protease. The alternative pathway is initiated by the spontaneous hydrolysis of C3 to C3(H_2O). Factor B is then cleaved by factor D to form the fluid phase C3 convertase C3(H_2O)Bb.

Complement activation occurs on cell surfaces. It can be triggered by antibody bound on target cell (classical pathway), direct recognition of microbial cell-surface patterns by complement factors (classical and lectin pathways) or spontaneous activation of complement factor C3 in the absence of complement inhibitors on microbial cell surface (alternative pathway) (Ricklin et al., 2010) (Fig.1).

All activation pathways lead to the assembly of a C3 convertase enzyme, which is covalently linked to the cell surface (Pangburn et al., 1981; Wallis et al., 2010). This convertase cleaves multiple molecules of C3, which can then form additional C3 convertases via the alternative pathway, establishing an amplification loop (Fig.2). This makes the complement factor C3 the most important and central factor in the complement cascade. In the absence of C3 all effector functions are blocked and deficiency in other alternative pathway components leads to severe immune dysfunction, whereas deficiencies in the other two activation pathways usually lead to relatively milder morbidities (Ghannam et al., 2008; Ram et al., 2010; Reis et al., 2006). Not surprisingly, the alternative pathway appears to be the evolutionarily oldest part of the complement system, the lectin and classical pathways being later modifications of an already functional system based on spontaneous activation of C3 (Rodriguez et al., 2012).

6.1.3 Effector functions of complement

The complement system has three major effector functions: opsonization, chemotaxis, and direct lysing of bacterial cells. The larger fragment of cleaved C3, called C3b, binds covalently to the target surface and can be recognized by complement receptors of phagocytes and other cells (Ricklin et al., 2010). This tagging, also known as opsonization, greatly enhances the ability of the phagocytes to kill the pathogen.

If the C3b molecule remains associated with the activating convertase, it modifies its enzymatic specificity to make it a C5 convertase (Pangburn and Rawal, 2002). This new enzyme complex cleaves C5 to soluble C5a and membrane-associated C5b, which initiates the assembly of the membrane attack complex (MAC). In essence, MAC is a short molecular tube, which inserts into the plasma membrane of a bacterial cell and kills it by allowing free diffusion of ions and small molecules through the membrane (Muller-Eberhard, 1985) (Fig.3).

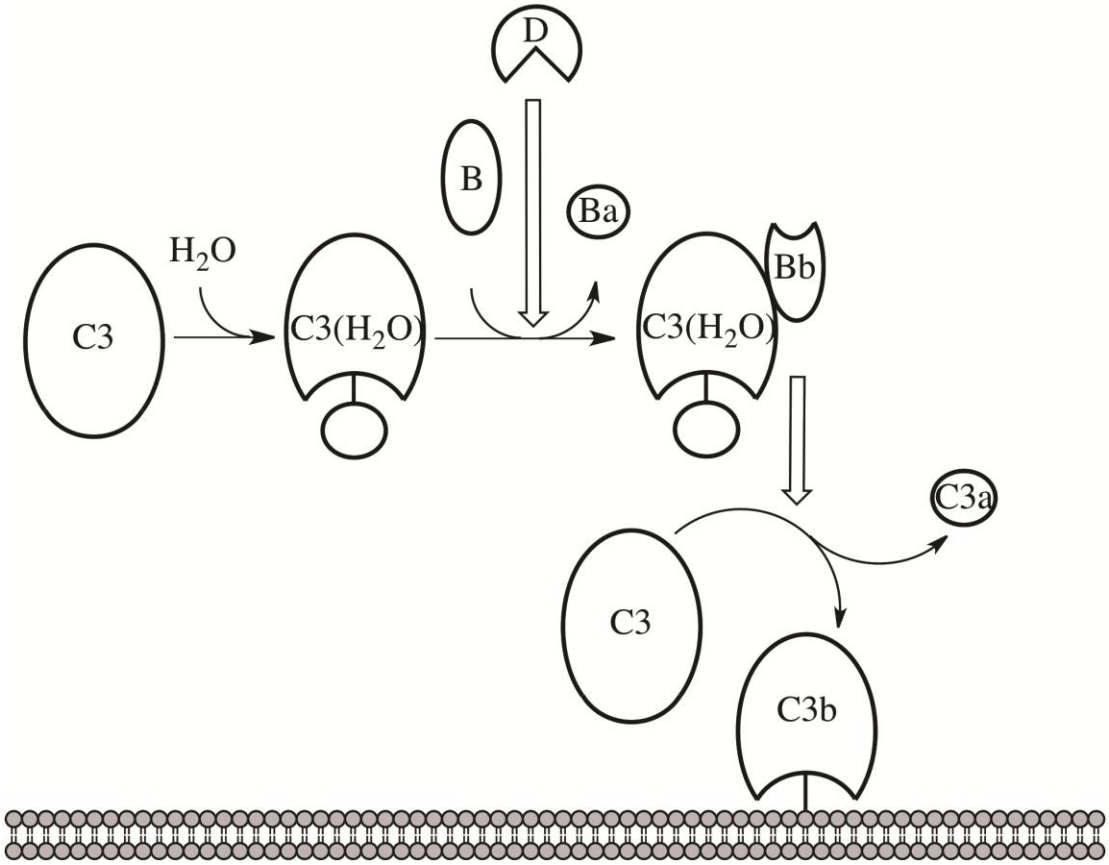


Figure 2. Activation of the alternative pathway. The alternative pathway of complement is activated by spontaneous hydrolysis of C3 to $C3(H_2O)$. Factor B binds to $C3(H_2O)$ and is then cleaved by factor D to Ba and Bb. The short-lived fluid phase C3 convertase $C3(H_2O)Bb$ cleaves additional C3 molecules to C3a and C3b. C3b binds rapidly to any nearby surface.

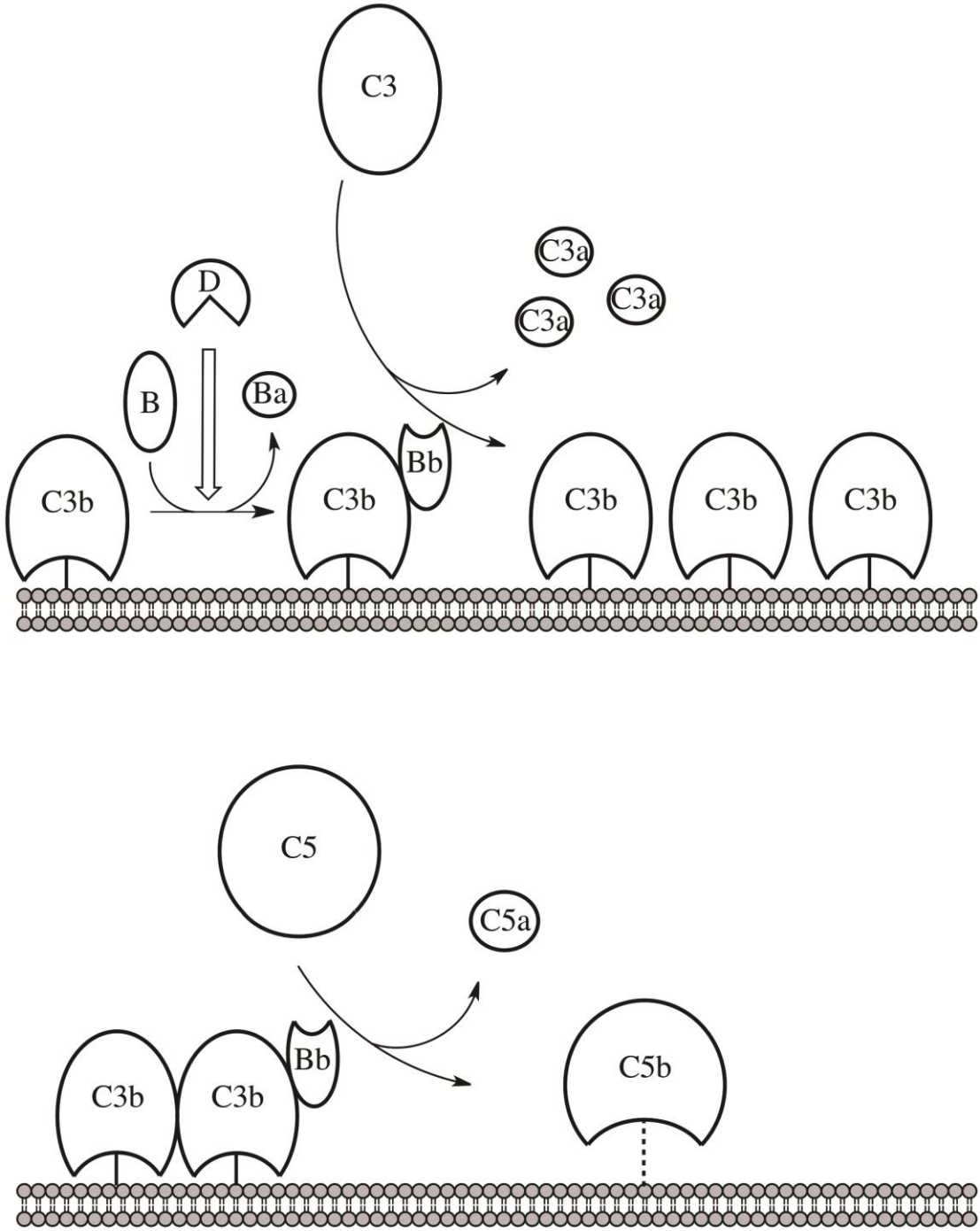


Figure 3. Amplification of complement activation. Membrane-bound C3b leads to amplification of the complement activation by forming a membrane-bound alternative pathway C3 convertase C3bBb, which cleaves additional C3 molecules (upper panel). The convertase can be further stabilized by properdin (omitted for clarity). If the newly formed C3b molecule remains associated with the convertase, it modifies the enzymatic activity, forming a C5 convertase C3bBb3b (lower panel).

The small soluble fragments, C3a and C5a, diffuse freely away and form a concentration gradient leading to the site of complement activation. They are called anaphylatoxins, since they have a strong ability to induce inflammation (Klos et al., 2009). They stimulate the endothelial cells of the local blood vessels to express leukocyte adhesion molecules and to increase permeability of the vessel wall, leading to recruitment of neutrophils and other leukocytes, and influx of plasma into the inflamed tissue. The plasma carries with it additional complement factors, which function to bolster the ongoing complement activation. Leukocytes use the anaphylatoxin gradient to find their way to the site of infection (chemotaxis). In addition, C5a triggers the mast cells to release inflammatory mediators from their preformed granules and the phagocytosis of C3b coated microbes is greatly enhanced when C5a binds to its receptor simultaneously with C3b binding to its own receptor on the surface of a macrophage (van Lookeren Campagne et al., 2007).

6.1.4 Regulation of complement

The rapid activation, amplification, and powerful effector functions of the complement system pose a threat of auto-reactivity to host cells. Therefore, complement activation on self is inhibited by various, often redundant, mechanisms (Zipfel and Skerka, 2009).

Activation of the classical and lectin pathways requires active recognition of the target by the initiating molecules (antibody, CRP or C1q for the classical pathway; MBL or ficolins for the lectin pathway). The alternative pathway, on the contrary, does not need a target structure for activation. The intrinsic tendency of C3 to be hydrolyzed leads to a constant production of small amounts of active C3b in all bodily fluids (Bexborn et al., 2008). Activated C3b has a very short half-life and it binds instantly to any membrane present (Pangburn et al., 1981). On host cell surfaces the bound C3b is immediately inactivated to iC3b by factor I and complement regulatory proteins factor H, complement receptor 1 (CR1) and membrane cofactor protein (MCP; CD46) that function as cofactors for factor I. The Bb fragment of Factor B associated with C3b on host cell surfaces is displaced from the convertase complex by factor H, CR1 and decay accelerating factor (DAF) to prevent further complement activation (Lambris et al., 1996). CRIg blocks the binding of the C3 substrate to the C3bBb enzyme complex (Wiesmann et al., 2006) (Fig.4).

Microbes lack these regulators and on their surfaces the activation continues and is rapidly amplified. Covalent binding of C3b to the cell surface ensures that the activation does not spread beyond the target. Together, these functions make the complement system capable of discriminating between self and non-self, which is a fundamental task of the immune system.

6.2 Complement C3 deficiency in humans

Total deficiency of complement C3 is rare in humans, with only 20 families described worldwide (Reis et al., 2006)(II). The pivotal role of the complement system in the early defense against bacterial pathogens and the central position of C3 in the complement cascade make the patients highly susceptible to invasive infections (Ram et al., 2010). Later in life, C3 deficiency often leads to different forms of autoimmunity (Reis et al., 2006).

6.2.1 Infectious complications

The severe infections caused by C3 deficiency are manifested usually in the first years of life. The patients are susceptible to recurrent invasive infections caused by Gram-negative bacteria, including *Neisseria meningitidis, Haemophilus influenzae* and *Escherichia coli,* and by Gram-positive bacteria, such as *Streptococcus pneumoniae*, *Streptococcus pyogenes* and *Staphylococcus aureus*. The infectious foci range from otitis media, sinusitis and urinary tract infections to pneumonia, meningitis and septicemia (Reis et al., 2006). The patients require regular prophylactic use of antibiotics and an extensive vaccination strategy to prevent infectious complications.

6.2.2 Autoimmunity

In the later years of life, patients with C3 deficiency commonly suffer from autoimmune manifestations. These are often associated with accumulating immune complexes of particulate antigen bound to immunoglobulin molecules, since C3 has a central role in their clearance (Klint et al., 2000). The kidney in particular is vulnerable to immune complex deposition leading to glomerulonephritis. Also IgA nephropathy and conditions resembling systemic lupus erythematosus have been diagnosed in C3 deficient patients (Reis et al., 2006).

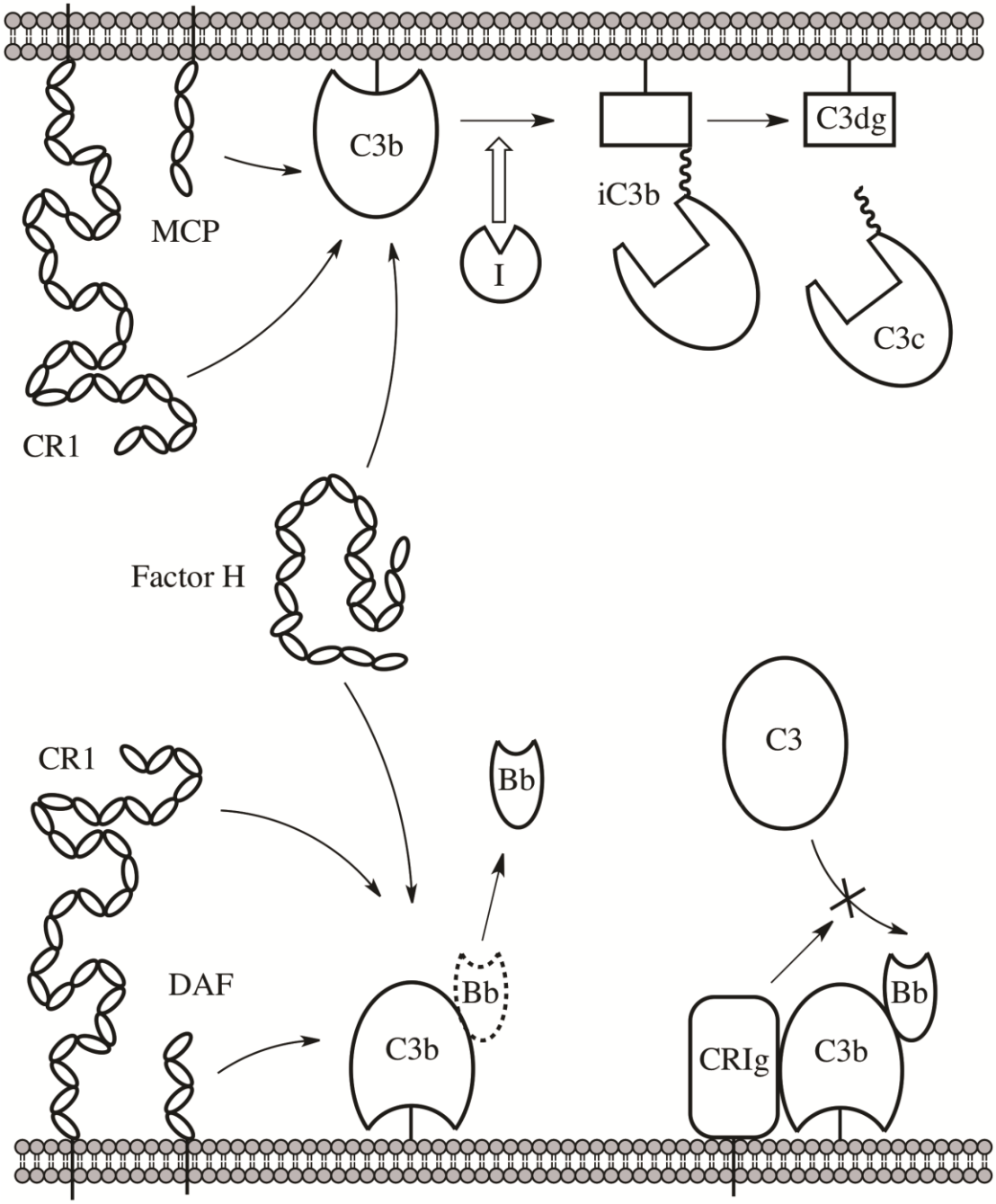


Figure 4. Inhibition of complement activation. Complement activation is regulated at the level of C3b in numerous ways. Factor H, CR1 and MCP facilitate the inactivation of C3b by factor I into iC3b and further to C3dg. Factor H, CR1 and DAF displace Bb from the C3bBb complex and CRIg blocks the binding of the C3 substrate to the C3bBb complex.

6.3 The innate immune response

6.3.1 Phagocytosis

Opsonization of pathogens with complement fragments would not make much sense without the existence of phagocytes equipped with appropriate complement receptors. Phagocytosis is an ancient defense method originating from the ability of large single-cell organisms to prey on smaller microbes by engulfing them. In humans, the most important phagocytes are the macrophages and immature dendritic cells (DCs) resident in tissues, and granulocytes, which are summoned to the tissue from the bloodstream once an intruder has been detected. The DCs are specialized to initiate an adaptive immune response, and their functions will be discussed later.

Macrophages are the most numerous phagocytes in healthy tissues. They differentiate continuously from monocytes leaving the circulation, and in the absence of infection serve as waste disposal units, degrading apoptotic cell debris marked for quiet removal by iC3b (Flierman and Daha, 2007). If a pathogen emerges, macrophages are among the first cells to react to the imminent threat. They have multiple sets of pattern recognition receptors (PRRs), which bind to pathogen associated molecular patterns (PAMPs) (Mukhopadhyay et al., 2009). PAMPs are conserved structures present on many microbes but not on the body's own cells (host cells). Phagocytic PRRs present on the macrophage cell surface, such as the scavenger receptors, induce internalization and degradation of the bound microbe.

Opsonization by complement facilitates phagocytosis of all microbes but it is crucial in the recognition of encapsulated pathogens, which have covered their surface with a thick polysaccharide capsule to evade direct recognition by phagocyte PRRs (Cunnion et al., 2003; Zaragoza et al., 2003). Phagocytosis of these pathogens relies on complement receptors CR1, CR3 and CR4 present on the cell surface of phagocytes.

6.3.2 Recognition of non-self: the danger-signal

In addition to phagocytic receptors, the macrophages and DCs have other PRRs that activate pro-inflammatory signaling cascades in the cell after binding to their ligands. These include Toll-like receptors (TLRs) present on the cell surface and membranes of intracellular vesicles, and multiple families of intracellular receptors capable of sensing bacterial products (Kersse et al., 2011), viral replication in the cytosol (Lappalainen et al., 2013) or general cellular stress (Sheedy et al., 2013).

The mammalian TLRs are the most profoundly studied class of the signaling PRRs and the function of the TLRs provides a feasible mechanistic explanation on the molecular level to the observation that the innate immune system can broadly recognize the type of an invading pathogen. This information is then passed on to the adaptive immune system to tailor the ensuing adaptive response to be as effective as possible against the particular invader (Abdelsadik and Trad, 2011; Qian and Cao, 2013).

In humans, 10 different TLRs have been characterized. Together, they recognize PAMPs from Gram-positive and Gram-negative bacteria, fungi and viruses. Those TLRs that are located on the cell surface recognize structures of extracellular

pathogens, such as the lipopolysaccharide (LPS) of Gram-negative bacteria (TLR-4) (Park et al., 2009) and the lipoteichoic acids of Gram-positive bacteria and fungal zymosan (TLR-1:TLR-2 and TLR-6:TLR-2 heterodimers) (Irvine et al., 2013). Intracellular TLRs present in the endosome membranes bind to viral molecules, such as single-stranded RNA (TLR-7) and DNA with unmethylated CpG (TLR-9) (Wei et al., 2009).

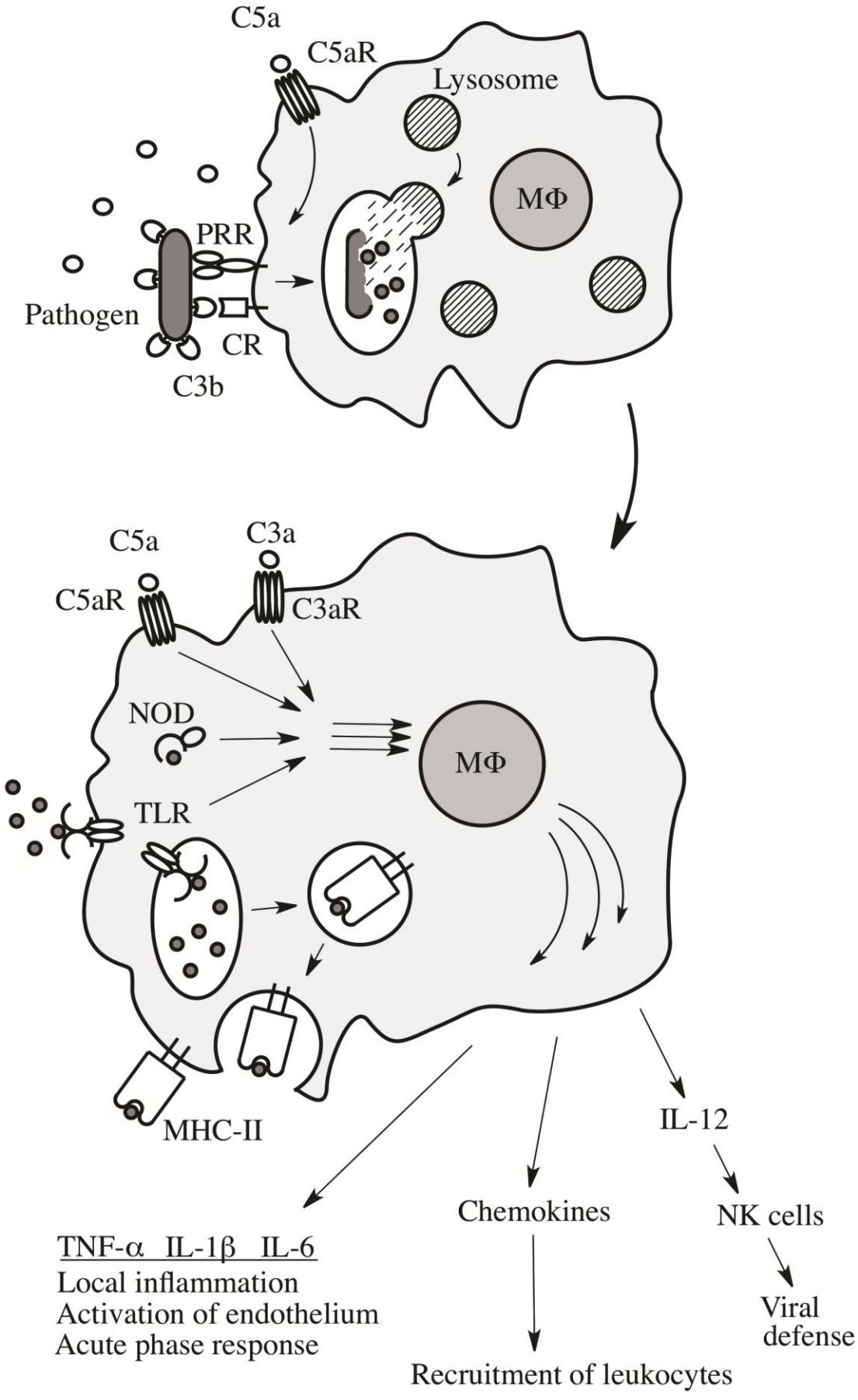
The expression of TLRs is not limited to the macrophages and DCs. Granulocytes express multiple types of TLRs and natural killer (NK) cells express TLRs with viral specificities, in accordance with their central role in viral defense. TLR-5 recognizing the flagellin of flagellated bacteria is present on the cell surface of macrophages and DCs but also on the basal surface of the intestinal epithelial cells (Abdelsadik and Trad, 2011).

Ligand binding to TLRs induces intracellular signaling cascades ultimately leading to activation of the cell and production of secondary messenger molecules, such as cytokines. The intracellular signaling initiated by TLRs can be modulated by complement, since the signaling cascades associated with C3aR and C5aR signaling share intracellular mediators with the TLR pathway (Song, 2012). Usually this probably is the case *in vivo*, because many TLR ligands, such as zymosan of yeast cell walls and lipopolysaccharide of Gram-negative bacteria, are also strong activators of the complement system (Harboe et al., 2012; Inzana et al., 1987). Coinciding complement activation can, for example, modulate the IL-12 and IL-10 production induced by TLR activation (Zhang et al., 2007) and, therefore, lead to profound changes in the ensuing adaptive immune response (Fig.5).

The complement receptors were introduced above as phagocytic receptors. However, they also serve a function in the recognition of non-self and danger-signaling. CR1 degrades target-bound C3b into iC3b and further to C3dg, both of which are ligands for the CR2 present on the B cell surface. Binding of the ligands to CR2 enhances B cell activation (Roozendaal and Carroll, 2007). CR3 and CR4 have multiple binding sites and in addition to binding to iC3b they function also as integrins, mediating cell-cell contacts and binding to the extracellular matrix and CR3 can induce phagocytosis by recognizing LPS and β-glucans with its lectin-binding domain (Petty et al., 2002). CR3 signaling has been shown to modulate IL-12 production by the APCs (Kim et al., 2004; Leon et al., 2006), thus regulating the T helper cell differentiation.

Complement activation and ligand binding to TLRs occurs simultaneously *in vivo*, so it is not surprising that the signaling pathways initiated by them synergize with each other. In addition to CR3, also the anaphylatoxin receptor C5aR and C3aR mediated responses modulate the cytokine production of TLR activated APCs.

Figure 5. Activation of macrophages. Macrophages recognize C3b-coated pathogens by complement receptors (CR) and pattern-recognition receptors (PRR). The pathogens are ingested and degraded in phagolysosomes. Phagocytosis is further stimulated by C5a binding to its receptor (C5aR) (upper panel). TLR and NOD molecules recognize pathogen associated molecular patterns and activate the macrophage. Signaling from complement anaphylatoxin receptors C3aR and C5aR synergize with TLR and NOD signaling leading to production of cytokines and chemokines by the macrophage. Peptides derived from the degraded pathogens are presented on the macrophage surface by MHC II molecules (lower panel).


C5a
C5aR
Lysosome
MΦ
PRR
Pathogen
CR
C3b
C5a
C3a
C5aR
C3aR
NOD
MΦ
TLR
MHC-II
IL-12
Chemokines
NK cells
TNF-α IL-1β IL-6
Local inflammation
Activation of endothelium
Acute phase response
Viral
defense
Recruitment of leukocytes

6.3.3 Induced innate immunity

Recognition of foreign structures by TLRs and other pro-inflammatory PRRs leads to synthesis and release of prostaglandins, leukotriens and pro-inflammatory cytokines by the macrophages and DCs. These mediators act in consort with complement anaphylatoxins C3a and C5a and histamine released from mast cells to activate the endothelium of the local tissue blood vessels (Movat, 1987). Within minutes from macrophage activation the endothelial cells start to express selectins, which recruit leukocytes to the tissue to fight the infection. The vessel dilates, blood flow is slowed down and the junctions between endothelial cells are loosened to allow the influx of plasma into the tissue, carrying with it additional complement proteins, antibodies and other defense molecules. The pathogen is prevented from spreading to the bloodstream by blood clotting (thrombosis) in the local vessels (Engelmann and Massberg, 2013). Together with the increased fluid in the tissues the partial occlusion of local blood vessels directs the flow of fluid into the lymph vessels and, eventually, to the local lymph node, where adaptive immunity will be initiated.

If the invader is an extracellular pathogen, for example a bacterium replicating in the interstitium, the macrophages attempt to phagocytose and kill the bacteria to clear or at least to limit the infection until adaptive immunity is ready to take command. Their numbers are increased by blood-borne monocytes differentiating to macrophages in the tissues but they are also aided by another cell type, the neutrophilic granulocytes, or neutrophils, which move rapidly from the bloodstream to the inflamed tissue. Compared to macrophages, the neutrophils are far less sophisticated in their actions; their mission is to engulf and kill as many pathogens as quickly as they can. Once this is accomplished, they die by apoptosis, only to be replaced with new neutrophils continuously arriving from the blood.

Phagocytosis is not effective against viruses hiding inside host cells. Recognition of viral structures by macrophages leads to the production of the cytokine IL-12 by the macrophages (Abdelsadik and Trad, 2011), leading to activation of the NK cells, which can directly kill virus-infected cells (Choi and Mitchison, 2013). Cytokines secreted by macrophages and NK cells also modulate the metabolism of the tissue cells to make them less susceptible to viral infection.

6.3.4 The acute phase and clinical signs of infection

In addition to the local effects, some cytokines secreted by macrophages have also systemic effects. TNF-α, IL-1β and IL-6 activate the liver to produce acute phase proteins, such as CRP, fibrinogen, and complement factors, and the hypothalamus reacts to these cytokines by increasing the body temperature to hamper the growth of pathogens. Reserves of neutrophils are released from the bone marrow into the circulation and tissue DCs are induced to mature and to migrate to the local lymph nodes to initiate an adaptive immune response. The classical clinical signs of infection, i.e. pain, redness, swelling and increased temperature, are actually signs of the function of the innate immunity.

6.4 Adaptive immunity and the role of T cells in the immune response

6.4.1 Antigen uptake and processing by antigen presenting cells

Adaptive immunity begins when a DC presents antigen to a naïve $CD4^+$ T cell (Itano and Jenkins, 2003). Preparation of this rendezvous starts when a resident DC senses foreign material with its PRRs in the tissue. The foreign antigen is phagocytosed and degraded by the DC and the fragments are bound to MHC II molecules in the phagolysosomes (Schulze and Wucherpfennig, 2012). Antigens derived from the cytosol are actively transported to the endoplasmic reticulum and bound to MHC I molecules (Hulpke and Tampe, 2013). Stimulation of TLRs and other PRRs induces maturation of the DC and expression of costimulatory molecules (Medzhitov et al., 1997; Steinman and Hemmi, 2006). The cell leaves the tissue and is carried in the lymph to a local lymph node, where the naïve T cells are waiting.

The most efficient method of antigen uptake is receptor-mediated phagocytosis via recognition of PAMPs or opsonins, such as C3b on the surface of the pathogen. If this is not possible, the pathogen can still be engulfed by macrophages and DCs by means of macropinocytosis, the passive internalization of interstitial fluid by these cells. B cells can recognize specific antigen with their antigen receptor and then process and present it in a similar manner (Itano and Jenkins, 2003).

Dendritic cells, macrophages and B cells are called professional antigen-presenting cells (APCs), because they present peptide fragments of extracellular antigens on MHC II-molecules to $CD4^+$ T cells and can express costimulatory molecules, such as B7, required for the activation of T cells (Itano and Jenkins, 2003). Between these cells there is a clear distribution of work. T cell activation is the ultimate goal of DCs, and they are normally the only cells capable of initiating a T cell response to a pathogen that is encountered for the first time (Byersdorfer and Chaplin, 2001; Ingulli et al., 1997). The strength and quality of signals from the PRRs determine the expression of costimulatory molecules on the surface of the DC and the cytokine pattern secreted by the cell (Yamane and Paul, 2012). Macrophages and B cells, on the contrary, require activating signals from T cells previously activated by DCs in order to express costimulatory molecules on their surface (Cassell and Schwartz, 1994; Chang et al., 1995).

Macrophages aim at efficient clearing of pathogens by phagocytosis and they present antigen to T cells to get help in this task. The help is provided in the form of activating cytokines and cell surface molecules, such as CD40L, which enhance the bactericidal activities of the macrophage. Generally, macrophages do not travel to the local lymph node after encountering a pathogen, but reside in the tissue. They are important in supporting the effector T cells arriving in the tissue. However, macrophages are continuously present also in the lymph nodes, where they engulf pathogens arriving in the afferent lymph and degrade apoptotic lymphocytes (Witmer and Steinman, 1984).

B cells need T cell help for the production of antibody and, most importantly, for the antibody class switching, which modulates the effector functions of the antibody produced. Due to the specificity of their antigen receptor, they are able to present to T cells soluble antigens that are present in low concentration. This is emphasized in the

case of toxins, which are soluble molecules produced by certain pathogens and are highly toxic to host cells even in low concentrations. In addition to the antigen specific B cell receptor (BCR) the B cells use CR2 to collect antigens tagged with iC3b and C3dg and they are able to transfer these antigens to follicular dendritic cells (FDCs) for presentation to other B cells (Phan et al., 2009).

All three types of APCs process the antigen in a similar manner for presentation. Phagocytosed material is taken up in endocytic vesicles, which fuse with lysosomes. The vesicles are then acidified, which activates proteases, resulting in degradation of proteins into peptides. Then MHC II molecules are transported to the endosomes from the endoplasmic reticulum. In the acidic environment, with the help of auxiliary proteins, the peptide-binding cleft of the MHC II molecule opens up and is able to bind peptides residing in the vesicle. Peptide-MCH II complexes are then transported to the cell surface (Vyas et al., 2008).

6.4.2 Antigen presentation to T lymphocytes

T cells do not recognize antigen directly. They only react to peptide fragments of antigen processed by other cells and presented on MHC class I or class II molecules. Moreover, the T cell antigen receptor (TCR) binds both to the MHC molecule and the peptide, restricting the ability of a given T cell to recognize peptides bound to one type of MHC only. CD8 and CD4 are coreceptors of the TCR and they bind to MHC I and MHC II molecules, respectively.

There are three types of human MHC class I molecules, called human leukocyte antigen (HLA)-A, HLA-B and HLA-C, and three types of MHC class II molecules, HLA-DP, HLA-DQ and HLA-DR. Of these, numerous alleles are present in the population, increasing the level of protection from infections on the population level.

All nucleated cells are able to present peptides on MHC I molecules. These peptides originate from the cytosolic proteins and are generated by continuous degradation of all proteins produced by the cell. This degradation is a regulated process and takes place in the proteasome, a highly specialized molecular apparatus in the cytoplasm (Basler et al., 2013). The peptides are then transported to the endoplasmic reticulum with the help of a transport protein called TAP, and then loaded onto the MHC I molecules (Panter et al., 2012). Usually the peptides generated by the proteasome are derived from the cell's own proteins but in the case of viral infection of the cell, also viral peptides will be produced and funneled for presentation by the MHC I molecules. In addition, cytokines produced in the presence of a viral infection increase the activity of the proteasome (Basler et al., 2013) and induce higher expression of the MHC I proteins, leading to effective recognition of the foreign antigen by activated $CD8^+$ cytotoxic T cells and killing of the infected cells.

6.4.3 Activation of T lymphocytes

In order to be activated, a naïve T cell must recognize its specific peptide presented to it by an APC (Itano and Jenkins, 2003). This is called signal 1 and it is the *sine qua non* of antigen specific T cell activation (Murphy, 2012). In addition to this, the same APC must deliver signal 2, or costimulatory signal, in the form of B7 to the naïve T cell (Guermonprez et al., 2002). These two signals induce proliferation of the T cell, producing more cells with the same antigen specificity. The type of the T cell response will be determined by signal 3 consisting of the cytokine milieu in the lymph

node during the activation and partly of the strength of the TCR signaling (Zhu and Paul, 2010)(Fig.6).

Memory T cells retain functional specialization from the original encounter with an antigen and a subgroup of them, the effector memory T cells, does not require costimulatory signals and can respond to the cognate antigen whenever it is presented, making memory responses to previously encountered antigens much faster (Mueller et al., 2013).

In addition to antigen specific activation, T cells can also be activated to some extent in a TCR-independent manner by the cytokines produced upon inflammation (Tough and Sprent, 1998). However, the effect of this bystander activation to the immune response and its regulation have not been completely sorted out.

6.4.4 $CD4^+$ T lymphocyte effector functions

Pathogens use diverse means for surviving inside the host, and, therefore, different defense strategies are needed for optimal clearance of different invaders. T cells are crucial in this process and the type of the ensuing immune response is largely dictated already in the first contact between a DC and a naïve T cell (Yamane and Paul, 2013). As noted before, the DC uses its TLRs and other PRRs to broadly recognize the type of the intruder and mediates this information to the activated T cells in the form of cytokines (Fig.7).

6.4.4.1 Regulatory T cells

In the absence of infection, the cytokine milieu in the lymph nodes is dominated by suppressive cytokines, mainly TGF-β secreted both by DCs and regulatory T cells (T_{reg}s) expressing the transcription factor FoxP3 (Josefowicz and Rudensky, 2009). The FoxP3 expressing T_{reg} cells can be divided in two main groups, the thymus derived 'natural' nT_{reg}s and the inducible iT_{reg}s arising from naïve T cells in the periphery. The nT_{reg}s are thought to have TCRs with relatively high affinity for self peptides (Liston and Rudensky, 2007) and their main role is to prevent autoimmunity. The iT_{reg}s have a different task: they develop in response to innocuous external antigens and commensal microbes to prevent unnecessary and potentially harmful responses at the outer limits of the body (Lehtimaki and Lahesmaa, 2013).

In the steady state, T_{reg} cells suppress the activation of naïve T cells and DCs by direct cell-cell contacts and secretion of suppressive cytokines (Sakaguchi et al., 2008). In addition, the DCs present self peptides on their MHC molecules in the absence of costimulatory signals. The naïve T cells recognizing the self-antigens are either deleted, made anergic (Hawiger et al., 2001), or directed to regulatory lineages, such as iT_{reg} or T_R1, producing IL-10 and more TGF-β and further suppressing activation of other T cells (Horwitz et al., 2008; Roncarolo et al., 2006).

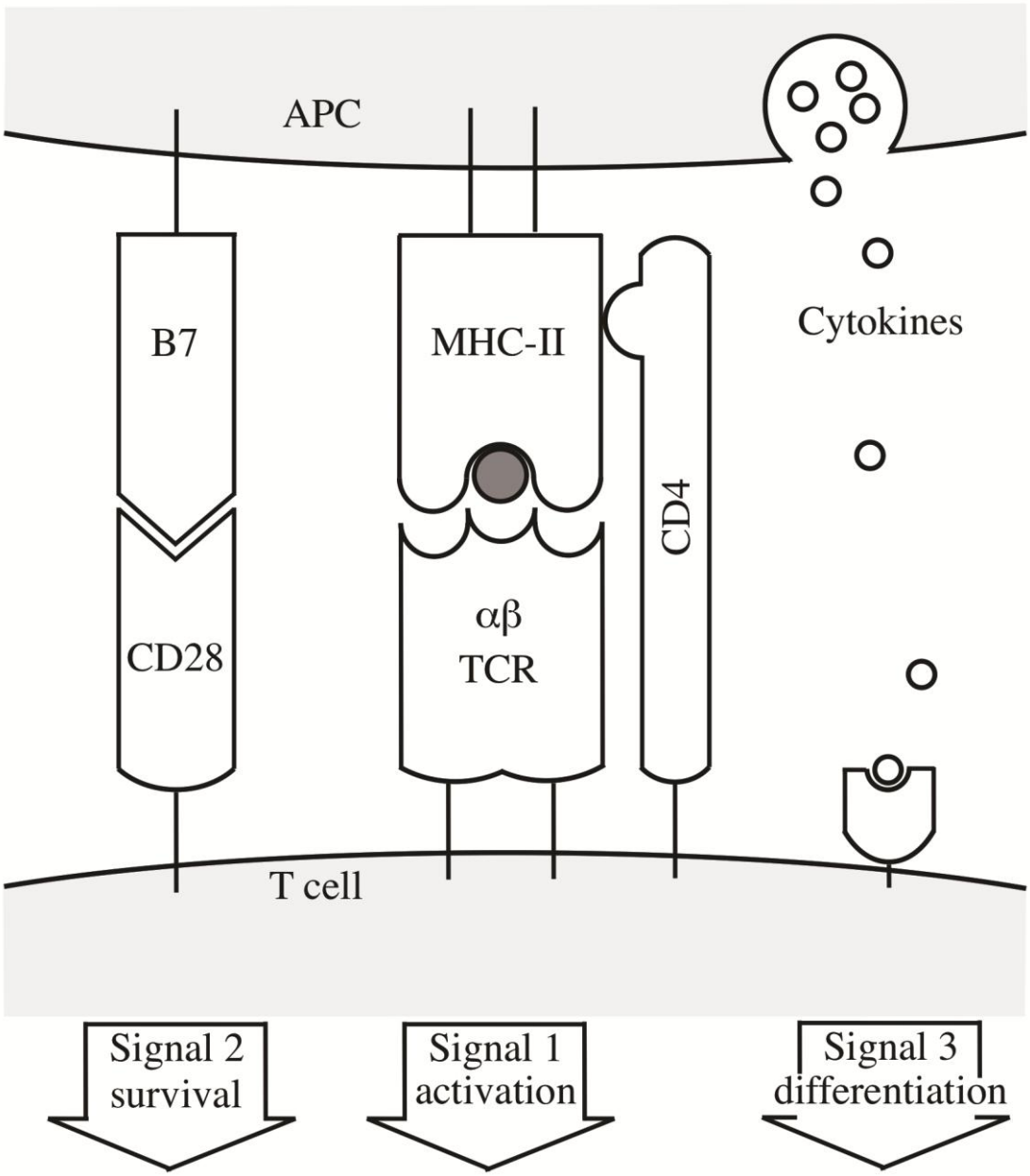


Figure 6. Antigen presentation to T cells. Antigenic peptide is presented to the T cell (in this figure a $CD4^+$ T helper cell) in an MHC molecule. Binding of the TCR to the peptide-MHC complex activates the T cell and the costimulatory signal in the form of B7 induces proliferation of the cell. Cytokines modulate the differentiation of the activated T cell.

6.4.4.2 T_H17 cells

Early in the immune response IL-6 is produced, especially if the pathogen is an extracellular, pyogenic bacterium. The combination of TGF-β and IL-6 induces the naïve $CD4^+$ T cells recognizing their cognate antigen to differentiate to T_H17 type helper T cells, which are efficient supporters of neutrophil activation and inflammation (Kroenke et al., 2008). T_H17 cells express the transcription factor RORγt and secrete members of the IL-17 family and IL-6 (Bettelli et al., 2008).

6.4.4.3 T_H1 cells

Intracellular pathogens, such as viruses and mycobacteria stimulate TLRs 3, 7 and 9, leading to production of IL-12 by the DCs and NK cells. This funnels the activated $CD4^+$ T cells to T_H1 type effector cells, characterized by the expression of the transcription factor T-bet and production of IFN-γ (Szabo et al., 2000). T_H1 immunity is cell-mediated, boosting macrophage function and $CD8^+$ T cell responses. T_H1 cells induce B cell class-switching to IgG1 and IgG3 in the human immune system and IgG2a and IgG3 in the murine system (Murphy, 2012).

6.4.4.4 T_H2 cells

T_H2 cells are induced by IL-4 and T_H2 responses are characterized by an IgE dominated humoral response and activation of mast cells, eosinophils and basophils but suppression of other cell-mediated immunity. T_H2 cells produce IL-4, IL-5 and IL-13 and express the transcription factor GATA-3 (Zheng and Flavell, 1997). This branch of immunity appears to be directed against helminths and other parasites but in the affluent countries it is better known for its role in allergic diseases (Murphy, 2012).

6.4.4.5 T_{FH} cells

In addition to the four lineages described above, others have also been suggested. The follicular T helper cell (T_{FH}) lineage has established itself in the recent literature, although it is still a matter of controversy, whether these cells arise directly from naïve $CD4^+$ T cells or from cells that have already adopted a T_H1, T_H17 or T_H2 phenotype. Nevertheless, T_{FH} cells are specialized to providing help to B cell responses in the B cell follicles (Fazilleau et al., 2009). IL-6 and IL-21 are associated with their induction, they secrete IL-21 and express the transcription factor Bcl-6 (Awasthi and Kuchroo, 2009).

6.4.4.6 Misguided T helper cell responses

T_H2 cells are notorious for their association with allergy but all Th lineages have a dark side of their own. T_H1 responses lead to classical autoimmune diseases, if the response is directed against self-antigens. Misguided T_H17 responses are also associated with autoimmunity, whereas excessive T_{reg} activity can lead to chronic infections (Murphy, 2012).

6.4.4.7 Other aspects of T_H differentiation

The cytokines elicit intracellular signaling in the T_H cells via the Janus tyrosine kinase (JAK) – Signal-transducing activator of transcription (STAT) pathway. Activation of STAT1 and STAT4 leads to T_H1 differentiation, whereas STAT6, STAT3 and STAT5 are associated with T_H2, T_H17 and iT_{reg} differentiation, respectively (Lönnberg et al., 2013). In addition to the cytokine milieu, another important factor affecting the T

helper cell lineage choice is the strength of the TCR signaling. Strong TCR signals tend to favor the induction of T_H1, T_H17 and T_{FH} lineages, whereas weaker TCR signaling due to low concentration of antigen or peptides binding to the TCR with low affinity tilt the balance towards the production of T_H2 or T_{reg} responses. However, the plasticity of the lineages makes it somewhat difficult to split the T helper cells into strict categories, since the lineage choice is not fixed: cells can invert to another lineage and intermediate phenotypes exist (O'Shea and Paul, 2010).

6.4.5 Effects of complement on T cell immunity

Complement activation or the lack of it can modulate T cell function in at least three different ways: by modifying the function of DCs and other APCs, by affecting chemotaxis of both APCs and T cells themselves (Ricklin et al., 2010) and directly by binding to receptors specific for different complement cleavage products present on the T cell surface (Fuchs et al., 2009; Heeger et al., 2005; Zaffran et al., 2001). On the systemic level, mice deficient in C3 produce impaired antiviral T cell responses (Fang et al., 2007; Kopf et al., 2002; Suresh et al., 2003) and display delayed rejection of skin allografts (Marsh et al., 2001; Peng et al., 2006). It has been suggested by some reports that C3 produced locally by the antigen presenting DC plays a crucial role in the induction of effector T helper cells (Peng et al., 2006).

Closer analysis of the influence of complement in the functional differentiation of T helper cells has revealed that T_H1 and T_R1 responses are particularly dependent on complement. Ligation of the complement regulator CD46 (membrane cofactor protein, MCP) simultaneously with TCR stimulation on human $CD4^+$ cells has been shown to induce differentiation to the T_H1 lineage or to T_R1 lineage, depending on the activation conditions (Cardone et al., 2010). CD46 has an intracellular signaling domain that can directly mediate changes in the cell (Astier et al., 2000) and recently it has also been suggested that CD46 forms a complex with a Notch-signaling pathway component Jagged-1 on the cell surface (Le Friec et al., 2012). Ligation of CD46 with complement cleavage products would then release Jagged-1 for activation of Notch-pathway. The four mammalian Notch proteins (Notch1-Notch4) are all expressed by $CD4^+$ T cells and there are two groups of ligands for them, the Delta-like ligands and the Jagged ligands. An in-depth review of the pathway is beyond the scope of this text. In brief, Notch ligands expressed by DCs affect the functional differentiation of $CD4^+$ T cells in many ways and the Delta-like ligands appear to favor differentiation to the T_H1 lineage, whereas the Jagged ligands promote T_H2 responses (Amsen et al., 2009).

Figure 7. T helper cell differentiation. The functional differentiation of activated $CD4^+$ T cells is directed by the local cytokine milieu. The crucial cytokines and signature transcription factors are presented in the figure. IL-12 drives the differentiation of T_H1 cells, which express T-bet and produce IFN-γ; IL-4 favors T_H2 differentiation, GATA-3 expression and production of more IL-4 by the T_H2 cells. TGF-β alone leads to differentiation of FoxP3 expressing T_{reg} cells, whereas TGF-β and IL-6 together induce the differentiation of Th17 cells expressing the transcription factor RORγt. The T_{FH} cells express the transcription factor Bcl-6 and produce IL-21. However, the independency of the T_{FH} lineage and the inducing cytokines (IL-6 and IL-21 have been suggested) are a matter of some controversy (see text for details).

naïve
CD4+
T cell
TFH
Bcl-6
IL-21
TGF-β
IL-6
Th17
RORγt
IL-17
Treg
FoxP3
TGF-β
IL-10
IL-12
IL-4
Th2
GATA-3
IL-4
Th1
T-bet
IFN-γ

The exact intracellular signaling mechanisms following CD46 ligation are unknown and its effects can be partly mediated by Notch-signaling, as described above. Nevertheless, it is clear that CD46 mediates signals of complement activation to T cells, functioning as a costimulator of TCR signaling and promoting the induction of the T_R1 phenotype. Intriguingly, the CD46-induced T_R1 cells also express mucosal homing markers, such as the integrin α4β7, suggesting a role for complement in regulating the balance between T effector responses and tolerance in the mucosal immune system (Alford et al., 2008; Kemper et al., 2003).

In the mouse CD46 expression is limited to the testis and another protein, Crry, carries out the complement regulatory MCP function of human CD46 (Li et al., 1993). Crry ligation has shown to be costimulatory to the murine T cells (Fernandez-Centeno et al., 2000) but analysis of its role in the induction of a regulatory phenotype in T cell has produced conflicting results (Alford et al., 2008; Ojeda et al., 2011).

6.4.6 The humoral immune response

Humoral immunity is based on the production of antigen-specific immunoglobulins or antibodies. They are soluble molecules that recognize and bind to their specific antigen with their Fab-sites, leaving a functional Fc part free for association with leukocyte Fc-receptors. Antibodies are produced by plasma cells differentiating from activated B cells. There are multiple subclasses of antibodies, which differ in their effector functions and the optimal clearance of a certain pathogen requires a particular set of antibodies (Schroeder and Cavacini, 2010).

IgG is the most abundant antibody class in human serum and it is further divided to subclasses of lowering concentration as follows: IgG1 binds readily to Fc receptors on phagocytes and other cells, inducing phagocytosis and strongly enhancing inflammatory effector mechanisms; IgG2 is mainly directed against polysaccharide antigens and has weaker binding affinity to Fc receptors; IgG3 is an efficient activator of the classical pathway of complement; IgG4 has negligible affinity to Fc receptors and it is associated with tolerogenic responses and repeated exposure to an antigen (Meiler et al., 2008; Nimmerjahn and Ravetch, 2008; Schroeder and Cavacini, 2010). Production of IgG1 and IgG3 is associated with a T_H1 type response, whereas IgG4 is associated with a T_H2 or regulatory T cell response (Aalberse et al., 2009; van de Veen et al., 2013).

IgM is produced early in the immune response and it can be produced to some extent without the help of T cells. The individual antigen binding sites of the IgM molecule have relatively low affinity for the antigen since the IgM producing B cells usually have not gone through the affinity maturation process (described below). The IgM molecule has a pentameric structure, which compensates for the lower affinity of the individual binding sites. Naïve B cells express IgM and IgD as their antigen receptors on the cell surface prior to the class switching process induced by T cells. IgD is not produced in soluble form and its role in the B cell function remains somewhat obscure (Schroeder and Cavacini, 2010).

The two remaining antibody classes are IgA and IgE. IgA is associated with mucosal surfaces and tolerance, and most of it is secreted as a dimer across the mucosal epithelia (Woof and Mestecky, 2005). IgE binds to mast cells and is associated with T_H2 type immunity and allergy (Erb, 2007).

6.4.6.1 The Role of T cells in immunoglobulin class switching

B cell affinity maturation and immunoglobulin class-switching are driven by helper T cells. During the follicular reaction, activated B cells continuously compete with each other for T cell help in the form of CD40L (Lane et al., 1992). Proliferating B cells accumulate random mutations in their antigen receptor genes, leading to changes in the affinity of the receptor to the antigen (Shlomchik and Weisel, 2012). Those B cells that gain a higher affinity to antigen are able to collect more antigen and present its fragments to helper T cells, getting more CD40 signaling and increasing proliferation. This leads to increased affinity of the produced antibodies but at the same time also the function of the antibodies is modified in a process called immunoglobulin class switching. Cytokines produced by the helper T cells make the B cells to change the constant region of the antibody molecule from the IgM initially expressed by naïve B cells to other subtypes. T_H1 cells usually induce production of IgG1 and IgG3 in humans and IgG2a and IgG3 in mice, whereas T_H2 cells induce switching to IgE production by the B cells (Murphy, 2012) (Fig.8).

6.4.6.2 Complement, antigen, and follicular dendritic cells

B cell activation is facilitated by complement in two ways. First, C3b degradation fragments bind to CR2 receptors on the B cell, delivering a costimulatory signal to the B cell upon antigen binding to BCR (Dempsey et al., 1996). Second, antigen bound to CR2 receptors on follicular dendritic cells (FDCs) enhances B cell affinity maturation, antibody class-switching and generation of B cell memory (Roozendaal and Carroll, 2007).

6.5 Regulation of adaptive immunity

6.5.1 Development of T cells and central tolerance

T cells develop in the thymus from lymphocyte precursors originating from the bone marrow. Upon arrival in the thymus the cells are committed to the T cell lineage in response to signals delivered by the thymic stromal cells (Zlotoff and Bhandoola, 2011) and are thereafter called thymocytes, until they leave the thymus as naïve T cells.

The function of T cells is based on the ability of the T cell population to recognize peptides from any foreign intruder but remaining tolerant to self. Each T cell has a single, MHC restricted functional specificity, but due to the extreme diversity of the TCR repertoire of the T cell population, peptides derived from almost any foreign protein can be recognized by some T cells (Arstila et al., 1999). These characteristics of the T cell population stem from the elaborate development of the thymocytes in the thymus.

Thymocyte development begins with the recombination of the TCR gene segments. The TCR β-locus is recombined first and the ensuing protein product is tested for its ability to pair with a surrogate α-chain, pTα, on the cell surface (Brady et al., 2010). The random recombination process creates great diversity in the produced β-chains but also a lot of wasting; most of the recombinations are nonproductive, leading to apoptosis of the cell. The γδ T cells, which are beyond the scope of this text, also diverge from the αβ-lineage at this point.

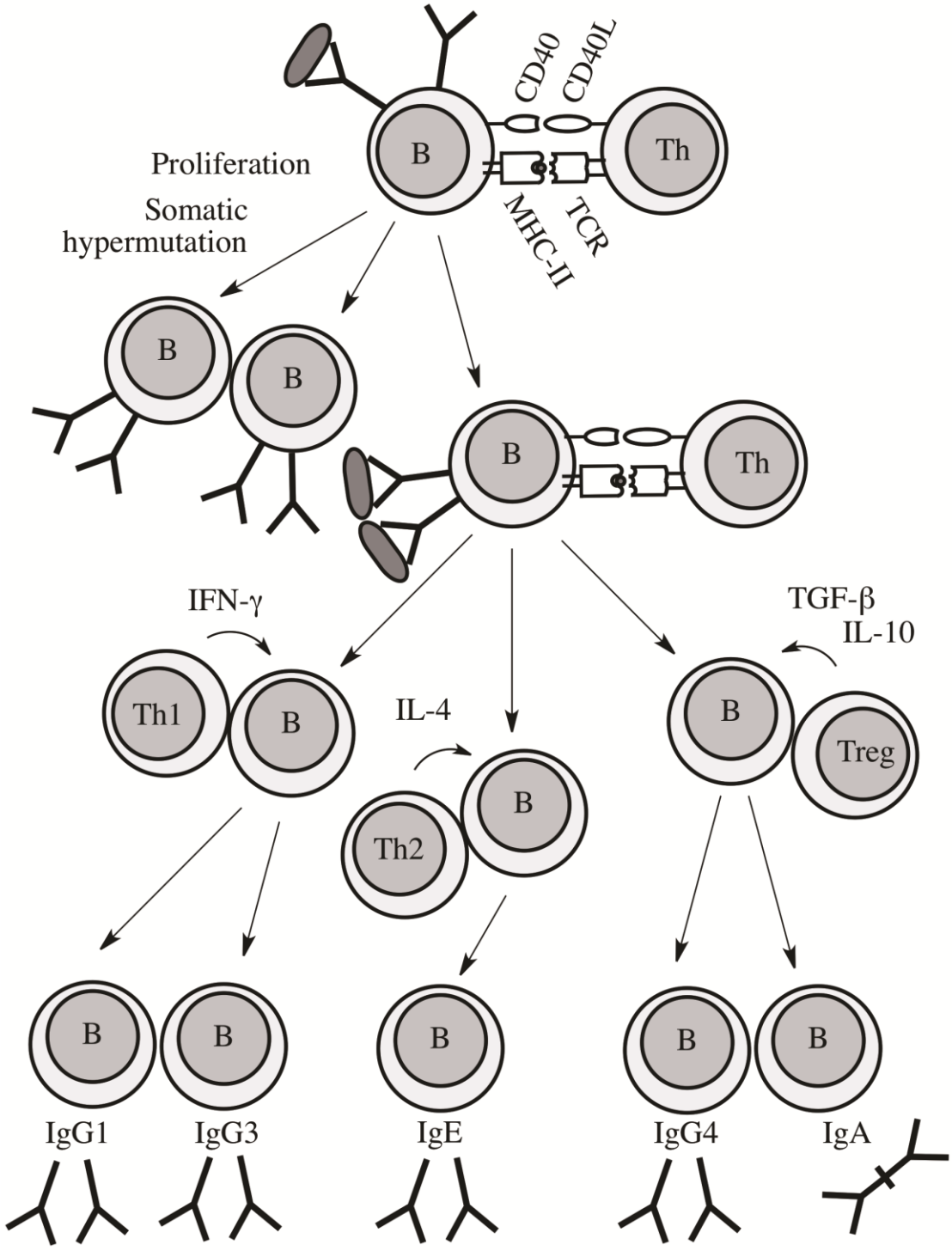


Figure 8. B cell affinity maturation and immunoglobulin class switching. B cell affinity maturation is driven by T helper cells. The proliferating B cells continuously compete for T cell help in the form of CD40 signaling and cytokines. Those B cells that manage to increase the affinity of their BCR for the antigen are able to capture and present more antigen to the T cells, leading to higher rate of proliferation of those B cells. Simultaneously, the cytokines produced by T cells drive the immunoglobulin class switching by B cells.

Thymocytes with successfully recombined TCR β-chains then proliferate for a few cycles and start to express CD4 and CD8 on their surface. At this point they are called double-positive (DP) thymocytes and they start to recombine the TCR α-locus (Carpenter and Bosselut, 2010). In the α-locus multiple successive recombinations can be made and the recombination process continues until the cell dies by neglect in the absence of signals or is positively selected by the binding of the αβ TCR to a self peptide-MHC complex. If the recognized MHC molecule is type I, then the thymocyte loses the expression of CD4 and becomes a CD8 single-positive (SP) cell, whereas recognition of an MHC II molecule leads to a CD4 SP phenotype. Positive selection also leads to the cessation of the recombination of the α-locus.

Positive selection is required for the testing of the functionality of the TCR, but the thymocytes are also tested for their potential autoreactivity in a process called negative selection (Klein et al., 2009). In addition to the proteins necessary for cell function, the thymic epithelial cells produce peripheral proteins for presentation by thymic DCs to the thymocytes (Derbinski et al., 2001). Thymocytes binding with high affinity to a self peptide-MHC complex are negatively selected and die by apoptosis, purging the T cell repertoire of highly autoreactive cells and leading to central tolerance.

However, there lies a thin line between purging autoreactivity and loss of functionality. Too vigorous elimination of all autoreactivity from the mature TCR repertoire would lead to decreased ability to respond to foreign peptides with minor differences from self peptides. Therefore, the TCR repertoire includes also autoreactive clones but these are usually kept in check by peripheral mechanisms. In addition, some autoreactive thymocytes are programmed to the T_{reg} lineage, suppressing other autoreactive T cells in the periphery (Sakaguchi et al., 2010).

Humans have a functional T cell population already at birth and the thymus continues to produce T cells after birth, peaking at puberty. After this the thymus begins to involute and the T cell population is thought to maintain its numbers by low-level proliferation in the periphery. In the mouse the T cell population is established during the first few days after birth; thymectomy at birth results in an almost complete lack of T cells in the mouse.

B cells develop in the bone marrow, where recognition of interstitial self antigen by the BCR leads to death of the B cell precursor. This negative selection will remove cells recognizing native extracellular self antigens from the B cell repertoire, but the cells are not tested for reactivity against intracellular antigens or fragmented self antigens (Hentges, 1994).

6.5.2 Peripheral tolerance

The central tolerance cannot prevent all autoimmunity, and peripheral measures are important in avoiding self-reactivity. Perhaps most importantly, naïve lymphocytes are isolated from tissue antigens by preventing their entry to tissues (Mueller, 2010). Instead, they recirculate continuously in the bloodstream and enter peripheral lymphoid tissues, searching for their cognate antigen presented by a DC. If the DC has not been activated by recognition of PAMPs, it will induce the T cells recognizing peptides carried on its MHC molecules to adopt a regulatory phenotype or to go to a permanent state of unresponsiveness called anergy (Hawiger et al., 2001).

Furthermore, self peptides presented by DCs activate thymus-derived T_{reg} to secrete suppressive cytokines in the peripheral lymph nodes (Sakaguchi et al., 2008), further bolstering the tolerogenic state (Fig.9). The importance of the peripheral tolerance is highlighted in the mucosal immune system, which is the topic of the next section.

6.6 Mucosal immunity

6.6.1 The original vertebrate immune system

It has been proposed that the adaptive immune system of the vertebrates has originally evolved in the mucosal tissues (Rodriguez et al., 2012) and it has been estimated that up to 70% of all lymphoid cells reside in the mucosal immune system (Pabst et al., 2008). The gastrointestinal tract is a particularly challenging environment for adaptive immunity due to the continuous exposure to commensal microbes and protein antigens derived from food. The intestinal immune system should remain tolerant to these innocuous antigens but still keep up the ability to respond rapidly to pathogens lurking among the abundant harmless antigens.

6.6.2 Organization of the intestinal immune system

The organized lymphoid tissues of the intestine include the Peyer's patches of the small intestine and isolated lymphoid follicles, which are found both in the small and the large intestine. The epithelium covering these structures contains highly specialized epithelial cells known as the M-cells, capable of transporting antigen from the gut lumen to the underlying lymphoid tissue (Mowat, 2003). The subepithelial dome of the Peyer's patch is rich in DCs, T cells and B cells and below it there are B cell follicles surrounded by T cell areas (Debard et al., 2001). The isolated lymphoid follicles are smaller than Peyer's patches and contain mainly B cells (Hamada et al., 2002)(Fig.10).

In addition to the organized lymphoid tissues, lymphoid cells are found scattered all over the intestinal tissue. DCs, macrophages, T cells, B cells and plasma cells are present in the connective tissue (lamina propria) underlying the intestinal epithelium and lymphocytes are found also in the epithelium. Lymphatic vessels collect lymph originating from the lamina propria, the Peyer's patches and the lymphoid follicles to the mesenteric lymph nodes, which connect the mucosal immune system to the systemic immunity (Wagner et al., 1998).

6.6.3 Mucosal tolerance

T cells are the key to tolerance also in the mucosal system. In the human system, the T cell population is already established at birth, so the central tolerance cannot purge the T cell repertoire from cells recognizing antigens derived from food or commensal microbes. Therefore, mucosal tolerance relies mainly on peripheral mechanisms, most importantly the induction of iT_{reg}s and T_R1 cells. Dendritic cells in the lamina propria are conditioned by TGF-β and retinoic acid (RA) to favor tolerance and once they travel to the mesenteric lymph nodes, they induce naïve T cells to differentiate to regulatory cells with mucosal homing markers integrin α4β7 (Alpan et al., 2001; Meyer et al., 2012). These T cells then travel to the lamina propria, where they are further supplied and replenished by signals from macrophages sampling the gut lumen for antigen (Hadis et al., 2011).

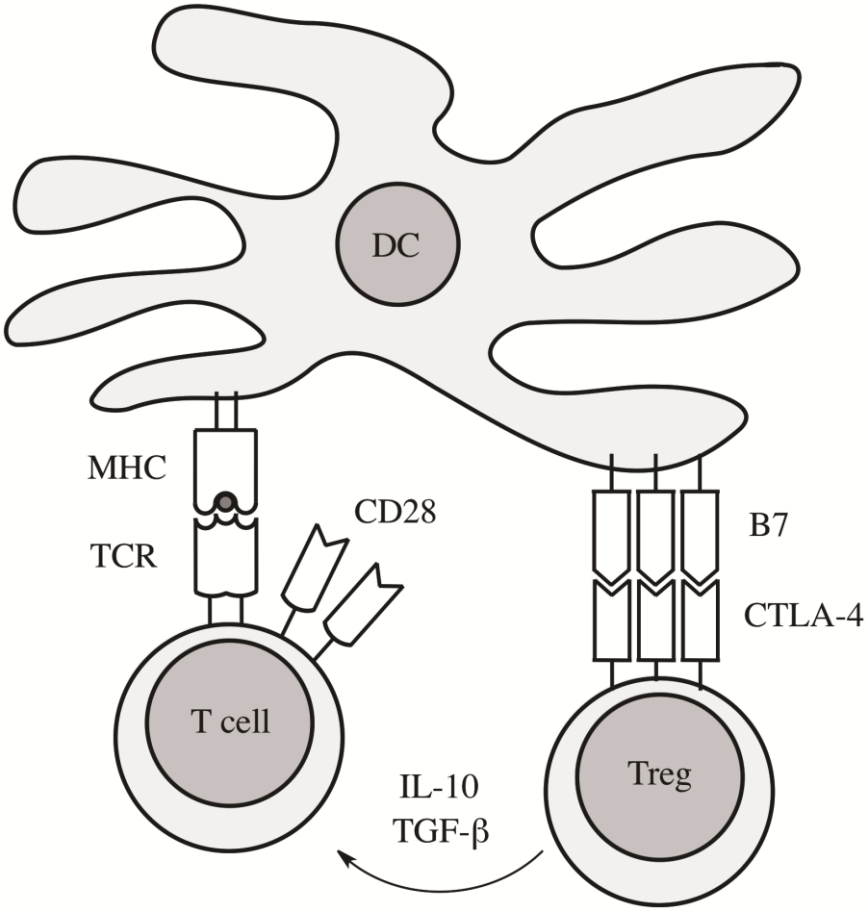


Figure 9. Inhibition of T cell activation by regulatory T cells. Treg cells inhibit the activation of autoreactive T cells by binding to B7 molecules with their CTLA-4 molecules, blocking the B7-CD28 costimulatory signaling and by secreting suppressive cytokines TGF-β and IL-10.

6.6.4 Oral tolerance

Oral tolerance is a special form of tolerance, leading to both intestinal and systemic unresponsiveness of the immune system to a protein antigen administered orally before systemic challenge. The phenomenon was first described in 1946 (Chase, 1946) and has thereafter been extensively studied in murine models (Mowat, 2003). It can be reached with repeated small doses of antigen leading to a regulatory T cell response, or with a single high dose of antigen, leading to deletion and apoptosis of the antigen-responding cells. In the mouse, oral tolerance manifests as reduced antigen-induced T cell proliferation and lower levels of antigen-specific immunoglobulins, IgE in particular (Weiner et al., 2011).

The underlying mechanism of the systemic tolerance in oral tolerance is not fully understood. It is possible that the protein antigen disseminates systemically via the bloodstream and ends up in peripheral lymph nodes or even thymus. In the absence of danger-signals, this would lead to tolerogenic presentation of the antigen by DCs and induction of regulatory T cells. Another possibility is that some of the mucosally induced regulatory T cells spread to other lymphoid tissues either after leaving the mesenteric lymph nodes or after a round of further proliferation in the lamina propria (Hadis et al., 2011).

The ability to induce tolerance by the oral route is a promising way for treating autoimmune diseases. Experimental mouse models of human disease have been successfully treated with oral antigen, leading to amelioration of the symptoms or even prevention of the onset of the disease (Mayer and Shao, 2004; Zhang et al., 1991).

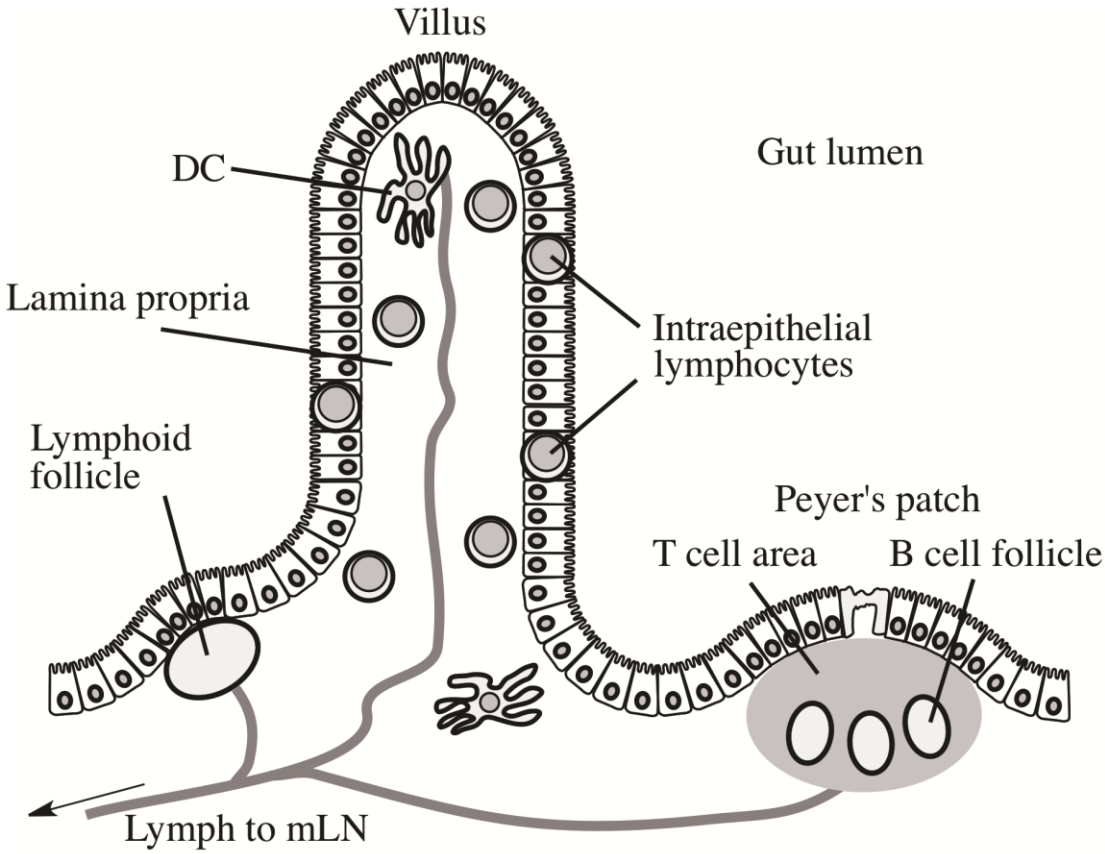
Only quite recently have experiments with human subjects shown that similar mechanisms of tolerance to orally administered protein antigen function in humans. Based on results from experimental work with the neoantigen KLH, it would appear that human oral tolerance centers more on T cell responses, whereas antibodies to oral antigen are still produced (Husby et al., 1994).

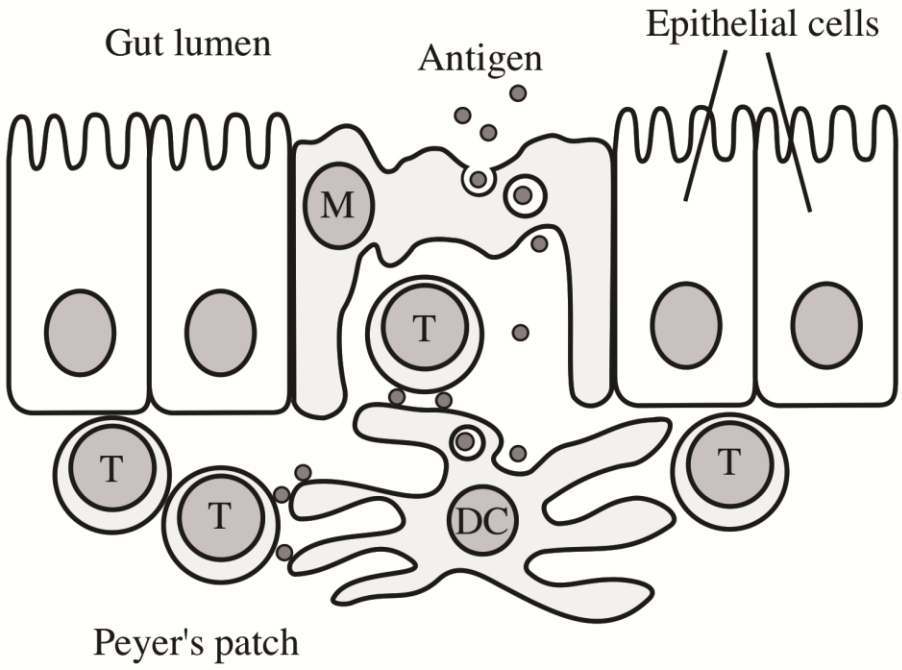
Unfortunately, attempts to treat human autoimmune disease with oral tolerization have failed. Oral administration of insulin to patients with type 1 diabetes has failed to induce clinically significant tolerance (Monetini et al., 2004; Pozzilli et al., 2000). One explanation to this failure is that murine models of autoimmune disease have usually been treated with oral antigen before or at the induction of disease, whereas in humans the treatment comes at a time when a full immune response has already developed. However, even a study, where relatives of diabetic patients were treated with oral insulin, failed to reduce the incidence of diabetes in this high-risk group (Hanninen and Harrison, 2004).

6.6.5 Tolerance to commensal organisms

Tolerance to the intestinal commensal organisms is based on both anatomical and immunological factors. The commensal bacteria do not possess virulence factors required for penetration of the mucosal epithelium and are normally confined to the gut lumen (Sansonetti, 2011). Their antigens are presented to the immune system in the Peyer's patches by the means of M cell mediated transportation. The DCs in the Peyer's patches present the commensal antigens to T cells, inducing differentiation to the T_{reg} lineage and expression of mucosal homing markers. B cells are programmed to produce IgA and to home to mucosal tissues (Kelsall, 2008). Since the produced IgA is mostly transported to the gut lumen and the responding lymphocytes are kept in the mucosal tissues, the systemic immunity ignores the commensal antigens in the steady state. Therefore, the systemic immunity remains capable of responding to the commensal organisms, in case they would spread into the circulation (Slack et al., 2009).

Figure 10. Organization of the mucosal immune system.
The organized lymphoid tissues of the intestine include Peyer's patches, isolated lymphoid follicles and the mesenteric lymph nodes (mLN, not shown). The Lymphoid follicles contain mainly B cells, whereas the Peyer's patches have a T cell area and B cell follicles. The subepithelial dome of the Peyer's patch (lower panel) is rich in DCs and the overlying epithelium contains M cells specialized for transport of luminal antigens. In addition to the organized lymphoid tissues, also the lamina propria contains DCs and numerous lymphocytes. Lymphocytes are also present in the epithelium.


Villus
DC
Gut lumen
Lamina propria
Intraepithelial
lymphocytes
Lymphoid
follicle
Peyer's patch
T cell area
B cell follicle
Lymph to mLN


Gut lumen
Antigen
Epithelial cells
M
T
T
T
T
DC
Peyer's patch

7 AIMS OF THE STUDY

In this study, we wanted to clarify the role of the complement system in shaping and regulating the adaptive immunity on the systemic level. The specific aims were:

(1) To study the role of complement system in the induction of an adaptive immune response to a protein antigen in a strongly proinflammatory context in a C3 deficient mouse model.

(2) To define the effect of complement on the induction of oral tolerance in the C3 deficient mouse model.

(3) To characterize the state of the mucosal tolerance in two Finnish C3 deficient human patients.

(4) To analyze the differentiation pattern of T helper cells and the adaptive immunity in response to vaccination with tetanus and diphtheria toxoids on the systemic level in a unique set of samples from eight C3 deficient human patients.

8 MATERIALS AND METHODS

8.1.1 Patient samples

Blood samples from the studied two Finnish C3 deficient patients were collected during their regular follow-up hospital admissions. Archived serum samples collected from these two patients and six other C3 deficient patients from different countries were used for measurements of serum levels of complement proteins and immunoglobulins. Blood and sera collected from healthy donors were used as controls. The controls were age-matched with the patients whenever possible, but due to the young age of the patients the matching was not perfect in every occasion.

8.1.2 Mice

The animals used in the study were C3-KO mice in the C57bl/6 background with wild-type C57bl/6 mice as controls. The knockout mouse strain has been generated by professor Marcela Pekna and colleagues, and is described in detail elsewhere (Bykov et al., 2006; Pekna et al., 1998). In brief, embryonic stem cells from the 129Ola mouse strain were transfected with a gene construct, where the exon 24 of the mouse C3 gene had been replaced with a neomycin resistance cassette (*neo*). Cells that had incorporated the construct in their genome were selected by culturing them in the presence of the antibiotic G418, which blocks protein synthesis in cells not expressing the *neo*. Surviving stem cells were injected into C57bl/6 blastocysts and implanted into the uterus of pseudopregnant C57bl/6 mice. The resulting chimeric offspring were bred with C57bl/6 mice and the heterozygous C3+/- mice in the next generation were intercrossed to produce homozygous C3-/- mice with a mixed 129Ola/C57bl/6 background. These mice were then backcrossed to the C57bl/6 genetic background for multiple generations.

8.1.3 Induction of oral tolerance in the experimental animals

To induce oral tolerance in the mouse model, the animals were given repeated intragastric doses of 1mg ovalbumin (OVA, Grade V, Sigma-Aldrich) in physiological saline or saline only. The intragastric gavage was repeated altogether eight times during a period of four weeks.

8.1.4 Immunization of the experimental animals

After a resting period of two weeks the OVA-fed animals were immunized with 50µg OVA in complete Freund's adjuvant and boosted with 50µg OVA in incomplete Freund's adjuvant. For immunization studies, the mice were immunized in a similar manner but with varying doses of 50µg, 100µg and 150µg of OVA and without prior oral administration of antigen.

8.1.5 Immunohistochemistry

Local mucosal T cell response in the OVA-fed mice was assessed by staining sections of the jejunum with anti-CD3 antibody. CD3 is part of TCR co-receptor complex and is expressed by all T cells. Tissue samples were embedded in Tissue Tek OCT-compound (Sakura Finetek) and frozen immediately in liquid nitrogen. Sections of the samples were fixed on slides in -20°C acetone and stained with a monoclonal rat anti-mouse antibody (Caltag) followed by a secondary antibody and peroxidase based detection of positively stained cells. The density of $CD3^+$ cells in the lamina propria

was counted by using an Olympus BX50 microscope (Olympus), and expressed as cells per square millimeter.

8.1.6 Cell isolation

Mouse spleens were collected aseptically and homogenized mechanically. Red blood cells were lysed by incubating the cells in RBC-lysing buffer (Sigma-Aldrich) or aqua. Cells from peripheral lymph nodes were released from the tissue mechanically.

Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples with Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation. The cells were either used directly for analysis by various methods described below or cryopreserved for later use with the CTL-Cryo™ ABC Media Kit (Cellular Technology Limited).

8.1.7 Cell culture

Primary cell culture with mouse splenocytes and human PBMCs was performed on 96- and 24-well tissue culture plates. Mouse splenocytes were stimulated with OVA (10μg/ml), plate-bound anti-mouse CD3 mAb (BD Biosciences), or anti-mouse Crry mAb (BD Biosciences) or both antibodies. Human cells were stimulated with plate-bound anti-human CD3 mAb (BD Biosciences) with or without soluble anti-human CD28 mAb (BD Biosciences) or with soluble tetanus toxoid (TT).

Antigen-specific stimulation of mouse splenocytes with OVA was performed in Dulbecco's modified Eagle medium (DMEM; in-house product, Haartman Institute) supplemented with 10% fetal calf serum (FCS; Invitrogen), 10 mM HEPES, 2 mM L-glutamine, 50μM 2-mercaptoethanol, 100 μg/ml streptomycin, and 100 U/ml penicillin (all from Sigma–Aldrich), whereas CD3/Crry stimulation was carried out in serum-free conditions with CTL-wash medium (Cellular Technology Limited) supplemented with glutamine, mercaptoethanol and antibiotics as above.

Medium for human PBMC stimulation assays was RPMI supplemented with 10% pooled inactivated human AB-serum. HEPES, glutamine, mercaptoethanol and antibiotics were included as above.

8.1.8 Proliferation assay

After 3 days (anti-CD3 stimulations) or 5 days (antigen-specific stimulations) of cell culture the wells were pulsed with tritiated thymidine (3.7×10^4 Bq per well; GE Healthcare). The samples were harvested 6 hours later with a Skatron harvester (Newington), and the amount of thymidine incorporated into the DNA of proliferating cells analyzed with a Microbeta liquid scintillation counter (Wallac) using OptiScint HiSafe scintillation fluid (PerkinElmer). The results are shown either as counts per minute (cpm) so that the background cpm value observed in unstimulated wells has been subtracted from the cpm of stimulated wells, or as a stimulation index, where the cpm value measured in the stimulated wells has been divided with the cpm of unstimulated wells.

8.1.9 Flow cytometry

The flow cytometry experiments presented in this study have been carried out with three different cytometers: the FACScan and the FACSAria (BD Biosciences) and the Cyan ADP (DAKO Cytomation). Analyses were carried out from mouse splenocytes

and human PBMCs both freshly after isolation and after the above-mentioned stimulations in cell culture.

The surface markers on the target cells were first stained with monoclonal antibodies conjugated to fluorescent molecules either directly or in some cases via the use of secondary antibodies or biotinylated primary antibodies followed by streptavidin with a fluorescent label. The cells were then permebilized to allow staining of intracellular antigens. For staining panels targeted on antigens residing in the cytosol or endoplasmic reticulum and intracellular vesicles, such as cytokines, the IC-fix buffer set (eBioscience) was used. For staining of nuclear antigens, such as transcription factors, the FoxP3 fixation and permeabilization kit (eBioscience) was used.

In mouse experiments the studied surface antigens were CD4 (T helper cells), CD8 (Cytotoxic T cells) and CD19 (B cells). Intracellular staining of murine splenocytes was carried out with anti-mouse T-bet (T_H1 cells), GATA-3 (T_H2 cells), FoxP3 (T_{reg} cells) and IL-17a (T_H17 cells). In some experiments, mouse splenocytes were labeled with CFSE (CellTrace CFSE Cell Proliferation Kit, Invitrogen) prior to stimulation to allow the identification of proliferating cells with the flow cytometer at the end of the cell culture.

For human PBMCs, monoclonal antibodies to CD4, CD8, integrin α4 and β7 (mucosally homing cells), CD69 (activated cells), CD45RO (activated/memory cells), CD25 (activated/regulatory cells) and CD127 were used for surface staining and intracellular staining for FoxP3, CTLA-4, IL-10, IFN-γ, IL-4, IL-17 and TGF-β1 were carried out in various combinations.

8.1.10 RNA isolation and cDNA synthesis

Mouse splenocytes, cells isolated from peripheral lymph nodes and tissue samples were lysed by using TriPure Isolation Reagent (Roche) and mechanical homogenization. Total RNA was then isolated from the solution with RNeasy MiniKit columns (Qiagen). First-strand cDNA was synthesized by using AMV-reverse transcriptase enzyme (Finnzymes) and oligo-dT-primer (Sigma-Aldrich).

8.1.11 Quantitative real-time PCR

Quantitative real-time PCR analysis was performed with the iCycler-IQ instrument (Bio-Rad Laboratories). Assays for mouse transcription factors T-bet, GATA-3, RORγt and FoxP3, mouse cytokines IL-4, IL-10, IL-12a (p35) and IFN-γ, and for the house-keeping gene HPRT were commercially available, whereas mouse TCR Cα was an assay-by-design product consisting of the primers 5'-CAA AGA GAC CAA CGC CAC CTA and 5'-CGG TCA ACG TGG CAT CAC, and probe 5'-6FAM-CCA GTT CAG ACG TTC CC-quencher. All assays were intron-spanning primer-probe assays purchased from Applied Biosystems (Foster City, CA). Relative expression levels were normalized against HPRT or TCR Cα expression, as indicated.

8.1.12 Mouse immunoglobulin and cytokine measurements

Mouse serum was collected at the end of the animal experiments and OVA-specific immunoglobulin subclasses were measured with enzyme linked immunosorbent assay (ELISA). Microtitre plates were coated over night with OVA (2μg/ml) and samples were then added on wells, diluted in PBS supplemented with 1% bovine serum albumin (BSA). Bound antibodies were detected with isotype-specific biotinylated

anti-mouse antibodies followed by streptavidin-horseradish peroxidase (HRP) (all from BD Biosciences) and peroxidase substrate (Kirkegaard & Perry Laboratories). The results are shown as optical density (OD) at 405 nm. Mouse IL-10 levels in cell-culture supernatants were measured with a commercial ELISA reagent set (Mouse IL-10 ELISA Ready-SET-Go; eBioscience) following manufacturer's instructions.

8.1.13 Microbiology

Strains of commensal microbes (*Saccharomyces cerevisiae*, *Bacteroides fragilis*, *Eschericia coli*, *Klebsiella oxytoca*, *Acinetobacter sp*., *Enterobacter cloacae*, *Corynebacterium sp.*, *Proteus mirabilis*, *Staphylococcus epidermidis* and *Streptococcus viridans*) were isolated from clinical samples or healthy volunteers, and grown in suitable broth to mid-log phase. Microbes were then washed twice and the concentration adjusted to 0,6 OD at 600 nm in phosphate buffered saline (PBS). This suspension was used for coating of a MaxiSorp microtitre plate (Nunc Thermo Fisher Scientific), which was let dry over night at +37°C before use for the measurement of human anti-commensal serum immunoglobulin levels.

8.1.14 Human anti-commensal immunoglobulin measurements

For human commensal microbe ELISA, sera were heat-inactivated at +56°C for 30 minutes prior to use. Serum samples from C3 deficient patients and healthy controls were titrated for optimal dilution in PBS. Microbe-coated plates were washed with PBS containing 0.05% Tween and diluted serum then incubated in the wells for 1 h at room temperature. After washing, bound IgG and IgA were detected by incubation with HRP-conjugated anti-human IgG or IgA (Jackson) 1:5000 in PBS. OPD substrate solution (Dako) supplemented with hydrogen peroxide was then added on wells and reaction stopped with 0.5 M H_2SO_4. The amount of bound antibody was measured as OD at 492 nm.

8.1.15 Vaccine responses and measurements of serum immunoglobulins

The levels of antigen specific immunoglobulin subclasses were measured in serum samples from C3 deficient patients (n=8) and healthy controls (n=38). Two vaccine antigens (tetanus toxoid and diphtheria toxoid) were used for these measurements. Microtitre plates regularly used for clinical measurements of tetanus and diphtheria toxoid responses were kindly provided by the HUSLAB immunology department. Sera diluted in PBS with 1% BSA were incubated over night in the wells followed by isotype specific mouse anti-human Ig antibodies. HRP-conjugated anti-mouse Ig antibody was then added, followed by OPD substrate. The results were measured as OD at 492 nm.

Levels of total immunoglobulins (IgM, IgA, IgG, IgG1, IgG2, IgG3, IgG4, IgE) in human serum samples and vaccine responses to serogroups of *Streptococcus pneumoniae* and to tetanus toxoid were measured following standard diagnostic ELISA procedures (HUSLAB and National Institute for Health and Welfare, Helsinki, Finland).

8.1.16 Serum cytokine measurements

Milliplex magnetic bead panel (Millipore) was used for the detection of cytokines with the Luminex xMAP system (Luminex) following the manufacturer's instructions. The beads coupled with mAbs to cytokines were sonicated, mixed, and diluted to bead diluents. The beads were then incubated with serum samples at 4°C

overnight, followed by incubation with detection antibodies for 1 hour, after which streptavidin-PE was added. The assay was analyzed with the Luminex 200 instrument (Luminex) using Luminex xPONENET software (version 3.1).

8.1.17 Western blotting

To determine the presence of human C3, serum samples and PBMC lysates were run in a 10% SDS-PAGE gel under reducing conditions. The proteins were then transferred to a nitrocellulose membrane, unspecific binding blocked with 5% milk and polyclonal rabbit anti-human C3c antibody (1:10 000; Dako) was then added and incubated at +4°C overnight. HRP–conjugated goat anti-rabbit antibody (1:10,000; Jackson ImmunoResearch Laboratories) was used as a secondary antibody.

For mouse cell culture supernatants, Goat anti-mouse C3 antibody (1:2000; Bethyl Laboratories) was used to detect mouse C3 in undiluted supernatants, followed by HRP donkey anti-goat (1:5000; Jackson ImmunoResearch Laboratories) secondary antibody. Electrochemiluminescence was then used to detect the bound antibodies in both murine and human samples.

8.1.18 Other complement measurements

The hemolytic activity of the classical pathway of complement (CH100Cl) was measured with an enzyme immunoassay, and the serum concentration of C3 was measured with immunoturbidimetry, both according to standard diagnostic procedures (HUSLAB).

8.1.19 Statistics

The data are shown as means ± standard deviations (SDs) or as individual values. The statistical analysis was performed with the SPSS program, versions 19 and 20 (SPSS Inc.). For comparison of means in two groups the two-tailed Student's t-test was used and for comparison of multiple groups the one-way ANOVA test with Tukey HSD *post-hoc* analysis was used. The paired samples t-test was used for analysis of the response of mouse splenocytes to Crry-stimulation. Correlations between variables were assessed with the Pearson's correlation coefficient. In all analyses $p < 0.05$ was considered as limit of statistical significance. In some instances variation between different animal experiments was eliminated by normalizing the data in relation to variable mean value in the control group of each individual experiment, as indicated.

In those instances, where the *n* in the patient group was two, no statistical tests were used and the data are shown as individual values compared to mean values in healthy controls. In addition, SD in the control group is indicated.

8.1.20 Ethical considerations

Written informed consent was obtained from the patients and healthy control subjects or their parents, or both, before sampling. The study plan considering the work with human subjects was accepted by the ethics committee of the Joint Municipal Authority of the Pirkanmaa Hospital District and by the ethics committee for pediatrics at the Helsinki University Central Hospital.

The number of experimental animals used and the animal study protocol was approved by the Laboratory Animal Board of the Southern Finland Regional State Administrative Agency.

9 RESULTS

9.1 Immune response in C3 deficient mice

We started our work by studying the adaptive immune response to immunization with ovalbumin (OVA) in a C3 deficient mouse strain. Due to the central position of C3 in the complement cascade, activation of the complement system is blocked almost completely in this mouse model. The immunization was carried out with Freund's complete adjuvant, which is a potent inducer of cell-mediated adaptive immune responses and T_H1 type immunity. Wild-type animals of the background strain (C57bl/6) were used as controls (I).

9.1.1 T cell proliferative response

After immunization, splenocytes were stimulated in vitro with OVA and a proliferation assay was used to measure the antigen-specific proliferative response of memory T cells. In mice immunized with 50 μg OVA a clear proliferative response was observed in the WT group, whereas C3-KO mice had a significantly decreased response. When higher doses of OVA (100 μg and 150 μg) were used for immunization, also C3-KO mice showed a clear proliferative response but it remained lower than the response in WT mice immunized with the same dose of antigen. These data indicated that antigen-specific stimulation of T cells in the absence of C3 led to a reduced proliferative response (I).

9.1.2 Functional differentiation of T helper cells

Proliferation of T cells in response to an antigen is important for the initiation of adaptive immune responses but the simultaneous functional differentiation of $CD4^+$ cells to different T helper cell subsets is even more crucial for the outcome of the response. To analyze the impact of complement system on the induction of Th subsets we cultured splenocytes from mice immunized with 50 μg OVA for two days in the presence of OVA and measured the expression of Th lineage determining transcription factors on the mRNA level by quantitative PCR. In C3-KO mice the expression of T_H1 associated T-bet was significantly lowered but the T_H2, T_H17 and T_{reg} markers did not differ significantly between the study groups.

Next we labeled the splenocytes with CFSE and stimulated them with OVA for five days. The stimulated cells were then stained for CD4 and the intracellular Th lineage markers, and the OVA-induced upregulation of these markers in the proliferating $CFSE^{low}$ $CD4^+$ cells was determined by comparing their expression to cells from the same donors cultured in the absence of OVA. This flow cytometric analysis showed that in WT splenocytes the stimulation induced a significant increase in the expression of T-bet, GATA-3 and FoxP3. In C3-KO splenocytes T-bet expression was not increased but the other lineage markers, including IL-17a, did show a significant positive response to antigen-specific stimulation. In conclusion, the upregulation of the T_H1 associated transcription factor T-bet was impaired in C3-KO mice compared to WT mice both at the mRNA and protein level.

To further analyze the basis of the impaired T_H1 induction in C3-KO mice, we measured the expression of the T_H1 inducing cytokines IL-12 and IFN-γ and the T_H2 inducing IL-4 in the axillary lymph nodes of mice immunized with 150 μg OVA. The qPCR analysis of the mRNA isolated from these collecting lymph nodes of the

immunization area showed that the cytokine milieu was less well suited for T_H1 induction in the C3-KO mice. Both IL-12 and IFN-γ had significantly higher expression in the WT mice compared to C3-KO mice, whereas IL-4 expression showed no difference between the mouse strains (I).

9.1.3 Antigen specific antibody response

B cells require T cell help for antibody production and for immunoglobulin class switching in particular. Cytokines secreted by Th cells responding to an antigen largely dictate the antibody subclasses produced by the simultaneously activated B cells and the plasma cells differentiating from them. Therefore, we expected that the observed disturbance in the functional differentiation of T_H1 cells in our C3-KO mice would be reflected on the subclass profile of the antibody response elicited by the immunization. To test this, we measured the amount of OVA-specific IgG subclasses and IgE in the sera of the immunized animals.

In the mice immunized with 50 μg of OVA the concentrations of OVA-specific IgG1, IgG2a and IgG3 were all significantly lower in the sera of the C3-KO mice as compared to the corresponding values measured from the WT mice. However, OVA-specific IgE levels did not differ between the groups. The highest immunization dose of 150 μg OVA, which elicited a clear proliferative response also in the C3-KO mice, induced comparable IgG1 and IgG2a responses in the two mouse groups but the level of the T_H1 associated IgG3 was significantly lower in the C3-KO mice also with this immunization dose. IgE levels remained similar between the groups also with the 150 μg immunization dose.

The deficiency in antigen-specific IgG subclass production in the C3-KO mice was not total, since all measured Ig subclasses were significantly elevated in the immunized WT and C3-KO groups compared to non-manipulated controls of the same mouse strain tested with the same assay. This suggests a defect in class switch regulation instead of a complete deficiency in B cell class switching to the antibody subclasses in question (I).

9.1.4 Correlation of proliferation with the antibody response

To evaluate the dynamics of the adaptive immune response induced by the immunization with OVA on the systemic level, we analyzed the correlation between the T cell proliferation induced by OVA stimulation in vitro and the levels of OVA-specific IgG subclasses and IgE in the sera of the immunized animals. Pearson's correlation coefficient was used to define the strength of correlation and the analysis was performed to a combined dataset of the three immunization doses (50, 100 and 150 μg OVA).

The correlation analysis revealed that in the WT mice, T cell proliferation had a strong positive correlation with the T_H1 associated IgG subclasses IgG2a and IgG3 (Pearson's correlation coefficient 0.436 and 0.744; $P < 0.02$ and $P < 0.001$, respectively), whereas the proliferative response in the C3-KO mice correlated with the T_H2 associated IgE (Pearson's correlation coefficient 0.456; $P < 0.02$). No other significant correlations between the proliferative response and the antibody isotypes were found.

Taken together, these data indicated that immunization with OVA in Complete Freund's adjuvant resulted in a T_H1 dominated T cell and antibody response in the WT mice. In the C3-KO mice an OVA-specific T cell and humoral response was also observed but the T cell response was attenuated and the immune response in general was deviated towards a T_H2 type response (I).

9.2 Oral tolerance in C3 deficient mice

9.2.1 T cell response after oral administration of antigen

We studied the impact of the complement system on the induction of oral tolerance in the C3 deficient mouse model with a classical experiment of oral antigen administration followed by immunization with the same antigen. The mice were given repeated intragastral gavages of OVA in saline or saline only and then immunized with 50 ug OVA in Complete Freund's adjuvant. At the end of the experiment, the distribution of the main lymphocyte populations in the spleen was analyzed with flow cytometry.

In WT mice fed with OVA prior to immunization (WT_{OVA} group) the $CD4^+$ fraction of lymphocytes had increased compared to WT mice fed with saline (WT_{ctrl} group), with a concomitant decrease in the $CD19^+$ fraction (B cells). The $CD8^+$ fraction remained unchanged. The distribution of these fractions did not differ significantly between the WT and C3-KO mouse strains and the differences between C3-KO mice fed with OVA ($C3_{OVA}$ group) and C3-KO mice fed with saline ($C3_{ctrl}$ group) did not reach statistical significance.

To test the induction of oral tolerance by the administration of oral antigen we then stimulated the splenocytes for five days with OVA in vitro and measured the proliferative response to the antigen stimulation. Splenocytes isolated from the WT_{ctrl} mice proliferated readily in response to OVA stimulation, whereas the proliferative response in WT_{OVA} splenocytes was clearly reduced, indicating successive induction of oral tolerance in the WT mice. On the contrary, the proliferative response in the $C3_{OVA}$ group did not differ significantly from the response in the $C3_{ctrl}$ group. However, in line with the previous findings (I), the proliferative response also in the $C3_{ctrl}$ group was low, preventing the drawing of definitive conclusions on the tolerization in the C3-KO mice.

Administration of OVA into the gastrointestinal tract of the experimental animals should dampen also the local mucosal response to the systemic immunization with OVA. We analyzed the local gut-associated response to OVA-immunization by measuring the density of $CD3^+$ cells in the wall of the jejunum. The saline-fed WT_{ctrl} and $C3_{ctrl}$ mice had similar numbers of $CD3^+$ cells in the jejunum, whereas the OVA-fed WT_{OVA} and $C3_{OVA}$ groups differed significantly from each other, with no decrease in the response in the $C3_{OVA}$ mice. These data suggested that in the absence of functional complement, the local T-cell response to immunization could not be prevented by prior mucosal exposure to OVA (II).

9.2.2 Inhibition of the antibody response by oral antigen

Attenuated T cell proliferative response is a classical sign of oral tolerance but also the humoral immune response is tolerized. To analyze the humoral response to immunization in the four experimental mouse groups, we measured the levels of

OVA-specific IgG1, IgG2a, IgG3 and IgE in the mouse sera at the end of the experiment. In the WT mice the oral administration had clearly induced oral tolerization of the antibody response. In WT_{OVA} mice all the measured immunoglobulin subclasses had significantly lower levels compared to WT_{ctrl} mice. In contrast, the OVA-feeding had failed to induce tolerance in the C3-KO mice. There were no statistically significant differences in the OVA-specific immunoglobulin subclasses between the $C3_{OVA}$ and $C3_{ctrl}$ groups.

The failure of the C3-KO mice to respond to oral antigen by inducing tolerance of the humoral response could not be explained by the generally weaker antibody response in this mouse strain, since the levels of OVA-specific antibodies of all the measured classes were significantly higher in all experimental groups compared to those seen in nonimmunized animals of the same mouse strain, indicating a broad OVA-specific B-cell response to immunization also in the C3-KO mice. Furthermore, the T_H2 associated IgE levels were similar in the saline-fed WT_{ctrl} and $C3_{ctrl}$ groups, but OVA feeding resulted in a significant decrease only in the WT mice (II).

9.2.3 Cytokine profile in vitro and in the mouse intestine

In the recent years, the modulation of T cell responses by direct binding of complement fragments, and C3 cleavage products in particular, to receptors expressed on T cell surface has been an area of active research. The most interesting result is the discovery that on human T cells, the ligation of the widely expressed complement regulator membrane cofactor protein (MCP/CD46) in conjunction with TCR stimulation induces a suppressive T_R1 phenotype in the activated T cells after an initial costimulatory effect (Cardone et al., 2010). The induced regulatory phenotype is characterized by production of IL-10 and, interestingly, also the expression of mucosal homing markers (Alford et al., 2008).

In the mouse, CD46 expression is limited to the testis and the MCP function is carried out by Crry, a membrane protein expressed also on T cells. Ligation of mouse Crry has been shown to mediate a costimulatory signal to T cells, but its possible regulatory function is largely unknown, with only two reports linking it to IL-10 production and regulatory T cell phenotype in a mouse model of arthritis (Banda et al., 2003; Ojeda et al., 2011).

We asked if the mouse membrane cofactor protein Crry had a role in the induction of intestinal tolerance. First we analyzed the costimulatory and immunoregulatory functions of Crry ligation by an anti-Crry mAb on WT mouse splenocytes. The experiments were performed in a serum-free cell culture to exclude possible confounding effects of the complement proteins present in normal serum-based culture medium. Furthermore, the possible production of C3 by the cultured cells themselves was excluded by a sensitive Western blot assay of the culture supernatants; no C3 was detected.

The costimulatory effect of the anti-Crry mAb was clear. The T cell proliferation induced by plate-bound anti-CD3 mAb was greatly enhanced when anti-Crry mAb was also present (stimulation index 11 in wells with anti-CD3 only, and 23 in wells with both mAb:s, n=4), showing that the ligation of Crry in this assay was functional, with a clear effect on intracellular signaling.

Next, we tested the consequences of Crry ligation in the absence of immunostimulatory signals by culturing the WT splenocytes with plate-bound anti-Crry mAb alone. This stimulation did not induce proliferation, but it changed the cytokine balance in the cells, leading to significantly enhanced expression of IL-10 mRNA and significantly reduced expression of IFN-γ mRNA, measured by qPCR. When the culture supernatants were analyzed for the presence of IL-10 with ELISA, the level of IL-10 was higher in the Crry stimulated wells compared to nonstimulated control wells, although the difference did not reach statistical significance. However, analysis of the anti-CD3 stimulated wells showed that when used in conjunction with TCR stimulation, the anti-Crry antibody clearly increased the production of IL-10, from 148 ± 64 pg/mL with anti-CD3 alone to 211 ± 102 pg/mL ($P < 0.02$).

Finally, we moved back to the four experimental mouse groups fed with OVA or saline and immunized with OVA, and analyzed the local cytokine balance in tissue samples from the jejunum with qPCR. At the time of sampling, Peyer's patches had been excluded from these tissue samples. The balance of expression of IL-10 and IFN-γ showed a significant shift towards the suppressive IL-10 in WT_{OVA} mice, whereas there was no difference between the $C3_{ctrl}$ and $C3_{OVA}$ groups. We also tested the balance between IL-10 and IL-17, another proinflammatory cytokine. Also this analysis indicated a shift in the cytokine balance away from the tolerogenic IL-10 and towards the proinflammatory IL-17 expression in $C3_{OVA}$ mice compared with that seen in WT_{OVA} mice.

In conclusion, the mouse experiments indicated a dual role for the complement system in the regulation of T cell responses. On the one hand, complement activation is a proinflammatory signal enhancing T cell proliferation and T_H1 differentiation. On the other, complement plays a role in the establishment of T cell tolerance. With this interesting finding in mind, we turned to study the interrelations of complement and T cells in the human system (II).

9.3 Identification of two C3 deficient patients

Patients with C3-deficiency usually display attenuated responses to immunization and their ability to produce a long-term antibody response has been suggested to be impaired. At the cellular level, the maturation of dendritic cells has been reported to be defective and the induction of T_R1 cells from $CD4^+$ T cells by simultaneous activation of CD3 and CD46 in the presence of IL-2 has been shown to be impaired. We identified two Finnish patients with permanent and total lack of serum C3.

9.3.1 Clinical characteristics of Patient 1

An 18 month old male patient (Patient 1) was referred to specialist consultation as a suspected case of primary immunodeficiency. He had suffered from prolonged pneumonia, which had, regardless of appropriate antimicrobial treatment, led to establishment of a pleural effusion and, ultimately, to pneumococcal sepsis (serogroup 6B). Upon examination of the immunological status he was found to have undetectable total complement hemolytic activity due to a total and permanent absence of the C3 protein in serum. Serum immunoglobulin levels, including IgG subclasses, were within the reference values, although IgG3 and IgG4 levels were low. Previously, he had suffered from otitis media once at the age of 10 months.

Four months later, at the age of 22 months, Patient 1 was hospitalized again due to pneumococcal sepsis (serogroup 35F). At this point prophylactic treatment with 20mg/kg amoxicillin once a day was started. Thereafter, he has had no further invasive infections (II).

9.3.2 Clinical characteristics of Patient 2

Due to family history, the index patient's younger male sibling (Patient 2) was also examined at the age of 11 months. He had had no preceding clinical symptoms. He was also found to have a total and permanent absence of the C3 protein. His IgG, IgA and IgM levels were normal, but IgG3 and IgG4 levels were abnormally low at the time of diagnosis. Prophylactic treatment with amoxicillin was started.

Patient 2 suffered from atopic dermatitis in infancy and at the age of 4 years he was examined due to nightly wheezing and coughing. A panel of prick-tests to common allergens revealed positive reactions to cat and dog dander. However, the wheezing eased off without specific treatment. For the atopic dermatitis local glucocorticoids have been used successfully.

Patient 2 was hospitalized once at the age of 18 months due to fever. Previously he had had rhinitis and coughing and upon clinical examination was found to have body temperature of 39.0°C, low total leukocyte count (2.9) and low CRP. Intravenous cefuroxime was started, followed by intravenous ceftriaxone, and the fever responded well to this treatment. However, CRP concentration in plasma remained low and blood culture revealed no causative agent. Later, at the age of 3 years, he had an episode of 3 days of fever up to 38.5°C with no focal symptoms. The fever eased off without specific treatment. At the age of 5 years he had an H1N1 influenza infection. No specific treatment nor hospitalization was needed and the infection cleared up well (II).

9.3.3 The parents

The non-consanguineous parents of the patients both had lowered levels of C3 in sera (0.5 g/L in the mother and 0.6 g/L in the father; reference value 0.71-1.41 g/L) but normal total hemolytic complement activity. Their clinical history showed no autoimmune morbidity or abnormal infectious diseases (II).

9.4 Mucosal tolerance in C3 deficient patients

9.4.1 Mucosally homing activated T cells

The results obtained from our experiments on oral tolerance in the C3 deficient mice opened an interesting view on the role of the complement system in the mucosal immune homeostasis. Therefore, we tested if the C3-deficient patients would also have signs of abnormal mucosal immunity. The available sample material was limited to blood samples drawn from the Finnish C3 deficient brothers when they were 8.5 and 7 years old. PBMCs were isolated from these samples and age-matched controls, and the expression of the mucosal homing markers integrin α4 and β7 was used to identify cells destined for the gut-associated lymphoid tissues. The activation status of $CD4^+$ and $CD8^+$ lymphocytes was analyzed by staining the cells for CD45RO and the early activation marker CD69.

The intestinally homing $\alpha4^+\beta7^+$ lymphocytes of the C3 deficient patients had much higher frequency of $CD69^+$ cells than the healthy control lymphocytes. This was true both for $CD4^+$ and $CD8^+$ cells, and the difference was most pronounced when compared to healthy children, as adult samples tended to have higher frequency of $CD69^+$ cells. The frequency of $CD45RO^+$ cells among the $\alpha4^+\beta7^+$ lymphocytes in C3 deficient patients was also higher than the average measured in the healthy control children, although most of the gut-homing lymphocytes were $CD45RO^+$ in the controls as well. Analysis of the $\alpha4^-\beta7^-$ T cells did not show differences in the CD69 and CD45RO expression, indicating that the observed changes were specific to T cells destined for the gut-associated lymphoid tissues (II).

9.4.2 Humoral immune response to commensal organisms

The mucosal immune system is continuously exposed to harmless foreign antigens derived from food proteins and commensal organisms and avoidance of wasteful and potentially harmful immune responses to them is obviously beneficial to the host. The animal models of experimental oral tolerance used by us and other immunologists reflect this tendency of the intestinal immune system to avoid productive immune responses to foreign antigens encountered in the steady state (Weiner et al., 2011). In humans, defects in mucosal tolerance have been linked to mishandling of antigens derived from the commensal flora residing in the gut (Israeli et al., 2005; Russell et al., 2009), leading to abnormal immune responses directed against them.

To analyze the pattern of reactivity to commensal organisms in the C3 deficient patients, we measured the serum levels of IgG and IgA specific to several species of commensal flora, especially those residing in the gut. The serum samples used for this analysis were taken from the patients when they were 2.5 years old. At this time the levels of total IgG and IgA in the patient sera were within the reference values.

Compared to age matched controls, the serum levels of IgG against most of the studied microbes of the commensal gut flora were elevated in the C3 deficient patients. Notably, this was also true for IgG specific for the fungal commensal *Saccharomyces cerevisiae*, which is used as a biomarker for disease activity in patients with inflammatory bowel disease (Russell et al., 2009).

Analysis of IgG levels against *Staphylococcus epidermidis*, which is associated with the skin, and *Streptococcus viridans*, which is associated with the oral cavity, showed no increase. For the most part, the levels of IgA specific for the commensal microbes were similar or slightly decreased in the C3 deficient patient sera compared to levels in healthy controls (II).

9.5 Vaccination responses in C3 deficient patients

9.5.1 Anti-pneumococcal response

The studied two Finnish C3 deficient patients have been vaccinated broadly and repeatedly to prevent infectious complications. Analysis of the antibody responses elicited by the vaccinations showed that most of the vaccine antigens have induced adequate responses in the patients. However, when the antibody response against serogroups of *Streptococcus pneumoniae* was measured from sera collected from the patients at the age of 2.5 years, patient 1 had a protective level of specific antibodies against only one of the studied seven serogroups, despite the multiple vaccinations

with a 7-valent conjugate vaccine (Prevenar, Pfizer; hereafter ‘pneumococcal vaccine’) and the clinical history of invasive pneumococcal infections. On the contrary, patient 2 displayed a relatively good serological response to the pneumococcal vaccination (II).

9.5.2 Response to vaccination with tetanus- and diphtheria toxoids

Our observations of the impaired T_H1 immune response in the C3 deficient mice and of the perturbed mucosal tolerance in the absence of C3 in both mice and human subjects made us to ask, whether the human C3 deficiency would affect the dynamics of the systemic immune responses in a similar manner as in the mouse. To broaden our analysis from the case study of the two Finnish C3 deficient patients, we collected archived samples from other C3 deficient patients and ended up having sera from altogether eight patients. Although the clinical records obtained from these patients were not always perfect, we used this unique set of samples to evaluate the balance of the Th immune responses in the absence of C3 by a meticulous analysis of the antibody response to tetanus toxoid (TT) and diphtheria toxoid (DT). The antigen-specific immunoglobulin subclasses reflect T cell immune responses due to the central role of Th cells in the stimulation of B cell responses and the instruction of immunoglobulin class-switching of the antigen specific B cells in particular.

We measured the levels of antibodies of the IgA and IgG classes specific for TT and DT in the patient and control sera. For TT, we also measured the specific IgE and total IgE levels in the sera. TT and DT specific IgG levels did not differ significantly between the study groups, showing that vaccination of the C3 deficient patients had produced a clearly measureable antibody response comparable to that observed in the control sera. Also antigen specific IgA levels were similar in the study groups for both of the studied antigens. However, TT specific IgE levels were significantly lower in the patient sera and five out of eight patients had no measureable TT specific IgE. Levels of total serum IgE did not differ significantly between patients and controls but the two Finnish patients had higher serum IgE compared to other C3 deficient patients.

Further analysis of the IgG subclasses IgG1, IgG2, IgG3 and IgG4 specific for TT and DT showed no difference between the study groups in the levels of antigen specific IgG1, whereas the other subclasses displayed clearly differing patterns between the groups. For both antigens, the T_H1 associated IgG3 was present at significantly higher levels in the patient sera, whereas the tolerance-associated IgG4, which was highly expressed in the control samples, was practically non-existent in the patient sera. Also antigen-specific IgG2 was present at significantly lower levels in the patient sera (III).

9.5.3 Dynamics of the PDT antibody response

From our index case, patient 1, we possessed multiple serum samples collected at various time points before and after vaccinations. This allowed us to further dissect the dynamics of the antibody response to the TT and DT antigens. At the age of 24 months, the patient received a booster PDT-vaccination. Serum collected at the day of vaccination showed that the previous PDT vaccinations given in the infancy had resulted in a clearly measureable TT-specific IgG3 response, whereas the serum levels of other IgG subclasses were relatively low. Four months prior to the PDT-booster, the patient had received a pneumococcal vaccine for the first time (boosted three weeks before the PDT-booster) and also a HiB-booster vaccination. The latter

contains TT as protein conjugate but, interestingly, this vaccination had not induced a measureable humoral response to the TT.

Two months after the PDT booster, the levels of TT-specific IgG, IgG1, IgG2 and IgG3 had clearly risen in response to the vaccination but there was no measurable specific IgG4 in the serum. At the age of 31.5 months (7.5 months after the PDT-booster), however, the TT-specific antibody levels had already fallen clearly.

Next, we measured the DT specific antibody response to the same PDT-booster. Also for DT, the previous vaccinations had induced a clear IgG3 response, whereas other DT-specific IgG subclasses were low in the serum at the age of 20 months. The two pneumococcal vaccinations, containing DT as the protein conjugate and given between the age of 20 and 24 months, had resulted in a DT-specific antibody response so that at the time of the PDT-booster all the DT-specific IgG subclasses were already on the rise. The PDT-booster induced a further rise in all DT-specific IgG subclasses.

Similar to the TT-response, and despite a further pneumococcal vaccine-booster, also the DT-specific antibody levels had started to fall 7.5 months after the PDT-booster vaccination, suggesting a failure in the ability of the C3-deficient immune system to maintain a long-term humoral response to these protein antigens.

The last sample collected at the age of 8.5 years (103 months) is the one included in the larger analysis of the IgG subclass distribution presented above, further underlining the tendency of the C3-deficient immune system to produce TT- and DT-specific IgG3 and no IgG4. In the years between, the patient had received multiple rounds of immunizations with PDT and other vaccines containing TT and DT as protein conjugates (III).

9.5.4 Serum cytokine profile

The cytokine milieu in the organized lymphoid tissues guides the proliferating lymphocytes to adopt distinct effector functions associated with the different Th lineages (T_H1, T_H2, T_H17 or T_{reg}). Cytokines are efficient at mediating signals locally in the lymph nodes and other tissues but they spread also systemically in the bloodstream and can influence the function of the immune system also in other parts of the body. Therefore, the analysis of cytokines present in the serum gives information on the overall balance of the immune system.

We measured the levels of a set of proinflammatory and suppressive cytokines in the C3 deficient patient sera and compared it to the levels found in control sera. In line with our previous results indicating a failure of tolerance in the C3 deficient system, the suppressive cytokines were generally expressed at lower levels in the patient sera and, furthermore, proinflammatory cytokines were expressed at higher levels. However, the individual variation was high and due to the small sample size only two cytokines, IL-12 and IL-21, both present at higher levels in the patient sera, displayed a statistically significant difference between the groups. As noted above, IL-12 is the most important inducer of T_H1 differentiation and IL-21 is a potent activator of B cell responses. IL-12 is mostly produced by DCs and macrophages, whereas IL-21 is produced by T cells, especially T_{FH} cells.

Although not statistically significant, the lover average level of the suppressive IL-10 in the patient sera and the higher average level of the proinflammatory IL-17A and IL-6 in the patient sera support the conclusion that the in the absence of C3 the human immune system is in a continuous proinflammatory status.

The analysis of correlations between the cytokines and the TT and DT specific antibody subclasses revealed a connection between the elevated IL-12 and IL-21 and the difference in the levels of IgG3 and IgG4 in the patient and control sera. Serum IL-12 levels had a statistically significant positive correlation with both TT- and DT-specific IgG3 levels and a statistically significant negative correlation with TT- and DT-specific IgG4 levels. Serum IL-21 levels correlated statistically significantly and positively with the TT-specific IgG3 levels and had a borderline positive correlation with DT-specific IgG3 levels. With IgG4, the IL-21 levels showed a statistically significant negative correlation for both TT- and DT-specific IgG4 (III).

Table 1. Summary of the results and comparison of the characteristics of C3 deficiency in human and mouse

	Human	Mouse
T cell proliferation	-	Decreased
T_H1 cytokines/transcription factors	Increased (serum)	Decreased (cell culture)
T_H1 associated serum IgG	Increased	Decreased
Serum IgE	Normal/increased	Normal/increased
Serum IgG4	Decreased	-
IL-10	Decreased (serum)	Decreased (cell culture)
Mucosal T cells	More mucosally homing activated T cells	More T cells in the lamina propria
Mucosal antibody response	More IgG against commensals	Failure of experimental oral tolerance

10 DISCUSSION

10.1 Immune response in C3 deficient mice

The first part of this thesis (I) focused on the immune response induced by immunization with OVA in C3 deficient mice. The adjuvant used was Complete Freund's adjuvant, which contains mycobacterial antigens and usually favors a T_H1 type response (Shibaki and Katz, 2002). Both C3-KO mice and the WT controls developed a clear OVA-specific immune response but several differences in the quantity and quality of the components of the immune response were seen between the mouse strains.

The splenocytes isolated from WT mice proliferated more in response to OVA compared to C3-KO splenocytes and the antigen-specific stimulation induced an increase in the expression of the T_H1 signature transcription factor T-bet in the WT but not in the C3-KO splenocytes. The profile of the OVA-specific serum antibodies showed lower levels of the T_H1 associated IgG2a and IgG3 in the C3-KO mice, whereas the T_H2 associated IgE levels were not affected by C3 deficiency. In addition, the C3-KO mice had higher levels of serum total IgE. Together, these observations pointed at a deficient induction of a T_H1 immune response and deviation to T_H2 type response in the C3-KO system. This conclusion was strengthened by the positive correlation between T cell proliferative response and IgG2a and IgG3 in the WT mice, whereas T cell proliferation correlated with IgE levels in the C3-KO mice. In other words, the antigen specific T cell response in the WT mice was associated with production of T_H1 associated immunoglobulins but with T_H2 associated immunoglobulins in the C3-KO mice. Moreover, the collecting lymph nodes of the immunization area had lower mRNA expression levels of the T_H1 inducing cytokines IL-12 and IFN-γ in the C3-KO mice, suggesting that alteration of the cytokine milieu at the site of the induction of the immune response was one of the causes for the T_H1 impairment (I).

The importance of complement in stimulating B cell responses has been known for a long time (Carroll and Isenman, 2012), and in our study setting the C3 deficiency clearly affected the humoral response to OVA-immunization. However, the parallel impairment of the T_H1 type T cell response and the T_H1 associated immunoglobulins indicated that the observed change in the humoral response in the C3-KO mice was at least partly secondary to the lack of T cell help (I).

Complement activation can affect T cell responses in several ways, including direct binding of complement factors to complement regulatory proteins with signaling capacity on the T cell surface (Kemper and Atkinson, 2007). The local production of C3 by the DC and the T cell itself during the DC-T cell signaling in the lymph node has been suggested to be important for the efficient induction of T helper cell responses and of T_H1 responses in particular (Cope et al., 2011; Peng et al., 2006). However, the study setting does not allow the separation of the effects of the local and systemic production of C3, and given the importance of complement for APC function in general, it is probable that the APCs, such as DCs, form the crucial link between complement and T cells in our experimental setting.

Antigen presentation and cytokine production are the central APC functions required for T cell activation. Both of these functions are modulated by complement activation products and are likely to be compromised in the C3 deficient system. The anaphylatoxins C3a and C5a are important in recruiting immune cells to the site of antigen exposure and in the general activation of the immune system. Opsonization of foreign particles by C3b promotes effective phagocytosis and this is further enhanced by C5a binding to its receptor on the APC. Therefore, the lack of functional complement will lead to deficient phagocyte migration and reduced antigen uptake, resulting in decreased antigen presentation to T cells in the C3-KO mice. This may have an impact on the Th balance, since previous studies have shown that antigen presentation at low concentration favors T_H2 type responses (Grakoui et al., 1999). In our study setting, increasing the amount of OVA used in the immunization elicited a measureable proliferative response also in the C3-KO mice, suggesting that a higher concentration of antigen can partly compensate for the impaired antigen presentation in the C3 deficient system.

In addition to the indirect effects of complement activation on antigen presentation, the anaphylatoxins C3a and especially C5a have more direct effects on the cytokine production of the APCs. Intracellular signaling from C3aR and C5aR synergizes with TLR signaling and modulates the expression of cytokines and the functional differentiation of Th cells (Drouin et al., 2002; Hawlisch et al., 2004). C5a signaling via C5aR and a more recently identified second receptor, C5L2, can either stimulate or downregulate the TLR induced IL-12 production by APCs (Hawlisch et al., 2005; Hawlisch et al., 2004; Karp et al., 2000), whereas C5aR deficient DCs have been shown to promote the differentiation of T_H17 and T_{reg} cells (Weaver et al., 2010). The adjuvant used in our immunization protocol contains antigens from killed mycobacteria, which activate multiple TLR pathways, the combined effect of which leads to the production of T_H1 inducing cytokines, such as IL-12. The lack of anaphylatoxins in the C3-KO mice may disturb this process, leading to the lower IL-12 production observed in our study setting. Interestingly, we did not see differences in the IL-17 or FoxP3 expression in our mouse model (I).

Taken together, the immunization experiments with the C3-KO mice (I) showed that functional complement is required for the normal differentiation of T helper cells in mice, and that the lack of complement activation impaired the T_H1 differentiation in particular. T_H1 type immunity is mainly targeted against intracellular pathogens, such as viruses, whereas complement is an extracellular defense system present in the serum and the interstitial fluid. Therefore, the dependency of T_H1 immune responses on complement activation may seem paradoxical. However, the intracellular pathogens are susceptible to complement attack during the extracellular phases of infection, and there are numerous examples of complement evasion strategies by intracellular pathogens, such as viruses. For example, HSV and HIV virions protect themselves from complement attack by coating their surface with hijacked host complement regulators (Lubinski et al., 2002; Speth et al., 2003). Also intracellular bacteria, such as Yersinia, use diverse methods for complement evasion, further highlighting the role of complement in the T_H1 dominated defense against intracellular infections (China et al., 1993). Interestingly, another common feature of the mentioned pathogens is their mucosal route of infection, suggesting a special role for complement in the mucosal immunity.

10.2 Complement in mucosal tolerance

To study the role of complement in the induction of oral tolerance, we used the same C3 deficient mouse model and immunization protocol as for the general studies on the systemic immune response. However, in this study setting the mice were given repeated doses of OVA or control saline solution into the gastrointestinal tract prior to the immunizations (II).

In the WT mice, oral antigen suppressed the subsequent antigen-specific adaptive immune response clearly. The proliferation response of splenocytes was dampened and the local T cell response in the gut attenuated. The levels of antigen-specific IgG subclasses and IgE were significantly lowered in the serum of the OVA-fed mice at the end of the experiment. On the contrary, oral antigen failed to suppress the systemic T cell response and the humoral response in the C3-KO mice. Furthermore, the local cytokine balance in the jejunum was shifted from the expression of tolerogenic IL-10 to the inflammatory IFN-γ and IL-17 in the C3-KO mice (II).

IL-10 has a central role in intestinal tolerance (Cong et al., 2002) and the simultaneous ligation of CD46 and CD3 on human $CD4^+$ T cells has previously been shown to induce IL-10 production, regulatory T_R1 phenotype and the expression of mucosal homing integrin α4β7 *in vitro* (Alford et al., 2008). The functional homolog of human CD46 in mice is Crry (Li et al., 1993), and we tested the response of WT splenocytes to Crry stimulation alone and combined with CD3 stimulation. Although previous work on the regulatory role of Crry in the murine system has provided conflicting results, in our hands the effect of Crry ligation on IL-10 production was clear: when simulated with anti-Crry mAb alone, the expression of IL-10 mRNA was significantly increased and the expression IFN-γ mRNA was significantly decreased in the splenocytes. When used simultaneously with anti-CD3 mAb, the Crry stimulation resulted in significantly increased IL-10 protein levels in the culture supernatants. These results suggested that Crry has an analogous role to human CD46 in promoting IL-10 production in mouse T cells (II).

T_R1 cells are crucial to the maintenance of peripheral tolerance and they are especially important in the regulation of the intestinal immunity (Cong et al., 2002). In contrast to the thymic nT_{reg} and inducible iT_{reg} cells, the T_R1 regulatory cells do not express FoxP3 and they probably do not represent a fixed lineage of T helper cells but rather a temporary functional phenotype dependent on external signals. Consistent with this and in line with the results from the immunization experiments, the samples collected from C3-KO mice and WT controls of the oral tolerance experiment did not differ significantly in the expression of FoxP3 and we did not see changes in FoxP3 expression after Crry stimulation *in vitro*.

A rare opportunity to broaden our analysis to the human system occurred with the diagnosis of two C3 deficient patients (II). The experimental setting was necessarily limited by the available sample material. We studied the expression of activation markers CD69 and CD45RO and the mucosal homing markers integrin α4β7 on T cells from peripheral blood to assess the activation status of T cells destined for the intestinal tissues and analyzed the levels of serum IgG and IgA specific for commensal microbes. The analysis revealed that the population of mucosally homing T cells included a higher frequency of activated cells in the C3 deficient patients compared to healthy controls. IgG levels against intestinal commensal organisms

were elevated in the patient sera and the IgA levels were similar or slightly lowered compared to healthy controls (II).

In inflammatory bowel diseases, a shift in the response to commensal organisms from local IgA production to IgG and productive immunity has been documented in earlier reports. In particular, the antibodies against the fungal commensal, *Saccharomyces cerevisiae*, which was also included in our analysis, have been used as a marker of the disease activity in these conditions (Israeli et al., 2005; Russell et al., 2009). The CD46-induced differentiation of T_R1 has been demonstrated to be impaired in C3 deficient patients (Ghannam et al., 2008), and our results suggested a shift in the balance of the mucosal immune system away from tolerance and towards productive immunity in the C3 deficient human subjects (II). Although the patients have not suffered from gastrointestinal symptoms, the tendency of the C3 deficient patients to develop autoimmune manifestations in the later age (Reis et al., 2006) could be associated with chronic stimulation of the immune system by mishandled commensal antigens.

10.3 Systemic immunity in C3 deficient patients

To assess the immune response elicited by parenteral antigen challenge in the C3 deficient patients we analyzed the profile of serum antibodies specific for TT and DT in samples collected from eight C3 deficient patients (III) and compared it to the corresponding values in a control group of 38 healthy Finnish school children aged 7-10 years (19 male and 19 female subjects). The vaccination history of the control subjects was not exactly known but according to the Finnish vaccination protocol almost every child is given a PDT vaccination at the age of 3, 5 and 12 months and boosted at the age of 4 years (Rapola, 2007).

The analysis of the antigen-specific IgG levels in the control subjects confirmed a successful immunization of all control subjects to both studied antigens. Some of the controls also displayed a TT specific IgE response (III).

The TT and DT specific IgG levels in the sera from C3 deficient patients did not differ significantly from the levels measured in control sera, indicating a successful immunization also in the patient group. However, the analysis of the IgG subclasses revealed a qualitative disparity between the patients and controls. The T_H1 associated IgG3 levels were significantly higher in the patient sera and the tolerance associated IgG4 levels were below detection limit in almost all samples, for both antigens. Also IgG2 levels were significantly lower in the patient samples, whereas IgG1 levels did not differ between the groups. TT specific IgE levels were low in the patient sera but the importance of this observation is somewhat questionable due to the low number of patients and the high prevalence of negative results also in the control subjects (III).

IgG4 production is associated with repeated antigen exposure, T_R1 responses and tolerance (Meiler et al., 2008; van der Neut Kolfschoten et al., 2007). Therefore, the lack of antigen-specific IgG4 response in the C3 deficient patients (III) is in line with our previous observations of impaired tolerance in the C3 deficient patients and C3-KO mice (II). However, the higher levels of the T_H1 associated antigen-specific IgG3 in the patient sera (III) contrasts sharply with our results of attenuated T_H1 immunity in the C3-KO mice (I). Of human IgG subclasses, IgG3 has the strongest potential for classical pathway complement activation. It can be speculated that higher IgG3 levels

could compensate for the C3 deficiency by inducing the activation of the early components of the classical pathway, resulting in production of the C4 cleavage products C4b, which is a weak opsonin, and C4a, which is a weak peptide mediator of inflammation. This hypothesis could be tested by measuring C4b deposition on bacterial cells in C3 deficient patient serum. However, a mechanistic explanation for such a compensatory process is difficult to imagine.

Next, we measured the serum levels of a set of cytokines in the patient and control sera (III). As mentioned above, cytokines have a central role in the signaling events leading to the functional differentiation of T cells and the immunoglobulin class switching by B cells. In this analysis the inflammatory cytokines IL-12 and IL-21 were present at significantly higher concentrations in the patient sera compared to healthy controls. IL-12 is produced by DCs and it drives the differentiation of the activated T helper cells to the T_H1 lineage (Yamane and Paul, 2013). T_{FH} cells produce IL-21 and it has been shown to drive the production of the IgG1 and IgG3 antibody subclasses by the B cells (Pene et al., 2004). The positive correlation of the IL-12 and IL-21 levels with the antigen specific IgG3 levels and the negative correlation with antigen specific IgG4 levels further highlighted the T_H1 deviation of the immune response on both the cytokine and antibody levels in the C3 deficient patients (III).

Complement activation is mainly an immunostimulatory event and complement cleavage products activate APCs via various pathways. However, the binding of the anaphylatoxins C3a and C5a to their receptors on the APC surface activate signaling cascades that share intracellular signaling factors with the TLR signaling pathways, leading to modulation of the TLR induced production of IL-12 (Hawlisch et al., 2004; Song, 2012). Interestingly, previous studies have indicated that the combination of TLR signaling and C5a receptor signaling can lead to either increased or decreased production of proinflammatory cytokines (Raby et al., 2011; Weaver et al., 2010; Zhang et al., 2007). Subtle differences in the regulation of the APC-T_H cell signaling between mice and men and the dissimilarity of the immunization conditions in our study settings probably explain why C3-KO mice displayed reduced IL-12 levels in the collecting lymph nodes after immunization and a generally impaired T_H1 response to immunization (I), whereas the C3 deficient human subjects had increased IL-12 levels in the serum and produced significantly higher levels of the T_H1 associated IgG3 in response to parenteral antigen challenge (III). The importance of CD46 signaling (discussed below) to tolerogenic T cell responses in the human system and the lack of systemic expression of CD46 in the mouse is probably one explanation to the observed differences between the species.

The modulation of the adaptive immune responses by complement is not limited to effects on APCs. In fact, the function of all cell types of the adaptive immunity, including the natural and inducible T_{reg} cells (Kwan et al., 2013; Strainic et al., 2013), can be modulated by complement, either directly or indirectly. Most importantly, the maintenance of normal memory B cell responses and long term antibody production is dependent on C3b-mediated retaining of antigen on FDC:s in B cell follicles and the CR2 on B cells plays an important role in lowering the B cell activation threshold (Carroll and Isenman, 2012; Dempsey et al., 1996). The lack of C3 probably leads to impaired FDC function, which most likely explains the abnormally quick decay of antigen-specific serum antibody levels in the C3 deficient patients observed in our

index case (III) and in previous reports (Ghannam et al., 2008). The impaired activation of B cells in the absence of CR2 signaling offers a plausible explanation for the general attenuation of the antibody response in C3 deficient patients.

Immunoglobulin class switching is strictly controlled by T helper cells, and, therefore, the observed changes in the antigen-specific IgG subclasses (III) suggest that there are changes in the functional differentiation of T helper cells in the C3 deficient patients. Direct binding of complement fragments on T cells can mediate either stimulatory or suppressive signals (Kemper and Atkinson, 2007), but in the light of the recent literature, the suppressive signals appear to dominate. Ligation of CD46 (MCP) on human T cells can mediate a costimulatory signal (Karsten and Kohl, 2010), but it can also lead to a T_R1 phenotype (Kemper et al., 2003). In addition, the initial costimulatory effect and IFN-γ production is converted to IL-10 production, when the follow-up time is extended (Cardone et al., 2010). Other complement receptors CD55 (DAF) and CD59, which blocks MAC formation, mediate suppressive signals to the T cell (Heeger et al., 2005; Longhi et al., 2006). It can be speculated that the lack of these suppressive signals in the T cell population of the C3 deficient patients could lead to impaired differentiation of T_R1 cells and lower production of IL-10. Switching to IgG4 requires IL-10 (Jeannin et al., 1998; van de Veen et al., 2013), and antigen specific IgG4 was unmeasureably low in almost all C3 patient samples, whereas the PDT vaccination had induced clear TT and DT specific IgG4 responses in most of the studied control subjects. Although not statistically significant, the lower level of IL-10 in the patient sera compared to controls supports this hypothesis (III). Further studies of isolated T cells from the C3 deficient patients are required to clarify this point.

In conclusion, the analysis of the immune response in the unique set of samples from eight C3 deficient patients further confirmed the importance of complement to the induction of T cell tolerance and for the T helper cell differentiation in general. Although the failure of tolerance was clear both in the C3-KO mouse model and the C3 deficient patients, the lack of functional complement had opposing results for the T_H1 immune response in mouse and human systems. In the mouse, T_H1 immunity was impaired, whereas in the human system it was strengthened in the absence of C3.

11 CONCLUDING REMARKS

The work presented in this thesis underlines the role of the complement system as one important factor in the regulation of the functional differentiation of the T helper cells and modulation of adaptive immunity in general. Earlier studies on complement deficient mouse models have demonstrated that T cell responses are attenuated in C3 deficient mouse models (Fang et al., 2007; Peng et al., 2006), and our results showed that on the systemic level, T_H1 immunity is impaired and the T_H2 response is favored in the C3-KO mouse model. A novel finding was the role of the complement system as a regulator of intestinal tolerance both in the mouse model and in human subjects. Recognizing this role of the complement system will have an impact on the treatment strategies for inflammatory bowel diseases and food allergies.

In the third publication we analyzed the systemic immune response in a unique set of eight C3 deficient patients. In addition to the failure of the mucosal tolerance presented in the second publication, the results indicated that in the human system, C3 deficiency leads also to a systemic impairment in tolerance.

The interdependency of the lymphocyte responses and the complement system may at first seem surprising. Based on the traditional view of the immune system, the innate and adaptive responses are separated both in space and time; the innate immunity is like a garrison in the tissue, delaying the invasion of the overwhelming pathogen army and waiting for the cavalry of the adaptive immunity to arrive and turn the tide of the battle. Numerous recent publications have obsoleted this view, highlighting the interplay of the innate and adaptive arms of the immune system. Also the phylogeny of the adaptive immunity indicates that it has been built on the foundations of the evolutionarily older innate immunity. In the context of our results indicating a role for complement system in the mucosal T cell tolerance, it is intriguing to notice that the intestinal tissues, where innocuous foreign material is abundant and complement activation can be assumed to be continuous, appear to be the place of origin of the evolution of the adaptive immunity.

In the course of evolution, the advent of the adaptive immunity coincides with the appearance of the jawed vertebrates. Compared to the invertebrates, the vertebrates have a slower growth rate and they reach reproductive maturity later, which makes them more vulnerable to infections. In addition, the ability to chew probably led to a rapid diversification of the diet, introducing a wider variety of foreign structures and pathogens to the gastrointestinal tract. Together, these changes necessitated a more complex immune system, especially in the mucosal tissues. The ontogeny of the thymus and the avian bursa of Fabricius, an organ specialized for B cell development, further support the mucosal association of the adaptive immunity; they both derive from pouches of the embryonic gut.

Although our results clearly define complement as an important factor in the regulation of the adaptive immune responses and immune tolerance in particular, it would be exaggeration to claim that complement alone decides between tolerance and productive immunity. However, the presence of various sets of complement receptors on all cells of the immune system, including the T and B lymphocytes and APCs, renders to the complement system an ability deliver such a versatile set of signals to

the adaptive immune system that it is difficult to predict the results of the signaling based on the *in vitro* study of isolated cell lines or single receptor proteins. The decision between immunological tolerance and the range of different forms of productive immunity must be based on the combination of signals from the TLRs and other PRRs, complement receptors and lymphocyte antigen receptors and the complex and partly overlapping intracellular signaling events following the external stimuli.

The results presented in this thesis contribute novel information on the collaboration of the innate and adaptive immunity on the systemic level, emphasizing the seamless interplay of the different arms of immunity in the mammalian immune homeostasis.

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13 REFERENCES

Aalberse, R.C., S.O. Stapel, J. Schuurman, and T. Rispens. Immunoglobulin G4: an odd antibody. *Clin Exp Allergy* 39:469-477. 2009.

Abdelsadik, A., and A. Trad. Toll-like receptors on the fork roads between innate and adaptive immunity. *Hum Immunol* 72:1188-1193. 2011.

Alford, S.K., G.D. Longmore, W.F. Stenson, and C. Kemper. CD46-induced immunomodulatory CD4+ T cells express the adhesion molecule and chemokine receptor pattern of intestinal T cells. *J Immunol* 181:2544-2555. 2008.

Alpan, O., G. Rudomen, and P. Matzinger. The role of dendritic cells, B cells, and M cells in gut-oriented immune responses. *J Immunol* 166:4843-4852. 2001.

Amsen, D., A. Antov, and R.A. Flavell. The different faces of Notch in T-helper-cell differentiation. *Nat Rev Immunol* 9:116-124. 2009.

Arstila, T.P., A. Casrouge, V. Baron, J. Even, J. Kanellopoulos, and P. Kourilsky. A direct estimate of the human alphabeta T cell receptor diversity. *Science* 286:958-961. 1999.

Astier, A., M.C. Trescol-Biemont, O. Azocar, B. Lamouille, and C. Rabourdin-Combe. Cutting edge: CD46, a new costimulatory molecule for T cells, that induces p120CBL and LAT phosphorylation. *J Immunol* 164:6091-6095. 2000.

Awasthi, A., and V.K. Kuchroo. Immunology. The yin and yang of follicular helper T cells. *Science* 325:953-955. 2009.

Banda, N.K., D.M. Kraus, M. Muggli, A. Bendele, V.M. Holers, and W.P. Arend. Prevention of collagen-induced arthritis in mice transgenic for the complement inhibitor complement receptor 1-related gene/protein y. *J Immunol* 171:2109-2115. 2003.

Basler, M., C.J. Kirk, and M. Groettrup. The immunoproteasome in antigen processing and other immunological functions. *Curr Opin Immunol* 25:74-80. 2013.

Bettelli, E., T. Korn, M. Oukka, and V.K. Kuchroo. Induction and effector functions of T(H)17 cells. *Nature* 453:1051-1057. 2008.

Bexborn, F., P.O. Andersson, H. Chen, B. Nilsson, and K.N. Ekdahl. The tick-over theory revisited: formation and regulation of the soluble alternative complement C3 convertase (C3(H2O)Bb). *Mol Immunol* 45:2370-2379. 2008.

Brady, B.L., N.C. Steinel, and C.H. Bassing. Antigen receptor allelic exclusion: an update and reappraisal. *J Immunol* 185:3801-3808. 2010.

Byersdorfer, C.A., and D.D. Chaplin. Visualization of early APC/T cell interactions in the mouse lung following intranasal challenge. *J Immunol* 167:6756-6764. 2001.

Bykov, I., S. Junnikkala, M. Pekna, K.O. Lindros, and S. Meri. Complement C3 contributes to ethanol-induced liver steatosis in mice. *Ann Med* 38:280-286. 2006.

Cardone, J., G. Le Friec, P. Vantourout, A. Roberts, A. Fuchs, I. Jackson, T. Suddason, G. Lord, J.P. Atkinson, A. Cope, A. Hayday, and C. Kemper. Complement regulator CD46 temporally regulates cytokine production by conventional and unconventional T cells. *Nat Immunol* 11:862-871. 2010.

Carpenter, A.C., and R. Bosselut. Decision checkpoints in the thymus. *Nat Immunol* 11:666-673. 2010.

Carroll, M.C., and D.E. Isenman. Regulation of humoral immunity by complement. *Immunity* 37:199-207. 2012.
Cassell, D.J., and R.H. Schwartz. A quantitative analysis of antigen-presenting cell function: activated B cells stimulate naive CD4 T cells but are inferior to dendritic cells in providing costimulation. *J Exp Med* 180:1829-1840. 1994.
Chang, M.D., E.R. Stanley, H. Khalili, O. Chisholm, and J.W. Pollard. Osteopetrotic (op/op) mice deficient in macrophages have the ability to mount a normal T-cell-dependent immune response. *Cell Immunol* 162:146-152. 1995.
Chase, M.W. Inhibition of experimental drug allergy by prior feeding of the sensitizing agent. *Proc Soc Exp Biol Med* 61:257-259. 1946.
China, B., M.P. Sory, B.T. N'Guyen, M. De Bruyere, and G.R. Cornelis. Role of the YadA protein in prevention of opsonization of Yersinia enterocolitica by C3b molecules. *Infect Immun* 61:3129-3136. 1993.
Choi, P.J., and T.J. Mitchison. Imaging burst kinetics and spatial coordination during serial killing by single natural killer cells. *Proc Natl Acad Sci U S A* 110:6488-6493. 2013.
Cong, Y., C.T. Weaver, A. Lazenby, and C.O. Elson. Bacterial-reactive T regulatory cells inhibit pathogenic immune responses to the enteric flora. *J Immunol* 169:6112-6119. 2002.
Cope, A., G. Le Friec, J. Cardone, and C. Kemper. The Th1 life cycle: molecular control of IFN-gamma to IL-10 switching. *Trends Immunol* 32:278-286. 2011.
Cunnion, K.M., H.M. Zhang, and M.M. Frank. Availability of complement bound to Staphylococcus aureus to interact with membrane complement receptors influences efficiency of phagocytosis. *Infect Immun* 71:656-662. 2003.
Debard, N., F. Sierro, J. Browning, and J.P. Kraehenbuhl. Effect of mature lymphocytes and lymphotoxin on the development of the follicle-associated epithelium and M cells in mouse Peyer's patches. *Gastroenterology* 120:1173-1182. 2001.
Dempsey, P.W., M.E. Allison, S. Akkaraju, C.C. Goodnow, and D.T. Fearon. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* 271:348-350. 1996.
Derbinski, J., A. Schulte, B. Kyewski, and L. Klein. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat Immunol* 2:1032-1039. 2001.
Drouin, S.M., D.B. Corry, T.J. Hollman, J. Kildsgaard, and R.A. Wetsel. Absence of the complement anaphylatoxin C3a receptor suppresses Th2 effector functions in a murine model of pulmonary allergy. *J Immunol* 169:5926-5933. 2002.
Engelmann, B., and S. Massberg. Thrombosis as an intravascular effector of innate immunity. *Nat Rev Immunol* 13:34-45. 2013.
Erb, K.J. Helminths, allergic disorders and IgE-mediated immune responses: where do we stand? *Eur J Immunol* 37:1170-1173. 2007.
Fang, C., T. Miwa, H. Shen, and W.C. Song. Complement-dependent enhancement of CD8+ T cell immunity to lymphocytic choriomeningitis virus infection in decay-accelerating factor-deficient mice. *J Immunol* 179:3178-3186. 2007.
Fazilleau, N., L. Mark, L.J. McHeyzer-Williams, and M.G. McHeyzer-Williams. Follicular helper T cells: lineage and location. *Immunity* 30:324-335. 2009.
Fernandez-Centeno, E., G. de Ojeda, J.M. Rojo, and P. Portoles. Crry/p65, a membrane complement regulatory protein, has costimulatory properties on mouse T cells. *J Immunol* 164:4533-4542. 2000.

Flierman, R., and M.R. Daha. The clearance of apoptotic cells by complement. *Immunobiology* 212:363-370. 2007.

Fuchs, A., J.P. Atkinson, V. Fremeaux-Bacchi, and C. Kemper. CD46-induced human Treg enhance B-cell responses. *Eur J Immunol* 39:3097-3109. 2009.

Ghannam, A., M. Pernollet, J.L. Fauquert, N. Monnier, D. Ponard, M.B. Villiers, J. Peguet-Navarro, A. Tridon, J. Lunardi, D. Gerlier, and C. Drouet. Human C3 deficiency associated with impairments in dendritic cell differentiation, memory B cells, and regulatory T cells. *J Immunol* 181:5158-5166. 2008.

Grakoui, A., D.L. Donermeyer, O. Kanagawa, K.M. Murphy, and P.M. Allen. TCR-independent pathways mediate the effects of antigen dose and altered peptide ligands on Th cell polarization. *J Immunol* 162:1923-1930. 1999.

Guermonprez, P., J. Valladeau, L. Zitvogel, C. Thery, and S. Amigorena. Antigen presentation and T cell stimulation by dendritic cells. *Annu Rev Immunol* 20:621-667. 2002.

Hadis, U., B. Wahl, O. Schulz, M. Hardtke-Wolenski, A. Schippers, N. Wagner, W. Muller, T. Sparwasser, R. Forster, and O. Pabst. Intestinal tolerance requires gut homing and expansion of FoxP3+ regulatory T cells in the lamina propria. *Immunity* 34:237-246. 2011.

Hamada, H., T. Hiroi, Y. Nishiyama, H. Takahashi, Y. Masunaga, S. Hachimura, S. Kaminogawa, H. Takahashi-Iwanaga, T. Iwanaga, H. Kiyono, H. Yamamoto, and H. Ishikawa. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J Immunol* 168:57-64. 2002.

Hanninen, A., and L.C. Harrison. Mucosal tolerance to prevent type 1 diabetes: can the outcome be improved in humans? *Rev Diabet Stud* 1:113-121. 2004.

Harboe, M., P. Garred, J.K. Lindstad, A. Pharo, F. Muller, G.L. Stahl, J.D. Lambris, and T.E. Mollnes. The role of properdin in zymosan- and Escherichia coli-induced complement activation. *J Immunol* 189:2606-2613. 2012.

Hawiger, D., K. Inaba, Y. Dorsett, M. Guo, K. Mahnke, M. Rivera, J.V. Ravetch, R.M. Steinman, and M.C. Nussenzweig. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J Exp Med* 194:769-779. 2001.

Hawlisch, H., Y. Belkaid, R. Baelder, D. Hildeman, C. Gerard, and J. Kohl. C5a negatively regulates toll-like receptor 4-induced immune responses. *Immunity* 22:415-426. 2005.

Hawlisch, H., M. Wills-Karp, C.L. Karp, and J. Kohl. The anaphylatoxins bridge innate and adaptive immune responses in allergic asthma. *Mol Immunol* 41:123-131. 2004.

Heeger, P.S., P.N. Lalli, F. Lin, A. Valujskikh, J. Liu, N. Muqim, Y. Xu, and M.E. Medof. Decay-accelerating factor modulates induction of T cell immunity. *J Exp Med* 201:1523-1530. 2005.

Hentges, F. B lymphocyte ontogeny and immunoglobulin production. *Clin Exp Immunol* 97 Suppl 1:3-9. 1994.

Horwitz, D.A., S.G. Zheng, and J.D. Gray. Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other. *Trends Immunol* 29:429-435. 2008.

Hulpke, S., and R. Tampe. The MHC I loading complex: a multitasking machinery in adaptive immunity. *Trends Biochem Sci* 38:412-420. 2013.

Husby, S., J. Mestecky, Z. Moldoveanu, S. Holland, and C.O. Elson. Oral tolerance in humans. T cell but not B cell tolerance after antigen feeding. *J Immunol* 152:4663-4670. 1994.

Ingulli, E., A. Mondino, A. Khoruts, and M.K. Jenkins. In vivo detection of dendritic cell antigen presentation to CD4(+) T cells. *J Exp Med* 185:2133-2141. 1997.
Inzana, T.J., M.F. Tosi, S.L. Kaplan, D.C. Anderson, E.O. Mason, Jr., and R.P. Williams. Effect of Haemophilus influenzae type b lipopolysaccharide on complement activation and polymorphonuclear leukocyte function. *Pediatr Res* 22:659-666. 1987.
Irvine, K.L., L.J. Hopkins, M. Gangloff, and C.E. Bryant. The molecular basis for recognition of bacterial ligands at equine TLR2, TLR1 and TLR6. *Vet Res* 44:50. 2013.
Israeli, E., I. Grotto, B. Gilburd, R.D. Balicer, E. Goldin, A. Wiik, and Y. Shoenfeld. Anti-Saccharomyces cerevisiae and antineutrophil cytoplasmic antibodies as predictors of inflammatory bowel disease. *Gut* 54:1232-1236. 2005.
Itano, A.A., and M.K. Jenkins. Antigen presentation to naive CD4 T cells in the lymph node. *Nat Immunol* 4:733-739. 2003.
Jeannin, P., S. Lecoanet, Y. Delneste, J.F. Gauchat, and J.Y. Bonnefoy. IgE versus IgG4 production can be differentially regulated by IL-10. *J Immunol* 160:3555-3561. 1998.
Josefowicz, S.Z., and A. Rudensky. Control of regulatory T cell lineage commitment and maintenance. *Immunity* 30:616-625. 2009.
Karp, C.L., A. Grupe, E. Schadt, S.L. Ewart, M. Keane-Moore, P.J. Cuomo, J. Kohl, L. Wahl, D. Kuperman, S. Germer, D. Aud, G. Peltz, and M. Wills-Karp. Identification of complement factor 5 as a susceptibility locus for experimental allergic asthma. *Nat Immunol* 1:221-226. 2000.
Karsten, C.M., and J. Kohl. The complement receptor CD46 tips the scales in T(H)1 self-control. *Nat Immunol* 11:775-777. 2010.
Kelsall, B. Recent progress in understanding the phenotype and function of intestinal dendritic cells and macrophages. *Mucosal Immunol* 1:460-469. 2008.
Kemper, C., and J.P. Atkinson. T-cell regulation: with complements from innate immunity. *Nat Rev Immunol* 7:9-18. 2007.
Kemper, C., A.C. Chan, J.M. Green, K.A. Brett, K.M. Murphy, and J.P. Atkinson. Activation of human CD4+ cells with CD3 and CD46 induces a T-regulatory cell 1 phenotype. *Nature* 421:388-392. 2003.
Kersse, K., M.J. Bertrand, M. Lamkanfi, and P. Vandenabeele. NOD-like receptors and the innate immune system: coping with danger, damage and death. *Cytokine Growth Factor Rev* 22:257-276. 2011.
Kim, S., K.B. Elkon, and X. Ma. Transcriptional suppression of interleukin-12 gene expression following phagocytosis of apoptotic cells. *Immunity* 21:643-653. 2004.
Klein, L., M. Hinterberger, G. Wirnsberger, and B. Kyewski. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol* 9:833-844. 2009.
Klint, C., B. Gullstrand, G. Sturfelt, and L. Truedsson. Binding of immune complexes to erythrocyte CR1 (CD35): difference in requirement of classical pathway components and indication of alternative pathway-mediated binding in C2-deficiency. *Scand J Immunol* 52:103-108. 2000.
Klos, A., A.J. Tenner, K.O. Johswich, R.R. Ager, E.S. Reis, and J. Kohl. The role of the anaphylatoxins in health and disease. *Mol Immunol* 46:2753-2766. 2009.
Kopf, M., B. Abel, A. Gallimore, M. Carroll, and M.F. Bachmann. Complement component C3 promotes T-cell priming and lung migration to control acute influenza virus infection. *Nat Med* 8:373-378. 2002.

Kroenke, M.A., T.J. Carlson, A.V. Andjelkovic, and B.M. Segal. IL-12- and IL-23-modulated T cells induce distinct types of EAE based on histology, CNS chemokine profile, and response to cytokine inhibition. *J Exp Med* 205:1535-1541. 2008.

Kwan, W.H., W. van der Touw, E. Paz-Artal, M.O. Li, and P.S. Heeger. Signaling through C5a receptor and C3a receptor diminishes function of murine natural regulatory T cells. *J Exp Med* 210:257-268. 2013.

Lambris, J.D., Z. Lao, T.J. Oglesby, J.P. Atkinson, C.E. Hack, and J.D. Becherer. Dissection of CR1, factor H, membrane cofactor protein, and factor B binding and functional sites in the third complement component. *J Immunol* 156:4821-4832. 1996.

Lane, P., A. Traunecker, S. Hubele, S. Inui, A. Lanzavecchia, and D. Gray. Activated human T cells express a ligand for the human B cell-associated antigen CD40 which participates in T cell-dependent activation of B lymphocytes. *Eur J Immunol* 22:2573-2578. 1992.

Lappalainen, J., J. Rintahaka, P.T. Kovanen, S. Matikainen, and K.K. Eklund. Intracellular RNA recognition pathway activates strong anti-viral response in human mast cells. *Clin Exp Immunol* 172:121-128. 2013.

Le Friec, G., D. Sheppard, P. Whiteman, C.M. Karsten, S.A. Shamoun, A. Laing, L. Bugeon, M.J. Dallman, T. Melchionna, C. Chillakuri, R.A. Smith, C. Drouet, L. Couzi, V. Fremeaux-Bacchi, J. Kohl, S.N. Waddington, J.M. McDonnell, A. Baker, P.A. Handford, S.M. Lea, and C. Kemper. The CD46-Jagged1 interaction is critical for human TH1 immunity. *Nat Immunol* 13:1213-1221. 2012.

Lehtimaki, S., and R. Lahesmaa. Regulatory T Cells Control Immune Responses through Their Non-Redundant Tissue Specific Features. *Front Immunol* 4:294. 2013.

Leon, F., N. Contractor, I. Fuss, T. Marth, E. Lahey, S. Iwaki, A. la Sala, V. Hoffmann, W. Strober, and B.L. Kelsall. Antibodies to complement receptor 3 treat established inflammation in murine models of colitis and a novel model of psoriasiform dermatitis. *J Immunol* 177:6974-6982. 2006.

Li, B., C. Sallee, M. Dehoff, S. Foley, H. Molina, and V.M. Holers. Mouse Crry/p65. Characterization of monoclonal antibodies and the tissue distribution of a functional homologue of human MCP and DAF. *J Immunol* 151:4295-4305. 1993.

Liston, A., and A.Y. Rudensky. Thymic development and peripheral homeostasis of regulatory T cells. *Curr Opin Immunol* 19:176-185. 2007.

Longhi, M.P., C.L. Harris, B.P. Morgan, and A. Gallimore. Holding T cells in check--a new role for complement regulators? *Trends Immunol* 27:102-108. 2006.

Lönnberg, T., Z. Chen, and R. Lahesmaa. From a gene-centric to whole-proteome view of differentiation of T helper cell subsets. *Brief Funct Genomics* 12:471-482. 2013.

Lubinski, J.M., M. Jiang, L. Hook, Y. Chang, C. Sarver, D. Mastellos, J.D. Lambris, G.H. Cohen, R.J. Eisenberg, and H.M. Friedman. Herpes simplex virus type 1 evades the effects of antibody and complement in vivo. *J Virol* 76:9232-9241. 2002.

Marsh, J.E., C.K. Farmer, S. Jurcevic, Y. Wang, M.C. Carroll, and S.H. Sacks. The allogeneic T and B cell response is strongly dependent on complement components C3 and C4. *Transplantation* 72:1310-1318. 2001.

Mayer, L., and L. Shao. Therapeutic potential of oral tolerance. *Nat Rev Immunol* 4:407-419. 2004.
Medzhitov, R., P. Preston-Hurlburt, and C.A. Janeway, Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388:394-397. 1997.
Meiler, F., S. Klunker, M. Zimmermann, C.A. Akdis, and M. Akdis. Distinct regulation of IgE, IgG4 and IgA by T regulatory cells and toll-like receptors. *Allergy* 63:1455-1463. 2008.
Meyer, T., R. Ullrich, and M. Zeitz. Oral tolerance induction in humans. *Exp Mol Pathol* 93:449-454. 2012.
Monetini, L., M.G. Cavallo, E. Sarugeri, F. Sentinelli, L. Stefanini, E. Bosi, R. Thorpe, and P. Pozzilli. Cytokine profile and insulin antibody IgG subclasses in patients with recent onset type 1 diabetes treated with oral insulin. *Diabetologia* 47:1795-1802. 2004.
Movat, H.Z. The role of histamine and other mediators in microvascular changes in acute inflammation. *Can J Physiol Pharmacol* 65:451-457. 1987.
Mowat, A.M. Anatomical basis of tolerance and immunity to intestinal antigens. *Nat Rev Immunol* 3:331-341. 2003.
Mueller, D.L. Mechanisms maintaining peripheral tolerance. *Nat Immunol* 11:21-27. 2010.
Mueller, S.N., T. Gebhardt, F.R. Carbone, and W.R. Heath. Memory T cell subsets, migration patterns, and tissue residence. *Annu Rev Immunol* 31:137-161. 2013.
Mukhopadhyay, S., A. Pluddemann, and S. Gordon. Macrophage pattern recognition receptors in immunity, homeostasis and self tolerance. *Adv Exp Med Biol* 653:1-14. 2009.
Muller-Eberhard, H.J. The killer molecule of complement. *J Invest Dermatol* 85:47s-52s. 1985.
Murphy, K.P. 2012. Janeway's immunobiology 8th edn. Garland Science, Taylor & Francis Group, New York.
Nimmerjahn, F., and J.V. Ravetch. Fcgamma receptors as regulators of immune responses. *Nat Rev Immunol* 8:34-47. 2008.
O'Shea, J.J., and W.E. Paul. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science* 327:1098-1102. 2010.
Ojeda, G., E. Pini, C. Eguiluz, M. Montes-Casado, F. Broere, W. van Eden, J.M. Rojo, and P. Portoles. Complement regulatory protein Crry/p65 costimulation expands natural treg cells with enhanced suppressive properties in proteoglycan-induced arthritis. *Arthritis Rheum* 63:1562-1572. 2011.
Pabst, R., M.W. Russell, and P. Brandtzaeg. Tissue distribution of lymphocytes and plasma cells and the role of the gut. *Trends Immunol* 29:206-208; author reply 209-210. 2008.
Pangburn, M.K., and N. Rawal. Structure and function of complement C5 convertase enzymes. *Biochem Soc Trans* 30:1006-1010. 2002.
Pangburn, M.K., R.D. Schreiber, and H.J. Muller-Eberhard. Formation of the initial C3 convertase of the alternative complement pathway. Acquisition of C3b-like activities by spontaneous hydrolysis of the putative thioester in native C3. *J Exp Med* 154:856-867. 1981.
Panter, M.S., A. Jain, R.M. Leonhardt, T. Ha, and P. Cresswell. Dynamics of major histocompatibility complex class I association with the human peptide-loading complex. *J Biol Chem* 287:31172-31184. 2012.

Park, B.S., D.H. Song, H.M. Kim, B.S. Choi, H. Lee, and J.O. Lee. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature* 458:1191-1195. 2009.

Pekna, M., M.A. Hietala, T. Rosklint, C. Betsholtz, and M. Pekny. Targeted disruption of the murine gene coding for the third complement component (C3). *Scand J Immunol* 47:25-29. 1998.

Pene, J., J.F. Gauchat, S. Lecart, E. Drouet, P. Guglielmi, V. Boulay, A. Delwail, D. Foster, J.C. Lecron, and H. Yssel. Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells. *J Immunol* 172:5154-5157. 2004.

Peng, Q., K. Li, H. Patel, S.H. Sacks, and W. Zhou. Dendritic cell synthesis of C3 is required for full T cell activation and development of a Th1 phenotype. *J Immunol* 176:3330-3341. 2006.

Petty, H.R., R.G. Worth, and R.F. Todd, 3rd. Interactions of integrins with their partner proteins in leukocyte membranes. *Immunol Res* 25:75-95. 2002.

Phan, T.G., J.A. Green, E.E. Gray, Y. Xu, and J.G. Cyster. Immune complex relay by subcapsular sinus macrophages and noncognate B cells drives antibody affinity maturation. *Nat Immunol* 10:786-793. 2009.

Pozzilli, P., D. Pitocco, N. Visalli, M.G. Cavallo, R. Buzzetti, A. Crino, S. Spera, C. Suraci, G. Multari, M. Cervoni, M.L. Manca Bitti, M.C. Matteoli, G. Marietti, F. Ferrazzoli, M.R. Cassone Faldetta, C. Giordano, M. Sbriglia, E. Sarugeri, and G. Ghirlanda. No effect of oral insulin on residual beta-cell function in recent-onset type I diabetes (the IMDIAB VII). IMDIAB Group. *Diabetologia* 43:1000-1004. 2000.

Qian, C., and X. Cao. Regulation of Toll-like receptor signaling pathways in innate immune responses. *Ann N Y Acad Sci* 1283:67-74. 2013.

Raby, A.C., B. Holst, J. Davies, C. Colmont, Y. Laumonnier, B. Coles, S. Shah, J. Hall, N. Topley, J. Kohl, B.P. Morgan, and M.O. Labeta. TLR activation enhances C5a-induced pro-inflammatory responses by negatively modulating the second C5a receptor, C5L2. *Eur J Immunol* 41:2741-2752. 2011.

Ram, S., L.A. Lewis, and P.A. Rice. Infections of people with complement deficiencies and patients who have undergone splenectomy. *Clin Microbiol Rev* 23:740-780. 2010.

Rapola, S. National immunization program in Finland. *Int J Circumpolar Health* 66:382-389. 2007.

Reis, E.S., D.A. Falcao, and L. Isaac. Clinical aspects and molecular basis of primary deficiencies of complement component C3 and its regulatory proteins factor I and factor H. *Scand J Immunol* 63:155-168. 2006.

Ricklin, D., G. Hajishengallis, K. Yang, and J.D. Lambris. Complement: a key system for immune surveillance and homeostasis. *Nat Immunol* 11:785-797. 2010.

Rodriguez, R.M., A. Lopez-Vazquez, and C. Lopez-Larrea. Immune systems evolution. *Adv Exp Med Biol* 739:237-251. 2012.

Roncarolo, M.G., S. Gregori, M. Battaglia, R. Bacchetta, K. Fleischhauer, and M.K. Levings. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev* 212:28-50. 2006.

Roozendaal, R., and M.C. Carroll. Complement receptors CD21 and CD35 in humoral immunity. *Immunol Rev* 219:157-166. 2007.

Russell, R.K., B. Ip, M.C. Aldhous, M. MacDougall, H.E. Drummond, I.D. Arnott, P.M. Gillett, P. McGrogan, L.T. Weaver, W.M. Bisset, G. Mahdi, D.C. Wilson, and J. Satsangi. Anti-Saccharomyces cerevisiae antibodies status is

associated with oral involvement and disease severity in Crohn disease. *J Pediatr Gastroenterol Nutr* 48:161-167. 2009.
Sakaguchi, S., M. Miyara, C.M. Costantino, and D.A. Hafler. FOXP3+ regulatory T cells in the human immune system. *Nat Rev Immunol* 10:490-500. 2010.
Sakaguchi, S., T. Yamaguchi, T. Nomura, and M. Ono. Regulatory T cells and immune tolerance. *Cell* 133:775-787. 2008.
Sansonetti, P.J. To be or not to be a pathogen: that is the mucosally relevant question. *Mucosal Immunol* 4:8-14. 2011.
Schroeder, H.W., Jr., and L. Cavacini. Structure and function of immunoglobulins. *J Allergy Clin Immunol* 125:S41-52. 2010.
Schulze, M.S., and K.W. Wucherpfennig. The mechanism of HLA-DM induced peptide exchange in the MHC class II antigen presentation pathway. *Curr Opin Immunol* 24:105-111. 2012.
Sheedy, F.J., A. Grebe, K.J. Rayner, P. Kalantari, B. Ramkhelawon, S.B. Carpenter, C.E. Becker, H.N. Ediriweera, A.E. Mullick, D.T. Golenbock, L.M. Stuart, E. Latz, K.A. Fitzgerald, and K.J. Moore. CD36 coordinates NLRP3 inflammasome activation by facilitating intracellular nucleation of soluble ligands into particulate ligands in sterile inflammation. *Nat Immunol* 14:812-820. 2013.
Shibaki, A., and S.I. Katz. Induction of skewed Th1/Th2 T-cell differentiation via subcutaneous immunization with Freund's adjuvant. *Exp Dermatol* 11:126-134. 2002.
Shlomchik, M.J., and F. Weisel. Germinal center selection and the development of memory B and plasma cells. *Immunol Rev* 247:52-63. 2012.
Slack, E., S. Hapfelmeier, B. Stecher, Y. Velykoredko, M. Stoel, M.A. Lawson, M.B. Geuking, B. Beutler, T.F. Tedder, W.D. Hardt, P. Bercik, E.F. Verdu, K.D. McCoy, and A.J. Macpherson. Innate and adaptive immunity cooperate flexibly to maintain host-microbiota mutualism. *Science* 325:617-620. 2009.
Song, W.C. Crosstalk between complement and toll-like receptors. *Toxicol Pathol* 40:174-182. 2012.
Speth, C., H. Stoiber, and M.P. Dierich. Complement in different stages of HIV infection and pathogenesis. *Int Arch Allergy Immunol* 130:247-257. 2003.
Steinman, R.M., and H. Hemmi. Dendritic cells: translating innate to adaptive immunity. *Curr Top Microbiol Immunol* 311:17-58. 2006.
Strainic, M.G., E.M. Shevach, F. An, F. Lin, and M.E. Medof. Absence of signaling into CD4(+) cells via C3aR and C5aR enables autoinductive TGF-beta1 signaling and induction of Foxp3(+) regulatory T cells. *Nat Immunol* 14:162-171. 2013.
Suresh, M., H. Molina, M.S. Salvato, D. Mastellos, J.D. Lambris, and M. Sandor. Complement component 3 is required for optimal expansion of CD8 T cells during a systemic viral infection. *J Immunol* 170:788-794. 2003.
Szabo, S.J., S.T. Kim, G.L. Costa, X. Zhang, C.G. Fathman, and L.H. Glimcher. A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100:655-669. 2000.
Tough, D.F., and J. Sprent. Bystander stimulation of T cells in vivo by cytokines. *Vet Immunol Immunopathol* 63:123-129. 1998.
van de Veen, W., B. Stanic, G. Yaman, M. Wawrzyniak, S. Sollner, D.G. Akdis, B. Ruckert, C.A. Akdis, and M. Akdis. IgG4 production is confined to human IL-10-producing regulatory B cells that suppress antigen-specific immune responses. *J Allergy Clin Immunol* 131:1204-1212. 2013.

van der Neut Kolfschoten, M., J. Schuurman, M. Losen, W.K. Bleeker, P. Martinez-Martinez, E. Vermeulen, T.H. den Bleker, L. Wiegman, T. Vink, L.A. Aarden, M.H. De Baets, J.G. van de Winkel, R.C. Aalberse, and P.W. Parren. Anti-inflammatory activity of human IgG4 antibodies by dynamic Fab arm exchange. *Science* 317:1554-1557. 2007.

van Lookeren Campagne, M., C. Wiesmann, and E.J. Brown. Macrophage complement receptors and pathogen clearance. *Cell Microbiol* 9:2095-2102. 2007.

Vyas, J.M., A.G. Van der Veen, and H.L. Ploegh. The known unknowns of antigen processing and presentation. *Nat Rev Immunol* 8:607-618. 2008.

Wagner, N., J. Lohler, T.F. Tedder, K. Rajewsky, W. Muller, and D.A. Steeber. L-selectin and beta7 integrin synergistically mediate lymphocyte migration to mesenteric lymph nodes. *Eur J Immunol* 28:3832-3839. 1998.

Wallis, R., D.A. Mitchell, R. Schmid, W.J. Schwaeble, and A.H. Keeble. Paths reunited: Initiation of the classical and lectin pathways of complement activation. *Immunobiology* 215:1-11. 2010.

Weaver, D.J., Jr., E.S. Reis, M.K. Pandey, G. Kohl, N. Harris, C. Gerard, and J. Kohl. C5a receptor-deficient dendritic cells promote induction of Treg and Th17 cells. *Eur J Immunol* 40:710-721. 2010.

Wei, T., J. Gong, F. Jamitzky, W.M. Heckl, R.W. Stark, and S.C. Rossle. Homology modeling of human Toll-like receptors TLR7, 8, and 9 ligand-binding domains. *Protein Sci* 18:1684-1691. 2009.

Weiner, H.L., A.P. da Cunha, F. Quintana, and H. Wu. Oral tolerance. *Immunol Rev* 241:241-259. 2011.

Wiesmann, C., K.J. Katschke, J. Yin, K.Y. Helmy, M. Steffek, W.J. Fairbrother, S.A. McCallum, L. Embuscado, L. DeForge, P.E. Hass, and M. van Lookeren Campagne. Structure of C3b in complex with CRIg gives insights into regulation of complement activation. *Nature* 444:217-220. 2006.

Witmer, M.D., and R.M. Steinman. The anatomy of peripheral lymphoid organs with emphasis on accessory cells: light-microscopic immunocytochemical studies of mouse spleen, lymph node, and Peyer's patch. *Am J Anat* 170:465-481. 1984.

Woof, J.M., and J. Mestecky. Mucosal immunoglobulins. *Immunol Rev* 206:64-82. 2005.

Yamane, H., and W.E. Paul. Cytokines of the gamma(c) family control CD4+ T cell differentiation and function. *Nat Immunol* 13:1037-1044. 2012.

Yamane, H., and W.E. Paul. Early signaling events that underlie fate decisions of naive CD4(+) T cells toward distinct T-helper cell subsets. *Immunol Rev* 252:12-23. 2013.

Zaffran, Y., O. Destaing, A. Roux, S. Ory, T. Nheu, P. Jurdic, C. Rabourdin-Combe, and A.L. Astier. CD46/CD3 costimulation induces morphological changes of human T cells and activation of Vav, Rac, and extracellular signal-regulated kinase mitogen-activated protein kinase. *J Immunol* 167:6780-6785. 2001.

Zaragoza, O., C.P. Taborda, and A. Casadevall. The efficacy of complement-mediated phagocytosis of Cryptococcus neoformans is dependent on the location of C3 in the polysaccharide capsule and involves both direct and indirect C3-mediated interactions. *Eur J Immunol* 33:1957-1967. 2003.

Zhang, X., Y. Kimura, C. Fang, L. Zhou, G. Sfyroera, J.D. Lambris, R.A. Wetsel, T. Miwa, and W.C. Song. Regulation of Toll-like receptor-mediated inflammatory response by complement in vivo. *Blood* 110:228-236. 2007.

Zhang, Z.J., L. Davidson, G. Eisenbarth, and H.L. Weiner. Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin. *Proc Natl Acad Sci U S A* 88:10252-10256. 1991.
Zheng, W., and R.A. Flavell. The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells. *Cell* 89:587-596. 1997.
Zhu, J., and W.E. Paul. Peripheral CD4+ T-cell differentiation regulated by networks of cytokines and transcription factors. *Immunol Rev* 238:247-262. 2010.
Zipfel, P.F., and C. Skerka. Complement regulators and inhibitory proteins. *Nat Rev Immunol* 9:729-740. 2009.
Zlotoff, D.A., and A. Bhandoola. Hematopoietic progenitor migration to the adult thymus. *Ann N Y Acad Sci* 1217:122-138. 2011.