Regulation of B cell Responses by the Innate Immune System

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Doctoral Thesis UPF - 2013

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THESIS ABSTRACT

Immunoglobulin (Ig) diversification is essential for the generation of protective immune responses against intruding microbes. B cells require cognate interaction with T cells to generate protective antibodies, although signals from innate cells play also a key role in helping B cell responses. We have characterized that B cells of the human upper respiratory mucosa undergo T cell dependent and T cell independent IgM-to-IgD class switching. In addition to enhancing mucosal immunity, crosslinking of IgD on innate cells including basophils stimulates release of immunoactivating, proinflammatory and antimicrobial mediators, defining IgD as an important immunomodulator. Signals capable of inducing IgM-to-IgD class switching include not only T cell dependent signals, but also BAFF and APRIL, two CD40L-related factors released by innate immune cells. We have studied the signalling pathway of the BAFF and APRIL receptor TACI, which binds the adaptor MyD88 and induces class switching by triggering NF-κβ activation. Overall, we elucidate novel cellular and signaling pathways required for the induction of Ig diversification and production.

RESUM DE LA TESI

La diversificació de les immunoglobulines és essencial per a la generació de respostes immunes protectores contra els patògens. Els limfòcits B requereixen interacció afí amb els limfòcits T per tal de generar anticossos protectors, tot i que les senyals de cèl.lules innates també juguen un paper clau en les respostes dels limfòcits B. Hem caracteritzat que en els limfòcits B de la mucosa del tracte respiratori superior humà tenen lloc respostes tant depenents com independents de limfòcits T que donen lloc al canvi d'isotip de IgM a IgD. A més a més de millorar la immunitat de la mucosa, la unió de la IgD a cèl.lules innates, incloent basòfils, estimula l'alliberament de molècules immunoactivadores i de mediadors proinflamatoris i antibiòtics. Senyals que poden induir el canvi d'isotip de IgM a IgD inclouen no només senyals dependents de limfòcits T, sinó també BAFF i APRIL, dos factors relacionats amb el CD40L produïts per les cèl.lules innates del sistema immunitari. Hem estudiat la via de senyalització de TACI, receptor de BAFF i APRIL, i hem observat que s'uneix l'adaptador MyD88 per induir el canvi d'isotip de les immunoglobulines mitjançant l'activació de NF-βκ. En general, hem dilucidat noves vies cel.lulars de senyalització necessàries per a la inducció de la diversificació i la producció de les immunoglobulines.

PREFACE

Immunoglobulin D (IgD) is not only co-expressed with IgM on the surface of the majority of mature B cells as a transmembrane antigen receptor, but is also found as a secreted Ig in blood and mucosal secretions. Cognate interaction between T and B lymphocytes of the adaptive immune system is essential for the production and diversification of antibodies against microbes and the establishment of long-term immunological memory. Growing evidence shows that - in addition to presenting antigens to T and B cells - cells of the innate immune system provide activating signals to B cells, as well as survival signals to antibody-secreting plasma cells. Understanding this crosstalk provides a deeper insight on the mechanisms that help B cell responses.

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INTRODUCTION AND AIMS

Chapter I

Introduction

1. THE IMMUNE RESPONSE

The immune system is comprised of a series of highly integrated physical structures and biological processes that protect our body against disease. This protection involves the recognition and clearance of foreign agents also referred to as antigens that range from toxic molecules to complex microorganisms, including viruses, bacteria, fungi and parasites. In addition, the immune system recognizes and eliminates abnormal host's cells capable of inducing inflammation or tumor growth (1). All these protective functions require a sophisticated system of "sensors" (receptors) that discriminate molecules associated with healthy cells from molecules associated with foreign, dead or abnormal cells (2-3). Any perturbation of this discriminatory capacity can lead to the onset of autoimmunity, inflammation or cancer (4). As exemplified by the epithelial surfaces that separate the sterile milieu of our body from the external environment, another remarkable property of our immune system relates to its ability to generate multiple layers of innate and adaptive defences that have increasing specificity (5).

Components of the **innate immune response** are the first to react to pathogen invasion and are found at sites of injury or infection within minutes. Granulocytes, monocytes, macrophages, dendritic cells (DCs) and natural killes (NK) cells promote innate immune responses by recognizing and responding to microbes through nonspecific germline-encoded pattern recognition receptors (PRRs), including the Toll-like receptors (TLRs) (3).

T and B cells promote **adaptive immune responses** by recognizing microbes through specific somatically recombined antigen receptors known as T cell receptor (TCR) and B cell receptor (BCR), respectively. A remarkable feature of T and B cell responses is that they generate long-lived memory cells (6). These cells patrol both circulation and lymphoid organs for

years after the initial infection and mount very rapid and robust secondary responses upon encountering antigen for a subsequent time.

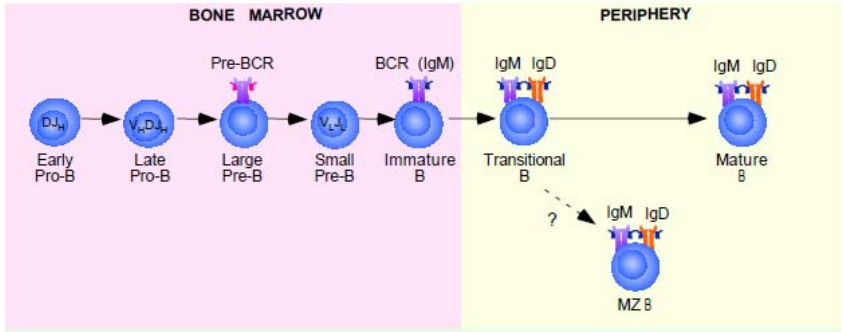
2. B CELL DEVELOPMENT

B cells are a subset of lymphocytes that play a key role in the humoral immune response. This response provides immune protection by producing immunogloblulin (Ig) molecules commonly known as antibodies that target specific antigenic determinants (or epitopes) associated with intruding microbes. B cells were originally designated from the bursa of Fabricius, which is the site of B cell maturation in birds and chickens (7). This nomenclature turned out to be very appropriate, because the bone marrow is the major site of B cell development in several mammalian species.

In humans, B cells develop from hematopoietic stem cells in the fetal liver during gestation and in the bone marrow after birth (8). Each newly generated B lymphocyte carries a transmembrane Ig receptor that is comprised of two identical Ig heavy chain (H) molecules and two identical Ig light chain (L) molecules, which can be either Igκ or Igλ (9). The IgH and IgL chain molecules include an antigen-binding variable region encoded by recombined V_HDJ_H and V_LJ_L genes, respectively. The V, D and J segments of these genes are organized in multiple families within the IgH and IgL loci and their assembly into in-frame V_HDJ_H and V_LJ_L exons requires an antigen-independent diversification process known as V(D)J recombination (10-11). B cell development proceeds through several intermediate stages (Fig. 1) that can be distinguished on the basis of the expression of various cell surface markers and ordered patterns of IgH and IgL chain gene rearrangement (12-13). Progression through these stages involves a cross-talk between B cell precursors and bone marrow stromal cells, which guide B cell development through the expression of both membrane-bound and soluble growth and

differentiation factors, including interleukin (IL)-7, fms-related tyrosine kinase 3 (FLT3) ligand and thymic stromal lymphopoietin (TSLP) (14). Several transcription factors regulate the early steps of B cell development, including the Pax5 protein (15).

Figure 1. Development of early B cells in the bone marrow. Early B cell development occurs in an antigen-independent manner through phenotypically distinct differentiation stages characterized by specific Ig gene rearrangement events. Pro-B cells emerge from common lymphoid precursor cells and include early pro-B (or pre-pro-B) and late pro-B (or pro-B) cells that undergo DJH and V-DJH gene rearrangements, respectively. These DNA recombination events require RAG1 and RAG2 endonucleases and are associated with D gene diversification by nucleotide addition via the enzyme terminal deoxyribonucleotidil transferase (TdT). Late pro-B cells differentiate to large pre-B cells that express a surface pre-BCR molecule composed of a VH-Cμ IgH chain and a pseudo (or surrogate) IgL chain formed by the Vpre-B and λ5 proteins. Large pre-B cells with in-frame VHDJH rearrangements undergo positive selection and further differentiate to small pre-B cells, which down-regulate surface pre-BCR expression, contain cytoplasmic VH-Cμ protein and undergo VL-JL recombination via RAG proteins. Subsequent assembly of two IgH and two IgL chains leads to the formation of a surface BCR in immature B cells. In the presence of strong BCR signals from self-antigens, immature B cells undergo negative selection by clonal deletion. However, some autoreactive immature B cells can be rescued through receptor editing, which requires a new up-regulation of RAG protein expression. Then, immature B cells differentiate to transitional B cells that express both surface IgM and IgD through alternative splicing of a long VHDJH-Cμ-Cδ mRNA. Transitional B cells exit the bone marrow and further differentiate to either mature naïve B cells or mature MZ B cells in secondary lymphoid organs. Naïve and MZ B cells initiate antibody production after differentiating to plasma cells in response T cell-dependent or T cell-independent antigens, respectively.



Proliferation		Yes	No	Yes	No	No	No	No	No
RAG expression		Yes	Yes	No	Yes	Low	Low	No	No
TdT expression		Yes	Yes	No	Yes	No	No	No	No
BCR rearrangement	IgH	$D\text{-}J_H$	$V_H\text{-}DJ_H$	V_HDJ_H	V_HDJ_H	V_HDJ_H	V_HDJ_H	V_HDJ_H	V_HDJ_H
	IgL	Germline	Germline	Germline	$V_L\text{-}J_L$	V_LJ_L (κ or λ)	V_LJ_L (κ or λ)	V_LJ_L (κ or λ)	V_LJ_L (κ or λ)
BCR expression		None	None	Pre-BCR	None	IgM	IgM, IgD	IgM, IgD	IgM, IgD
Phenotype		IL-7R$^+$ CD10$^+$ CD19$^-$ CD21$^-$ CD24$^+$ CD27$^-$ CD34$^+$ CD38$^+$ Ig$^-$	IL-7R$^+$ CD10$^+$ CD19$^+$ CD21$^-$ CD24$^+$ CD27$^-$ CD34$^+$ CD38$^+$ Ig$^-$	IL-7R$^+$ CD10$^+$ CD19$^+$ CD21$^-$ CD24$^+$ CD27$^-$ CD34$^-$ CD38$^+$ Ig$^-$ ($V_H\text{-}C\mu^+$, Vpre-B$^+\lambda5^+$)	IL-7R$^+$ CD10$^+$ CD19$^+$ CD21$^+$ CD24$^+$ CD27$^-$ CD34$^-$ CD38$^+$ Ig$^-$ ($V_H\text{-}C\mu^+$)	IL-7R$^-$ CD10$^+$ CD19$^+$ CD21$^+$ CD24$^+$ CD27$^-$ CD34$^-$ CD38$^+$ IgM$^+$	IL-7R$^-$ CD10$^+$ CD19$^+$ CD21$^+$ CD24$^+$ CD27$^-$ CD34$^-$ CD38$^+$ IgM^{hi}IgD^{lo}	IL-7R$^-$ CD10$^-$ CD19$^+$ CD21^{high} CD24$^+$ CD27$^+$ CD34$^-$ CD38$^-$ IgM$^+$IgD^{lo}	IL-7R$^-$ CD10$^-$ CD19$^+$ CD21$^+$ CD24$^-$ CD27$^-$ CD34$^-$ CD38$^-$ IgM^{lo}IgD^{hi}
Response to antigen		None	None	Positive selection	None	Negative selection or receptor editing	None	Yes	Yes

V(D)J Recombination

Pro-B cells initially recombine D and J segments in the IgH locus to form a DJ segment that subsequently recombines with a V segment to assemble a complete V_HDJ_H gene (Fig. 2). These recombination events randomly target individual members of multiple V, D and J gene families and require the induction of double-stranded DNA breaks in specific recombination signal sequences by a heterodimeric recombination activating gene (RAG) complex that includes RAG1 and RAG2 proteins (16-17). RAG1 and RAG2 recombinases are also required for the rearrangement of TCR gene segments and indeed deleterious mutations of RAG1 or RAG2 lead to severe combined immunodeficiency (SCID), which is characterized by the lack of both B and T cells (18). The enzyme terminal deoxynucleotidyl transferase (TdT) increases the diversity of Ig genes genes by adding N-nucleotides at the DJ junction of a recombined V_HDJ_H exon (19). Productive V_HDJ_H recombination stops the expression of RAG proteins (11), leading to the transcription of the V_HDJ_H gene together with the constant (C) heavy chain gene μ (Cμ) gene to form a complete IgH chain (20). Subsequent assembly of the IgH chain with surrogate invariant IgL proteins encoded by V-preB and λ5 gene segments is followed by transient surface expression of a pre-B cell receptor (pre-BCR) complex that also includes invariant Igα and Igβ subunits with signaling function (21). Signals emanating from the pre-BCR are a critical checkpoint for B cell development as they regulate the expansion of pre-B cells and their further differentiation into immature B cells (22). Immature B cells re-express RAG proteins to initiate the rearrangement of V and J segments from the IgL locus and form a complete Ig molecule (20). Of note, RAG proteins target the Igλ locus when the Igκ locus fails to generate an in-frame (or productive) VJ rearrangement. Assembly of the IgL chain with the IgH chain is followed by the expression of a fully competent IgM receptor that functions as a surface BCR (23).

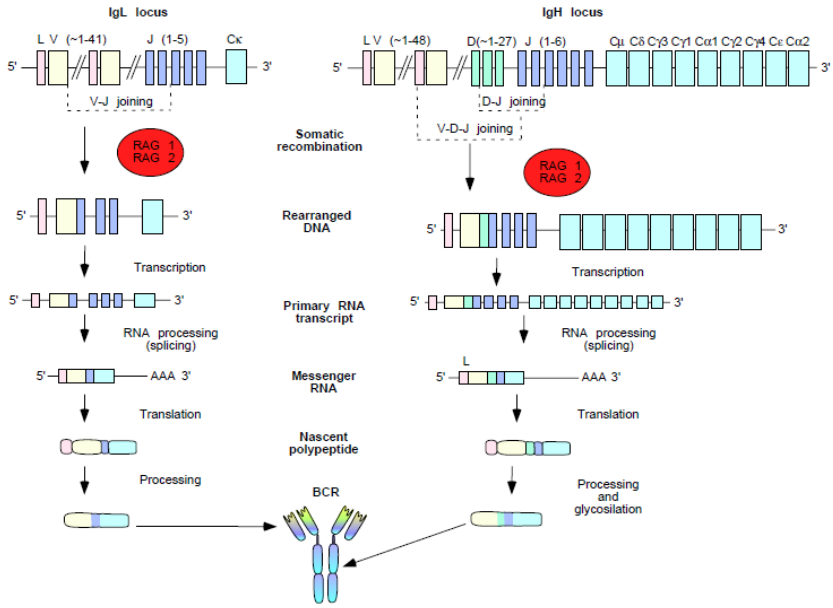


Figure 2. Mechanism underlying the generation of antibody molecules. The Ig loci include multiple cassettes of V, D and J (IgH locus) and V and J (IgL loci termed Igκ and Igλ) gene segments that undergo random rearrangement during B cell development in the bone marrow through a process involving RAG proteins. In the IgL loci, a VL gene segment rearranges with a JL gene segment to generate a VLJL exon. Transcription of VLJL and subsequent splicing of VLJL mRNA to a Cκ or Cλ mRNA generates a VLJL-CL mRNA that is polyadenilated and then translated into a mature IgL chain protein. In the IgH locus, similar recombination and transcription events lead to the formation of a VHDJH-CH (Cμ in the earliest stages of B cell differentiation) mRNA that undergoes splicing and polyadenilation to generate a mature IgH protein. In each Ig locus, V gene transcription initiates at the level of a leader (L) sequence positioned upstream of each V segment. Ultimately, two identical IgH and two identical IgL chain proteins are assembled to generate a membrane-bound heterotetrameric protein termed BCR.

Checkpoints and Transitional B Cells.

Following Ig gene rearrangement, immature B cells that express an autoreactive BCR undergo receptor editing, which involves the replacement of the V segment in the recombined V_LJ_L gene with an upstream V segment through a RAG-mediated reaction (24). An immature B cell unable to edit its autoreactive BCR is eliminated by apoptosis through a process referred to as

clonal deletion (25). After progressing through this tolerance checkpoint, immature B cells leave the bone marrow as transitional B cells that co-express IgM and IgD through a process of alternative splicing of a long RNA containing V_HDJ_H as well as Cμ and Cδ exons (26). Transitional B cells are typically short-lived and functionally immature and express high levels of the developmentally regulated molecules CD24 and CD38, but not the memory B cell molecule CD27 (27). These transitional B cells become fully mature naïve B cells expressing unmutated V(D)J genes, IgM, IgD and CD19, but not CD24, CD27 and CD38 after colonizing peripheral lymphoid organs. Here, stromal cells provide mandatory survival signals that support the maintenance of a highly diversified and fully functional repertoire of peripheral B cells (28-29).

3. B CELL ACTIVATION

Naïve B cells undergo additional differentiation steps after encountering native antigens in secondary lymphoid organs such as spleen, lymph nodes and mucosal associated lymphoid tissues (MALT). Naïve B cells recognize and internalize antigen by utilizing surface IgM and IgD receptors (30-31). Large antigens usually require the interaction of B cells with antigen-sampling macrophages and DCs located in the subcapsular sinus and paracortical areas of lymph nodes or in the MZ of the spleen, whereas small soluble antigens gain access to B cells after entering the follicle through a specialized transport system known as the follicular conduit network (32-36). This structure communicates with afferent lymphatic vessels and consists of collagen fiber cores surrounded by myofibroblast-like cells known as fibroblastic reticular cells (37-39). After receiving activating signals from the BCR, B cells down-regulate the expression of surface IgD, process the internalized antigen to form an MHC-II-peptide complex and migrate to the boundary of the follicle with the T cell zone, also known as the T-B border

(40-41). There, B cells present antigen to with T follicular helper (T_{FH}), a professional B cell helper subset of $CD4^+$ T cells that express CD40 ligand (CD40L) and cytokines such as IL-4, IL-10, IL-21 and interferon (IFN)-γ (42-43). These T_{FH} cells originate from naïve $CD4^+$ T cells recognizing antigen on IL-12-producing DCs (44-46). After establishing a cognate interaction with early T_{FH} cells, antigen-activated B cells differentiate along one of two alternative pathways (47). The extrafollicular pathway generates short-lived plasmablasts that secrete IgM (48), whereas the follicular pathway yields germinal center B cells known as centroblasts and centrocytes that mediate antibody diversification, selection and production (Fig. 3) (49-51).

Germinal Center Reaction

After increasing the expression of the chemokine receptor CXCR5, early T_{FH} cells and activated B cells move to the follicle in response to the chemokine CXCL13, a CXCR5 ligand produced by follicular DC (FDCs) (52-54). Early T_{FH} cells become germinal center T_{FH} cells by entering a Bcl6-dependent genetic program that is induced by signals present in the follicular environment (55-56). By expressing high levels of CD40L and IL-21, germinal center T_{FH} cells sustain the proliferation, differentiation, diversification and selection of centroblasts and centrocytes (57-59). Similar to T_{FH} cells, these germinal center B cells express Bcl-6, a transcription factor essential for the maintenance and development of the germinal center reaction (50). Centroblasts undergo extensive clonal expansion in the dark zone of the germinal center, thereby pushing naive IgM^+IgD^+ B cells to a peripheral area of the follicle called mantle zone (50). Centroblasts undergo clonal expansion as well as somatic hypermutation (SHM) and class switch recombination (CSR), two Ig-diversifying processes that are highly dependent on the enzyme activation-induced cytidine deamionase (AID) (60-63).

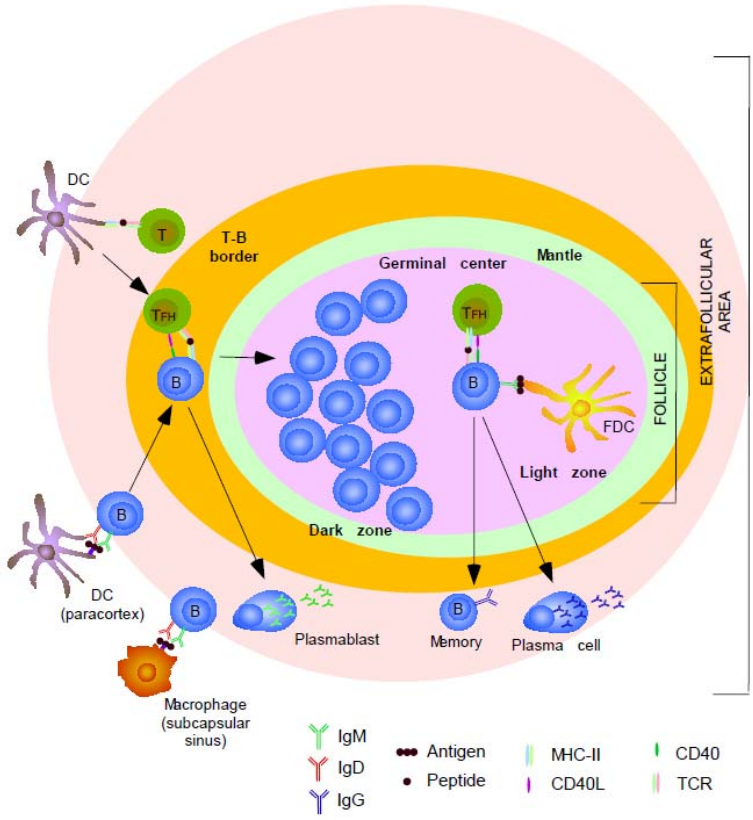


Figure 3. The germinal center reaction. Naïve B cells capture native antigen from subcapsular sinus macrophages and paracortical DCs through the BCR (both IgM and IgD molecules) and subsequently establish a cognate interaction with T_{FH} cells located at the boundary between the follicle and the extrafollicular area. After activation by T_{FH} cells via CD40L and cytokines such as IL-21, B cells enter either an extrafollicular pathway to become short-lived IgM-secreting plasmablasts or a follicular pathway to become germinal center centroblasts. In the dark zone of the germinal center, centroblasts undergo extensive proliferation, express AID and induce somatic hypermutation (SHM) and class switch recombination (CSR) from IgM to IgG, IgA or IgE (the figure only shows IgG). After exiting the cell cycle, centroblasts differentiate into centrocytes that interact with FDCs located in the light zone of the germinal center. FDCs expose immune complexes containing native antigen to the BCR and centrocytes with low-affinity for antigen die by apoptosis, whereas centrocytes with high affinity for antigen differentiate to long-lived memory B cells or plasma cells expressing high-affinity and class-switched antibodies. Memory B cells recirculate, whereas plasma cells migrate to the bone marrow.

Then, centroblasts exit the cell cycle to become smaller and non-dividing centrocytes that recognize native antigen trapped on the surface of FDCs using their newly hypermutated BCR (57-59, 64-65). After binding antigen, centrocytes establish a cognate interaction with germinal center T_{FH} cells (57-59, 66). This interaction mainly occurs in the light zone of the germinal center and contributes not only to the maintenance and selection of high-affinity and class-switched centrocytes, but also to the differentiation of centrocytes into memory B cells or plasma cells (65, 67-70). Centrocytes expressing a low-affinity BCR die by apoptosis and then are engulfed by resident phagocytes known as tingible body macrophages (42, 50).

Centroblasts and centrocytes express IgM or IgG or IgA together with CD19, CD27, CD38 and CD10, but not IgD and CD24. Of note centroblasts also express CD77, whereas centrocytes do not (71-72). Furthermore, centroblasts and centrocytes contain highly mutated V(D)J genes, express the germinal center-associated transcription factor Bcl-6, and contain molecular footprints of ongoing CSR and SHM, including strong AID expression (50). Unlike naïve B cells from the follicular mantle, centroblasts and centrocytes lack the intracellular anti-apoptotic factor Bcl-2 and instead express intracellular Bcl-2 family members with pro-apoptotic activity (71, 73-74). This feature renders germinal center B cells highly susceptible to apoptosis, which allows their elimination in the absence of engagement of BCR by high-affinity antigens. T_{FH} cells expressing FasL further increase the elimination of low-affinity germinal center B cells (75-76). Indeed, germinal center B cells up-regulate the expression of the death-inducing receptor Fas upon engagement CD40 by CD40L on T_{FH} cells (77-80). In the presence of CD40 signaling, the death-inducing signals emanating from Fas are overridden by strong "rescue" signals generated by the BCR. This mechanism promotes the survival of germinal center B cells expressing a BCR with high affinity for antigen.

Plasma Cell and Memory B Cell Differentiation

The germinal center reaction leads to the formation of long-lived antibody-secreting cells plasma cells that migrate to the bone marrow, and memory B cells that enter the circulation and lymphoid organs to screen peripheral lymphoid organs for the presence of antigen (6, 81-82). Plasma cells accumulate IgM, IgG, IgA or IgE in their cytoplasm and usually express mutated V(D)J genes, CD19, high levels of CD27 and CD38, but not IgD (except some plasmablasts from the upper respiratory tract) or CD24 (82-83). Short-lived plasmablasts from extrafollicular areas or peripheral blood also express the proliferation molecule Ki-67 as well as some levels of surface Ig receptors (48). Instead, long-lived plasma cells from the bone marrow usually lack Ki-67 and surface Ig receptors, but typically express the syndecan-1 molecule CD138. In the bone marrow, stromal cells, eosinophils and megakaryocytes provide powerful survival signals to plasma cells (84-86).

Memory B cells are critical to mount quick secondary humoral responses to recall antigens. In addition to entering the circulation, memory B cells form IgG-expressing extrafollicular aggregates and IgM-expressing follicle-like structures in draining lymph nodes (6, 68). After a subsequent exposure to recall antigen, IgG-expressing memory B cells rapidly generate antibody-secreting plasmablasts, whereas IgM-expressing memory B cells initiate a secondary germinal center reaction. All these responses are characterized by rapid B cell activation, proliferation, differentiation and secretion of high-affinity antibodies (87). Memory B cells express mutated V(D)J genes, IgG, IgA or (more rarely) IgM as well as CD19, CD24 and CD27, but not IgD or CD38 (88). The signals involved in the long-term survival of memory B cells remain unclear. Some studies point to antigen-independent polyclonal signals from B cell-intrinsic TLRs, whereas others point to antigen-dependent signals involving T cells and basophils (86).

4. SECONDARY Ig GENE DIVERSIFICATION

Mature B cells emerging from the bone marrow further diversify their Ig genes through two antigen-dependent processes known as SHM and CSR (Fig.4). These processes require AID, a DNA-editing enzyme strongly expressed by centroblasts and centrocytes (61, 89). While CSR diversifies the effector functions of an Ig molecule, SHM provides a structural substrate for the selection of Igs with higher affinity for antigen.

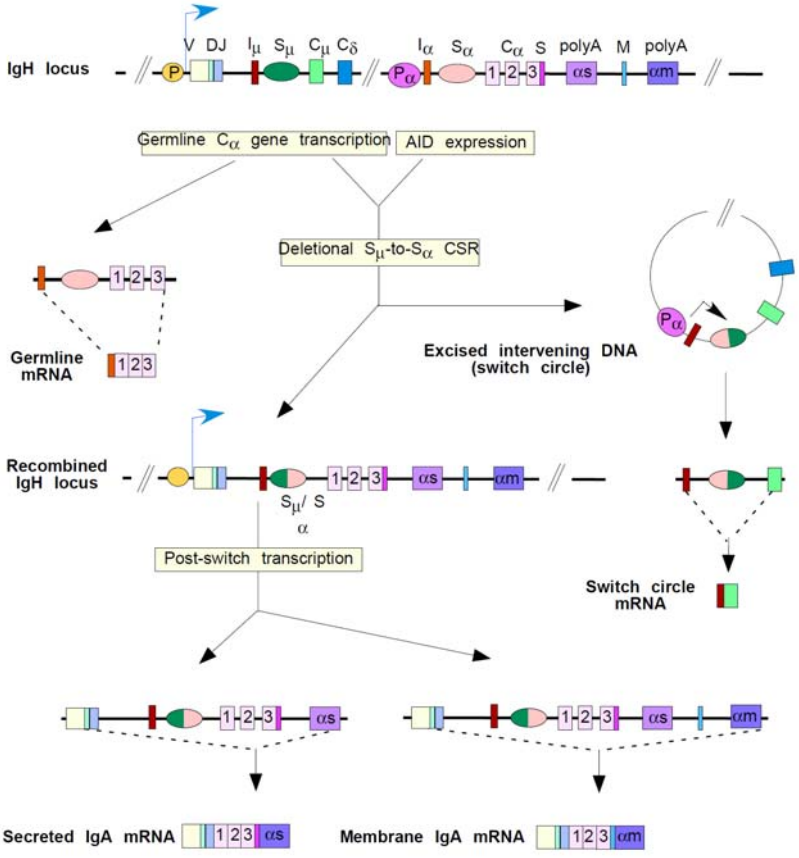


Figure 4. Mechanism underlying antibody class switching. The IgH locus contains a rearranged VHDJH exon encoding the antigen-binding domain of an immunoglobulin. Following rearrangement of the IgL locus, B cells produce intact IgM and IgD through a transcriptional process driven by a promoter (P) upstream

of VHDJH (blue arrow). Production of downstream IgG, IgA or IgE with identical antigen specificity but different effector function occurs through CSR. The diagram shows the mechanism of IgA CSR, but a similar mechanism underlyes IgG and IgE CSR. Appropriate stimuli induce germline transcription of the Cα gene from the promoter (Pα) of an intronic α (Iα) exon (black arrow) through an intronic switch α (Sα) region located between Iα and Cα exons. In addition to yielding a sterile Iα-Cα mRNA, germline transcription renders the Cα gene substrate for AID, an essential component of the CSR machinery. AID expression occurs following activation of B cells by helper signals from TFH cells (in the TD pathway) or innate immune cells (in the TI pathway). By generating and repairing DNA breaks at Sμ and Sα, the CSR machinery rearranges the IgH locus, thereby yielding a reciprocal deletional DNA recombination product known as Sα-Sμ switch circle. This episomal DNA transcribes a chimeric Iα-Cμ mRNA under the influence of signals that activate Pα. Post-switch transcription of the IgH locus generates mRNAs for both secreted and membrane IgA proteins. Cα1-3, exons encoding the Cα chain of IgA; S, 3' portion of Cα3 encoding the tailpiece of secreted IgA; M, exon encoding the transmembrane and cytoplasmic portions of membrane-bound IgA; αs, polyadenylation site for secreted IgA mRNA; αm, polyadenylation site for membrane-bound IgA mRNA.

Class Switch Recombination

This processs is an irreversible DNA recombination event that replaces the Cμ gene encoding the C_H region of the IgM molecule with the Cγ1, Cγ2, Cγ3, Cγ4, Cα1, Cα2 or Cε gene encoding the C_H region of IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 or IgE, respectively (90-91). CSR targets intronic switch (S) regions located upstream of each C_H gene and initiates with the germline transcription of a specific C_H gene in response to cooperative CD40L and cytokine signals (91-92). Cytokine signals are important to target specific C_H genes. Thus, while IL-4 and IL-13 preferentially activate Cγ4 and Cε, TGF-β predominantly induces Cα1 and Cα2 genes (93-95). Moreover, IL-4, IL-10 and IL-21 mostly target Cγ1, Cγ2 and Cγ3 genes (96-99). Germline transcription yields a primary transcript that encompasses the S region and its downstream C_H gene (100). Although later spliced into a non-coding germline, the primary transcript plays a central role in CSR (101). Indeed, this RNA physically associates with the template DNA strand of the

targeted S region to form a stable DNA-RNA hybrid that becomes substrate of AID, a DNA-editing enzyme induced by CSR-inducing signals (61). AID deaminates cytosine residues on both DNA strands of the actively transcribed S region to generate multiple DNA lesions that are subsequently processed by a complex DNA repair machinery to form double-stranded DNA breaks (91). Fusion of double-stranded DNA breaks via the non-homologous end-joining pathway induces looping-out deletion of the intervening DNA with subsequent replacement of Cμ with a downstream C_H gene. The resulting juxtaposition of the recombined VDJ gene with a Cγ, Cα or Cε gene permits B cells to acquire an Ig with novel effector functions but identical specificity for antigen (91).

Somatic Hypermutation

This process introduces point mutations in the recombined V(D)J exons encoding the antigen-binding V region of an antibody (102-103). In the germinal center of secondary lymphoid follicles, these point mutations provide the structural correlate for the selection of high-affinity B cells by antigen exposed on FDCs. During affinity maturation, the point mutations induced by AID mostly generate amino acids replacements in complementarity determining regions, which play a key role in the formation of the antigen-binding pocket formed by the V regions of IgH and IgL chains (91, 103). SHM includes an initial phase that requires the mutagenic activity of AID, followed by a second phase that involves the error-prone repair of AID-induced mutations (104). Of note, these mutations preferentially target specific hotspots, including the DGYW motif, where D stands for G, A or T nucleotides, Y for C or T nucleotides, and W for A of T nucleotides (103). Error-prone DNA repair is performed by members of a family of low-fidelity translesional DNA polymerases that recognize DNA lesions and bypass them by inserting bases opposite to the lesion (105-106).

Amino acid replacements brought about by SHM increase the affinity and fine specificity of an antibody, but do not modify the framework regions, which regulate the structural organization of Ig molecules. Similarly, SHM does not induce amino acid replacements in the promoter and intronic enhancer, which regulate the transcriptional activity of the Ig locus.

5. TI PATHWAYS FOR Ig PRODUCTION

Although predominantly occurring in germinal center B cells engaged in a T cell-dependent (TD) antibody response against protein antigens, CSR and SHM can also occur in extrafollicular B cells engaged in a T cell-independent (TI) antibody response against carbohydrate or lipid antigens (Fig. 5) (107-108). In spite of generating immune protection and memory, this TD pathway is relatively slow and needs to be integrated with a faster TI pathway that activates extrafollicular B cells through CD40L-like factors released by cells of the innate immune system, including B-cell-activating factor of the tumor necrosi factor (TNF) family (BAFF) and a proliferation-inducing ligand (APRIL) (109-110). These mediators cooperate with microbial BCR and TLR ligands to induce early CD40-independent antibody responses to highly conserved carbohydrate and lipid antigens (111-114).

Extrafollicular B cells strategically positioned at the mucosal interface and in the marginal zone (MZ) of the spleen typically respond to TI carbohydrate and glycolipid antigens captured by macrophages and DCs (48, 115-116). Such "frontline" B cells include B-1 cells and MZ B cells (117-119). In the mouse, B-1 cells constitute a distinct lineage of self-renewing B cells that are produced during fetal life and are mostly localized in the peritoneal cavity, spleen and intestine (117). B-1 cells generate innate (or "natural") adaptive immunity by spontaneously releasing polyspecific IgM but also IgA and IgG antibodies that provide a first line of defense against viral and bacterial

infections (115). Recent work has identified a small subset of B cells functionally equivalent to mouse B-1 cells in the circulation of humans, but further studies are needed to confirm these findings (120). Similar to B-1 cells, MZ B cells express polyspecific antibodies that recognize TI antigens with low affinity, at least in mice (115). In humans, MZ B cells can also produce monospecific antibodies to TI antigens (119). Classically, TI antigens are classified into type-1 (TI-1) antigens, which include microbial TLR ligands such as lipopolysaccharide (LPS), and type-2 (TI-2) antigens, which include bacterial cell wall polysaccharides. Additional TI antigens include microbial glycolipids recognized by MHC-I-like CD1 molecules expressed by MZ B cells (121).

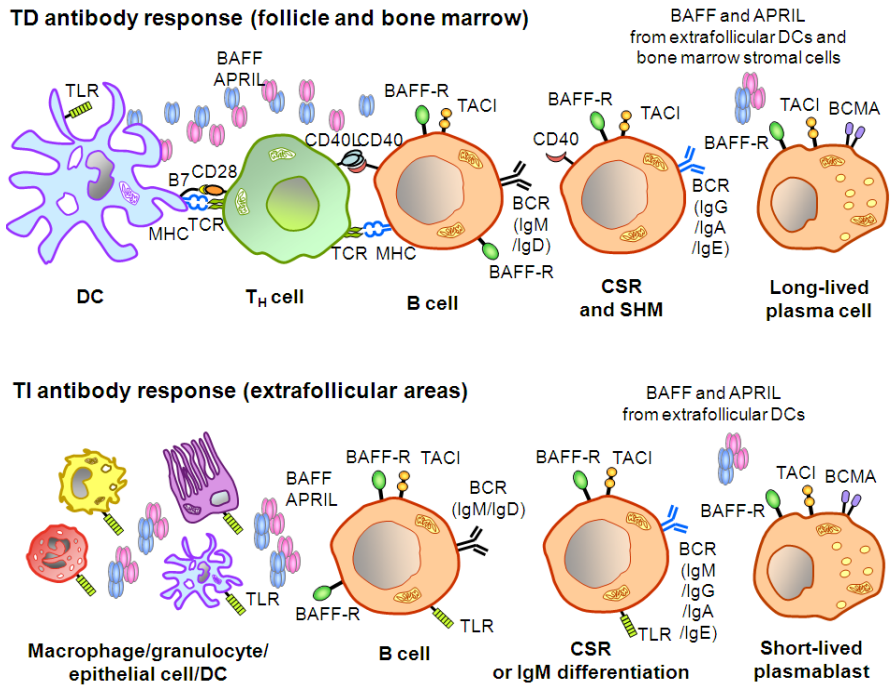


Figure 5. T cell-dependent (TD) and –independent (TI) antibody responses. TD antibody responses involve the ligation of BCR and the engagement of CD40 on follicular or bone marrow B cells by CD40L expressed on CD4+ TH cells that have been activated by antigen presentation on MHC-II molecules from those B cells. CD40L-CD40 interaction activates CSR and SHM, which eventually leads to Ig class

switching from IgM to IgG, IgA or IgE and the differentiation of these B cells into Ig-producing plasma cells (PCs). This process mostly takes place in the germinal centers of secondary lymphoid organs, such as lymph nodes, spleen and Peyer's patches. BAFF and APRIL secreted by DCs can further augment TD antibody responses. TI antibody responses involve the activation of B cells by BCR ligands as well as BAFF and APRIL secreted by multiple immune cell types, such as DCs, monocytes, macrophages, epithelial cells (ECs) and granulocytes. This process mostly takes place in extrafollicular areas such as those in the splenic marginal zone and intestinal lamina propia. BAFF and APRIL induce CSR of these B cells by binding to their receptor TACI, which induces signals that activate NF-κB signaling and AID function. The B cells can undergo CSR from IgM to IgG, IgA and IgE and differentiate into Ig-secreting PCs. BAFF secreted by the various cell types can promote the survival of these PCs by binding to BAFF-R expressed on these cells.

Both B-1 and MZ B cells are characterized by a state of active readiness that involves elevated expression of nonspecific TLRs (111-112, 114, 117) and poorly diversified BCR molecules capable of recognizing multiple microbial products (118, 121). B-1 and MZ B cells also show elevated expression of the transmembrane activator and calcium-modulating cyclophilin-ligand interactor (TACI), a receptor that triggers CSR and antibody production in response to BAFF and APRIL (115, 122). These CD40L-like factors are released by cells of the innate immune system such as macrophages, DCs, granulocytes and epithelial cells after sensing the presence of microbes via TLRs (86, 114, 123-125).

BAFF and APRIL Signaling

BAFF and APRIL are structurally related molecules produced as transmembrane ligands or soluble trimers by DCs, monocytes, macrophages, neutrophils, eosinophils, basophils, FDCs, endothelial cells, epithelial cells and stromal cells (110, 113, 123, 125-128). TACI on B cells undergoes extensive crosslinking by high-order BAFF and APRIL oligomers that are released by innate immune cells in response to intruding microbes (109, 129). Aggregation of TACI receptors activates nuclear factor-kappa β (NF-κB) through a mechanism involving the recruitment of TNF receptor-associated

factor (TRAF)2, TRAF3, TRAF5 and TRAF6 to the cytoplasmic tail of TACI. Although highly expressed by MZ and B-1 B cells, TACI is also expressed by follicular B cells and therefore contribute to both TI and TD antibody responses. In addition to inducing class switching, antibody production and plasma cell differentiation, TACI seems to regulate the size of the peripheral B cell pool by either controlling the amount of BAFF available for signalling through BAFF-R or limiting the amount of TRAF available to transmit BAFF-R signals (130-131).

In addition to engaging TACI, both BAFF and APRIL interact with the B cell maturation antigen (BCMA) receptor to promote plasma cell survival. BCMA is mostly expressed by antibody-producing plasmablasts and plasma cells and its engagement by BAFF or APRIL causes NF-κB activation through a canonical TRAF-dependent pathway similar to that induced by CD40 and TACI (132).

Unlike APRIL, BAFF also engages BAFF receptor (BAFF-R or BR3), a protein widely expressed by B cells at any stage of differentiation, except plasma cells (132-134). BAFF-R is typically activated by soluble BAFF trimers that are continually released by innate immune cells and stromal cells under homeostatic conditions. Engagement of BAFF-R by BAFF elicits the recruitment of TRAF3, which is followed by the degradation of TRAF-3 through a mechanism involving TRAF2, cellular inhibitor of apoptosis protein (c-IAP) and MALT1 (135-136). TRAF3 degradation causes activation of the enzyme NF-κB-inducing kinase (NIK) and induction of a non-canonical NF-κB pathway that up-regulates the expression of intracellular anti-apoptotic Bcl-2 family proteins, including Bcl-2, Bcl-xL and Mcl-1 (137). BAFF-R also triggers down-regulation of intracellular pro-apoptotic Bcl-2 family proteins, such as Bax, Bid and Bad. Of note, this pathway may cooperate with survival signals from the BCR (134). In general,

BAFF-R is essential for the survival of peripheral B cells, but some evidence indicates the additional involvement of BAFF-R in TI antibody production (123, 138-139).

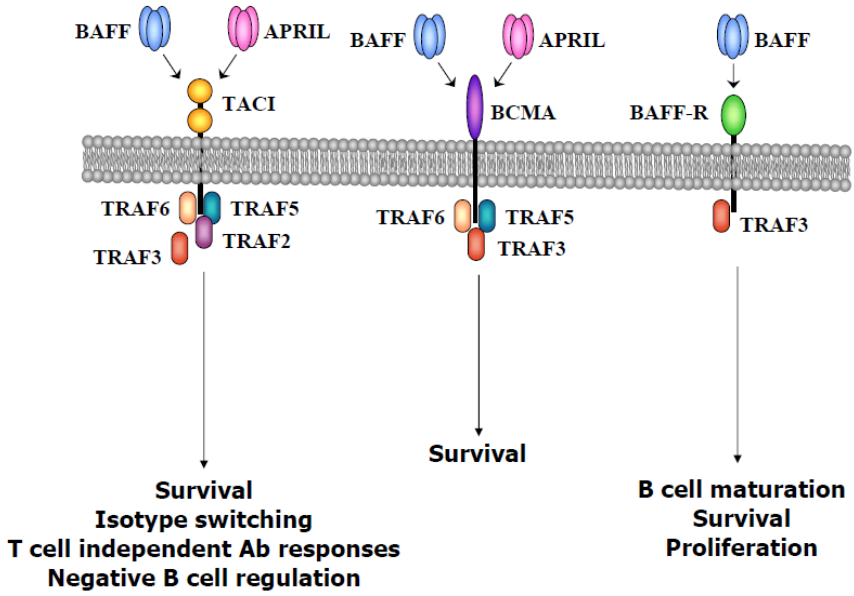


Figure 6. BAFF and APRIL signaling pathways. BAFF and APRIL activate B cells through at least three receptors known as TACI, BCMA and BAFF-R. Thorough the recruitment of TRAF proteins and the activation of different transcription factors, several mechanisms are activated in B cells.

5. THE IgD PUZZLE

IgD Discovery and Evolution

IgD is among the least understood isotypes of vertebrate Igs, although it has been discovered for over 50 years. Physicians David Rowe and John Fahey identified and characterized an unusual myeloma protein with electrophoretic and metabolic properties distinct from the known Ig classes at that time (IgM, IgG and IgA) while studying the disease multiple myeloma in 1964 (140-141). The myeloma protein displayed no reactivity to the anti-sera against IgM, IgG or IgA. In addition, an antigenically related form of this protein was detected at low concentrations in the serum of healthy subjects, but not of newborn, or agammaglobulinaemia patients. It did not possess the antigenic determinants characteristic of IgM, IgG or IgA, thus does not appear to be a subclass of any of these three Ig classes. Those evidences collectively suggested that this myeloma protein represented a member of a novel Ig class in humans, named IgD.

Studies of IgD in other species in the late 1970s and early 1980s identified IgD only in primates, rodents and selected species of mammals, including dog, mouse, rat, rabbit, guinea pig, whereas it was undetectable in other mammals, such as swine and birds (142-146). The discordance of the spotty presence of IgD with the animal phylogeny and evolution had lead to the general view that IgD was a recently evolved Ig class selected for certain species-specific functions in the respective host. Recent studies have discovered that IgD or its homologs and orthologs in a wide spectrum of species that are evolutionarily much more ancient, such as cartilaginous fishes, bony fishes, amphibians, and reptiles, with the exception of birds (91, 147-148). The most primitive species that have an adaptive immune system, i.e., the cartilaginous fishes, appeared on earth as many as some 470 million

years ago when jawed vertebrates first evolved (Fig. 7). These findings demonstrated that IgD is an ancient Ig isotype selected throughout evolution, and suggested that IgD or its homologs and orthologs has important immunological functions and probably confers the host some critical survival advantage.

IgD exhibits a high structural diversity thoughout vertebrate evolution, which could infer the possible selection of IgD as a structurally flexible locus to backup and complement the functions of IgM. The presence of IgD may ensure the preservation of essential immune functions in case of IgM defects, and the structure flexibility of IgD may provide additional immune functions in a species-specific manner.

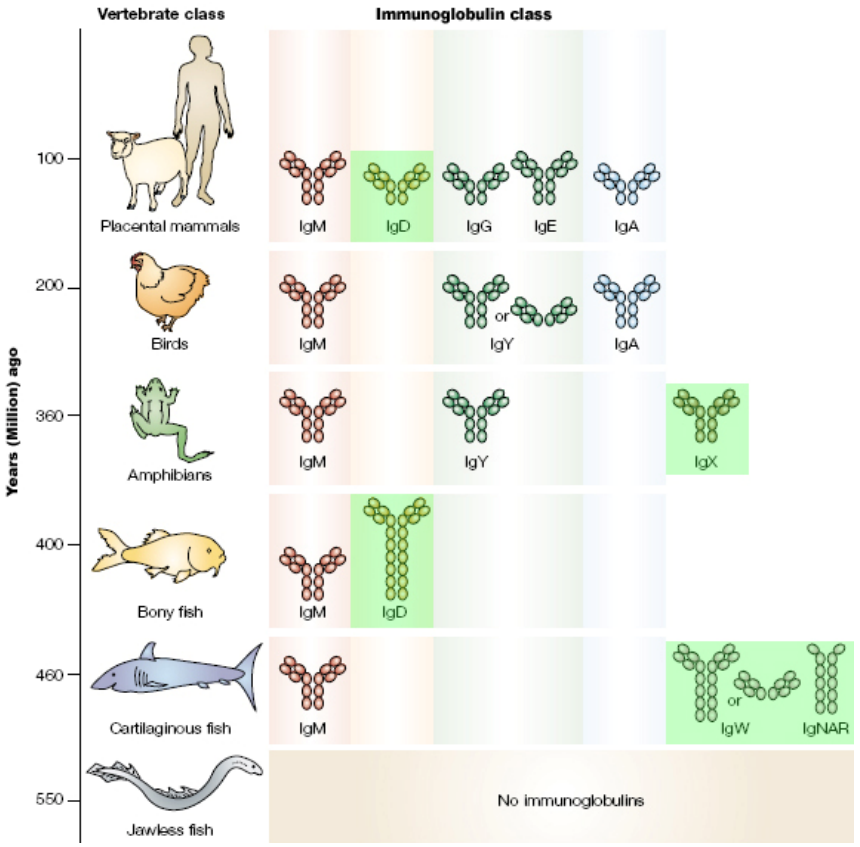


Figure 7. Evolution of IgD in vertebrates. Recombination-activating gene (RAG)-mediated DNA rearrangement of Ig genes to generate combinatorial diversity is one of the hall-marks of the adaptive immune system, which first appeared some 470 million years ago in jawed vertebrates. The Ig classes identified in each species group are shown. IgD and its homologs and orthologs in various groups are shaded in green. Fine differences among IgD and its homologs and orthologs in different species are discussed in the text below. Ig classes in the same column are believed to have common ancestry. Numbers on the left indicate the time when the particular vertebrate group emerged in evolution. IgNAR, immunoglobulin new antigen receptor. Modified from (149), © Nature Publishing Group.

IgD Distribution

The distribution of IgD in species other than human and mouse, especially in non-mammals, is poorly characterized. This is at least in part due to the unavailability of antibodies against IgD and the low abundance of IgD in those species. This section therefore focuses on the discussion of the distribution of IgD in humans.

Soluble IgD. The median serum IgD concentrations generally range from 20 to 50 μg/ml, with considerable variation even among people of the same age and sex. No consistent correlation with sex or age was found. The normal serum range of IgD is wider than that of any other Ig isotype. Individuals can be high or low producers, and low producers can convert to high producers in the setting of certain infections and immune activation. Therefore, the large variability and lack of correlation with demographic parameters of serum IgD might be contributed by the distinct history of immunological exposure in different individuals. In addition to blood, IgD is also present in human nasal, lacrimal, salivary, mammary, bronchial, pancreatic, and cerebrospinal fluids (39–45), and in the amniotic fluid of pregnant women with concentrations progressively increasing during the first half of pregnancy (46). Only trace amounts of IgD are present in intestinal mucosal secretions (39, 43, 47–49). The distribution of soluble IgD largely correlates with the distribution of IgD-producing B cells. Intestinal mucosa,

liver, peripheral lymph nodes, spleen, and bone marrow contain very few IgD-producing B cells, while tonsils, adenoids, salivary, and lachrymal glands, and nasal mucosa harbor abundant IgD-producing B cells (39, 43–45, 50–53). IgD-producing B cells can account for up to 20% of all Ig-secreting cells in human tonsils (53–55). The reason why they are rarely found in bone marrow and gut-associated lymphoid tissues in healthy individuals is probably because they are not normally generated there and they express a homing profile not in favor of the intestinal mucosa ($\alpha4\beta7$int / lowCCR9lowCCR7highCD62Lhigh) (56). The numbers of IgD-producing B cells in the upper aerodigestive mucosa are drastically increased in patients with IgA deficiency (57–59). Despite the fact that IgD-producing B cells express abundant J-chain (43, 45, 47, 51), IgD is generally recognized not to associate with J-chain or the secretory component and not to cross epithelium, placenta, or blood–brain barrier (58). IgD found on the surface of the epithelium and in mucosal secretions, in cerebrospinal fluid, as well as in the cord blood of some pregnant women (60–63) is thought to result from paracellular diffusion through cell junctions and production of IgD in the fetus. Nonetheless, evidence supporting the transepithelial and transplacental movements of IgD has been documented (64).

Cell-associated IgD. Cell-associated IgD includes transmembrane IgD, intracellular IgD, and secreted IgD bound to various cell types. Transmembrane IgD is expressed by mature naive B cells prior to antigenic stimulation and CSR and by IgD-producing B cells in the upper aerodigestive MALTs and peripheral blood of healthy individuals. B cells, in addition to expressing transmembrane IgD, can also bind secreted IgD (66). The studies of interaction of secreted IgD with T cells postulated a putative IgD receptor on a small fraction of T cells in human peripheral blood and mouse peripheral lymphoid organs, which binds to N-linked carbohydrate moieties of IgD (67, 68). Crosslinking of IgD receptor on T cells was shown

to protect T cells from apoptosis (69). It has also been shown that this IgD receptor could promote the formation of immune synapse between cognate T cells and naive B cells that express transmembrane IgD and thereby augment antigen presentation and antibody production (70, 71). The expression of this receptor was detected on CD4+ T cells in mice within minutes after oligomeric or aggregated but not monomeric IgD injection into mouse and was inhibited by the administration of tyrosine kinase inhibitors (67). However, many data were not readily explainable. IgD receptor-expressing T cells did not immediately show an activated phenotype, such as the expression of CD25. Coadministration of T-cell-specific activating agents with oligomeric IgD did not enhance IgD receptor expression on T cells. The IgD receptor on T cells has not been identified. One reason that may have given rise to the many intriguing data is that this putative IgD receptor may not normally be expressed by T cells but rather be released by other cell types and binds to T cells after oligomeric IgD injection into mice. The release of the IgD receptor and its binding to T cells would be very rapid in response to oligomeric IgD injection, which may explain the rapid appearance of IgD receptor on T cells.

IgD Expression

Expression by alternative splicing. While IgM is first expressed by pre-B cells, IgD emerges later during B cell ontogeny, being mostly expressed at the transitional and mature B cell stage, at least in rodents and primates (148, 150). In mammals, the Cδ gene is positioned immediately downstream of the Cμ gene in the same transcriptional unit, allowing these two primordial Ig isotypes to be coordinatedly regulated at the transcriptional level (Fig.8). In mature B cells, IgM and IgD are generated by alternative splicing of a long primary mRNA transcript containing the rearranged VDJ exons and the Cμ and Cδ exons. The recombined VDJ exons are spliced to the first Cμ exon

to generate IgM, or to the first Cδ exon to generate IgD, but the transcriptional ratio of Cμ and Cδ exons varies widely in different types of B cells (151). The mechanisms regulating the ratio of Cμ to Cδ exon usage are poorly understood, but are likely to involve the post-translational modification of RNA polymerase II and the induction of factors that regulate mRNA polyadenylation and splicing in response to antigenic stimulation and cellular differentiation. In plasma cells, the transcriptional elongation factor ELL2 associates with the carboxy-terminal portion of RNA polymerase II and with the polyadenylation factor CstF-64 to promote skipping of downstream exons through preferential usage of upstream mRNA cleavage and polyadenylation sites (152-153). A similar transcriptional repression mechanism could explain the down-regulation of IgD expression that typically occurs in most antigen-activated B cells, except $IgM^{-}IgD+$ B cells.

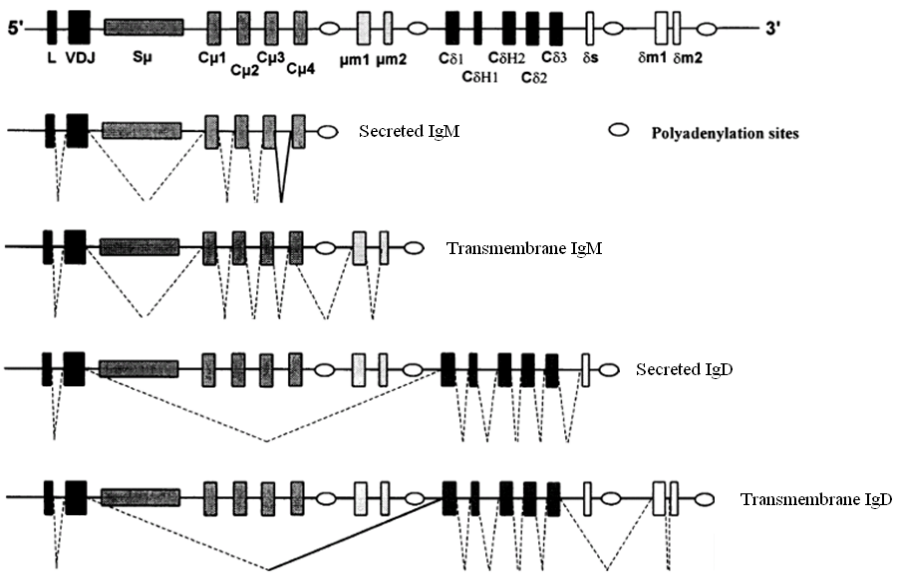


Figure 8. Expression of IgD and IgM by alternative splicing in mature B cell. Exons encoding the rearranged VDJ region and the various domains (including the

membrane and secreted portions) of IgM and IgD are shown in boxes. Polyadenylation sites are represented by ovals. Dotted lines show the various splicing configurations of primary transcripts to yield secreted and membrane-bound forms of IgM and IgD. Modified from (Preud'homme et al., 2000), © Elsevier.

Expression by class switching. In humans, a small subset of B cells express IgD but not IgM after undergoing an unconventional form of CSR (154). These IgM$^-$IgD+ B cells are found in the circulation as well as in tonsils, nasal cavities, lachrymal glands and salivary glands (150), but are rarely detected in non-respiratory mucosal districts. The specific topography of IgM $^-$IgD+ B cells may result from the expression of tissue homing receptors that do not favor colonization of extra-respiratory mucosal sites such as the intestine (155). Interestingly, IgM $^-$IgD+ B cells are also found in channel catfish [13], but are not generated through IgM-to-IgD CSR. Indeed, although expressing AID (156), catfish B cells seem to lack recognizable switch (S) regions (157-158), suggesting that IgM $^-$IgD+ B cells originate from antigen-induced transcriptional inactivation of the IgM locus.

The mechanism of this unconventional form of CSR remains unclear. S regions are highly repetitive intronic DNA sequences with G-rich non-template strands that precede each Cμ, Cγ, Cα and Cε gene and guide the process of CSR (90-91). Upstream of each S region, there is a promoter associated with a short intronic (I) exon that mediates germline transcription (90-91). While germline transcription of Cμ occurs in a constitutive manner, germline transcription of Cγ, Cα and Cε occurs after exposure of B cells to specific cytokines (90-91). Germline transcription is crucial for CSR, as it renders the targeted S region substrate of AID, a DNA-editing enzyme essential for CSR (61, 90-91). Germline transcription of a given C_X gene yields a primary I_X–S_X–C_X transcript that is later spliced to form a secondary non-coding germline I_X–C_X transcript (90-91). The primary transcript physically associates with the template strand of the S region DNA to form a

stable DNA–RNA hybrid (90-91). Such a structure generates R loops, in which the displaced non-template strand exists as a Grich single-stranded DNA (90-91). AID deaminates cytosine residues on both strands of S region DNA, thereby generating multiple DNA lesions that are ultimately processed into double-stranded DNA breaks (90-91). Fusion of double-stranded DNA breaks at donor and acceptor S^X regions through the non-homologous endjoining pathway induces looping-out deletion of the intervening DNA, thereby juxtaposing the recombined V_HDJ_H exon encoding the antigen-binding V_H region of the rearranging Ig molecule to a new C_X gene (90-91).

In contrast to other Ig isotypes, in mammals, there is no canonic S region 5' to the C_δ gene. Only rudimentary switch sequences are present in human and mouse. Consequently, CSR from μ to δ is traditionally considered a very rare event. Study of human and murine myeloma and hybridoma cells evidenced homologous recombinations involving two 443-bp repeat regions located 5' and 3' to the C_μ gene followed by the deletion of C_μ (148). Study of human normal tonsillar and leukemic B cells revealed that a region called σ_δ, which is located in the intron between C_μ and C_δ exons and contains G-rich pentameric repeats, is able to serve as an actual switch region for C_δ to mediate CSR with S_μ and result in the deletion of C_μ (159) (Fig. 9). Therefore, B cells that are class switched to IgD and capable of secreting large amounts of IgD do exist in normal individuals. Indeed, they are quite abundant in the upper aerodigestive MALTs, such as tonsil, adenoid and nasal mucosa, as discussed earlier, which corresponds to the abundant IgD found in these tissues.

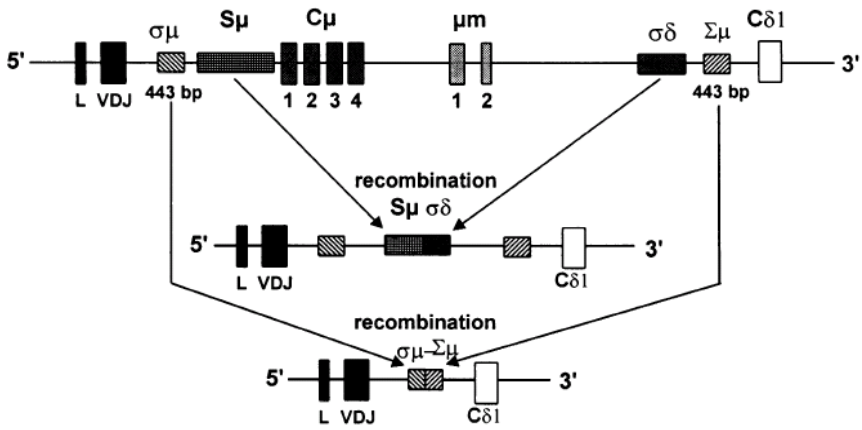


Figure 8. Expression of IgD by CSR. Schematic representation of Cμ-to-Cδ CSR in human, which has been found to occur between Sμ and σδ regions and between σμ and Σμ regions in normal and malignant B cells. Modified from (Preud'homme et al., 2000), © Elsevier.

Chapter II

Aims

This project has been developed to study the innate signals that induce the immune response of B cells. The main objectives of the project were:

- To study the IgD class switch recombination mechanism and production

- To analyse the signalling pathway of TACI and TLRs in class switching and antibody production

PART II

RESULTS

Chapter III

Immunoglobulin D enhances immune surveillance by activating antimicrobial, pro-inflammatory and B cell-stimulating programs in basophils

Kang Chen, Weifeng Xu, Melanie Wilson, Bing He, Norman W. Miller, Eva Bengten, Eva-Stina Edholm, Paul A. Santini, Poonam Rath, April Chiu, Marco Cattalini, Jiri Litzman, James Bussel, Bihui Huang, Antonella Meini, Kristian Riesbeck, Charlotte Cunningham-Rundles, Alessandro Plebani, & Andrea Cerutti

Nature Immunology 2009. Volume 10, pages 889-898.

Chapter VI

The transmembrane activator TACI triggers immunoglobulin class switching by activating B cells through the adaptor MyD88.

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Nature Immunology 2010 Volume 11, pages 836-845.

PART III

DISCUSSION AND CONCLUSION

Chapter V

Discussion

We have carried out extensive investigation of the localization, phenotype and generation of IgD^+IgM^- B cells that produce IgD, and we have also explored multiple functions of IgD in aspects regarding mucosal and systemic immune defense. Human B cells release IgD antibodies in the blood as well as respiratory, salivary, lacrimal and mammary secretions (160-165). IgD plasmablasts originated *in situ* from an active process of S_μ-to-σ_δ CSR that involved germline I_μ-C_δ transcription, required AID expression, and occurred through either a TD follicular pathway involving engagement of CD40 on B cells by CD40L on T cells or a TI extrafollicular pathway involving engagement of TACI on B cells by BAFF or APRIL from innate immune cells, possibly including epithelial cells and DCs (166-169).

Given that TACI is the major CSR-inducing receptor in the BAFF/APRIL system, we have also studied the molecular mechanism underlying this process. We have shown that TACI is functionally intertwined with TLRs, a family of innate antigen receptors capable of initiating both innate and adaptive immune responses after sensing highly conserved microbial products. Indeed, in addition to stimulating BAFF and APRIL release by DC and macrophage, signals from TLRs up-regulate the expression of TACI on B cells. Our studies have shown that this TI pathway involves the interaction of TACI with MyD88, which is followed by activation of IRAK-1 and IRAK-4 kinases, recruitment of TRAF6, induction of IKK via TAK1, and nuclear translocation of NF-κB by triggering phosphorylation and degradation of IkBα. TACI further activates NF-κβ by recruiting TRAF2, an adaptor protein that plays an important role in CSR. In general, these findings suggest that TACI and TLRs converge on MyD88 and TRAF adaptor proteins to optimize Ig diversification and production in frontline B cells.

Mucosal IgD^+IgM^- plasmablasts produce both polyreactive and monoreactive IgD antibodies encoded by unmutated and mutated V(D)J genes, respectively (165), possibly reflecting the need of the upper respiratory MALTs to mount maximally diversified IgD responses for optimal frontline defense. Such IgD responses likely entail the stimulation of specific λ^+ B cell precursors by a unique cocktail of mucosal signals comprising IL-21 and IL-2 or IL-21 and IL-15. Together with CD40L, BAFF and APRIL, these cytokines might account for the massive V(D)J gene diversification and oligoclonal expansion of IgD^+IgM^- B cells previously observed in tonsils (162, 170-171). The secretion of this cocktail of IgD-inducing signals results from the complex interplay between microbes and the various cell types present in tonsils that can produce these cytokines, such as monocytes, DCs, epithelial cells, T_{FH} and T_H17 cells (168, 172-174).

The interplay among mibrobes and cytokines produced by innate cells is also intrinsic in B cells, where the crosstalk between TACI and TLRs induces B cell activation and antibody production. TACI interacts with MyD88 through a conserved cytoplasmic region upstream of the TRAF2-binding domain motif that is distinct from the TIR domain by which MyD88 interacts with TLRs. Having defined the residues involved in the TACI-MyD88 interaction, it is possible to identify small molecules that disrupt TACI-MyD88 interaction by using a high-throughput screening of small chemical compound libraries. This approach may be used to attenuate CSR and antibody production in specific conditions, i.e. to treat autoimmune diseases associated with pathogenic CSR and BAFF or APRIL dysregulated expression.

Our findings on the signals and pathways involved in IgD CSR and production have important implications in the design of protective respiratory mucosal antibody vaccines. In particular, our result that BAFF is

more efficient than CD40L in inducing IgD production when combined with IL-2 or IL-15 and IL-21 highlights the importance of TI pathways in mucosal antibody responses. IL-15 is derived from TI sources, such as DCs and monocytes (175-177), and other reports showed that it is also produced by fibroblast-like stromal cells (178-180) in the bone marrow and melanoma cells in the skin (181). The finding that hyper-production of IgD in HIV-infected people becomes the most pronounced during the AIDS stage when T cell responses become defective further strengthens the importance of TI pathways (182).

Our results showing that TACI triggers CSR via MyD88 and the fact that MyD88 is usually associated with TLRs also suggests that a vaccine formulation simultaneously activating both pathways could be important for the development of vaccines, i.e. to boost mucosal IgA responses in humans.

Beside the importance of $IgD^{+}IgM^{-}$ plasmablasts in the mucosa, a population of these cells is constant present in the systemic circulation. This population is likely in transit from inductive sites in the upper respiratory mucosa to distant mammary, salivary, lacrimal, respiratory, tubal and auditive sites (155, 163, 183). At these effector sites, soluble IgD antibodies might enhance immune protection against local pathogens and commensal bacteria (184-190) by taking advantage of its poor complement-inducing activity to mediate immune exclusion in a non-inflammatory fashion, a feature perfectly compatible with mucosal antibody responses (164). Alternatively, IgD may confine its defensive functions to the subepithelial area of the upper aerodigestive MALTs.

Furthermore, this circulating population of $IgD^{+}IgM^{-}$ plasmablasts is clearly able to secrete IgD into blood. Although we have demonstrated the abundance of $IgD^{+}IgM^{-}$ plasmablasts in upper aerodigestive MALTs, we do

not wish to create or reinforce the false impression that IgD is predominantly a mucosal Ig isotype. IgD is produced in mucosal tissues in response to mucosal antigen stimulation, but the majority of our body's IgD is in the systemic circulation, in fact more than the fraction of IgG and IgA that are in circulation. In this regard, IgD is ideally suited to serve as the link between systemic immune system and the mucosal immune system in the upper part of our body.

Circulating IgD interact with basophils through a calcium-fluxing receptor that induce antimicrobial, opsonizing, inflammatory and immunostimulating factors such as cathelicidin, pentraxin-3, IL-1, IL-4 and BAFF. We have shown the dysregulation of IgD class-switched B cells and IgD-armed basophils in autoinflammatory syndromes, including Hyper-IgD syndrome (HIDS), cryopyrin-associated periodic fever syndrome (CAPS), TNF receptor associated periodic fever syndrome (TRAPS), and periodic fever aphtous stomatitis pharyngitis and cervical adenitis (PFAPA) syndrome.

Consistent with an important role of TACI in Ig diversification and production, some individuals with deleterious TACI substitutions suffer from recurrent infections by encapsulated bacteria and have less serum IgM, IgG and IgA (hypogammaglobulinemia) and impaired IgG responses to TI antigens such as capsular polysaccharides. Unlike patients with deleterious TACI substitutions, patients lacking MyD88 or IRAK-4 (a kinase downstream of MyD88) have recurrent infections with pyogenic bacteria, including encapsulated bacteria, but do not develop hypogammaglobulinemia and their responses to TI antigens such as capsular polysaccharides are impaired only sporadically *in vivo* (191-192). However, human TLRs and TACI recruit both MyD88 and IRAK-4 to trigger TI Ig diversification and production *in vitro* (112). One possibility is that humanTLRs and TACI use a MyD88-independent pathway to initiate TI Ig responses and a MyD88-

dependent pathway to sustain TI Ig responses over time. Alternatively, MyD88-dependent pathways might be important to optimize the class and affinity of TI Ig responses. A better understanding of these issues would require a systematic analysis of intestinal and respiratory Ig responses in patients with deleterious TACI, MyD88 or IRAK-4 substitutions. In mice, lack of TLRs or MyD88 impairs intestinal TI IgA production (193-194), whereas lack of TACI impairs systemic TI IgM, IgG and IgA production [108], but has unclear effects on respiratory TI IgA production (195). In light of these findings, it is likely that the contribution of TLR and TACI to mucosal immunity varies depending on the type of antigen (viral versus bacterial, soluble versus particulate) and the route of immune challenge.

Circulating IgD is significantly elevated in patients with systemic lupus erythematosus (SLE), a common autoimmune disease associated with severe morbidity, high mortality and prolonged loss of productivity and life quality of patients. Although this dysregulation in the IgD compartment of SLE patients is poorly understood, numerous clinical and immunological observations of IgD in SLE patients (including increased production, frequent and intense reactivity to many SLE-associated autoantigens and its strong potential to induce inflammatory responses) suggest that IgD is an important but neglected component of SLE pathogenesis. Further studies are needed to dissect the cellular and molecular basis of the IgD dysregulation in IgD patients. We recently found a population of IgD class-switched B cells in the mouse, allowing IgD's pathogenic function to be investigated in vivo crossing $IgD^{-/-}$ mice with various lupus mouse models. Knowing that a fraction of the autoantibodies against double-stranded DNA, histones and ribonucleoproteins in SLE patients are IgD, the next step would be to study the regulation and reactivity of these IgD autoantibodies.

BAFF and APRIL are not only important in the induction of IgD class switching, but their expression is upregulated by basophils upon IgD cross-linking *in vitro*. This finding provides a mechanistic explanation to prior studies showing that IgD deficiency leads to a contraction of the peripheral B cell compartment in mice (196). It has been reported that SLE abnormally augments myeloid cell release of BAFF and APRIL. MyD88 has been shown to have a central role in SLE, and lupus-prone T cell-deficient mice require MyD88 to develop IgG autoantibodies in response to a BAFF transgene. In this regard, further studies would be required to understand the role of TACI and MyD88 in the disregulation of CSR events in SLE. Inhibitors of TACI-MyD88 interaction may alleviate inflammation in SLE by attenuating CSR, SHM and autoantibody production in autoreactive B cells.

In conclusion, our data reveal a complex scenario in which T independent pathways and a poorly characterized Ig isotype are critical in the development and progression of B cell responses.

Chapter VI

Conclusions

1. B cells of the human upper respiratory mucosa generate local and circulating IgD+IgM- plasmablasts from an active process of CSR that involves germline transcription and requires AID expression.

2. IgD CSR occurs through either a T cell-dependent follicular pathway involving engagement of CD40 on B cells by CD40L on T cells, or through a T cell-independent extrafollicular pathway involving engagement of TACI on B cells by BAFF or APRIL from innate immune cells.

3. Circulating IgD interact with basophils through a calcium-fluxing receptor that induces antimicrobial, opsonizing, inflammatory and immunostimulating factors such as cathelicidin, pentraxin-3, IL-1, IL-4 and BAFF.

4. IgD class-switched B cells and "IgD-armed" basophils are dysregulated in patients with HIDS and autoinflammatory syndromes.

5. BAFF and APRIL promote the recruitment of MyD88 to a conserved cytoplasmic motif of TACI distinct from the TIR domain of TLRs.

6. TACI-MyD88 interaction induces CSR by triggering NF-κβ activation, germline C_H gene transcription and AID expression.

7. TACI-MyD88 CSR is induced through a TIR-independent pathway impaired in mice and humans lacking MyD88 or the IL-1R-associated kinase IRAK4, a signal transducer that binds MyD88.

ANNEX 1

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ANNEX 2

Abbreviations

AID	Activation-induced cytidine deaminase
APRIL	A proliferation-inducing ligand
BAFF	B-cell–activating factor of the TNF family
BAFF-R	BAFF receptor
BCMA	B-cell maturation antigen
BCR	B-cell receptor
CAPS	Cryopyrin-associated periodic fever syndrome
CD40L	CD40 ligand
c-IAP	Cellular inhibitor of apoptosis protein
CSR	Class switch recombination
DC	Dendritic cell
FDC	Follicular dendritic cell
FLT3	Fms-related tyrosine kinase 3 ligand
HIDS	Hyper-IgD syndrome
IFN	Interferon
Ig	Immunoglobulins
IL	Interleukin
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
MZ	Marginal zone

NF-κβ	Nuclear factor-kappa β
NIK	NF-κβ –inducing kinase
NK	Natural killer
PFAPA	Periodic fever aphtous stomatitis pharyngitis and cervical adenitis
PRR	Pattern recognition receptor
RAG	Recombination activation gene
SCID	Severe combined immunodeficiency
SHM	Somatic hypermutation
SLE	Systemic lupus erythematosus
TACI	Calcium-modulating cyclophilin ligand interactor
TCR	T-cell receptor
TD	T-cell–dependent
TdT	Terminal deoxyribonucleotidyl transferase
TH	T helper
TI	T-cell–independent
TNF	Tumor necrosis factor
TLR	Toll-like receptor
TRAF	TNF receptor-associated factor
TRAPS	TNF receptor associated periodic fever syndrome
TSLP	Thymic stromal lymphopoietin

ANNEX 3

Publications

1. Chen K, Xu W, Wilson M, He B, Miller NW, Bengten E, Eva-Stina Edholm ES, Santini PA, Rath P, Chiu A, Cattalini M, Litzman J, Bussel J, Huang B, Meini A, Riesbeck K, Cunningham-Rundles C, Plebani A, & **Cerutti A.** Immunoglobulin D enhances immune surveillance by activating antimicrobial, pro-inflammatory and B cell-stimulating programs in basophils. Nature Immunology 2009, 10: 889-898.

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