

Regulatory Properties of Dendritic cells and B cells in Adaptive Immunity

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ORIGINAL PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals (I-IV).

- I Johansson, B., Ingvarsson, S., Björck, P., & Borrebaeck, C. A. K. 2000. Human interdigitating dendritic cells induce isotype switching and IL-13-dependent IgM production in CD40-activated naive B cells. *J Immunol.* **164**:1847-1854.

- II Johansson-Lindbom, B., & Borrebaeck, C. A. K. 2002. Germinal center B cells constitute a predominant physiological source of IL-4: Implication for Th2 development *in vivo*. *J Immunol.* **168**:3165-3172.

- III Lindstedt, M., Johansson-Lindbom, B., & Borrebaeck, C. A. K. 2002. Global reprogramming of dendritic cells in response to a concerted action of inflammatory stimuli. *Int. Immunol. In press.*

- IV Johansson-Lindbom, B., Ingvarsson, S., & Borrebaeck, C. A. K. 2002. Th2-development within germinal centers. *Manuscript.*

ABBREVIATIONS

Ab	Antibody
AID	Activation-induced cytidine deaminase
APC	Antigen presenting cell
BCR	B cell receptor
CD	Cluster of differentiation
C _H	Constant domain of the immunoglobulin heavy chain
CSR	Class switch recombination
DC	Dendritic cell
Dp	<i>Dermatophagoides pteronyssinus</i>
DTH	Delayed type hypersensitivity
FDC	Follicular dendritic cell
GC	Germinal center
HEV	High endothelial venules
IC	Immune complex
IDC	Interdigitating dendritic cell
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LC	Langerhans cell
LPS	Lipopolysaccharide
MALT	Mucosa associated lymphoid tissues
MHC	Major histocompatibility complex
NK cells	Natural killer cells
PDC	Plasmacytoid dendritic cell
PNA	Peanut agglutinin
TCR	T cell receptor
Th cell	T helper cell
Tr1	Regulatory Th cell
V	Variable

I INTRODUCTION

The immune system of vertebrates is divided into innate and adaptive compartments, providing the host with different but yet interrelated protective responses against pathogens. Besides the physical boundaries towards the external environment, including the skin and mucosal surfaces, the innate immune system is composed of protective cells and molecules which rapidly become activated upon provocation. Some examples are natural killer cells (NK cells), scavenging macrophages and the complement system. To accommodate an immediate response innate immunity is compromised in terms of specificity and flexibility and can neither improve nor tailor its responses against a given antigen.

In marked contrast to the innate immune response, adaptive immunity is highly specific and exhibits a capacity to adapt host responses to the nature of infectious agents. B and T lymphocytes provide specific recognition of an immense variety of environmental antigens by expressing a diverse repertoire of clonally distributed antigen-specific receptors. This functional diversity is not present in germ-line configuration of the genome but is intrinsically created only among B and T cells by DNA-recombination of disconnected gene segments (Alt *et al.*, 1992). During immune responses B and T cells displaying receptors with appropriate specificity are clonally expanded. In addition, B cells are subjected to somatic mutations within the genes encoding the antigen-binding site of the receptor, selected on the basis of improved affinity, and after that expanded (MacLennan and Gray, 1986; Wagner and Neuberger, 1996). Adaptive immunity is consequently both qualitatively (increased affinity) and quantitatively (expanded pool of specific cells) improved during the course of an immune response and give rise to B cells producing specific and high affinity antibodies (Ab), CD4⁺ T cells providing help for B cell differentiation (including Ab-production) and, finally, CD8⁺ T cells participating in direct lysis of target cells. Furthermore, after clearance of the infectious agent specific B and T lymphocytes are maintained at increased steady-state levels, exhibit lower thresholds for re-activation and hence provide the host with an immunological

memory, which facilitate a faster and more robust recall response (Gray, 1994). Also memory is an unique property of adaptive immunity.

The clonal selection theory of Ab responses evolved during the late 1940's and the beginning of the 1950's and was put forward in its whole context by F. Macfarlane Burnet (1959). Briefly, he proposed that individual B cells display unique Ab on their surface, and if these B cell receptors (BCR) encounter and bind the corresponding ligand (antigen) they start to divide and accordingly give rise to a large clone of B cells producing Abs with identical specificity. The clonal selection theory could not by it self explain why self-reactive clones not continuously were generated and caused autoimmunity. In 1960 F. Macfarlane Burnet and Peter B. Medawar were awarded the Nobel Prize in Medicine for their discovery of "acquired immunological tolerance", a process by which self reactive clones are deleted instead of activated. This negative selection of lymphocytes was suggested to occur during early embryonic life and since B cells with BCRs against "self-components" were deleted, activation of the specific immune response could be triggered by direct recognition of "non-self" components by the remaining pool of lymphocytes. However, it has become increasingly clear that in spite of the fact that self-reactive clones continuously are deleted also during adult life, this elimination is far from complete and new potentially self-reactive cells are also continuously generated, not the least by the process of somatic mutation. Instead, activation of the adaptive immune response depends upon a number of cellular and molecular events which must be coordinated in a spatial and temporal fashion. A highly specialized subset of innate leukocytes called dendritic cells (DC) have been revealed to constitute the critical switch for turning on these consecutive events (Banchereau *et al.*, 2000). Therefore innate immunity plays a crucial role in the recruitment of the adaptive immune response and in particular DCs possess the capacity to regulate the activation of specific T and B lymphocyte.

Immune regulation does not only concern whether specific responses shall be raised or not but also involves a polarization of effector functions. For example, a small virus and a multicellular metazoan parasite cannot be efficiently defeated by the same mechanisms. To accommodate a tailored response, T and B cells cannot only provide specificity but they can also secrete different kinds

of cytokines and Abs, respectively, and thus recruit different categories of effector cells (Romagnani, 1994). An example that in a clear way illustrates the importance of an appropriate immune class regulation is the inappropriate polarization of CD4⁺ T cells and the disproportional secretion of IgE Abs which cause the clinical manifestations of allergic disease (Corry and Kheradmand, 1999).

The work presented in this thesis covers aspects of (i) how lymphocytes are activated by DCs (Paper I and III) and (ii) how the class of an immune response is determined (Paper I, II and IV).

2 SECONDARY LYMPHOID ORGANS

Adaptive immune responses are initiated within the secondary lymphoid organs (MacLennan *et al.*, 1997). These are the spleen, the peripheral lymph nodes, and the mucosa associated lymphoid tissues (MALT), including Peyer's patches in the small intestine and Waldeyer's ring of tonsils in the upper respiratory tract (Perry and Whyte, 1998). Whereas the spleen filters the blood and accounts for induction of immune responses against blood-borne antigens, the different lymph nodes and MALT are strategically positioned to guard peripheral tissues facing the external environment. Thus extra cellular fluid from most part of the body is drained into the afferent lymphatics and shuttled to the nearest situated lymph node. The different structures of MALT do not possess afferent lymphatics, and antigens are instead transported directly across the mucosal epithelia, predominantly by specialized epithelial cells called M cells (Neutra *et al.*, 1996). Although the separate lymphoid organs exhibit anatomical variations (mostly as a consequence of how antigens are delivered and lymphocytes recruited), architectural features which are of crucial importance for induction of specific immunity appear to be rather conserved among them. Since a detailed anatomical description of lymphoid tissues is beyond the scope of this thesis, I will only give a summary of mechanisms involved in the development of this shared architecture, which provide the playground for recruitment of the adaptive immune response.

The rational basis of lymphoid architecture

The lymphocyte pool in the human body contains cells at a magnitude of 10^{12} . The potential diversity of different TCR-specificity has been calculated to approximately 10^{15} (Davis and Bjorkman, 1988), which obviously do not reflect the actual TCR-usage. Instead Arstila *et al.* (1999) employed an experimental approach to estimate the existent diversity among naïve T cell, which in human was found to potentially range from 2.4×10^7 - 10^8 different α/β TCRs. Accordingly, the precursor frequency of a given TCR-specificity does not exceed 2.4×10^{-7} . However, epitope-specific T cell responses do not typically recruit a single TCR-specificity but rather involve different T cell clones (although a quite limited number) (Maryanski *et al.*, 1996). This fact is reflected

in a recent work by Blattman *et al.* (2002), in which the precursor frequency of a CD8⁺-restricted T cell response to a peptide antigen was estimated in mice to 5 x 10⁻⁶. Although based on a single peptide-epitope instead of an intact protein-antigen (which under physiological conditions will generate several peptide-epitopes and hence mobilize more T cell clones), the results provided by Blattman and coworkers clearly demonstrate an outermost challenging puzzle that the immune system has to deal with: to bring together APCs with exceptionally rare specific T and B lymphocytes. The solution to this problem is a tightly controlled migration of leukocytes and a highly ordered micro-architecture of the secondary lymphoid organs.

The molecular basis of lymphoid architecture

Naïve lymphocytes do not traffic to extralymphoid sites, under neither homeostatic nor inflammatory conditions (Picker and Butcher, 1992). Instead they continuously re-circulate through the secondary lymphoid organs, and while temporarily retained within these tissues, B and T cells become physically segregated resulting in separate B and T cell zones. The naïve B cells form follicular structures dispersed in cortex, which is situated beneath the anatomical compartments providing portal entry of antigens and APCs (*e.g.* subcapsular sinus in lymph nodes and the subepithelial dome in Peyer's patches). T lymphocytes become localized adjacent to the follicles in cortex where they form the T cell zones, in lymph nodes also referred to as paracortex (Rouse *et al.*, 1984).

The entrance of lymphocytes into the lymphoid organ and their subsequent zonal distribution within the tissue is largely governed by homeostatic chemokines and their receptors. Chemokines are low molecular weight chemotactic proteins. They commonly have a high isoelectric point resulting in electrostatic interaction with *e.g.* sulfated proteins and proteoglycans present on connective tissue. As a consequence they form concentration gradients, originating from the cellular sources of secretion (Cyster, 1999). Chemokine CCL21 (SLC) is constitutively produced at high levels by specialized high endothelial venules (HEVs). It triggers lectin-dependent firm adhesion of lymphocytes to the luminal side of the endothelium by signaling through counter-receptor CCR7 (Campbell *et al.*, 1998; Gunn *et al.*, 1998b).

Subsequently, CCR7⁺ lymphocyte pass through the HEV into surrounding T cell zones. CCR7 is expressed on all naïve and a subset of memory T cells (Sallusto *et al.*, 1999) and is critical for T cells extravasation through the HEVs (Forster *et al.*, 1999). B cells are not equally restrictive in their usage of receptors and can enter lymph nodes and peyer's patches both in a CCR7- and CXCR4-dependent manner. Endothelial cells appear not to produce the CXCR4 ligand CXCL12 themselves but clearly display CXCL12 on their luminal surface, probably as a result of transcytosis of CXCL12 from the abluminal side where the protein is produced. In addition, in peyer's patches, but not in lymph nodes, B cells can utilize a high level expression of chemokine receptor CXCR5 to directly enter B cell areas through HEVs present in these locations (Okada *et al.*, 2002). The corresponding ligand for CXCR5, CXCL13, is produced by stromal cells, including FDCs, only in the B cell follicles of lymphoid organ and has not been detected in non-lymphoid tissues (Gunn *et al.*, 1998a; Legler *et al.*, 1998). Presently it is not known whether HEVs produce CXCL13 themselves, but the chemokine has been detected on follicular endothelium both in murine peyer's patches and in human tonsil (Okada *et al.*, 2002; Schaerli *et al.*, 2000).

Regardless of which route B cells utilize for their entrance into the lymphoid tissue, their high levels of CXCR5-expression will ultimately localize them to the follicles. The important role for CXCR5 in the follicular homing is evident in mice with a targeted disruption of the CXCR5 gene. In these animals the various lymphoid organs are either completely degenerated or display severe architectural alterations including a complete absence of defined follicular structures. Moreover, transferred B cells from CXCR5-deficient mice fail to enter B cell areas in wild-type recipients, demonstrating the mandatory role for this receptor in follicular homing (Forster *et al.*, 1996). Naïve and resting memory T cells do not express CXCR5 and do accordingly not migrate into follicles (Ansel *et al.*, 1999). Instead they are retained in the T cell zones by CCL21, and yet a second ligand for CCR7, CCL19, being produced by stromal cells and DCs in this particular area (Cyster, 1999). By analogy with the CXCR5-deficient B cells lymphocytes transferred from CCR7 knock-out mouse are excluded from the T cell zones in the wild-type recipient (Forster *et al.*, 1999). To summarize, a zonal production of homeostatic chemokines in the

lymphoid tissue forms boundaries for B and T cells, which become segregated as a result of their differential expression of appropriate counter receptors.

The primary immune response

The primary immune response is characterized by a number of consecutively ordered events where APCs, T cells and B cells coordinate their efforts to provide protection against a previously not encountered antigen. The possibility to adoptively transfer large numbers of receptor-transgenic lymphocytes into syngenic recipients has bypassed limitations imposed by the otherwise low precursor frequencies *in vivo* and made it possible to visualize the kinetics, nature, and consequences of these cellular interactions. Within 18 hours after immunization of mice with a protein antigen in adjuvant DCs migrate from peripheral sites into the T cell zones of lymphoid organs where they establish antigen-specific interaction with CD4⁺ T cells (Ingulli *et al.*, 1997; Stoll *et al.*, 2002). The tight association between DCs and T cells is long-lived, and when Stoll *et al.* (2002) used laser scanning confocal microscopy for continuous imaging of intact explanted lymph node, T cells remained attached to the DCs throughout a 15-hour observation period. Within two days after immunization the T cells starts to divide and the magnitude of proliferation in the paracortex appear to peak approximately at day 4-5 (Garside *et al.*, 1998; Kearney *et al.*, 1994). With kinetics similar to the onset of cell division, the T cells also start to differentiate into separate subsets expressing new panels of chemokine receptors and adhesion molecules. A proportion of them upregulate tissue-selective adhesion molecules and receptors for inflammatory chemokines and thereby leave the lymphoid organ via the efferent lymphatics (Campbell and Butcher, 2002). These tissue homing cells, which also relatively rapidly start to produce effector cytokines (preferentially IFN- γ), reappear in the circulation roughly three days after immunization (Campbell and Butcher, 2002; Campbell *et al.*, 2001) and directly home to interstitial spaces of inflamed tissues and organs (Reinhardt *et al.*, 2001). Consistent to the mouse models, a large fraction of transitional CD45RA/RO double positive T cells isolated from human lymph nodes and tonsils express the skin-homing receptor cutaneous lymphocyte-associated antigen (CLA) (Picker *et al.*, 1993)

CD4⁺ T cells specialized to provide help for GC-formation and B cell differentiation seem to evolve from the naïve T precursor cells with even faster kinetics. Already one day after *in vitro* stimulation with LPS-activated DCs, human naïve T cells are induced to express CXCR5, and after two days of activation the majority of T cells in these cultures have acquired high level expression of CXCR5 (Schaerli *et al.*, 2001). In the adoptive transfer models T cells move into the edges of the B cell follicles where they physically interact with antigen-specific B cells 2 days after immunization (Garside *et al.*, 1998). In agreement with a critical role for CXCR5 in the follicular homing (Forster *et al.*, 1996), migration of T cells towards B cell areas coincide with their induced expression of CXCR5 (Ansel *et al.*, 1999). Instead, antigen activated B cells show a reverse pattern of alterations and gain expression of CCR7, which permit their redistribution towards the follicular rim (Reif *et al.*, 2002). In humans an antigen-dependent decrease in CXCR5 levels may also contribute to the movement towards the zonal boundary (Casamayor-Palleja *et al.*, 2002). Therefore, in an immense landscape with lymphocytes equipped with antigen-receptors of irrelevant specificities, cognate interaction between exceptionally few specific T and B cells is arranged by a zonal production of homeostatic chemokines and a flexible expression of their counter receptors.

3 GERMINAL CENTERS

GCs are critical for affinity maturation and isotype switching of antibody responses as well as for development of B cell memory (Berek *et al.*, 1991; Jacob *et al.*, 1991; Liu *et al.*, 1989; Pascual *et al.*, 1994).

Morphological progression

GCs arise within the follicles as a consequence of extensive oligoclonal proliferation of B cells which have bound, internalized, and presented processed antigen on MHC II for cognate T cells at the follicular edge (Garside *et al.*, 1998; Jacob and Kelsoe, 1992; Jacobson *et al.*, 1974). Cell division among these GC-founder B cells is evident already at day 2-3 after immunization with protein antigens in adjuvant (Garside *et al.*, 1998; Liu *et al.*, 1991). After that a vigorous B cell proliferation give rise to an intra-follicular cluster of B cell blasts that is easily visualized at day 3-5 by staining tissue sections with the plant lectin peanut agglutinin (PNA) (Garside *et al.*, 1998; Jacob and Kelsoe, 1992). Approximately at the same time as PNA⁺ GCs start to appear, the B cells blasts (termed centroblasts) start to accumulate mutations in their rearranged antibody variable (V) region genes (Jacob *et al.*, 1991; McHeyzer-Williams *et al.*, 1993). The onset of somatic mutation is in turn linked to differentiation into non-dividing centrocytes. As the centroblasts continue to proliferate and steadily give rise to new centrocytes, the interior of the follicle is divided into two distinguishable zones. Centroblasts occupy the dark zone proximal to the T cell zone whereas centrocytes become localized to the opposite pool, referred to as the light zone. Naïve B cells which do not participate in the GC-formation are excluded from the interior of the follicle and form the surrounding mantel zone, most dense distal to the T cell zone. The morphological progression of GCs in immunized mice was in detail analyzed and described by *e.g.* Liu *et al.* (1991) and has made it possible to identify and characterize the discrete stages of GC-associated B cell differentiation. The two important molecular events taking place within the microenvironment of this anatomical structure are somatic mutation and class switch recombination (CSR) of the Ig genes. Somatic mutation accumulate nucleotide insertions, deletions, and point mutations in the V exon of the Ig genes (Jacob *et al.*, 1991; Ohlin and Borrebaeck, 1998; Wilson *et al.*,

1998), and this genetic diversification is then tested for functional improvement where centrocytes expressing high affinity Ig on their surface are selected by limited amounts of antigens (Liu *et al.*, 1997). This means that B cells within GCs depend on exogenous signals for their survival, and ultimately such signals are only delivered to cells with an maintained or improved capacity to bind antigen. A high propensity to undergo apoptosis precedes the onset of somatic mutation and is indeed one of the first detectable features of GC-founder B cells (Lebecque *et al.*, 1997). CSR does not affect Ig specificity or affinity but instead deletes the pre-existing constant C_μ gene and positions a new C_H gene proximal to the V gene (Harriman *et al.*, 1993). This results in isotype switching of expressed Igs from IgM to either IgG, IgA or IgE. Each of these new isotypes displays different capability to fix complement, they bind to separate subsets of effector cells (as a result of differential expression of Fc- and complement-receptors on these cells) and they also vary in their ability to pass through mucosal epithelium (Heyman, 2000; Mostov, 1994). Therefore isotype switching largely governs the nature of an immune response against a given antigen and the regulation of CSR constitute a central and crucial event for the recruitment of appropriate arms of the immune system.

Somatic mutations and class switch recombination

Since somatic mutation (in centroblasts) was shown to precede CSR (in centrocytes), the two events were first considered as independent (Liu *et al.*, 1996). However, both of them display a high degree of resemblance in their prerequisite for activation of the mutating/recombinase activities. Both processes depend on substantial transcription from the target locus (Peters and Storb, 1996; Xu *et al.*, 1993), formation of DNA/RNA hybrids (Tian and Alt, 2000b; Tracy *et al.*, 2000) and double stranded DNA breaks (Bross *et al.*, 2000; Tian and Alt, 2000b). The recent finding that both processes are also induced by the same protein, activation-induced cytidine deaminase (AID), has clearly disproved the independence theory of somatic mutation and CSR (Muramatsu *et al.*, 2000). The discovery of AID was even more surprising in terms of its activity. Whereas the mutation/recombinase machinery modifies DNA sequences, AID was found to be an RNA-editing enzyme (Muramatsu *et al.*, 1999). It has therefore been suggested that AID modifies mRNAs encoding the mutating/recombinase enzymes (Muramatsu *et al.*, 2000). An alternative

interpretation is that AID acts directly on the DNA/RNA hybrid formation, which appear prior to DNA cleavage (Tian and Alt, 2000a). In any way, AID is expressed only in GCs of lymphoid tissues, and expression is absolutely necessary for induction of somatic mutation and CSR (Muramatsu *et al.*, 2000). Most important, transfection of AID into fibroblasts results in mutations in an artificial substrate, demonstrating that no other B cell-specific co-factors account for the selective onset of V gene mutations within GCs (Yoshikawa *et al.*, 2002). Whereas the extracellular signals that regulates expression of AID (and hence also somatic mutations and CSR) are poorly defined (discussed in next section), induced AID-expression may be sufficient for triggering the V gene mutations but clearly not for directing CSR. Switching of isotypes depends on cytokine-induced transcription from a specific intron promoter located upstream of each individual C_H gene, producing a germline encoded “sterile transcript” (Stavnezer-Nordgren and Sirlin, 1986). After processing, this sterile transcript forms the needed DNA/RNA hybrid with the complementary C_H locus and in this way makes the so called switch box accessible for site specific recombination (Snapper *et al.*, 1997). Accordingly, also cytokines regulate CSR but are possibly redundant for somatic mutations. It is widely accepted that these cytokines are preferentially provided by the CD4⁺ T cells within GCs. We have, however, in two separate works addressed cytokine production from DCs and from the GC B cells themselves (Paper I and III respectively). In paper I we demonstrate that human *in vivo*-derived DCs can produce several cytokines, including IL-13, which are known to regulate isotype switching and in accordance these DCs were found to trigger a switch to both IgG and IgA in CD40-activated naïve B cells. In paper III we provide evidence for GC B cells secreting IL-4. IL-4, together with IL-13, are critically needed for the CSR to the allergy-associated IgE isotype and has in addition been suggested to play an important role for the development of GC in MALT (Corry and Kheradmand, 1999; Vajdy *et al.*, 1995). Therefore our results indicate that GC B cells can in an autocrine manner direct their own IgE CSR and, furthermore, that their IL-4 production may be important for the maintenance of the GC-reaction.

Signals that promote, shape, and maintain the GC-reaction

The extra-cellular signals which trigger the process of somatic mutation are poorly defined. Under normal physiological conditions help from CD4⁺ T cells

is required, and in this T-B cell contact the CD40 ligand (L) displayed on activated T cells costimulates B cell growth and GC-formation by triggering signaling from CD40 expressed by the B cells (Castigli *et al.*, 1994; Foy *et al.*, 1994; Kawabe *et al.*, 1994). Mutations or other imposed alterations that prevent CD40-CD40L interaction, inhibit or severely impair the development of GCs (and therefore also somatic mutations and isotype switching) (Castigli *et al.*, 1994; Ferguson *et al.*, 1996; Fournier *et al.*, 1997; Kawabe *et al.*, 1994; McAdam *et al.*, 2001). These findings invariably support the idea that CD40L-expressing T cells provide non-redundant signals for the GC-reactions. Nonetheless, transgenic mice that contain a large pool of B cells with a high affinity receptor against the T cell-independent antigen NP-Ficoll, display initial formation of GCs (de Vinuesa *et al.*, 2000) and somatic mutations (Toellner *et al.*, 2002) after NP-Ficoll immunization. Therefore neither T cells nor CD40L provide unique signal(s) for these two events. However, the GCs arising after NP-Ficoll immunization dramatically abort at the time when centrocytes normally are selected by T cells, demonstrating an overall indispensable role for T cells in affinity maturation and development of B cell memory. In accordance to this conclusion, the rescuing of centrocytes from apoptosis and the entry of B cells into the memory cell pathway are critically dependent on CD40-ligation (Gray *et al.*, 1994; Liu *et al.*, 1989).

FDCs have for long been recognized for their extraordinary capability to retain antigens *in vivo* in the form of immune complexes (ICs) (Tew and Mandel, 1979). Similar to T cells FDCs also appear to have dual functions in the GC-reaction. They co-stimulate centroblast proliferation (Li *et al.*, 2000; Zhang *et al.*, 2001), and thereafter they may present ICs with intact antigen for centrocytes, possibly providing means to rescue them from apoptosis and to select for improved affinity (Liu *et al.*, 1989; MacLennan and Gray, 1986; Tew *et al.*, 1997). Whether such FDC-held ICs are necessary for GC-development and affinity maturation, does however represent a controversial issue. By creating an experimental *in vivo* system where FDC lack ICs, Hannum *et al.* (2000) demonstrated a normal formation of GCs and an ordinary frequency and pattern of Ig V gene mutations (indicative of a functional affinity maturation of Abs) in the complete absence of FDC-bound ICs.

By which mechanisms do then FDCs possibly contribute to early formation of GCs and V gene mutation? When Burton *et al.* (1993) isolated high density B cells from nude mice and stimulated these with anti- μ -dextran (thereby cross-linking surface IgM present on naïve B cells), they found a 2-3-fold augmentation in B cell proliferation if also FDCs were added to the cultures. Therefore FDCs appear to provide a microenvironment in B cell follicles favorable for proliferation of also recently antigen-activated B cells. This property may reside in FDC-expressed molecules which can co-stimulate antigen-experienced B cells, including molecules 8D6 (Li *et al.*, 2000) and CD137 (Pauly *et al.*, 2002). In addition, Szakal *et al.* (1988) demonstrated that FDC-associated immune complexes were formed *in vivo* within one day after primary immunization, presented for follicular B cells, and thereafter endocytosed by the B cells. In this way FDCs sequester and concentrate antigens on their surface, perhaps allowing B cells to present MHC II-associated peptides for T cells even under physiological conditions when the soluble antigen-concentration is limiting (Batista and Neuberger, 2000; Kosco-Vilbois *et al.*, 1993). Moreover, these accessory cells are key producers of CXCL13 and hence are critically involved in the organization of primary follicles and zonal distribution of lymphocytes (Ansel *et al.*, 2000; Ngo *et al.*, 1999). Therefore FDCs facilitate the encounter between rare specific T cells and cognate B cells. It is to be noted that in the T cell-independent response against NP-Ficoll described by Toellner *et al.* (2002) Ig V gene mutations were detected also in extra-follicular foci of plasma cells. Somatic mutations can therefore be triggered in the absence of both T cells and FDCs.

Apart from T cells and FDCs, also the complement system regulates GC-associated immune responses. Covalent attachment of activated products of complement C3 to antigen enhances antigen-immunogenicity (Dempsey *et al.*, 1996). Consistently, a number of reports have clearly demonstrated impaired Ab-responses after *in vivo* interference with C3 or the receptors CD21 and CD35, which bind the C3 products C3b and C3d (Gustavsson *et al.*, 1995; Hebell *et al.*, 1991; Heyman *et al.*, 1990; Pepys, 1972). A predominant mechanism for C3b/C3d deposition on antigen in non-immune subjects appears to be antigen-recognition by pre-formed IgM. Such IgM-based ICs dramatically enhance T cell-dependent immune responses in a C3-dependent manner when

administered in mice (Heyman *et al.*, 1988). By creating mice specifically lacking B cell complement receptors Croix *et al.* (1996) could demonstrate that a direct interaction of C3 products with CD21 and/or CD35 expressed on B cells was necessary for T cell-dependent B cell responses. Two separate mechanisms account for the complement-dependent nature of Ab-responses. First, complement-deposition on antigen mediates cross-linking of the BCR and CD21, leading to enhanced signaling from the receptor complex (Croix *et al.*, 1996). Therefore complement lowers the threshold for B cell activation. Secondly, CD21 appears to provide a signal that is independent of antigen-BCR interaction and is required for survival of B cells within GCs (Fischer *et al.*, 1998). Also FDCs express CD21 and CD35, and follicular retention of ICs by FDCs does indeed depend on these receptors (Fang *et al.*, 1998). Regarding FDCs involvement in the complement-dependent enhancement of Ab-responses, there is an apparent conflict between different reports (Ahearn *et al.*, 1996; Fang *et al.*, 1998; Hannum *et al.*, 2000). One possible explanation for such discrepancies may be the use of different immunization regimes since, for example, adjuvant appears to overcome some of the observed impairments in CD21/CD35-deficient mice (Wu *et al.*, 2000).

Taken together it is difficult to distinguish separate mechanism which specifically trigger GC-formation and somatic mutation. I think that these events are regulated in a redundant fashion and mainly take place as a consequence of vigorous antigen-dependent B cell proliferation. This suggestion is also supported by a recent work of Bergthorsdottir *et al.* (Bergthorsdottir *et al.*, 2001), demonstrating that somatic mutations can be triggered *in vitro* after BCR, CD40 and CD38-ligation, a signal-combination also promoting extensive B cell growth. *In vivo*, CD40-ligation is not an absolute requirement for these processes (as discussed above), and CD38 was found to be irrelevant. Under normal physiological conditions, however, the literature supports a scenario where antigen-activated B cells form GCs if activated in the presence of CD40L-expressing T cells, FDCs and activated complement. This does not exclude other regulatory components, and in this context we demonstrated a capacity of tonsil bone-marrow derived DCs to directly co-stimulate CD40-dependent proliferation, isotype switching, and Ab production during the

primary B cell response (Paper I). These results are discussed in Paper I and will also be addressed later on.

4 T HELPER CELL POLARIZATION

The Th1/Th2 paradigm and the continued diversification of T helper cells.

At the center of immune class regulation CD4⁺ T cells transmit, amplify and sustain polarized immune responses. The original division of CD4⁺ T cells into separate T helper (Th) 1 and Th2 subsets was done by Mossman *et al.* (1986) and was based on the finding that murine CD4⁺ T cell clones produced distinct patterns of cytokines in response to antigen or Con A stimulation. Essentially the same cytokine-signatures could be demonstrated for human CD4⁺ T cells, resulting in an establishment of the Th1/Th2 terminology (Romagnani, 1991). The regulatory properties of Th cells in immune responses have since then represented one of the most extensively studied fields within immunological research. The classification of disparate Th cell subsets has also been further diversified and today include Th1, Th2, Th0, Th3, and regulatory Th cells (Tr1), the two latter of which appear to be specialized in maintenance of peripheral tolerance (Groux, 2001).

Th1 cells produce IFN- γ and TNF- α . Th2 cells are distinguished by a robust production of IL-4, IL-5 and/or IL-13, and, importantly, by a complete absence of IFN- γ synthesis. Th0 cells are not so picky in their preference and consequently combine the Th1 and Th2 phenotypes (Romagnani, 1994). Th3 and Tr1 cells secrete TGF- β and IL-10, respectively, and can suppress both Th1 and Th2 responses (Bacchetta *et al.*, 1994; Barrat *et al.*, 2002; Groux *et al.*, 1997; Weiner, 2001). Even though many responses against infectious agents manifest both a Th1 and a Th2 component (Romagnani, 1994), a failure in directing Th cells towards a Th1- or a Th2-oriented response, respectively, can have severe consequences for host welfare. A by now classic example is the infection of different laboratory mouse strains with the intracellular protozoan *Leishmania major*. Infection of C57BL/6 mice provokes Th1 polarization and IFN- γ production which in turn activates macrophages to clear the infection. By contrast, BALB/c mice are susceptible to infection because of a genetic predisposition to develop a Th2-oriented response, where specific Th2 cells impair macrophage functions by producing IL-4. Therefore these mice develop progressive lesions that never heal (Louis *et al.*, 1998). Similar phenomenon can

also be seen among humans, where for example immunological resistance to *Mycobacterium leprae* is associated with a Th1-oriented cytokine profile but Th2-like responses cause progression of leprosy disease (Yamamura *et al.*, 1991). Inappropriate Th cell polarization and/or activation can also cause destructive inflammatory manifestations and tissue damage in response to otherwise innocuous environmental antigens. Allergic diseases represent the key example of such “unnecessary” Th cell responses and appear to evolve as a consequence of disproportional Th2-polarization and IgE isotype switching in genetically predisposed subjects (Parronchi *et al.*, 1991; Robinson *et al.*, 1992; Wierenga *et al.*, 1990). The original interpretation of the allergic phenotype was that atopic individuals developed Th2 responses to antigens which in non-atopic subjects caused Th1-polarization (Kapsenberg *et al.*, 1991; Romagnani, 1994). For example, Wierenga *et al.* (1990) reported that house dust mite *Dermatophagoides pteronyssinus* (Dp)-specific T cell clones established from patients with atopic dermatitis or allergic asthma displayed a Th2 phenotype whereas Dp-specific clones from non-atopic subjects displayed a Th1-like profile. More recent progress in this field indicate that clinical manifestations of allergic disease may not necessarily arise in atopic individuals because of a Th1 to Th2 deviation but may instead reflect a drift from a Th2 response supporting IgG4 class switching to a Th2 response supporting IgE production (Platts-Mills *et al.*, 2001). Interestingly, allergic patients undergoing immunotherapy display the reverse switch (*i.e.* levels of specific IgE drops and specific IgG4 levels increase), and this switch is accompanied by the appearance of allergen-specific and IL-10 producing Tr1 cells (Akdis *et al.*, 1998). It has therefore been suggested that the increased prevalence of atopic diseases in the industrialized world may be caused by an altered balance between Th2 and Tr1 cells in predisposed subjects (Yazdanbakhsh *et al.*, 2002). This further exemplifies the complexity in the regulatory network of Th cells and highlights the importance of understanding how Th cell development is regulated in the first place by *e.g.* APCs.

Effector functions

Th1 cells are primary responsible for DTH reactions. Their cytokine-profile support B cell isotype switching to IgG Abs that fix complement and bind complement receptors and Fc γ receptors preferentially expressed on

macrophages and NK cells. In this way Th1 cells target antigens to effector cells which are potent phagocytes and/or possess cytolytic activity. The Th1-associated secretion of IL-2, IFN- γ and TNF- α also promote differentiation of CD8⁺ T cells into active cytotoxic cells, recruits inflammatory leukocytes and trigger macrophage activation (including nitric oxide release).

Th2 cells support the allergic reaction, which can be an overall beneficial host response against *e.g.* metazoan parasites (round and flat worms) (Finkelman *et al.*, 1997). In general, antibody responses generated under Th2-oriented conditions appear to be more robust and characterized by the production of non-complement fixing isotypes (IgG4, IgE and IgA in humans and IgG1, IgE and IgA in mice). It is, however, important to emphasize that Th2 cells do not represent an unique “B cell-helper subset”, and that responses against several pathogens in mice are characterized by an exclusive production of the Th1- and IFN- γ -associated IgG2a isotype (Toellner *et al.*, 1998). The role for Th2 cells in pathogenesis of atopic diseases is neither limited to their high capacity to provide help for B cells and trigger IgE isotype switching. Indeed, Th2 cells can promote all signature features of allergic tissue inflammation, including IL-5 dependent recruitment and activation of eosinophils, IL-4-mediated activation of mast cells, and IL-4 and IL-13 induced expression of eotaxin from stromal cells. Finally, they can directly instigate the airway remodeling that occurs in patients suffering from allergic asthma (Romagnani, 2001). Therefore Th1 and Th2 cells are equally capable to migrate into peripheral target tissues and orchestrate inflammatory responses (Panina-Bordignon *et al.*, 2001). However, the natures of Th1 and Th2 induced responses are entirely different.

5 DENDRITIC CELLS

The first visualization of DCs, as described in the literature, was done by Langerhans (1868). He identified a cell population in epidermis with a morphological appearance which was not compatible with an epithelial origin. Instead, he suggested that these cells could represent sensory nerve endings, a highly understandable conclusion given the irregular shape of DCs. The realization of DCs as APCs awaited until the beginning of the 1970's when Steinman and coworkers in a number of publications described a novel cell population in the peripheral lymphoid organs of mice (Steinman *et al.*, 1975; Steinman and Cohn, 1973; Steinman and Cohn, 1974; Steinman *et al.*, 1974). These cells displayed a similar irregular and "branching" morphology as originally described for the Langerhans cells (LCs). They were bone marrow derived but could be distinguished from irregularly shaped phagocytes as having a low number of lysosomes and a poor capacity to internalize colloidal particles. It was also Steinman and coworkers who originally termed these cells "dendritic cells" (Steinman and Cohn, 1973) and demonstrated that DCs were superior to other cell types present in the mouse spleen to stimulate proliferation of allogenic T cells (Steinman and Witmer, 1978). The incorporation of LCs into the DC-family occurred gradually. In the context of DC-history it was early noted that LCs could selectively pick up antigens in the epidermal tissue (Shelley and Juhlin, 1977) and, furthermore, that they *in vitro* could stimulate antigen-specific and allogenic T cell proliferation with an efficacy at least similar to macrophages (Green *et al.*, 1980; Streilein *et al.*, 1980; Toews *et al.*, 1980). This led to the conclusion that LCs could represent DCs (Thorbecke *et al.*, 1980). A direct relationship between the LCs and the DCs present in the lymphoid organs was supported by the findings that lymph node DCs localized to the T cell zones (interdigitating DCs; IDCs) contained low levels of the LC-specific organelle Birbeck granule (Kamperdijk *et al.*, 1978). In further support for this notion, purified LCs were found to be immunologically immature, with only a very weak capacity to stimulate T cells, but after cultivation matured into highly efficient stimulators of allogenic T cells (Schuler and Steinman, 1985). The functional significance of these findings became clear when it was demonstrated that LCs and other immature DCs residing in peripheral tissues

acquired antigen at the site of its entry and migrated via the afferent lymphatics into the T cell zones where they provoked antigen-specific T cell activation (Fossum, 1988; Kripke *et al.*, 1990; Macatonia *et al.*, 1987; Moll *et al.*, 1993).

These early studies establish some of the key features of DC-function in relation to adaptive immunity. First, DCs display two functionally distinct stages, one being immature and non-immunogenic and one being mature and stimulatory, and can therefore regulate the activation of specific immunity. Secondly, the immature DCs sample the bodily tissues for the presence of antigens whereas their mature progenies selectively present the acquired antigens for T cells in the lymphoid organs. Accordingly, DCs physically link the peripheral tissues to the lymphoid organs. Finally, in the dose-response assays performed by Steinman and Witmer (1978), the number of DCs in the primary mixed leukocytes reaction (MLR) correlated closely with the MLR response, whereas other spleen leukocytes, including B cells and mononuclear phagocytes, could not support significant T cell proliferation. A widely accepted interpretation of these results is that only DCs can activate naïve T cells and therefore represent the only cells being capable of eliciting the primary immune response (Banchereau *et al.*, 2000).

Given the important role of DCs in the induction of primary immune responses, DC research during the past ten years has to a large extent focused on i) which signals trigger the process of DC-maturation, ii) the role of DCs in immune class regulation, iii) the role of DCs in induction and maintenance of tolerance (thymic and peripheral) and finally, iv) if such regulatory properties of DCs preferentially are conferred by different functions among disparate subsets or rather by a flexibility within single subsets.

Dendritic cell maturation

Immature DCs are strategically poised at the interface of the interior and external environments of the body. Thus they are especially frequent in the epidermis and dermis of the skin as well as in epithelial layers of mucosa. Also lymphoepithelial structures such as the human tonsil (Bjorck *et al.*, 1997) and murine Peyer's patches (Iwasaki and Kelsall, 2000) contain a large number of apparently immature DCs which are localized to the reticulated crypt epithelium

and subepithelial dome, respectively. In humans the intraepithelial DCs are CD1a⁺ LCs whereas dermis and subepithelial compartments contain CD1a⁻ dermal DCs (Cerio *et al.*, 1989; Fithian *et al.*, 1981; Nestle *et al.*, 1993). In Peyer's patches of mice the DC-population appears to be further diversified, but this will not be discussed here (Iwasaki and Kelsall, 2001).

The immature DCs are equipped with wide range of receptors which can mediate internalization of antigens. Some examples are receptors of the carbohydrate-binding lectin superfamily including Langerin (Valladeau *et al.*, 2000), DC-SIGN (Engering *et al.*, 2002; Geijtenbeek *et al.*, 2000), the mannose receptor (Sallusto *et al.*, 1995), and DEC-205 (Jiang *et al.*, 1995). Also Fc receptors are expressed on the DCs, allowing them to utilize antibodies for binding and uptake of antigens (Fanger *et al.*, 1997; Regnault *et al.*, 1999). Moreover, the immature DCs can efficiently phagocytose bacteria, yeast and other parasites (Guermonprez *et al.*, 2002). Internalized antigens are either processed along the endosomal/lysosomal pathway and thereafter redistributed to the cell surface as peptides associated to MHC class II, or alternatively transported into the cytosol for cross presentation on MHC class I (Guermonprez *et al.*, 2002; Heath and Carbone, 2001). More recent findings that DCs can engulf host apoptotic cells for subsequent presentation of antigens derived from these apoptotic cells on both MHC class I and II, demonstrate how these APCs, seemingly in a unique way, can elicit both CD4⁺ and CD8⁺ T cell responses against otherwise "hidden" antigens (Albert *et al.*, 1998; Russo *et al.*, 2000; Yrlid and Wick, 2000).

The process, by which DCs transform from the immature to the mature appearance, is referred to as maturation and is associated with an extensive transcriptional and functional reprogramming of the cells (Paper III and Huang *et al.*, 2001). Even though the nature of the maturation process can vary to a great extent, partially depending on intrinsic features of the stimuli, some key events are conserved regardless variations among the provocative agents. I think that a better way to put it is: some criteria must be met, if DCs shall be able to prime naïve T cells, and these criteria define DC-maturation. First, DCs must be able to interact with the naïve T cells within the T cell zones of lymphoid organs. As described above this interaction occurs with fast kinetics during the

primary immune response, and the directional migration of DCs into the T cell zones is mediated by an induced expression of CCR7 and hence acquired responsiveness to CC21, which besides the HEVs is also present on lymphatic vessels (Saeki *et al.*, 1999; Yanagihara *et al.*, 1998). Secondly, in order to prime naïve T lymphocytes DCs must provide co-stimulation (Jenkins and Schwartz, 1987), and this is achieved by up-regulation of co-stimulatory molecules such as CD40, CD80, and CD86 (Inaba *et al.*, 1994; Larsen *et al.*, 1992; Young *et al.*, 1992). Finally, these events must be coordinated in a temporal fashion to match optimal redistribution of peptide/MHC complexes from lysosomes to the cell surface (Lanzavecchia *et al.*, 1999; Pierre *et al.*, 1997). These three points, migration, co-stimulation, and peptide/MHC redistribution, are the most critical features of DC maturation. Apart from these the maturational process is accompanied with numerous alterations in DC phenotype, including a switch in the usage of adhesion molecules and induced expression of chemokines and pro-inflammatory cytokines (Banchereau *et al.*, 2000). Several of these aspects are covered in Paper III, in which we used gene chip technology to analyze in detail the nature and kinetics of the DC maturation being induced by inflammatory agents.

It is also important to emphasize that migration of DCs towards lymphoid organs occurs under steady state conditions in the absence of obvious maturation and that the net result of this spontaneous migration seems to be induction of T cell tolerance (Hawiger *et al.*, 2001). Since it has been shown that DCs in the Peyer's patches and mesenteric lymph nodes in rat contain DCs which have engulfed apoptotic intestinal epithelial cells, it is likely that the homeostatic migration of DCs into lymphoid tissues represent a physiological process involved in inducing and maintaining peripheral tolerance (Huang *et al.*, 2000).

Since DC maturation represents the key switch for induction of specific B and T cell responses, it is of critical importance to understand how the immature to mature DC transition is activated. In 1992 Janeway suggested that activation of adaptive immunity required recognition of common constituents of pathogenic microorganisms and furthermore wrote, "I consider that receptors for these structures have been selected over evolutionary time to provide broad-spectrum recognition of harmful foreign materials." This idea was based on the discovery

that APCs could be induced to provide co-stimulatory signals if exposed to bacterial lipopolysaccharide (LPS), influenza viruses, or polyinosinic-polycytidylic acid (poly-I:C) (Liu and Janeway, 1991). As a curiosity it can be mentioned that Janeway, in parallel to putting forward his theory of nonclonal recognition of pathogenic motives, also suggested that DCs were “sluggish” in antigen uptake and therefore could be, and were, constitutively active in co-stimulation. DCs were virtually limited to deal with viruses since these were “least likely to trigger innate immunity or induce co-stimulatory signals.” (Janeway, 1992). In 1997 Janeway and colleagues (Medzhitov *et al.*, 1997) described the cloning and characterization of human Toll-like receptor (TLR) 4 and further demonstrated that a constitutively active form of the receptor induced expression of CD80 and inflammatory cytokines. The natural ligand for TLR4 was then shown to be LPS (Poltorak *et al.*, 1998). Presently ten different TLRs have been identified in humans and mice, several of which have been shown to bind evolutionary conserved pathogenic motives (Janeway and Medzhitov, 2002). All these receptors have been identified among human DC subsets (Kadowaki *et al.*, 2001). **Therefore DCs provide the direct link between innate and adaptive immunity.**

Apart from pathogens, DCs can also be induced to mature in response to inflammatory mediators such as TNF- α and IL-1 α (Roake *et al.*, 1995; Sallusto and Lanzavecchia, 1994). This route of DC activation may represent an indirect sensing of pathogens because inflammation can be triggered by pathogen recognition receptors present on cells other than DCs including epithelial cells (Cario and Podolsky, 2000; Diamond *et al.*, 2000; Shuto *et al.*, 2001). Alternatively, intra-cellular constituents being released from *e.g.* necrotic cells may stimulate production of inflammatory cytokines. (Gallucci and Matzinger, 2001; Henson *et al.*, 2001; Matzinger, 1994). A third category of molecules which can mediate DC-maturation is type I interferons (*i.e.* IFN- α and IFN- β). These cytokines are released from virus-infected cells and are potent adjuvant for initiation of specific immunity (Blanco *et al.*, 2001; Le Bon *et al.*, 2001; Santini *et al.*, 2000).

After the discovery of the TLRs it has been put forward that these are essential for inflammatory responses and DC-maturation *in vivo* (Janeway and

Medzhitov, 2002). It has also been suggested that inflammatory mediators are not capable to stimulate complete DC-maturation (Granucci *et al.*, 2001). In paper III we demonstrate that a cocktail of inflammatory mediators (which may more accurately mimic *in vivo* inflammation as compared to experiments performed with TNF- α only) can induce a comprehensive and sustained reprogramming of DCs, in many aspect quite similar to the pathogen-induced DC-maturation (Huang *et al.*, 2001).

Human dendritic cell subsets and CD4⁺ T cell polarization

The unique capacity of DCs to activate naïve T cells has raised the question whether DCs also orchestrate primary Th cell polarization. Two alternative strategies of DCs to accommodate immune class regulation have been addressed. First, in both humans and mice, DCs are heterogeneous in terms of phenotype, function, and tissue distribution. This may lend evidence to the idea that the separate subsets of DCs divide the labor of class regulation, where one subset intrinsically promotes Th1 development and a second subset instructs Th2 differentiation. Secondly, a large number of different pathogen recognition receptors and other receptors involved in recognition of antigen on each of the various DC subsets indicate that DCs themselves are susceptible to functional modulation by antigens and/or their immediate microenvironment. Therefore there may be a “lineage component” and a “plasticity component” in DC regulation of T cell responses as suggested by *e.g.* Liu *et al.* (2001).

Hematopoietic CD34⁺ progenitors from cord blood, bone marrow, or blood differentiate along at least two separate developmental pathways in cultures supplemented with GM-CSF and TNF- α (Caux *et al.*, 1997; Caux *et al.*, 1996). One pathway involves the generation of CD11c⁺CD1a⁺ DCs continuously lacking expression of the monocyte related CD14 surface marker. Morphologically and phenotypically the CD1a⁺/CD14⁻ DCs resemble the epithelial LCs, and their generation is promoted by TGF- β , *in vivo* abundantly secreted by *e.g.* the epidermal keratinocytes. The second pathway furnishes a CD11c⁺CD1a⁻CD14⁺ intermediate which can further differentiate into CD11c⁺ DCs sharing several properties with the *in vivo* dermal and interstitial DCs. Consistent to the intermediate CD14⁺ phenotype of this second pathway, blood monocytes cultured with GM-CSF and IL-4 make up a second source for the

non-LC related DCs (Sallusto and Lanzavecchia, 1994). Interestingly, some degree of reversibility seems to exist in between the two pathways as supplementing the conditioned monocyte cultures with TGF- β allows the generation of LC-related DCs (Geissmann *et al.*, 1998). The physiological importance of TGF- β for LC development is evident from the selective lack LCs in TGF- β knock-out mouse (Borkowski *et al.*, 1996).

Besides the CD34-derived immature DCs, which appear to leave the bone marrow as committed CD11c⁺ DCs (Ito *et al.*, 1999), DCs are also generated in the periphery during inflammatory conditions (Liu, 2001). In accordance to the possibility to use monocytes as DC precursors *in vitro*, phagocytosing human monocytes migrating through a single layer of endothelial cells acquire properties of DCs (Randolph *et al.*, 1998), and inflammatory monocytes that phagocytose injected fluorescent microspheres in mice migrate to lymphoid organs where they express DC-restricted markers and high levels of costimulatory molecules (Randolph *et al.*, 1999). A second putative DC precursor that can perhaps develop DC-functions *in vivo* was first described, in the context of DCs, by Grouard *et al.* (Grouard *et al.*, 1997). These so called plasmacytoid cells are CD11c⁻, arise in the bone marrow (Olweus *et al.*, 1997), and migrate towards sites of inflammation, including inflamed tonsils (Grouard *et al.*, 1997), nasal mucosa of experimentally induced allergic rhinitis (Jahnsen *et al.*, 2000), and cutaneous lesions of lupus erythematosus (Farkas *et al.*, 2001). In addition, they have been found to accumulate at sites of tumor growth (Zou *et al.*, 2001). The lineage classification of the plasmacytoid cells represents a controversial issue with conflicting data supporting either a myeloid (Facchetti *et al.*, 1988; Olweus *et al.*, 1997; Prasthofer *et al.*, 1985) or a lymphoid origin (Res *et al.*, 1999; Spits *et al.*, 2000). Previous discrepancies may, however, be explained by more recent data, suggesting that they represent a phylogenetic unstable subset undergoing cell fate conversion (Comeau *et al.*, 2002). A prominent trait of these cells is their extensive production of IFN- α and β in response to viruses (Cella *et al.*, 1999; Siegal *et al.*, 1999). An analogous population of IFN- α producing cells has recently been demonstrated in mice and was found to be the principal IFN- α producers after *in vitro* exposure to herpes simplex virus (Bjorck, 2001) and influenza virus (Nakano *et al.*, 2001) as well as after *in vivo* infection with cytomegalovirus (Asselin-Paturel *et al.*, 2001).

The plasmacytoid cell may represent *in vivo* precursors for DC differentiation since *in vitro* culturing with IL-3 and CD40L generates cells with typical DC morphology, phenotype, and function (Grouard *et al.*, 1997). Also, viral stimulation can induce plasmacytoid DC-differentiation *in vitro*, perhaps representing a physiological pathway for their conversion into DCs (Kadowaki *et al.*, 2000). However, evidence of a DC-related function of these cells *in vivo* is still very limited (Cella *et al.*, 2000).

The most consistent feature of DCs is that they produce substantial amounts of IL-12 and/or IFN- γ in response to microbes or viruses and consequently direct Th1 differentiation (Barton and Medzhitov, 2002; Cella *et al.*, 2000; Macatonia *et al.*, 1995; Sousa *et al.*, 1997). Cognate interaction with CD40L expressing T cells or maturation in the presence of IFN- γ further enhance their IL-12 production (Cella *et al.*, 1996; Snijders *et al.*, 1998; Vieira *et al.*, 2000). However, the different DC subsets appear to differ intrinsically in their cytokine producing capacity. For example, in mice CD8 $^+$ DCs can readily secrete large amounts of IL-12 whereas CD8 $^-$ /CD11b $^+$ subsets generally appear to secrete lower levels (Pulendran *et al.*, 1997). In accordance to this, the CD8 $^+$ DCs promote production of IFN- γ dependent IgG2a *in vivo*, whereas CD8 $^-$ /CD11b $^+$ subsets facilitate production of the Th2-associated isotype IgG1 (Pulendran *et al.*, 1999). The picture becomes even more complicated since the various subsets of DCs express different patterns of TLRs, leading to different microbes activating different populations of DCs (Hornung *et al.*, 2002; Jarrossay *et al.*, 2001; Kadowaki *et al.*, 2001). Furthermore, a recent study addressing DC-responses against cytomegalovirus in mice revealed that IFN- γ producing CD8 $^+$ DCs suppressed IL-12 production from CD11b $^+$ DCs, establishing a cross-regulatory mechanism for *in vivo* regulation of cytokine production from these cells (Dalod *et al.*, 2002). Taken together, it is difficult to make conclusions regarding host-responses based on *in vitro* studies focused on a single DC population.

Concerning the occurrence of DC subsets in human being differentially prone to promote Th1 and Th2 responses, respectively, the CD11c $^-$ DCs generated *in vitro* from plasmacytoid cells (PDC) in the presence of IL-3 and CD40L were first described as poor producers of IL-12 and to preferentially support Th2-

differentiation *in vitro* (Pulendran *et al.*, 2000; Rissoan *et al.*, 1999). However, the interpretation of a Th2-skewing capacity of the PDCs was essentially based on the vigorous IL-10 production these cells triggered from naïve CD4⁺ T cells. In humans IL-10 does not represent a prototypic Th2 cytokine (Sornasse *et al.*, 1996). Instead, as mentioned in previous chapter, IL-10 can suppress both Th1 and Th2 associated proliferation and differentiation (Groux *et al.*, 1997). Subsequent studies have indeed confirmed that PDCs can render antigen-specific CD4⁺ T cells unresponsive (Kuwana *et al.*, 2001). In addition, they appear to be capable to assist in the generation of IL-10 producing CD8⁺ regulatory T cells (Gilliet and Liu, 2002). Finally, in direct discordance to the results published by Rissoan *et al.* (1999), Cella *et al.* (2000) showed that PDCs promoted strong Th1 polarization in response to both influenza virus and CD40L stimulation. In line with this finding CpG oligodeoxynucleotides, mimicking bacterial DNA, synergize with CD40L stimulation to induce IL-12 production from PDC (Krug *et al.*, 2001). Presently, there do not exist any solid and consistent evidence for a human DCs-lineage being intrinsically specialized in supporting Th2 differentiation.

The TLRs signals via adapter protein MyD88 and MyD88-deficient mice fail to raise Th1-responses after immunization with antigen mixed with bacterial adjuvant (Kawai *et al.*, 1999; Schnare *et al.*, 2001). Yet, in these mice B cells produce the IL-4-dependent IgG1 and IgE isotypes under identical conditions. In addition, the MyD88-deficient mouse can raise perfect (even increased) Th2-oriented responses when immunized with alum as adjuvant (non-bacterial stimuli) (Schnare *et al.*, 2001). These results demonstrate that TLRs are not essential for the Th2 development and suggest that DCs must utilize a different kind of receptors to be able to directly promote Th2-differentiation. In a pioneering study Fè d'Ostiani *et al.* (2000) demonstrated that this very well may be the case. By analyzing DC-function in relation to yeast and hyphae of the fungus *Candida albicans*, they provided most convincing evidence that DCs were able to discriminate between the two forms of the fungus. Both yeast and hyphae were phagocytosed but ingestion of yeast primed DCs for IL-12 production whereas phagocytosed hyphae did not induce IL-12 but instead activated DCs to produce IL-4. Consequently, when exposed to yeast form of *C. albicans* DCs primed IL-12 dependent Th1 differentiation *in vivo* and, in

marked contrast, when exposed to hyphae, IL-4-dependent Th-2 polarization. Therefore, although DCs appear to secrete IL-12 in response to most microorganisms, this study clearly demonstrate that these cells possess a certain degree of flexibility in their regulation of CD4⁺ T cell responses. Indications of similar capacity of DCs to modulate T cell responses in other murine experimental systems have later been reported. (Pulendran *et al.*, 2001; Whelan *et al.*, 2000). The contention that DCs directly can instruct Th2-differentiation was most recently further substantiated in a study with human cells, showing that thymic stromal lymphopoietin, secreted by epithelial cells at sites of allergic inflammation, matured CD11c⁺ DCs *in vitro* to promote a Th2 development characterized by substantial production of IL-5 and IL-13 but only limited amounts of IL-4 (Soumelis *et al.*, 2002).

Overall, I believe that it is important to consider the influence of DCs on CD4⁺ T cell polarization in the context of the spatial and temporal nature of the primary immune response. DCs rapidly migrate into the T cell zones of lymphoid tissues where they interact with specific T cells for a limited period of time, let us say 15 hours (Stoll *et al.*, 2002). Even if optimally primed for IL-12 production, DCs can only provide the cytokine at an early stage of this interaction since IL-12 synthesis displays a fast and transient kinetics with a peak in production at approximately eight hours after LPS-induced maturation (Langenkamp *et al.*, 2000). After that DCs rapidly downregulate their IL-12 production and in addition become refractory to further IL-12 inducing stimuli, *in vitro* (Kalinski *et al.*, 1999) as well as *in vivo* (Reis e Sousa *et al.*, 1999). Since stable polarization of T cells requires modulation of chromatin structure at the different cytokine loci (Agarwal and Rao, 1998) and this process generally occurs only after several cell cycles (Grogan *et al.*, 2001), T cells remain rather susceptible to further polarizing instructions, even after a prolonged period of time (Murphy *et al.*, 1996). This means that after their initial priming on DCs in the T cell zone, the naïve T cells may not be committed to differentiate towards a fixed cytokine-producing phenotype and therefore subsequent encounter with other APCs in the lymphoid organ can enhance or redirect the polarization process (Paper III and IV and, MacDonald and Pearce, 2002). Moreover, the demonstration of *in vitro* induced IFN- γ production in allergen-specific human Th2 cell lines suggests that T cells can be redirected in their cytokine production

at all stages of an immune response (Parronchi *et al.*, 1999). Moreover, the long-lasting persistence of antigen-presenting DCs in the inflamed airways of mice exposed to *Leishmania* LACK antigen may even indicate that T cells can be further polarized after having left the lymphoid tissue, perhaps in the periphery at sites of chronic inflammation (Julia *et al.*, 2002). Finally, although I, on purpose, have chosen to restrict the discussion concerning CD4⁺ T cell polarization to the influence of APCs and their cytokine production, it is important to emphasize that several other parameters accounts for the observed skewing of T cell responses. For example, different epitopes on a single protein antigen can elicit different Th cell responses (Parronchi *et al.*, 1998). Also a single point mutation in a TCR can dramatically alter the skewing towards Th1 or Th2, suggesting that the chemical and structural nature of the contact surface between the TCR and the peptide/MHC class II complex has an impact on the process of Th cell polarization (Blander *et al.*, 2000). Similar, a single point mutation in a protein antigen can completely redirect the Th cell response *in vivo* (Lee *et al.*, 2000). To summarize, DCs possess a remarkable ability to regulate Th cell polarization, in particular because of their robust but regulated IL-12 production. Also other DC derived molecules appear to directly modulate T cell responses, including IL-4 in mice. However, a number of parameters other than DCs play most important roles in CD4⁺ T cell polarization *in vivo*.

Dendritic cell – B cell interactions

As mentioned in chapter 3 somatic mutation and isotype switching occur within GCs and are to a large extent dependent on help provided by CD4⁺ T cells and in particular on CD40L expressed by these T cells. CD40L-expressing T cells are generated by DCs early during the primary immune response, and cognate interaction between specific T and B cells also take place at an early stage. Based on the extraordinary capacity of DCs to modulate primary immune responses as well as on the possibility that DCs, T cells and B cells could interact simultaneously during the primary immune response (MacLennan *et al.*, 1997), Dubois et al (1997) addressed the direct effect of DCs on CD40L-activated B cells. Their results demonstrated that DCs generated *in vitro* from CD34⁺ precursors 1) strongly enhanced the CD40-dependent proliferation of B cells and 2) increased the CD40-dependent Ab-secretion from both naïve (IgM) and memory B cells (IgG and IgA) in the presence of IL-2. An *in vivo* relevance

for these results was further supported by the finding that human IDCs isolated from tonsil had similar effect on the B cells (Bjorck *et al.*, 1997). Subsequent studies revealed that DC-produced IL-12 was synergistic with added IL-2 in driving plasma cell differentiation from the naïve B cells in the cultures based on the CD34⁺-derived DCs (Dubois *et al.*, 1998).

In Paper I we continued the investigation of tonsillar IDCs in relation to CD40-activated naïve B cells. Our results demonstrate that these CD11c⁺ DCs cannot only elicit IgM production from naïve B cells but also directly trigger CD40-dependent production of isotype switched IgG and IgA. These effects were strictly mediated by released molecules from the IDCs including IL-13 and possibly IL-6 and IL-10. We could not, however, detect secretion of IL-12 after CD40 stimulation (unpublished observations), suggesting that these cells at least partially differ from the *in vitro* generated DCs in the mechanism by which they promote B cell differentiation.

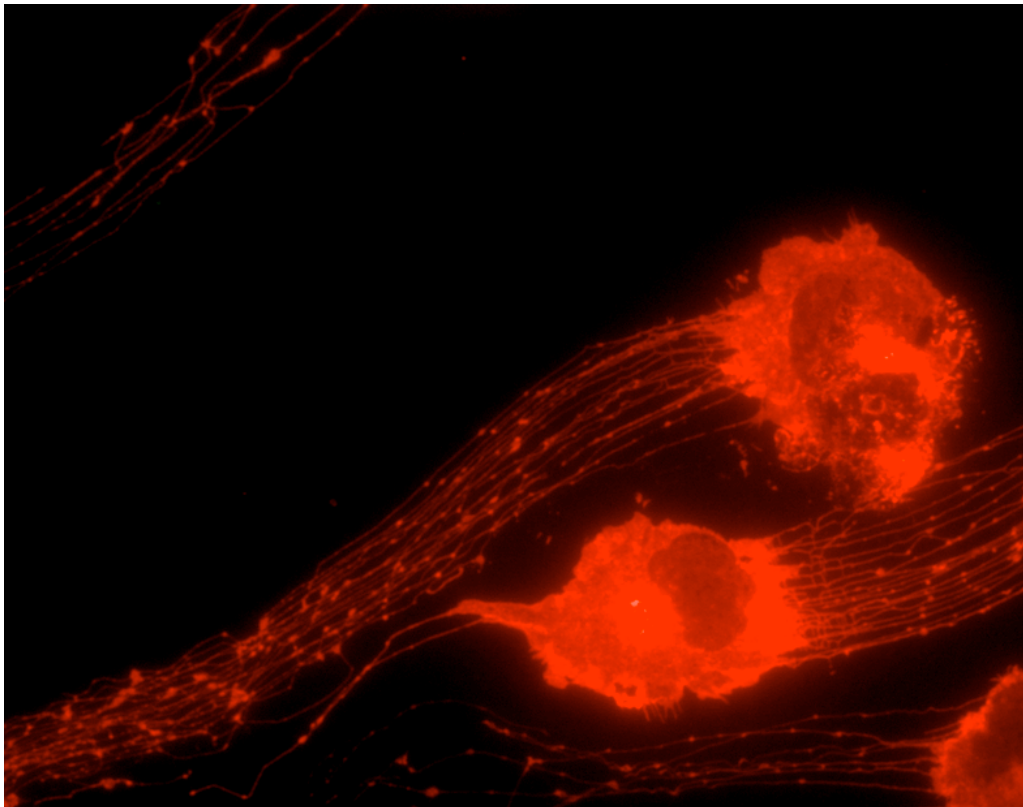


Figure 1 Human IDCs isolated from tonsil as described in Paper I. Cells were visualized by staining with an antibody against MCH class II

6 ANTIGEN PRESENTATION BY B CELLS

While DCs evidently are superior to other APCs in their capability to prime naïve T cells, B cells have been described to completely lack such capacity (Lassila *et al.*, 1988). Even though this represent a controversial issue (Constant *et al.*, 1995; Morris *et al.*, 1994), the probability that a specific B cell should encounter a specific naïve T cell must be considered as extremely low. B cells and T cells are segregated in the lymphoid organ and physical interaction between these cells depends upon a deviation from their homeostatic distribution. Naïve T cells change their migratory behavior first after antigen-specific interaction with APCs, preferentially DCs, in the T cell zone. The physical interaction between specific T and B cells therefore occurs in the context of a sequential T cell priming and under these circumstances B cells can efficiently support T cell proliferation and differentiation (Fuchs and Matzinger, 1992; Harris *et al.*, 2000). They may even be essential for the development of fully differentiated effector and memory T cells (Falcone *et al.*, 1998; Linton *et al.*, 2000).

Follicular helper T cells

Although GCs are mostly considered in terms of B cell proliferation and differentiation, they can also harbor an extensive growth of CD4⁺ T cells which are specific for the immunizing antigen (Fuller *et al.*, 1993; Gulbranson-Judge and MacLennan, 1996). As mentioned in chapter 2 the early priming of naïve CD4⁺ T cells on DCs in the T cell zone gives rise to at least two separate T cell differentiation pathways. One pathway generates a rapid development of tissue-homing effector cells, producing preferentially IFN- γ . The other pathway directs apparently non-polarized T cells towards the follicles in a CXCR5-dependent manner (Campbell and Butcher, 2002; Campbell *et al.*, 2001; Kim *et al.*, 2001). When mice are immunized with soluble protein antigens mixed with adjuvant, the initial T cell zone associated T cell division is accompanied by follicular homing of CD4⁺CXCR5⁺ T cells. This is followed by a gradual redistribution of T cell-proliferation from the T cell zone to the growing GC (Ansel *et al.*, 1999; Gulbranson-Judge and MacLennan, 1996; Kearney *et al.*, 1994). In fact, during these circumstances, T cells proliferate only transiently in the inter-follicular

areas and at the peak of the response essentially all specific CD4⁺ T cells are localized to the GCs where they continuously undergo cell division. Thereafter, the number of specific T cells in the lymphoid tissue starts to decrease in spite of the fact that a substantial number of proliferating T cells still can be detected within the GCs (Gulbranson-Judge and MacLennan, 1996; Kearney *et al.*, 1994). This imbalance clearly suggests that GC-localized T cells ultimately make up a second source of tissue-homing T cells, which accordingly exhibit a delay in their migration towards the peripheral tissues.

The magnitude of the GC-associated T cell proliferation varies considerably between different antigens and immunization regimes. First, if protein antigens are immunized intravenously without adjuvant, T cells proliferate in the paracortex of lymph nodes but fail to enter the B cell follicles (Kearney *et al.*, 1994). Although this protocol of immunization support the development of tissue-homing T cells, the overall response is short-lived and the few T cells which remain in the lymph nodes are refractory to a secondary antigenic challenge (Kearney *et al.*, 1994; Reinhardt *et al.*, 2001). The failure of T cells to enter the B cell follicles and the overall reduced T cell response, can both be explained by an insufficient maturation of DCs in the absence of microbial adjuvant (Ansel *et al.*, 1999). Induction of CXCR5 expression on T cells depends upon costimulatory signals provided by DCs (Brocker *et al.*, 1999; Flynn *et al.*, 1998; Walker *et al.*, 1999) and naïve T cells do not upregulate CXCR5 at high levels in response to TCR-triggering only (Schaerli *et al.*, 2001). Secondly, the degree of GC-associated T cell proliferation can also to a large extent vary between two different antigens which both provoke the development of fully differentiated effector and memory T cells (Luther *et al.*, 1997; Toellner *et al.*, 1998).

Transmission of a reverse signal – a role for GC B cells in the Th2 development?

Th2 cells have for long been recognized as the most important Th cell subset in relation to B cell differentiation and GC development. However, this interpretation has mainly been based on *in vitro* studies of established Th1 and Th2 clones or on demonstrations of a prominent role of Th2-derived cytokines in supporting *in vitro* B cell differentiation and Ab production (Paul and Seder,

1994). More recently, it has however been demonstrated that *in vitro* polarized Th1 and Th2 cells are equally capable of supporting B cell clonal expansion and Ab production *in vivo* (Smith *et al.*, 2000). Even more important, the follicular Th cells arising *in vivo* during a primary immune response are neither Th1 nor Th2 cells. Instead, as previously mentioned, they appear to represent non-polarized T cells at the time when they make cognate interaction with specific B cells. A meaningful question to ask is therefore; how do the B cells influence the cytokine profile of the follicular Th cells?

Th1 polarization is strongly promoted by the presence of IL-12 that can be produced in large quantities by *e.g.* DCs and macrophages. The most important factor in the Th2 development is IL-4 (Kopf *et al.*, 1993; Maggi *et al.*, 1992; Shimoda *et al.*, 1996). In Paper II we demonstrate that a predominant cellular source of IL-4 in human tonsil is the GC B cells. Based on this finding we suggested that GC B cells might be important for the Th2-development *in vivo*. In Paper IV we provide evidence for this suggestion by demonstrating that GC B cells can induce IL-4 and IL-13 production from CD4⁺ T cells *in vitro*. Consistent to this finding, we also characterize a Th2 precursor cell subset in the tonsil that uniformly display a GC associated phenotype (*i.e.* high levels of CXCR5 expression). Taken together, paper II and IV reveal an important role for the GC B cells in the development of Th2 effector cells. These papers thus shed new light on previous investigations, delineating an inherent property of B cells to preferentially support Th2 polarization (Macaulay *et al.*, 1997; Mason, 1996; Secrist *et al.*, 1995; Stockinger *et al.*, 1996). However, in contrast to these earlier works, we define (i) the mechanism by which the B cells instruct T cells to differentiate along the Th2 pathway and (ii) the physiological microenvironment in which the polarization process take place. GCs and GC B cells may accordingly be as important for the functional development of cytokine-producing T cells as the T cells are for the GC-associated B cell differentiation.

7 CONCLUDING REMARKS

This thesis is based upon four investigations addressing regulatory properties of DCs and B cells during adaptive immune responses. Both of these cell types can internalize, process, and present MHC class II associated antigens for CD4⁺ T cells but only DCs appear to be relevant APCs in the priming of naïve T cells. Two different reasons can explain the superior capacity of DCs, as compared to the B cells, to initiate primary T cell responses. First, DCs can mature into highly stimulatory APCs expressing high densities of MHC class II and costimulatory molecules. Even though also B cells upregulate these molecular entities the relative levels are seemingly not sufficiently high to prime naïve T cells. Secondly, the spatial and temporal nature of the primary immune response hinders the physical interaction between specific T and B cells unless the T cells not first are primed on DCs in the T cell zones of the lymphoid organs. Consequently, innate immunity and DCs are of critical importance when it comes to the triggering of the specific immune response.

Our data in paper I and III reinforce the important role of DCs during the early phase of adaptive immune responses and demonstrate that these innate cells directly can support also primary B cell responses. Moreover, in paper III we show that DCs potentially can trigger naïve T and B cells in the absence of pathogenic microbes since endogenously derived inflammatory agents can induce a comprehensive and sustained DC maturation. However, in contrast to their apparently unique function in the recruitment of the specific immune response, DCs are not the only cells participating in immune class regulation. The results presented in paper II and IV reveal how antigen-activated B cells within GCs instruct CD4⁺ T cells to differentiate along the Th2 pathway. Therefore Th cell polarization may be subjected to a counterbalanced regulation, where DC-produced IL-12 and/or IFN- γ / β promote Th1-differentiation and B cell-produced IL-4 furnish the Th2-development. Considering that Th2 cells and the cytokines they secrete are essential for the pathogenesis of allergic diseases, these results may have implications on future allergy-related research.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Människans immunsystem utgörs av en komplex sammansättning av celler och molekyler, som interagerar med varandra men även med kroppens övriga vävnader. Dess huvudsakliga uppgift är att försöka sin värd med ett försvar mot yttre och inre förhållanden, som kan orsaka denna skada, t.ex. bakterie- och virusinfektioner eller betingelser för utveckling av cancer. Denna försvarsaktivitet måste dock kontinuerligt balanseras på ett sådant sätt, att immunförsvaret inte angriper kroppsegna vävnader eller sätter igång reaktioner av onödig styrka eller missriktad karaktär. Immunförsvaret måste således uppvisa specificitet, aktiveras endast då uppenbara behov föreligger samt urskilja vilka försvarsmekanismer, som är lämpligast i varje enskilt fall. Vi kan t.ex. vanligtvis uthärda feber i samband med en maginfluensa men skulle knappast stå ut med motsvarande symptom varje gång vi åtar oss. Detta påstående kan tyckas vara naivt, men för t.ex. en nötallergiker kan maten bli betydligt mer besvärande än en enkel maginfluensa.

B- och T-lymfocyter utgör de celler som ger immunförsvaret dess specificitet. Varje enskild lymfocyt har ett antal identiska och för den enskilda lymfocyten unika receptorer på sin yta. Dessa receptorer kan känna igen en unik substans (antigen). Under en pågående immunrespons aktiveras endast de lymfocyter, som bär receptorer som kan binda till det aktuella främmande ämnet, t.ex. ett virus. Dessa lymfocyter kommer att öka kraftigt i antal och därefter utsöndra molekyler, som kan neutralisera smittämnet (antigenet). B-lymfocyten utsöndrar antikroppar och T-lymfocyten speciella signalsubstanser, s.k. cytokiner. Cytokiner kan i sin tur aktivera andra av immunförsvarets celler (inklusive B-lymfocyter), så att dessa också kan bidra till att eliminera det antigen, som utlöste reaktionen. Denna aktivering är alltså selektiv, och såväl B- som T-cellen bidrar till att endast vissa av immunförsvarets celler och molekyler rekryteras vid en given infektion.

Denna avhandling upptar fyra experimentella arbeten, i vilka olika mekanismer för reglering av B-cellens antikropsproduktion och T-cellens

cytokinproduktion har undersökts. B-cellers förmåga att styra T-celler har också studerats. Vidare har s.k. dendritiska celler analyserats i relation till hur dessa påverkar B- och T-cellers aktivitet. Slutsatser presenteras i de enskilda arbetena samt i den inledande summeringen av B-, T- och dendritiska cellers interaktioner under immunreaktioner.

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