STUDIES OF INTERLEUKIN-9 RECEPTOR EXPRESSION AND FUNCTION ON HUMAN TONSILLAR B CELLS

By

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Dedication

To my parents, who offered me the gift of unconditional and unselfish love, who believed in me and helped me realize my dreams

To my life companion Ehssan, who supported me every step of the way, with whom I share the quest for knowledge and the commitment to fulfill one's ambitions

To my wonderful daughter Zaynab whom I cherish, and my beloved son Mohammad

To my brother Tarek and his lovely daughter Catalina with all my love

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ABSTRACT

Interleukin-(IL)-9 is a pleiotropic cytokine secreted by activated Th2 cells. Interleukin-9 and the α-chain of the IL-9 receptor (IL-9Rα) have been shown to affect the differentiation pathway of various cell types including human T cells, eosinophils, and mast cells, all key players in allergic inflammation. However, there is little information regarding the differentiation effect of IL-9 on B cells. Specifically, there are no reports describing the expression of the IL-9Rα on human B lymphocytes. To gain a better understanding of the effect of IL-9 on human B lymphocytes and its role in the B-Th immune synapse in the germinal center, we sought to determine the expression of the IL-9Rα on human tonsillar B cells. This allows us to study the expression of the IL-9Rα on B cells at different stages of their antigen-driven maturation, in the context of B-T cell interactions. Human tonsillar B cells were fractionated, using a discontinuous percoll density gradient, into three populations representing different stages of B lymphocyte maturation: low density (LD) (recovered in the 30-50% Percoll fraction), which represents follicular mantle (FM) B cells and germinal center (GC) B cells, medium density (MD) (50-60%), which are primarily centroblasts and centrocytes, and high density (HD) (>60%) or more mature B cells. We have determined the expression of the IL-9Rα chain by immunocytochemistry, FACS analysis and immunohistochemistry. Immunocytochemistry was performed on freshly isolated purified human tonsillar B cells using a monoclonal antibody to the alpha chain of the IL-9R. This clearly demonstrated the presence of the IL-9Rα protein on tonsillar B cells. Using FACS analysis, unfractionated as well as fractionated B cells showed positive immunoreactivity. However, IL-9Rα expression was predominantly higher in the LD fraction of the B cells (39%), as compared to the MD (22%), and HD fractions (16%). These findings prompted us to further investigate the precise localization of the IL-9Rα positive B cells within the germinal centers. For this purpose, immunohistochemistry was performed on sections of human tonsils. Using double staining with anti-IL-9Rα and anti-CD20 (Alkaline Phosphatase anti-Alkaline Phosphatase APAAP) as well as with anti-IL-9Rα and anti-CD19 (immunofluorescence), we found that CD20+/CD19+ B cells within the secondary lymphoid follicles, and especially cells on the

edge of these follicles, displayed IL-9Rα. Furthermore, analysis of CD38 and IgD expression on IL-9Rα positive cells by three color flow cytometry showed that IL-9Rα was expressed on FM B cells (CD38-IgD+) and GC cells (CD38+IgD+). Upregulation of phosphotyrosine levels in LD cells stimulated with IL-9 as well as signal transducers and activators of transcription (STATs) phosphorylation was detected by immunoblotting and flow cytometry respectively. The results show a specific upregulation of STAT-5 and STAT3 phosphorylation in LD cells and not in total B cells, MD or HD cells. In order to better define the effect of IL-9 on follicular B cells, we hypothesized that IL-9 played a role in the B-Th immune synapse in GC by promoting germinal center isotype switching, and affecting IgE production consequently. In order to directly assess the effect of IL-9 on IgE production, LD cells, which are germinal center cells expressing the highest level of IL-9Rα in our analysis, were stimulated with an antibody to CD40 and IL-9 in the presence or absence of IL-4 and IgE production was measured by ELISA. Although IL-9 was unable to induce IgE secretion by itself, it potentiated IgE production mediated by IL-4. This suggests a synergistic role for IL-9 with IL-4 in modulating IgE production and B cell differentiation. This was accompanied by an upregulation of IL-9R and CD27 expression on LD cells throughout the culture period specifically following CD40 and IL-4 stimulation. No further increase in expression levels was observed in the presence of IL-9. Furthermore, CD27 upregulation occurs almost entirely on IL-9R positive cells, pointing to an important role for IL-9 downstream of CD40 and IL-4 signaling in B cell differentiation. The observed increase in IgE production was not due to an increase in cell number as IL-9 was not able to potentiate IgM-activated LD B cell proliferation, nor anti-CD40-activated LD B cell proliferation. Moreover, no synergistic effect for IL-9 on IL-4 induced LD B cell proliferation was observed. However, IL-9 protected LD B cells from Fas-induced apoptosis, suggesting an important role for IL-9 in B cell survival. In summary, IL-9 synergizes with IL-4 to augment IgE production in LD cells, it does so by enhancing cell survival rather than by increasing cell number.

These data show for the first time that a functional IL-9Rα chain is expressed on human follicular germinal center B cells. Taken together, our studies establish a new

understanding for the role of IL-9, a cytokine linked to increased susceptibility to allergy and asthma, in B cell maturation and IgE production.

SOMMAIRE

L'Interleukin-9 (IL-9) est une cytokine pléiotropique sécrétée par les lymphocytes T de type 2 (Th2). L'IL-9 et la chaîne α de son récepteur (IL-9Rα) sont impliquées dans la différentiation de divers types cellulaires incluant les lymphocytes T humains, les éosinophiles, les cellules 'mast', tous jouant un rôle dans l'allergie inflammatoire. Cependant, il existe peu d'information concernant l'effet de l'IL-9 sur la différentiation des lymphocytes B. Dans le but de mieux caractériser cet effet, nous avons déterminé l'expression du récepteur de l'IL-9 (IL-9Rα) sur les cellules B humaines des amygdales. Ce choix nous permet d'étudier l'expression de l'IL-9Rα sur les lymphocytes B à différentes étapes de leur maturation en périphérie suite à l'exposition à un antigène dans le contexte des interactions des lymphocytes B et T. Les lymphocytes B purifiés des amygdales ont été fractionné en utilisant un gradient sur Percoll (discontinuous percoll density gradient) en trois populations représentant différents stades de maturation des lymphocytes B: les lymphocytes B de faible densité (retrouvés dans la fraction de percoll 30-50%), représentant les cellules B germinales ou les cellules B 'LD', les lymphocytes B de densité moyenne (50-60%), qui sont principalement constitués de centroblastes et de centrocytes ou 'MD', et les lymphocytes B de haute densité récupérés à > 60% constitués de cellules matures inactives en majorité (HD). Nous avons déterminé l'expression de l'IL-9Rα par immunocytochimie, FACS et immunohistochimie. L'immunocytochimie sur des lymphocytes B d'amygdales fraîchement isolées et purifiées a été faite à l'aide d'un anticorps monoclonal dirigé contre la chaîne alpha du récepteur de l'IL-9. Ceci démontre clairement la présence de la protéine IL-9Rα sur les lymphocytes B des amygdales. L'analyse par FACS de cellules B fractionnées ou non sur un gradient de Percoll a montré la présence de l'IL-9Rα sur les cellules B. Nos résultats montrent une expression nettement élevée chez les cellules B germinales (39%), comparées aux cellules MD (22%), et les cellules HD (16%). Ces résultats nous ont incité à rechercher la localisation précise de l'IL-9Rα dans les centres germinaux. Pour réaliser cet objectif, nous avons effectué un double marquage de sections d'amygdales, en utilisant un anticorps dirigé contre l'IL-9Rα et un autre contre CD20 (APAAP) ainsi qu'un anti-IL-

9Rα et anti-CD19 (immunofluorescence). Nous avons montré que les cellules B CD20+/CD19+ dans les follicules lymphoïdes secondaires, et particulièrement les cellules aux bords de ces follicules, exprimaient l'IL-9Rα. De plus, l'analyse par cytométrie de flux de l'expression de CD38 et IgD sur les cellules positives pour l'IL-9Rα a montré que l'IL-9Rα est exprimé sur les cellules B folliculaires ou 'FM' B cells (CD38-IgD+) et les cellules B germinales GC (CD38+IgD+). L'augmentation en expression de la phosphotyrosine chez les cellules de faible densité (LD) ainsi que la phosphorylation des molécules STATs ont été montrées par la cytométrie de flux et par western blot. Les résultats montrent une augmentation de la phosphorylation de STAT5 et STAT3 uniquement chez les cellules LD et non chez les cellules B non fractionnées, les cellules MD ou HD. Afin de vérifier l'hypothèse selon laquelle l'IL-9 affecte les cellules B folliculaires en induisant un 'germinal center isotype switching', et par conséquent en influant la production d'anticorps IgE, nous avons décidé de vérifier directement l'effet de l'IL-9 sur la production IgE : les cellules LD, exprimant le plus haut niveau d'IL-9Rα selon notre analyse, ont été stimulées avec un anticorps dirigé contre CD40 en présence ou en absence d'IL-4 et la production d'IgE a été mesurée par ELISA. Bien que l'IL-9 était insuffisante pour induire la sécrétion d'IgE, elle a augmenté la production d'IgE médiée par l'IL-4. Ce qui suggère l'existence d'une synergie entre l'IL-9 et l'IL-4 dans la modulation de la production d'IgE et la différentiation des lymphocytes B. De plus, nous avons noté une augmentation de l'expression de l'IL-9Rα et de CD27 sur les cellules LD stimulées spécifiquement en présence de l'anti-CD40 et l'IL-4 au cours de la culture cellulaire. Il n'y a pas eu d'augmentation en présence de l'IL-9. L'uprégulation de CD27 a lieu presque spécifiquement sur les cellules IL-9R+, suggérant un rôle important de l'IL-9 suivant la signalisation par CD40 et IL-4 dans la différentiation des lymphocytes B. L'augmentation de la production d'IgE n'était pas due à un nombre de cellules accru car l'IL-9 n'a pas augmenté la prolifération des cellules LD stimulées par IgM ou CD40. De plus, l'IL-9, associée à l'IL-4, n'a pas augmenté la prolifération cellulaire. Par contre, l'IL-9 a un effet protecteur contre l'apoptose induite par Fas, impliquant l'IL-9 dans la survie des cellules B. En résumé, l'IL-9 synergise avec l'IL-4 afin d'augmenter la production d'IgE chez les lymphocytes B LD, et ce en affectant la survie cellulaire plutôt que la prolifération.

Cette étude démontre, pour la première fois, l'expression d'un récepteur fonctionnel de l'IL-9 sur les cellules B folliculaires germinales humaines. En effet, nos résultats établissent un nouveau rôle pour l'IL-9, une cytokine liée à la susceptibilité à l'allergie et l'asthme, dans la maturation des lymphocytes B et la production d'IgE dans le contexte de la synapse entre les cellules B et T dans les follicules secondaires.

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LIST OF ABBREVIATIONS

CD	**Cluster of differentiation**
MHC I/II	**Major histocompatibility complex class I or II**
p-MHC	**peptide-MHC**
APC	**Antigen presenting cell**
DC	**Dendritic cell**
BM	**Bone marrow**
BCR	**B cell receptor**
TCR	**T cell receptor**
IgH	**Immunoglobulin heavy chain**
IgL	**Immunoglobulin light chain**
Fas-R	**Fas receptor**
TNF	**Tumor necrosis factor**
IC	**Immune complex**
FDC	**Follicular dendritic cell**
Th	**T helper**
GC	**Germinal center**
SHM	**Somatic hypermutation**
CSR	**Class switch recombination**
IL	**Interleukin**
SL	**Surrogate light chain**
AICDA	**Activation induced cytidine deaminase**
CCR	**Chemokine receptor**
JAK	**Janus kinase**
STAT	**Signal transducer and activator of transcription**
ELISA	**Enzyme linked immunosorbent assay**
LD	**Low density**
MD	**Medium density**
HD	**High density**

STATEMENT OF ORIGINALITY

The following aspects described in this thesis are considered as contributions to original knowledge:

We have provided clear evidence for the expression of IL-9Rα on human LD tonsillar B cells, and demonstrated the ability of this receptor to transduce signals through activation of STAT-3 and STAT5 transcription factors. We also showed the ability of IL-9 to potentiate the production of IgE from LD cells in the presence of anti-CD40 and IL-4. This effect was shown to be accompanied by an upregulation of CD27 expression on IL-9Rα+ LD B cells which constitutes a new finding defining the phenotype of 'IgE producers' in the context of CD40 stimulation of B cells. Together, we provide clear evidence for IL-9's role in the immune synapse formation in the late phase of B cell differentiation in the secondary lymphoid follicles.

CHAPTER 1. INTRODUCTION

I. The Basis of the Immune Response to Infection

Our immune system's main function is to combat foreign living microorganisms that may invade our bodies. The response operates on two levels: an automatic unrefined process which cannot be further improved through learning known as 'the innate defense mechanism', and a memory based evolving response that acts in conjunction with the innate response to provide vigorous protection efficient in combating extracellular and intracellular infections known as 'the specific acquired response'. The key factors contributing to the initiation of these two types of immune responses are the pathogens, which when introduced to the host are recognized and eliminated. Based on their differential ability to detect the presence of infectious agents and dispose of the invaders, the two distinct immune-recognition systems have been broadly categorized as 'innate' and 'adaptive'.

I.A. Innate Immunity

From plants to humans, multicellular organisms are endowed with an inborn set of mechanisms that ensures their survival upon encounter with pathogens (bacteria, fungi, viruses, etc..). This is referred to as innate immunity. It is the initial response to infection that occurs immediately or within several hours after exposure to an antigen to eliminate microbes and prevent infection. It is mediated by antigen-nonspecific defense mechanisms. This phase is essential for the subsequent development of the adaptive immune response. As argued by Douglas T. Fearon of the University of Cambridge, U.K., and Richard M. Locksley of the University of California, San Francisco [1] ''Mammalian innate immunity is not merely a vestige of ancient antimicrobial systems that have been made redundant by the evolution of acquired immunity. Rather, it dictates the conduct of the acquired immune response.''

Understandably, the invading pathogens are responded to by the acquired immune system which cooperates with the first line of defense offered by the innate response to succeed in curtailing the spread of infection.

I.B Adaptive Immunity

The most distinguishing elements of this phase of the immune response are antigen specificity and memory. Acquired immunity has nearly infinite adaptability in responding to new pathogens: the T and B lymphocytes of the acquired immune response can rearrange the elements of their immunoglobulin and T-cell receptor genes to create different clones with distinct antigen receptors. B cells are the mediators of humoral immunity, they can generate up to 10^{14} different antibody with different specificities and different classes [2]. Once acquired immunity has dealt with a pathogen, some antigen-specific clones remain as memory cells that mediate a more rapid response the next time the pathogen is encountered.

I.B.1. Role of T Cells in Antigen Recognition and Initiation of the Immune Response

Antigen-specific T cells originate from thymus with receptors able to recognize peptides bound to major histocompatibility complex class I or class II molecules (MHC I and MHC II). Detection of a specific peptide–MHC (pMHC) complex expressed on APCs at a density of only 0.1–1 molecules/μm^2 is sufficient to induce robust T cell activation [3]. These thymus-derived lymphocytes circulate in the blood and lymphoid organs such as the lymph nodes, spleen, and the Peyer's patches of the intestinal mucosa until encounter with antigen-presenting cells (APCs) bearing specific MHC-peptide complexes. It is their peptide specificity that distinguishes these receptors, and their ability to interact with different affinities to the same peptide hence translating small quantitative differences in ligand binding into qualitatively different signals [3]. Dendritic cells are the most competent of all APCs in triggering T cell responses: they express a high level of MHC molecules along with a variety of 'accessory molecules' that help DCs to be more efficient in stimulating T cells [4]. However, a recent study using BM chimeras in mice allowing the depletion of MHCII molecules specifically in B cells has shown that cognate interaction through MHC-II on B cells was essential for both clonal expansion and differentiation of CD4 T cells. Hence, in vivo B cells were shown to provide essential

additional Ag presentation capacity to that provided initially by DC [5]. Two alternative paths may be initiated following ligand binding on T cells and APCs, one involving CD8+ T cell activation for the cells expressing antigen receptor of the same specificity, the other involving CD4+ T helper cell activation of other cell types, namely B cells (Figure 1). The induction of B cell proliferation and differentiation contributes to the establishment of humoral immunity.

What are the requirements for B cell activation, and what is the outcome of these interactions?

I.B.2. Antigen recognition by B cell receptors

The first comprehensive theory that addressed the function and production of antibodies was proposed by Ehrlich in his side-chain theory *(Ehrlich P. et al. The Croonian Lecture. On immunity with special reference to cell life. Proc Soc Lond Biol 1900; 66:424-448).* Although the amplitude of the repertoire of antigen specificity was not well appreciated, he postulated that naturally occurring antibodies described as side-chains acted as receptors on the cell surface. Upon antigen recognition and binding, these molecules were shed from the cell surface, and the cell compensated for this loss by producing and releasing more antibodies into the circulation.

It is not until more than fifty years later that the clonal selection theory (CST) was advanced by F. M., Burnet clarifying the nature of the cell producing antibodies as well as its specificity *(Burnet F. M. The Clonal Selection Theory of Acquired Immunity. Cambridge: Cambridge University Press, 1959).* Burnet proposed that a specific immune response can be explained in terms of the selection of a specific clone by a specific antigen. *One B cell: one antibody* supported the implications of this theory [6].

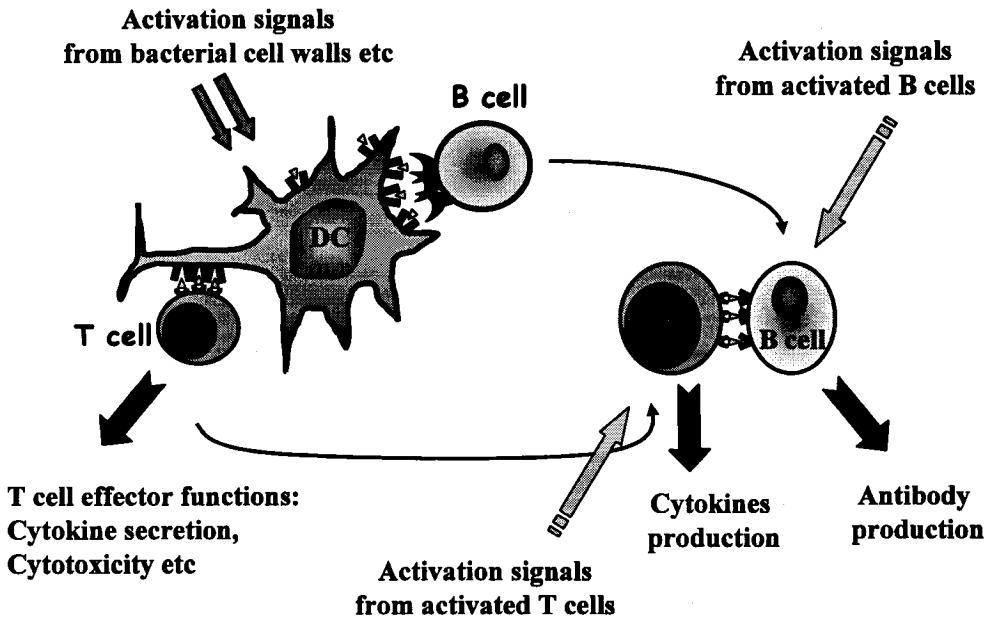


Figure 1: B and T cell activation.

Communication of the innate immune response with the effector cells of the adaptive immune response by synapse formation.

The ability to distinguish self from non-self and to initiate a specific immune reaction following recognition of non-self or foreign antigens constitutes the basis of this theory. This model has been modified over the years to include the need for a second signal for B and T cells to trigger a productive immune response and escape cell death [7,8]. In 1989, Janeway proposes the infectious non-self model (INS) whereby APCs do not co-stimulate T cells unless activated via PRRs (receptors for evolutionarily distant infectious non-self [9]. The self non-self model has been challenged by Matzinger, P. and colleagues in her danger theory proclaiming that the immune system's decision to react to a stimulus is determined by the 'danger' it poses to the host rather than its compliance with the self non-self model. Within this model, alarm signals released by stressed or injured cells are what triggers an immune response and not the identification of a 'foreign' antigen [10,11]. To date, scientists are still studying the different regulatory mechanisms that induce and modulate antibody production.

The expression of a functional BCR is a prerequisite for the initiation of an immune response leading to antibody production. B cells undergo an antigen-independent maturation process in the bone marrow with different checkpoints involving factors and signals from outside and inside of a B cell precursor that predict survival, growth and maturation of B lymphoid progenitors before the appearance of mature Ig receptors [12]. This developmental pathway depends on several transcription factors, including early B-cell factor, PU.1, E2A and paired box protein 5 (PAX5) [13]. Studies of primary immune deficiencies in humans, transgenic and gene inactivation models in mice, have allowed the characterization of progressive developmental checkpoints leading to the expression of a pre-BCR [14]. A model delineating these different stages in humans and mice is depicted in figure 2 [15-18]. The first checkpoint in B cell development relies on pre-BCR expression and signaling at the pro-B/pre-B transitional stage both in human and mouse.

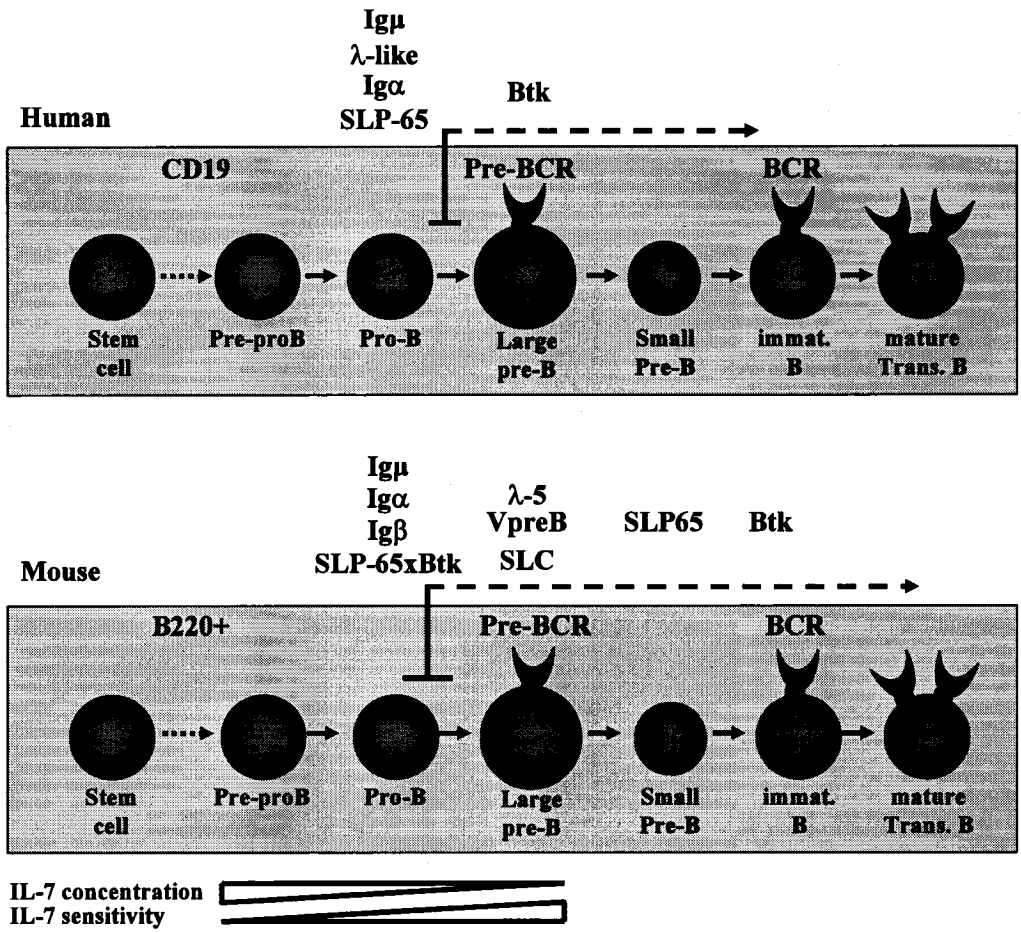


Figure 2: Human and mouse BM B cell development pathways.

Blockages in human and mouse B cell differentiation pathways in the bone marrow as a result of deficiencies in certain signaling proteins. Complete and incomplete blockages are indicated by full and dashed arrows, respectively. (*depicted from Espeli, M. et al. (14)*)

The pre-BCR, transiently expressed by developing precursor B cells, comprises the Ig μH chain, surrogate light (SL) chains VpreB and λ5, as well as the signal-transducing heterodimer Ig α/Ig β. Signaling through the pre-BCR regulates allelic exclusion at the Ig H locus, stimulates cell proliferation, and induces differentiation to small post-mitotic pre-B cells that further undergo the rearrangement of the Ig L chain genes [19]. Immature B cells subsequently express a complete IgM molecule on their surface. The second checkpoint is successfully passed in the presence of surface IgM, this allows negative selection to occur. B lymphocytes leave the bone marrow at the transitional B cell stage and complete their final development into mature B cells in the periphery [20]. Selccted B cells exiting the BM, undergo a progressive maturation from transitional B cells (T1) T1 B cells to T2 B cells to fully mature B cells. Different levels of surface markers distinguish these cellular subsets: T1 cells are IgM^{high} IgD^{-} $CD21^{-}$ $CD23^{-}$, whereas T2 B cells are IgM^{high} IgD^{+} $CD21^{+}$ and $CD23^{+}$ [21-23]. Immune Competence is acquired as B cells progress from T1 to T2 with a second round of negative selection to fully mature. The majority of B cells will recirculate in the spleen and other lymphoid organs until encountering their cognate antigen and undergoing antigen-dependent maturation in the periphery [24].

Initiation of the antibody response depends primarily on antigenic stimulation of the B cell receptor (BCR). Signals transmitted by the B cell Ag-receptor (BCR) influence not only cellular selection, maturation, and survival, but are imperative in generating the ultimate effector function of B cells, i.e. antibody production. Upon BCR triggering, a series of intracellular events take place leading to cell proliferation and differentiation [25,26]. The BCR also plays a role in antigen uptake, which, following intracellular processing, leads to presentation of antigen-derived peptides on MHC II molecules to T cells [27,28]. A BCR may interact with either soluble or membrane-associated antigens, however, membrane-bound antigens are most effective in promoting B cell activation [29]. As an example of the affinity of such interactions, soluble self-antigens of high-affinity induce anergy [30], whereas low-affinity membrane self-antigens lead to B cell deletion [31]. Other forms of soluble antigen bound to Fc portion of antibodies or complement forming an immune complex (IC) that is in a membrane-anchored form can induce the BCR,

constituting the most common form of antigen encountered by B cells [32]. Dendritic cells can present antigen to B cells [33]. Furthermore, in germinal centers, follicular dendritic cells have been shown to retain IC for presentation to B cells [34]. Presentation of intact antigen as a transmembrane protein triggers formation of a BCR-dependent immunological synapse (B synapse). The B cell synapse extracts intact antigen from the APC, processes it to generate peptide-MHC complexes for B-Th synapse formation [35]. Neuberger and colleagues have shown that immune complexes captured on FcR^+ cells can be presented to form a B synapse (Figure 3). The presentation of intact antigens as immune complexes on FDCs is thought to be important for the affinity maturation of antibodies [36]. As antigenic stimulation plays a role in DC activation and maturation, it is interesting to view the B-DC synapse and the potential role of cytokines in this interaction. Identification of cytokine receptors on DCs may elucidate their immunoregulation and characterize their role in B cell responses.

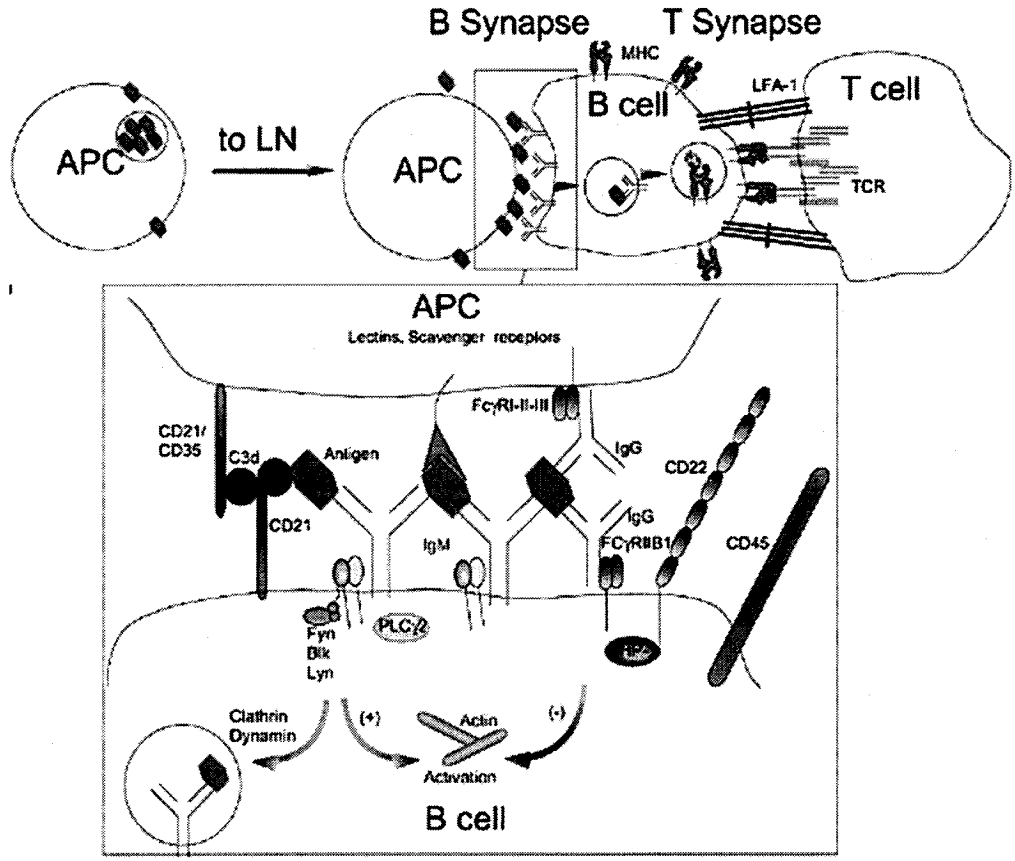


Figure 3: B cells and the APC immune synapse

In a first stage antigens or immune complexes captured by DCs are presented to B lymphocytes in the context of the B synapse. Consequently the B cell acts as a bridge relaying the extracted antigen and presenting it to helper T lymphocytes (T synapse). In the B synapse, molecules that negatively regulate the BCR signalosome are excluded from the synapsis area. (LN, lymph node; LFA-1, lymphocyte function–associated antigen 1). *(depicted from Dustin, M. et al. (36))*

II. The Humoral Immune Response:

The adaptive humoral immune response develops slowly, requiring at least one week and resulting in the production of monospecific, high-affinity antibodies, first of IgM and later, after class switching, of IgG, IgA and/or IgE type [37].

II.A. B-cell Activation by helper T cells

The dual role that B cells play in adaptive immunity involves antigen presentation and development of effector mechanisms following antigen exposure. Encounter of a naïve B cell with antigen prepares it to activate T cells and receive T cell-derived help signals. How the B cell prepares for such collaboration involves different stages that progress temporally, ultimately leading to the expression of cell-surface molecules important for productive interaction with T cells.

II.A.1.B-T cell interaction and the immunological synapses

The main form of communication between activated B cells and helper T cell clones is the immune synapse which impacts cell-fate decision in T-dependent B-cell responses. Figure 4 depicts the three synapses that occur within days of antigenic exposure over the course of the primary immune response within the secondary lymphoid follicles as described by McHeyzer-Williams, M. G. et al. [38]. Synapse I interactions occur concomitantly between activated DCs and naïve antigen-specific T cells [35], as well as antigen-primed DCs and naïve circulating B cells brought to T cell areas in the spleen or lymph nodes. This initial step allows maturation of the antigen presenting B cell by providing an efficient means for antigen and BCR internalization [38], and activation of the T cell to allow the provision of help to activated B cells.

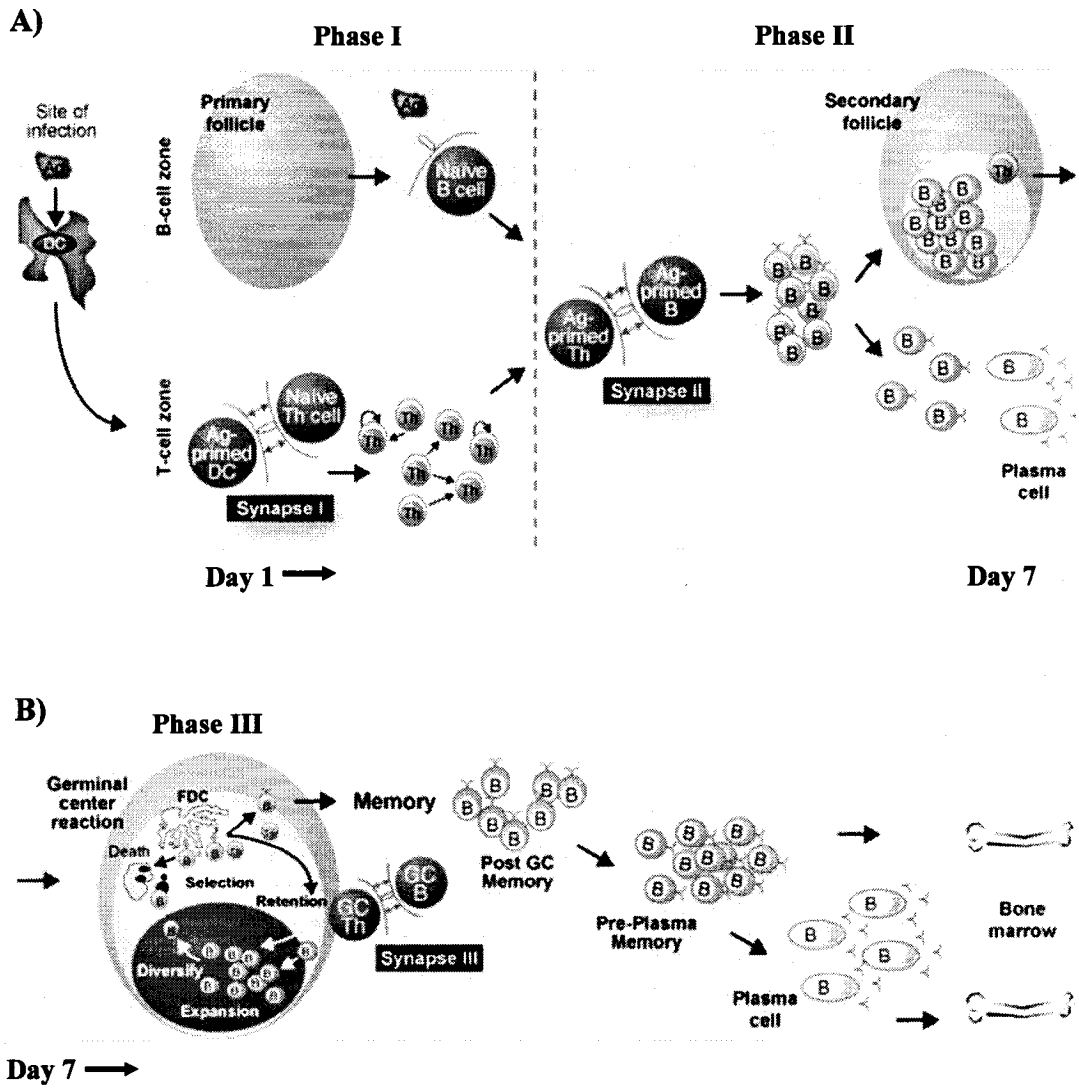

A)
Phase I
Phase II
Site of infection
Ag
DC
B-cell zone
Primary follicle
Naive B cell
T-cell zone
Naive Th cell
Ag-primed DC
Synapse I
Th
Ag-primed Th
Ag-primed B
Synapse II
Secondary follicle
Plasma cell
Day 1
Day 7
B)
Phase III
Germinal center reaction
FDC
Death
Selection
Retention
Diversity
Expansion
Memory
Post GC Memory
GC B
GC Th
Synapse III
Pre-Plasma Memory
Plasma cell
Bone marrow
Day 7

Figure 4: Immunological Synapses

Helper-T-cell regulated B-cell differentiation. Phase I begins at the site of infection with activation and emigration of DCs to the T-cell zones of the lymph nodes. Initiation of immune synapse I (Synapse I) occurs at this stage allowing the activated DCs to contact and trigger naïve Th cells expressing specific T-cell receptor (TCR). Studies have indicated that B cells can initiate an immune synapse with an activated antigen-bearing DC as well. Phase II occurs following clonal expansion, with cognate contact between antigen-specific B cells and antigen-activated Th cells within immune synapse II (Synapse II). Synapse II drives B-cell differentiation to either short-lived plasma-cell production, which progresses in the T-cell areas or movement into the follicular areas and the formation of secondary lymphoid follicles. Phase III begins with the GC reaction and polarization of secondary follicles into light-zone and dark-zone regions. Mechanisms of clonal expansion, SHM of the BCR, and antigen-specific selection for high affinity variants mark this phase leading to export of memory B cells. These memory B cells can either differentiate into long-lived plasma cells, or remain as non-secreting precursors for antigen recall. Synapse III interactions involve antigen-specific GC Th cells and GC B cells and are proposed to play a critical regulatory role in these late-stage B-cell developmental decisions. (*depicted from McHeyzer-Williams, M. G. et al.* [38])

Immune synapse II forms upon presentation of antigenic peptides in the context of MHC II proteins by antigen-specific B cells to cognate T cells, thus leading to B cell activation creating the first stage of antigen dependent B cell differentiation. Activated B cells either proliferate and terminally differentiate to antibody-secreting cells in the T-cell zone of the lymphoid organ, or after initial activation may migrate to the follicular area and initiate the formation of a GC. The role of CD40 and its ligand CD154 is crucial in modulating the outcome of this interaction and constitutes a prerequisite for GC development. It is in the GC that B cells activate somatic hypermutation of their immunoglobulin genes and then undergo a stringent selection for high affinity binding to antigen, resulting in the production of memory B cells and plasma cells secreting antibodies of high affinity.

II.B. The Germinal Center Response

Following activation, IgM^+ B cells migrate to the secondary lymphoid organs where they undergo antigen-driven maturation (Figure 5). Germinal centers (GCs) originate from a few founder B cells that undergo rapid clonal expansion [39,40]. To diversify the antibody repertoire, these proliferating B cells (centroblasts) undergo somatic hypermutation (SHM) of their immunoglobulin variable region genes within the GC dark zones [41-44] and give rise to centrocytes that are not in cell cycle and fill the light zone. SHM, in which point mutations are introduced into the variable region of immunoglobulin heavy and light chains, enables the selection of B cells that generate antibodies of higher affinity for antigen [45,46]. Selection based on the highest antigenic affinity and specificity occurs in the GC light zones by interaction of B cells with follicular dendritic cells (FDC) and GC T cells [47,48]. The light zone contains a rich network of follicular dendritic cells (FDC) that have the capacity to take up antigen and hold it on their surface. Centrocytes appear to be selected by their ability to interact with antigen held on FDC [49]. There is a high death rate among centrocytes in vivo, and when these cells are isolated in vitro, they undergo apoptosis within hours of culture. Long-term survival is achieved by signaling through their surface CD40. Both BCR and CD40 pathways, alone or together, contribute to B cell proliferation and differentiation [50]. B

cells subsequently differentiate to memory or plasma B lymphocytes [51]. This maturation process in the GC can be viewed as phases of B cell proliferation and mutation, followed by selective survival. Genetic analyses have shown that Activation Induced Cytidine Deaminase (AICD) is essential for both CSR and SHM [52,53]. It is by taking this journey within immunized peripheral lymphoid tissues, and via a progressive acquisition and loss of different surface markers, that the B cell matures and acquires its effector function. ***What are the different phenotypes that a maturing B cell displays throughout the GC reaction, and what molecules and/or cytokines orchestrate their expression?***

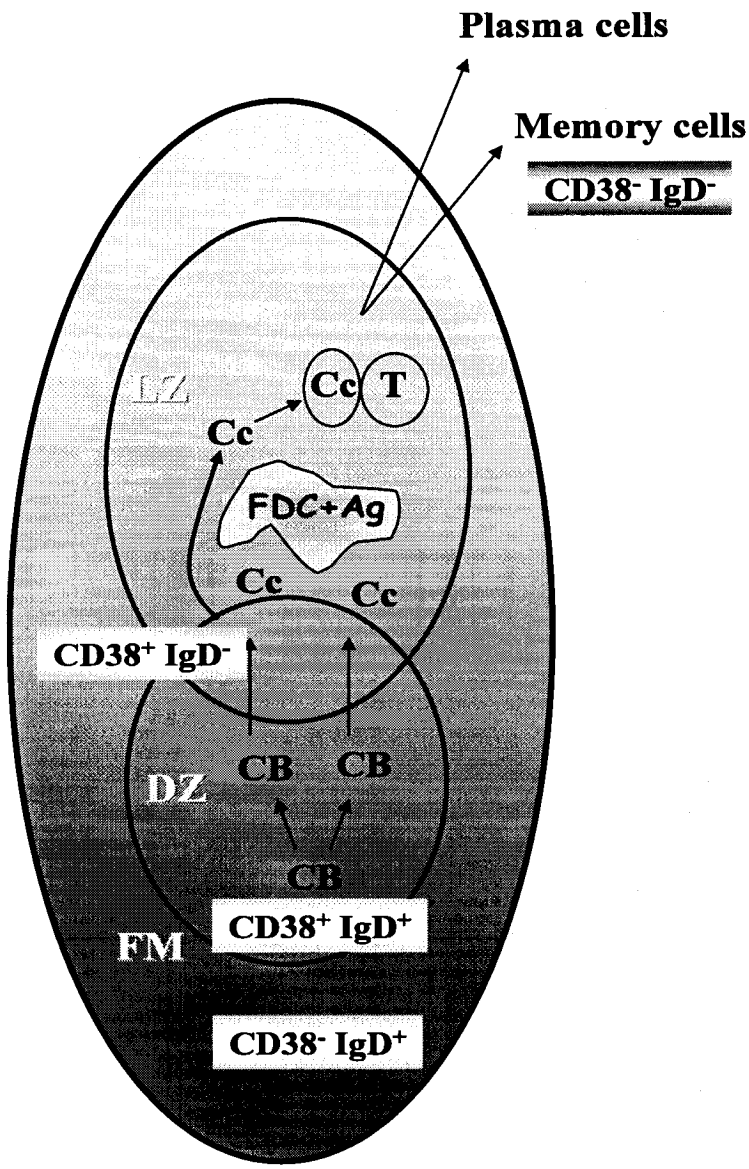

Plasma cells
Memory cells
CD38- IgD-
LZ
Cc
T
Cc
FDC+Ag
Cc
Cc
CD38+ IgD-
DZ
CB
CB
CB
FM
CD38+ IgD+
CD38- IgD+

Figure 5: The Germinal Center Reaction

Follicular mantle B cells ($CD38^{-}IgD^{+}$) are antigen-activated B cells that enter secondary follicules from periphery. Depending on their BCR affinities, a few are selected as founder GC cells ($CD38^{+}IgD^{+}$), which undergo somatic hypermutation of their IgV region genes and proliferate in the dark zone (DZ) of the follicle as centroblasts. Centrocytes are selected based on their affinity to antigen expressed by FDCs or T cells. Centrocytes with low affinity to antigen on FDC or T cells die by apoptosis. Centrocytes present peptides from FDC-derived Ag to local T cells. Selected Centrocytes may class switch their immunoglobulin genes and differentiate into plasma cells, memory B cells or go through receptor editing to enhance their affinity to antigen.

The definition of distinct subsets of B cells derived from human tonsillar lymphoid tissue representing different stages of peripheral B-cell development was done by Liu, YJ, Arpin, C. et al. [43]. Most other studies addressed memory development in humans in PBL which do not reflect the entire developmental process as is the case with tonsillar B cells. As human tonsils receive repeated antigen stimulation and may display all stages of peripheral B cell development [54]. Based on the work of Liu, YJ et al. B cells can be sorted into four subsets established using anti-IgD and anti-CD38 double immunofluorescence staining (Figure 6). Additional markers can be used to further subdivide each subset such as CD23 which separates naïve B cells into an $IgD^{+}CD38^{-}$ $CD23^{-}$ subset (Bm1), and an $IgD^{+}CD38^{-}CD23^{+}$ activated subset (Bm2). In turn, (GC) $IgD^{+}CD38^{+}$ cells can be separated into $CD77^{+}$ centroblasts (Bm3) and $CD77^{-}$ centrocytes (Bm4). A model representing localization of these subsets within GCs is depicted in figure 6.

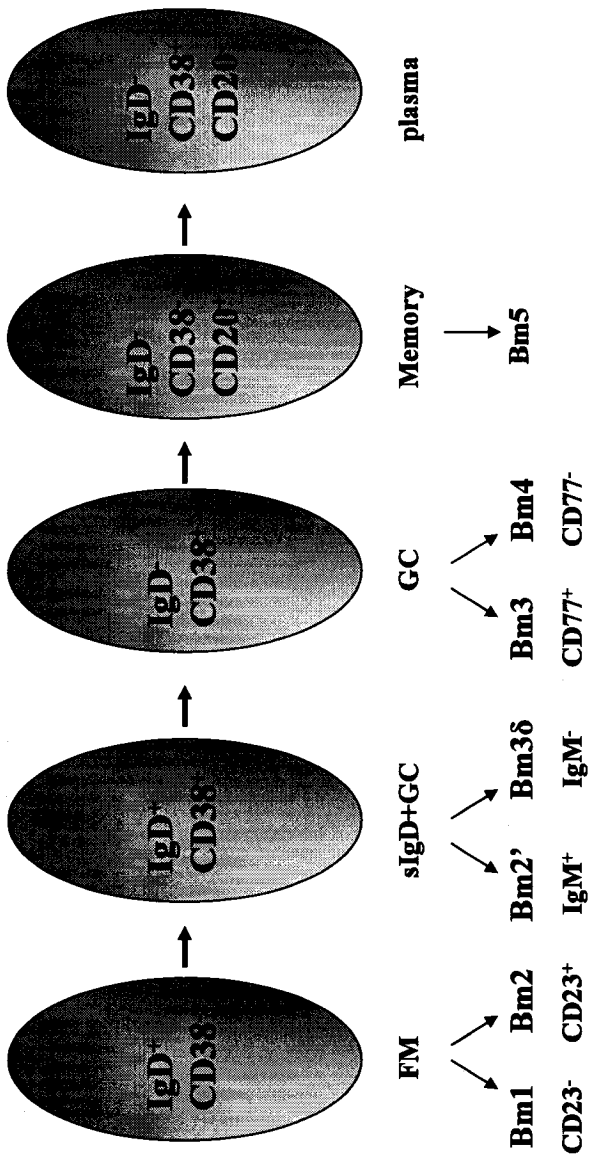


Figure 6. Phenotypic and functional characterization of human B-cell subsets

The five main developmental stages of differentiation of human peripheral B cell maturation in secondary lymphoid follicles as proposed by Liu et al developmental model [43].

The earliest GC B cells (GC founder cells) are $IgM^+IgD^+CD38^+$ B cells; they are characterized by the expression of naïve B cell markers IgM and IgD, and GC markers CD10, CD38 and CD71. They also express Fas/CD95 with little or no bcl-2 with a propensity to spontaneous apoptosis during culture [55]. The availability of apoptosis program early in GC formation ensures that only GC founder cells with the highest affinity for antigen, bearing unmutated antigen receptors, will be selected to undergo somatic mutation. Once entered in the GC dark zones these cells have to mutate and improve the affinity of their antigen receptors rapidly to escape cell death. Selection and somatic mutation occur concomitantly [56].

Analysis of centrocytes in the light zones of human tonsils showed a selective detection of sterile transcripts, a marker for the initiation of isotype switch [57-59]. As centrocytes are localized in the vicinity of T cell help in that area, the detection of isotype switching fits with the reported role of CD40-ligand [60,61] and secreted IL-4 and IL-10 [62] in modulating isotype switching. Other unidentified T cell expressed molecules and/or cytokines may also play a role as switching factors and affect the differentiation pathway of B cells at this stage.

In vitro studies have attempted to recreate the in vivo situation where high affinity GC B cells have picked up antigen from follicular dendritic cells [63] and present it to CD40-ligand-expressing GC T cells [60,61]. Upon culture of GC B cells with CD40-ligand-transfected L cells with IL-2 and IL-10 for three days, cells enter a proliferative phase and grow exponentially [64]. Two outcomes for GC B cells are obtained in vitro depending on the presence of CD40L for long term culture or its absence, memory or plasma cell differentiation. Findings from different culture systems confirmed the inhibitory effect of CD40L on B-cell differentiation to the plasma cell pathway [51,65,66]. The presence of cytokines allows the differentiation of B cells to secrete immunoglobulins.

II. C. Master Regulators of B cell differentiation

These successive differentiation steps are controlled by transcriptional regulators. Among these factors, PRDM1 the human homologue of Blimp-1, is a transcription

repressor acting to facilitate the germinal center response by promoting plasma cell differentiation [53]. This is accomplished by repressing the expression of other genes: cMYC which affects B-cell proliferation and cell cycle progression [67], Pax 5 with its role in B cell maturation and suppression of the secretory pathway [68], and BCL6 which arrests further development of GC cells [69]. Another factor involved in plasma cell differentiation is XBP-1 [70], a target of Pax 5. Initiation of the germinal center response following CD40 stimulation depends on NF-κB [71], and induction of activation-induced cytidine deaminase (AICDA) which is required for SHM and CSR [52]. BCL6 is also required for the formation of GC, where it acts in a B cell–autonomous manner to repress genes involved in the control of lymphocyte activation and cell cycle progression, allowing rapid cell proliferation [69]. BCL6 also arrests further development by acting in concert with PAX5 (BSAP) to repress genes involved in plasmacytic differentiation [72]. These genes play an important regulatory role for B cell maturation, any defect caused by chromosomal translocation is associated with disease leading to a variety of human non-Hodgkin's lymphomas [73].

II.D. Contact-mediated signals

The regulation of B-cell effector functions is mediated by the interaction of several receptor-ligand pairs. These contact-mediated signals are received from an activated T cell, and contribute to the development of affinity maturation and the humoral memory response of B cells (Table 1). The first receptor implicated in transduction of contact-dependent B cell activation signals to be described was MHC class II. In 1987, it was reported that engagement of MHC class II on naïve murine B cells induces the activation of Protein Kinase C molecules, elevation of intracellular cAMP, and occasionally apoptosis [74-76]. More recently, it was shown that triggering of MHC II with antigen in the presence of IL-4 leads to its association with immunoglobulin (Ig)-α/Ig-β (CD79a/CD79b) heterodimers, which function as signal transducers upon MHC class II aggregation by the T cell receptor (TCR) [77].

B cell receptor	T cell ligand	B cell effector functions
Class II MHC	TCR; CD4	Cooperates with other activation signals to stimulate proliferation, differentiation and enhanced antigen presentation
CD11a–CD18/CD54	CD54/CD11a–CD18	Cell adhesion, enhanced antigen presentation and enhanced activation
CD72	CD100	Development of B-1 B cells, production of high-affinity IgG response and enhanced antigen presentation
CD40	CD154	Proliferation, differentiation, isotype switching, cytokine production, protection from apoptosis, and germinal center and memory response development
CD134L/OX40L	CD134/OX40	Stimulation and enhancement of IgG response
CD137L/4-1BBL	CD137/4-1BB	Stimulation of T cells through CD137
CD27	CD70	Differentiation into plasma cells
CD30/CD153	CD153/CD30	Inhibition of B cell responses, such as isotype switching and plasma cell differentiation
CD95/Fas	CD95L/FasL	Induction of programmed cell death (apoptosis)

Table 1. B cell receptors
Contact-mediated interaction of B and T cells involves different B cell Transmembrane receptors and T cell ligands.

In addition to the vital interaction of B and T cells through MHC II, both B and T lymphocytes express various transmembrane adhesion molecules, whose surface expression is increased after activation signals. Both cell types express ICAM-1 (CD54) and LFA-1 (CD11a/CD18), which bind to each other and can thus mediate both homotypic and heterotypic adhesion which may amplify activation signals delivered by one cell type to the other [78]. It was shown that such signals can contribute to enhanced B cell antigen-presentation [79] and cooperate with CD40 mediated signaling [80]. Moreover, other costimulatory molecules are crucial for the germinal center reaction, namely ICOS whose deficiency results in a severe reduction of class-switched B cells in human patients leading to a clinical phenotype described as a common variable immunodeficiency [81].

II.D.1. The Role of CD40 Stimulation in B Cell Maturation

The most extensively studied B-cell surface molecule involved in the generation of T-dependent humoral responses following antigen challenge is the TNF-R family member CD40, which is constitutively expressed on mature B cells and, upon stimulation, recruits a family of adapter molecules called 'TNF-R-associated factors' [82] TRAFs 1, 2, 3, 5, and 6, leading to activation of NF-κB, JNK, p38, and PI3K [78]. The CD40 ligand (CD40L), CD154, is highly expressed primarily on the surface of activated T cells as a membrane-bound trimer, particularly following CD80/86-mediated CD28 coreceptor signaling [83]. Activation of B cells through CD40 occurs only following antigen encounter of B cells which upregulates CD80/86 expression and receiving T cell help. The CD40/CD154 interaction constitutes a crucial step in T cell-dependent B cell activation and differentiation as evidenced by the pathology associated with its genetic alteration leading to abnormal CD40L expression in humans suffering from the X-linked hyper-IgM syndrome [84]. This provides a molecular basis for immunoglobulin isotype switch defects observed in this immunodeficiency. The murine models of gene deficiency both in CD154 [85] and CD40 knock-out mice [86] demonstrate the important role of CD40/CD154 interaction in the humoral response and germinal center formation. The biological outcome of CD40 engagement on B lymphocytes being proliferation, antibody

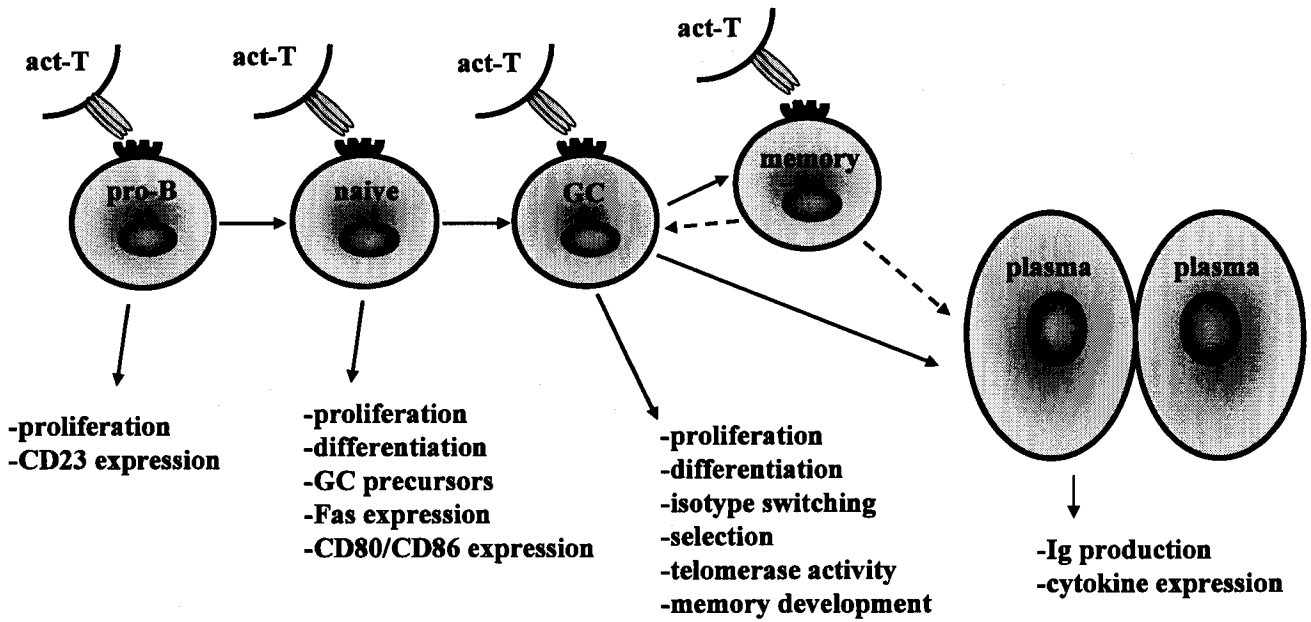
secretion, cytokine production, upregulation of different surface molecules involved in antigen presentation, isotype switching, development of germinal centers as well as a humoral memory response [87,88]. Signaling through CD40 elicits a broad variety of immune and inflammatory responses [87,89], and is not restricted to B lymphocytes only but can also be triggered in dendritic cells, monocytes, epithelial cells, endothelial cells, and fibroblasts [89]. Hence, abnormal CD40 signaling is associated with the pathogenesis of chronic inflammatory diseases such as autoimmune diseases [90], neurodegenerative disorders [91], graft-versus-host diseases [92], cardiovascular diseases [93], and cancer [94].

In mice, the role of CD40/CD154 interaction in B lymphocyte activation, proliferation, and differentiation has been addressed using CD40/CD154 knockout mice [85,86,95]. The dysfunctional CD40 signaling revealed its essential role in isotype switching, somatic hypermutation, generation of memory B lymphocytes, and Ig production. Since disruption of the gene blocked the development of a memory response [96] by preventing the GC reaction to be initiated, direct evidence for the effect of CD40 on B cell memory development was sought from wild-type in vivo models, whereby neutralization of CD40L with anti-CD154 inhibited the secondary humoral response to T-dependent antigens [97], strongly confirming the essential role of CD40 in memory B lymphocyte responses in mice.

In humans, patients suffering from the hyperIgM syndrome, presented deletions or mutations in the expression of CD40 [98]. This resulted in impaired B lymphocyte activation and Ig production. To determine the direct effect of CD40/CD154 interaction on human B cell memory development, studies in humans have been limited to in vitro systems. The impact of CD40 stimulation on B lymphocytes from peripheral blood or tonsils has been studied using different tools to stimulate CD40: cells transfected with CD154, anti-CD40 Abs, or soluble CD154 in the presence of IL-4 [99].

The first system to be described for in vitro stimulation of B cells the 'CD40 system' was developed by Banchereau, Rousset et al. [64]. Tonsillar B cells were cultured with CD32-transfected fibroblasts coated with anti-CD40 mAb and IL-4. Analysis of IgD

expression showed no decrease for its expression on cycling naïve B cells, and these cells had very modest soluble Ig synthesis [100], suggesting that B lymphocytes were blocked in their differentiation. However, a switch to IgE was detected subsequent to the addition of soluble anti-CD40 and IL-4 to B cell cultures [101]; other cytokines such as IL-10 enhanced IgG secretion [102], and stimulated B cell differentiation into morphologically typical plasma cells [103]. Hence, these results showed that the differentiation block observed with IL-4 could be overcome by other cytokines. However, a group reported that using this 'CD40 system' yielded differential outcomes whether a naive or a memory B cell was used independent of the cytokine added in the milieu (Figure 7A) [87]. Only memory B cells were induced to secrete Ig, thus pointing to the impact of the maturation stage of the B cell at the time of interaction with T cells on the outcome i.e proliferation vs differentiation and Ig production [99].


act-T
act-T
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naive
GC
memory
plasma
plasma
-proliferation
-CD23 expression
-proliferation
-differentiation
-GC precursors
-Fas expression
-CD80/CD86 expression
-proliferation
-differentiation
-isotype switching
-selection
-telomerase activity
-memory development
-Ig production
-cytokine expression

Figure 7A: Biological effects of CD40 activation on different stages of B cell development.

CD40 activation mediates maturation of B cells from pre-B cells to naive B cells, germinal center (GC) B cells, or memory B cells. CD40 activation of memory B cells can lead to reentry into the germinal center and/or accelerated differentiation into Ig-producing plasma cells (*depicted from van kooten, C. et al.* [87]).

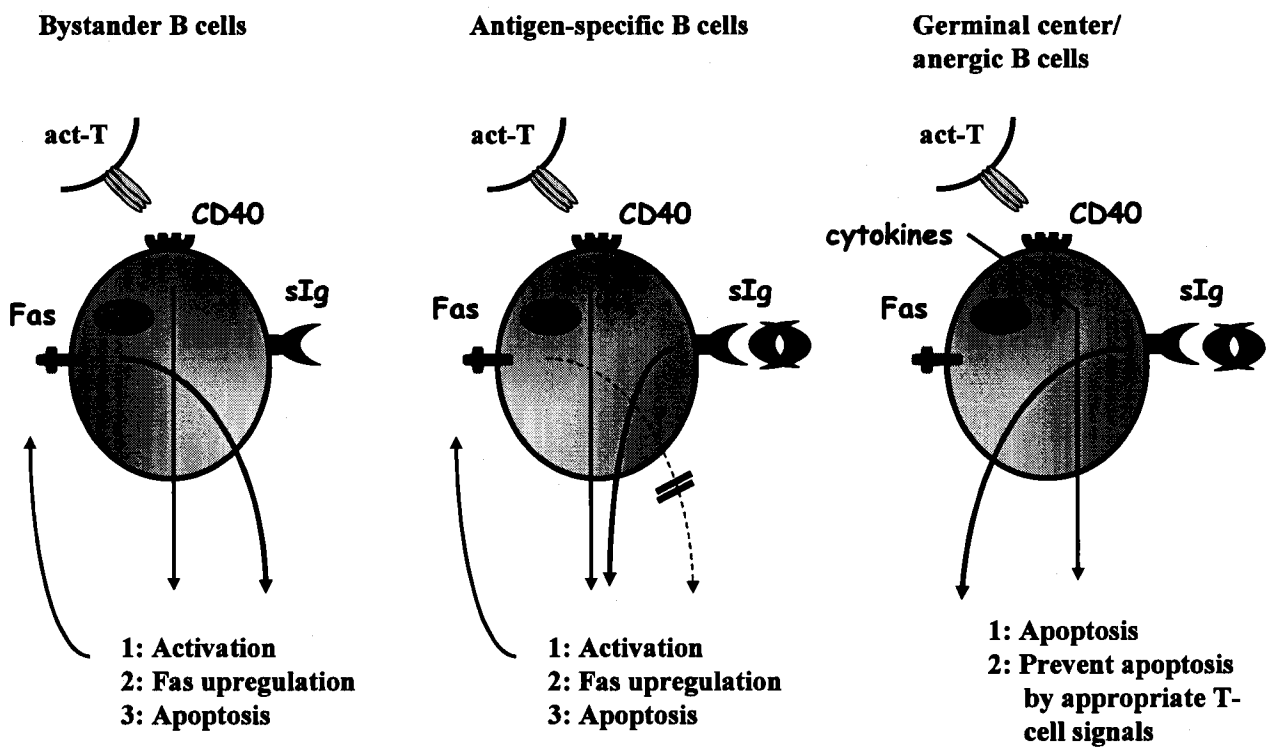

Bystander B cells
act-T
CD40
Fas
sIg
1: Activation
2: Fas upregulation
3: Apoptosis
Antigen-specific B cells
act-T
CD40
Fas
sIg
1: Activation
2: Fas upregulation
3: Apoptosis
Germinal center/
anergic B cells
act-T
cytokines
CD40
Fas
sIg
1: Apoptosis
2: Prevent apoptosis
by appropriate T-
cell signals

Figure 7B: Differential outcomes for CD40 activation depending on the maturation stage of B cells.

Model of the interactions between CD40, surface Ig (sIg), and Fas signaling and the different effects depending on the maturation stage of B cells. (A) CD40 activation of antigen-nonspecific bystander B cells results in cellular activation and induction of Fas expression. Interaction with Fas-ligand expressed on the activated T cell leads to B cell apoptosis. (B) Similarly, antigen-specific B cells are activated with cell activation and increased Fas expression. However, protection from apoptosis induction via Fas-FasL is mediated by a signal delivered by the surface Ig receptor (BCR), with subsequent activation and differentiation processes such as isotype switching. (C) Germinal center B cells, as well as anergic B cells obtained in different tolerance models, seem to be susceptible to apoptosis through BCR cross-linking. This induction of apoptosis seems to be independent of Fas-FasL. The B-T cell immune synapse in the presence of a combination of CD40L and cytokines (IL-4) prevents this form of apoptosis, allowing for further differentiation of these B cells (*depicted from van kooten, C. et al. (90)*).

II.D.2.CD27: Role in B Cell Maturation

Another important member of the TNF-R family for B lymphocytes activation is CD27. CD27 expression is restricted to a subpopulation of B lymphocytes and has been proposed as a marker for memory B cells [104]. Two populations in the human tonsils and PB have been differentiated based on CD27 expression [105]. Flow cytometric analysis revealed that CD27 molecules were expressed on GC B and memory B cells but not on naïve B cells [105-109]. Although several studies attribute its expression to memory B cells, one study describing its expression on different subsets of tonsillar B cells, showed that it was highly upregulated on plasma cells. However, they identified plasma cells based on CD38 and CD20 expression (CD38+CD20+) only. Defining the expression of IgD would have allowed differentiation of CD38+ plasma cells from CD38+IgD+ GC founder cells which could be present in their culture system. Morphological analysis reveals distinct features for CD27 positive cells which are larger cells with abundant cytoplasm compared to CD27 negative B cells which are smaller with scant cytoplasm [106]. The ligand for CD27, CD70, is a member of the TNF family. CD70 is expressed by a large fraction of activated T lymphocytes as well as activated B cells, and seems to play a role in the regulation of CD27 function at late stages of B cell differentiation into antibody-producing plasma cells [110,111]. In functional in vitro assays, $CD27^+$ B cells are rapidly activated and can produce higher levels of IgA, IgM, IgG and IgG subclasses compared to $CD27^-$ B cells [106,112]. Furthermore, CD27/CD70 interaction has been shown to specifically augment IgE secretion as the use of an anti-CD70 mAb completely abrogated this effect in B cell cultures. Only purified $CD27^+$ B cells secreted IgE following stimulation with IL-4 and anti-CD40. In addition, CD27/CD70 interaction enhanced B cell proliferation in the presence of IL-4 or IL-4 plus anti-CD40 [113]. CD27 signals through TRAF molecules (TRAF-2, -3 and -5) [82], and has common signaling pathways with CD40, such as NF-κB and c-Jun kinase [114,115]. Finally, the presence of somatic mutation, which is a definitive marker of memory B cells [116], has been described on $CD27^+$ IgD^+ PB B cells with the majority of cells carrying mutated V-region genes [107,116].

CD27 signaling is also important for the generation of plasma cells: $CD27^+$ memory B cells differentiate into plasma cells as a result of contact with CD27 ligand (CD70) transfectants in conjunction with interleukin-10 (IL-10) [112], *Staphylococcus aureus* Cowan strain (SAC) + IL-2 [117], or IL-4 + CD40 signaling [113]. This strongly suggests an important role for this molecule, which seems to be upregulated by CD40/CD154 interaction in PBL [99,118], in memory B cell differentiation and Ig production. Does CD27 synergize preferentially with a specific yet unidentified cytokine like CD40 with IL-4 to mediate B cell differentiation downstream of CD40?

A number of other B-cell molecules are also important for contact-dependent costimulation of T cells, without being direct transducers of B cell activation signals. These include: CD80, CD86, 4-1BBL, OX40L, and B7.h [78,119].

II.D.3.The Role of cytokines in mature B cell differentiation

Once B cells are activated by signals through costimulatory molecules and antigen receptor, cytokines play important roles in their subsequent differentiation. The fate of B cells upon activation also seems to depend on B-cell lineage and maturation stage [120]. The cytokine milieu is crucial for determining the outcome of an immune response after T-cell–B-cell interaction. Most studies have focused on the cytokines produced by T cells. It appears that B cells might have a more active role in determining the fate of immune responses as well [121-123]. It has been shown recently that Th cells direct secreted cytokines into two distinct pathways: one towards the immunological synapse, and another multidirectional pathway. Using intracellular cytokine staining, the authors show that the secretion of IL-2, IFN-γ, IL-3 and IL-10 is mainly concentrated at the immunological synapse in activated Th cells. By contrast, TNF, IL-4, CCL3, and CCL5 were observed to be scattered throughout the cell [124]. One of the best characterized cytokines produced by T cells in the context of a productive T-B collaboration is IL-4, which is required for IgG1 and IgE production in classic Th2 responses [125]. IL-4 is secreted by T cells into T-B synapses during cognate interaction with B cells [126]. Upon treatment with IL-4, naïve B cells have enhanced survival, increased expression of costimulatory CD80/86 molecules,

and are primed for MHC class II/Ig-α/β signaling [125,127]. The presence of IL-4 with B cells cultured on CD40 ligand-transfected cells induces a strong proliferative response in these cells [103]. Other T cell-derived cytokines involved in B cell differentiation include IL-2, IL-3, IL-6, and IL-10 [120]. IL-13, a Th2 cytokine, acts at different stages of the B cell maturation pathway: (a) it enhances the expression of CD23/Fc epsilon RII and class II MHC antigens on resting B cells; (b) it stimulates B cell proliferation in combination with anti-Ig and anti-CD40 antibodies; and (c) it induces IgE synthesis. Thus, the spectrum of the biological activities of IL-13 on B cells largely overlaps with the role ascribed to IL-4 [128]. It has also been demonstrated that IL-6 plays an important role in stimulating the proliferation and Ig secretion of isotype-switched B cells [129]. IL-9, a pleiotropic Th2 cytokine recently described, has been recently shown to modulate immunoglobulin production in PBL, however, its precise role in human B-T cell interactions remains to be addressed.

III.The Th1/Th2 Response

Once a B cell has 'made the choice' to become a memory or a plasma cell, what determines the activation requirements for the strength and direction of the effector immune response? Can a cytokine influence the outcome of a B-T cell interaction or is it the T cell that dictates the type of antibody produced? It is generally accepted that Th1 type responses include mainly cellular immune responses with antibodies produced of the IgG2a subtype and a prevalence of cytotoxic effector T cells, whereas Th2 immune responses influence humoral immunity relying on the production of IgE and IgG1 [130,131]. Cytokines are involved in modulating the immune response with a predetermined bias towards a Th1 or a Th2 response. IL-4 promotes Th2 clonal expansion and limits the proliferation of Th1 cells and, conversely, IFN-γ enhances Th1-cell growth, but decreases Th2-cell development (Figure 8) [132,133]. Several factors such as antigen type and/or load, route of infection, antigen presentation, the microenvironment at the time of infection or antigen exposure may alternatively greatly influence the type of immune response. The area is certainly open for debate with more than 6000 papers published thus far addressing Th1/Th2 regulation.

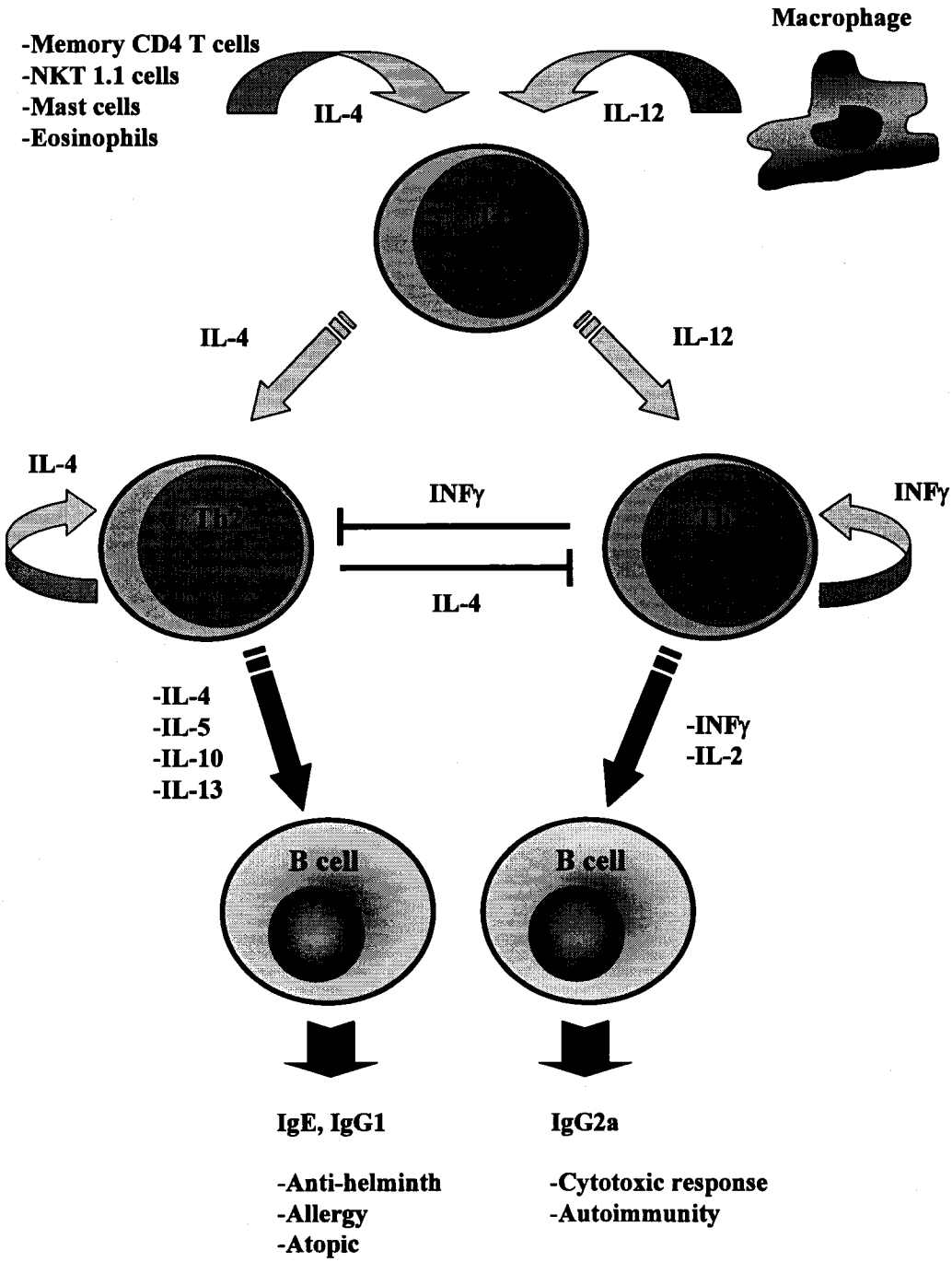

-Memory CD4 T cells
-NKT 1.1 cells
-Mast cells
-Eosinophils
IL-4
IL-12
Macrophage
IL-4
IL-12
IL-4
INFγ
IL-4
INFγ
-IL-4
-IL-5
-IL-10
-IL-13
-INFγ
-IL-2
B cell
B cell
IgE, IgG1
-Anti-helminth
-Allergy
-Atopic
IgG2a
-Cytotoxic response
-Autoimmunity

Figure 8: Th1 and Th2 immune responses

Depending on the cytokine 'milieu' Th cells can differentiate along the Th1 or Th2 pathway. IL-12 drives Th1 cells, whereas IL-4 promotes Th2 cells. The presence of IFN-γ and IL-4 plays a dual role as inhibitors of the other subset, and autocrine factors. Th1 enhances IgG2a synthesis by B cells through IFN-γ, whereas Th2 cells induce B-cell IgE and IgG1 production through IL-4. Th1 cells destroy intracellular pathogens and protect against organ-specific autoimmunity through IFN-γ. Th2 cells are anti-helminthic and increase allergic reaction through IL-4, IL-5 and IgE.

The provision of T cell help in the secondary lymphoid follicles is a crucial step in the development of memory B cells and their differentiation to antibody producing cells. Do Th1 and Th2 cells have equal chances in achieving contact with high affinity centrocytes and driving their selection and differentiation? Evidence obtained thus far remains controversial. Results published by Randolph, et al. have documented that differential expression of the chemokine receptor CCR7 on Th2 cells, which do not normally express the chemokine receptor, results in their localization in the follicles in a Th1-like pattern and inhibits their participation in B cell help in vivo but not in vitro [134]. Another study, published recently, argues against their findings that T cell help in the follicles is restricted to Th2 cells and presents data showing that in vivo polarized Th1 and Th2 cells clonally expand to similar levels and migrate into B cell follicles in which they support B cell clonal expansion and Ab production to a similar degree [135]. Moreover, they present direct evidence that in vivo polarized, IFN-γ secreting Th1 cells migrate into B cell follicles to interact with Ag-specific B cells. Other studies have also described the ability of Th1 cells to support B cell responses [136-138]. Critically, cytokines expressed in polarized immune responses contribute largely to peripheral B-cell development, with a potential exacerbation of the host's immune response leading to disease.

The expression of several diseases can depend on whether Th1 or Th2 lymphocytes predominate in response to an Ag challenge [139]. Robinson et al. [140] showed that allergic reactions involving IgE and mast cells could be due to the development and activation of allergen-specific TH2 cells. Subsequently, other clinical and experimental studies strongly implicated Th2 lymphocytes, and the cytokines they produce such as IL-4, IL-5, IL-13 and recently IL-9, in the pathogenesis of allergic asthma [141-143].

IV. Interleukin-9

IV.A. Cellular Source

IL-9 was first identified as a mouse T cell growth factor [144]. It was designated as P40, since its protein was purified at this molecular weight. It was isolated as a factor that

supported permanent Ag-independent growth of helper T cell clones [144-146], from the supernatant of helper T cell lines stimulated with Con A or phorbol esters. The cloning of the cDNA was performed following screening of the cDNA library obtained from these helper T cells clones with a probe prepared based on the sequence of the purified protein P40 [144]. In vitro expression studies have shown that Th2 lymphocytes could produce IL-9, adding it to the Th2 cytokine family [147]. Parallel studies had shown that bone marrow-derived mast cell lines (BMMC) proliferated and produced the inflammatory cytokine IL-6, in response to a mast cell growth-enhancing activity (MEA) [147]. More recently, murine BMMC were reported to produce a significant amount of IL-9 protein in response to stimulation with IL-1, and IgE-Antigen complexes. Moreover, the study shows that a dramatic potentiation of IL-9 mRNA and protein expression occurs following treatment with kit ligand or IL-10 [148]. A variety of other cell types including mast cells, eosinophils, and neutrophils were shown to produce IL-9. Further, upon stimulation of human PBMCs with anti-CD3 antibodies, IL-9's expression was induced [147,149,150]. The presence of IL-9 transcripts has been detected in human peripheral blood eosinophils, and eosinophil-differentiated HL-60 cell lines [150]. However, the release of the protein was significantly present only in eosinophils from asthmatic subjects. Moreover, stimulation of these cells with either TNF-α or IL-1 resulted in increased IL-9 levels in culture supernatants, whereas eosinophils from healthy control subjects did not show the same effect. Furthermore, IL-9 mRNA has been described in bronchial biopsy specimens of asthmatics [151]. Finally, although neutrophils from asthmatics were shown to express IL-9, the frequency of IL-9 mRNA+ neutrophils remained low [152].

IV.B Gene Structure

IL-9 belongs to a family of 4-helix bundle cytokines which also includes IL-2, IL-3, IL-4, IL-6, IL-7, and IL-15. The gene encoding human IL-9 maps to the Th2 cytokine gene cluster region that also includes IL-4, IL-5, IL-13, IL-3 and granulocyte-macrophage-colony stimulating factor (GM-CSF) on the long arm of chromosome 5 (5q31-35) (Figure 9) [153-156]. These factors have been implicated in allergic inflammation [157,158] These genes map to a syntenic region in mice on chromosome 11, except for the IL-

9 gene, which maps to mouse chromosome 13 [159]. Both human and murine IL-9 genes show similar genomic organization with 5 exons and 4 introns spanning approximately 4 kb [160]. In humans, the 5' flanking region contains specific binding sites for the activator protein 1 (AP-1) and (AP-2) transcription factors. The 5' promoter region was also shown to have a consensus sequence for IFN-regulating factor 1, as well as sequences common to IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, and GM-CSF genes suggesting common regulatory pathways [161].

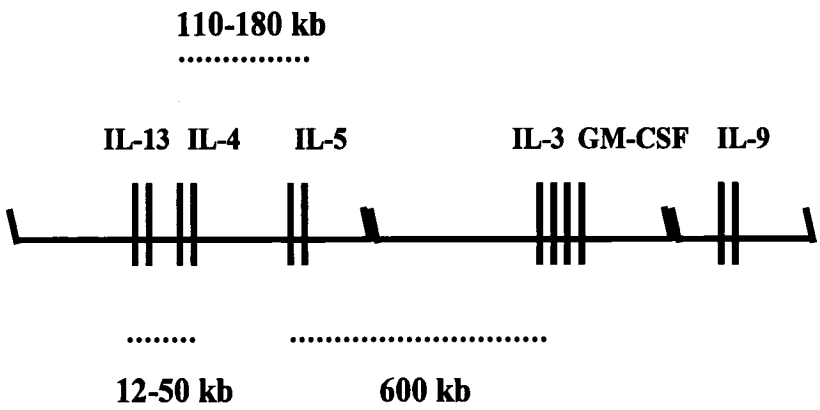


Figure 9: Map of the cytokine gene cluster on human chromosome 5

Human chromosome 5 (near segment q31), distances are approximate. The human IL-9 gene spans 4 kb on chromosome 5q31-q35 region; mIL-9 maps to chromosome 13; mIL-3, 4, and 5 are on chromosome 11. IL-9 cDNA sequence is composed of 5 exons and 4 introns.

Linkage studies performed on human populations [154,162] and in mice, as well as expression profiles [151] have proposed IL-9 as a candidate gene for asthma. The IL-9 gene expression seems to be under the control of different transcription factors. The IL-9 promoter region have been shown to bind to the activator of protein-1 (AP-1) and AP-2 transcription factors [160]. However, the minimum sequence required for IL-9 gene basal and inducible expression induction was described in human T cell leukemia virus type-1 transformed T cells, C5MJ2. It contained an NFκB site, an upstream 20 bp adjacent site, c-Jun, and potentially new proteins (35 kd) [163]. The expressed protein is a 14 kD glycoprotein. It has a signal sequence of 18 amino acids (aa), with a mature form of 144 aa. The IL-9 protein contains a high proportion of cationic aa residues and 10 cysteines and has 4N-linked glycosylation sites. The homology between human and murine IL-9 is 69% at the nucleotide level and 55% at the protein level [160].

IV.C. Regulation of IL-9 Expression

The requirement for T cell activation for IL-9 to be produced may be explained by the induction of IL-9 expression by IL-2 in human T cells [149,164]. In fact, a cascade of cytokines is involved in IL-9 expression: IL-2 induces IL-4 production, which in turn leads to IL-10 production when combined with IL-2, and consequently IL-2, IL-4 and IL-10 induce IL-9 production. The induction of IL-9 production in mast cells by cytokines such as IL-1, IL-10 and kit ligand, point to an important role played by IL-9 in the development of late asthmatic reaction [165,166]. In addition, a few reports have linked allergen challenge to an increase in IL-9 positive cells in the bronchoalveolar lavage fluid in patients with atopic asthma [167].

IV.D. The IL-9 Receptor

The human IL-9 receptor belongs to the hematopoietin receptor superfamily. IL-9 exerts its action by binding to the IL-9R, which is a heterodimer consisting of the IL-9Rα chain and the IL-2Rγ chain (Figure 10). This common γ chain is used by other cytokines such as IL-2, IL-4, IL-7 and IL-15 [168]. The human IL-9Rα gene is composed of 11 exons

and 10 introns ranging approximately over 17 kb [169]. Interestingly, a linkage for asthma and bronchial hyperresponsiveness has also been reported for the IL-9R gene locus [170].

In humans, expression of the IL-9Rα chain has been documented on different cell types involved in the pathogenesis of asthma. IL-9R alpha-chain protein expression was detected in cultured human airway smooth muscle (ASM) cells, primary cultured ASM cells, as well as bronchial smooth muscle cells within biopsies of asthmatics [171]. Polymorphonuclear neutrophils expressing the IL-9Rα chain have been detected in patients with airway dysfunction and damage [172-174]. In particular, RT-PCR analysis showed the presence of IL-9Ralpha-chain mRNA in PMN RNA preparations from asthmatic patients.

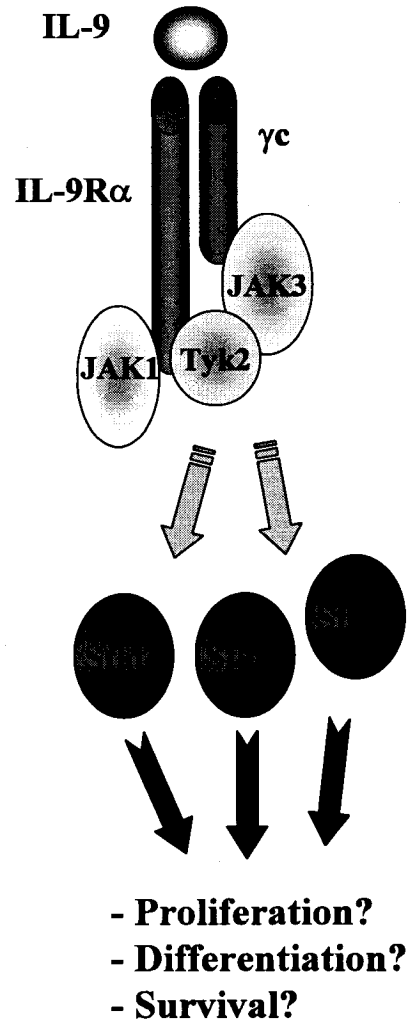


Figure 10: The IL-9R Complex and Signaling Components

IL-9 transduces its signal by sharing the common gamma chain with other cytokines IL-2, IL-4, IL-15, and using a specific alpha chain. Upon ligation with IL-9, JAK-1, JAK3, and Tyk2 can be activated and the signal is then propagated to STAT-1, 3, and 5 to induce proliferation, differentiation or survival.

Using FACS analysis, surface expression of IL-9Ralpha was detected on PMNs freshly isolated from asthmatics, and to a lesser extent on normal controls. In addition, protein expression of IL-9Ralpha was also detected in peripheral blood and bronchoalveolar lavage PMNs [175]. Finally, bronchial biopsy specimens from asthmatic patients had a higher level of IL-9R expression compared to controls thus confirming the potential role of IL-9 in asthma [151]. Moreover, a role for IL-9 in other inflammatory diseases is suggested from a study showing a significant increase in the expression of the IL-9R in the upper airways of cystic fibrosis patients compared to controls [176].

The first study to describe the presence of the IL-9R on murine cells shows no response for fresh non-activated or Concanavalin A-activated splenic B cells to stimulation with IL-9, along with no significant binding indicating either an absence or the presence of a low level of the receptor on these cells [177]. In another study in mice, transgenic expression of IL-9 leads to an expansion of the B-1 lymphocyte population with a majority of the expanded cells belonging to the B-1b subpopulation ($IgM^+Mac\text{-}1^+CD5^-$). Authors of the study demonstrate that the IL-9R is preferentially expressed on B1 cells both $CD5^+$ as well as $CD5^-$, in contrast, it is barely detectable at the surface of IgM+ cells from the spleen which are B2 cells [169]. Hence, these two studies seem to indicate that in mice IL-9R expression is mainly found on B1 cells which are characterized by their peritoneal and pleuropericardial location and their ability to respond to T-independent antigens [178].

IV.E. IL-9-induced Signalling

As depicted in figure 10, IL-9 signaling involves activation of Janus kinase (JAK)1 and JAK3 kinases which are associated with the IL-9 specific receptor and gamma chain respectively [179]. Jak kinases establish docking sites for SH2 and PTB domain containing signalling proteins such as STAT transcription factors and insulin receptor substrate (IRS) adaptor proteins by phosphorylation of the receptor tyrosine residues. Signal transducer and activator of transcription (STAT)1, STAT3 and STAT5 [179-182] are activated by IL-9. [3,5,8]STAT5 activation was shown to be required for IL-9

dependent growth and transformation of lymphoid cells. Upon mutation of the STAT5 binding site of the IL-9 receptor, STAT activation was greatly decreased and proliferation was inhibited. Expression of a constitutively active form of STAT5 results in IL-9 independent cellular growth of a transfected B cell line [183]. A specific role for STAT1 and STAT3 in differentiation gene induction has been shown by introducing point mutations in amino acids in the STAT binding region in the IL-9R [181]. In addition, IRS1 and IRS2 are required for mitogenic responses [180,184-187]. Phosphatidylinositol 3'-kinase (PI3K) and Grb-2 interact with tyrosine-phosphorylated IRS proteins subsequently. PI3K activity is necessary for the IRS-1/2-mediated proliferative effect of IL-9 [187]. In T cells and mast cells, IL-9 upregulated the expression of BCL3 gene in a JAK/STAT dependent pathway. BCL3 activates NF-κB leading to the transcription of a variety of genes implicated in stress and immune responses [188]. On the other hand, IL-9 signal attenuation seems to occur through induction of cytokine-inducible SH2-containing protein (CIS), and the suppressor of cytokine signaling (SOCS)-2 and 3 in a STAT-dependent manner. However, only SOCS-3 inhibited IL-9-induced signal transduction [189].

IV.F. Biological Effects of IL-9

Interleukin-9 and the α-chain of the IL-9 receptor (IL-9Rα) have been shown to affect the differentiation pathway of different cell types including human T cells [189], eosinophils [190], mast cells, and B cells [147,191].

IV.F.1. Effect on T cells

Using a chimeric human-mouse fetal thymic organ culture, a role for IL-9 in early thymic T cell development was demonstrated. The use of a neutralizing antibody to the IL-9Rα chain greatly reduced the number of thymocytes derived in vitro from human $CD34^+$ hemopoietic precursor cells, leading to decreased cell numbers of $CD4^+CD3^-CD8^-CD1^+$ progenitor cells and subsequently $CD4^+CD8^+$ double positive (DP) thymocytes [192]. However, as deletion of the IL-9 gene in mice did not affect T cell development as assessed by flow cytometry, whereby the expression of different T cell markers was

equivalent in wild type and knock-out mice, one may question whether human and murine systems are similar enough to allow us to conclude that IL-9 helps maintaining T cell survival but is not essential for T cell development [193]. Data obtained using transgenic mice indicate that IL-9 overexpression increases susceptibility to T-cell lymphoma development following irradiation or administration of low doses of mutagens [191,194] whereas in vitro, only murine T lymphoma and myeloid leukemia cells proliferate in response to IL-9 and not normal cells [191,195]. This evidence points to a role for IL-9 in T cell survival and tumor formation.

IV.F.2. Effect on Eosinophils

Interaction between IL-9 and eosinophils was examined in humans by purification of mature peripheral blood eosinophils. The expression of the IL-9Rα chain was detected, and eosinophils were protected from apoptosis in the presence of IL-9. In the presence of IL-5 and IL-3, IL-9 potentiated the development of eosinophils from human $CD34^+$ cord blood cells and human promyelocytic leukemia cell line (HL-60). *In vivo*, locally instilled IL-9 increases eosinophil count in BAL fluid [196]. Moreover, IL-9 transgenic mice were found to display significantly enhanced eosinophilic inflammation [197] suggesting that IL-9 may potentiate eosinophil function *in vivo*. IL-9 induced the expression of IL-5Rα on human $CD34^+$ cord blood progenitor cells [190]. Eosinophils were also found to be expanded in IL-9 transgenic mice in an IL-5 dependent pathway [198]. Strikingly, IL-9 knock-out mice were able to develop eosinophilic inflammation in a similar degree to their wild-type littermates [199], suggesting that in the murine system and possibly in humans redundancy exists with respect to IL-9's effects on inflammatory cells.

Eosinophils were also shown to have the capacity to synthesize and release IL-9 [200], this was observed in human peripheral blood eosinophil RNA preparations from subjects with atopic asthma, as well as in the eosinophil-differentiated HL-60 cell line. Moreover, cultured human peripheral blood eosinophils from asthmatic subjects synthesize and release IL-9 protein, which is upregulated on stimulation with TNF-alpha and IL-1beta.

IV.F.3. Effect on Mast cells

IL-9 was shown to affect other cell types such as mast cells, whereby IL-9 synergized strongly with stem cell factor to induce their growth and differentiation [201]. IL-9 was found to synergize with IL-3 in promoting the proliferation of bone marrow-derived mast cells [147]. IL-9 was not only found to potentiate mast cell growth but it also played a role as a differentiation factor. In vitro, IL-9 induced mouse mast cell proteases transcripts expression including mMCP-1 [202], mMCP-2, mMCP-4, as well as granzyme B [203]. Moreover, IL-9 increased the expression of the α-chain of the high affinity IgE receptor (FcεRIα) and stimulated IL-6 production [204]. It was also shown that BMMC were potent producers of IL-9 in response to IgE-allergen complexes challenge, thus implicating IL-9 in the regulation of the asthmatic inflammatory responses [166]. Studies on IL-9-transgenic mice have shown a mast cell expansion correlating with higher serum concentrations of mMCP-1 [205]. On the other hand, deficient IL-9 gene expression in mice decreases the number of mast cells in a model of *Schistosoma* infection [193].

IV.F.4. Effect on B cells

It has been shown that IL-9 can potentiate IL-4-induced IgG and IgE production from normal human PBMC, as well as from LPS stimulated murine B lymphocytes [206,207]. Moreover, transgenic mice that constitutively express IL-9 were shown to have an increase in both baseline and antigen-specific immunoglobulin concentration for all isotypes tested [169].

Allergen-exposed IL-9 transgenic mice exhibit many features of human asthma, and have increased IgE production [197]. In addition, intratracheally instilled IL-9 in lungs of C57BL/6 mice induced a significant elevation in total serum IgE in these mice [196]. However, it is not clear whether IL-9 specifically increases the production of IgE isotype or induces a global increase in Ig levels. These data strongly suggest a role for IL-9 in enhancing antibody production, and potentially modulating B cell growth and/or differentiation (Figure 11).

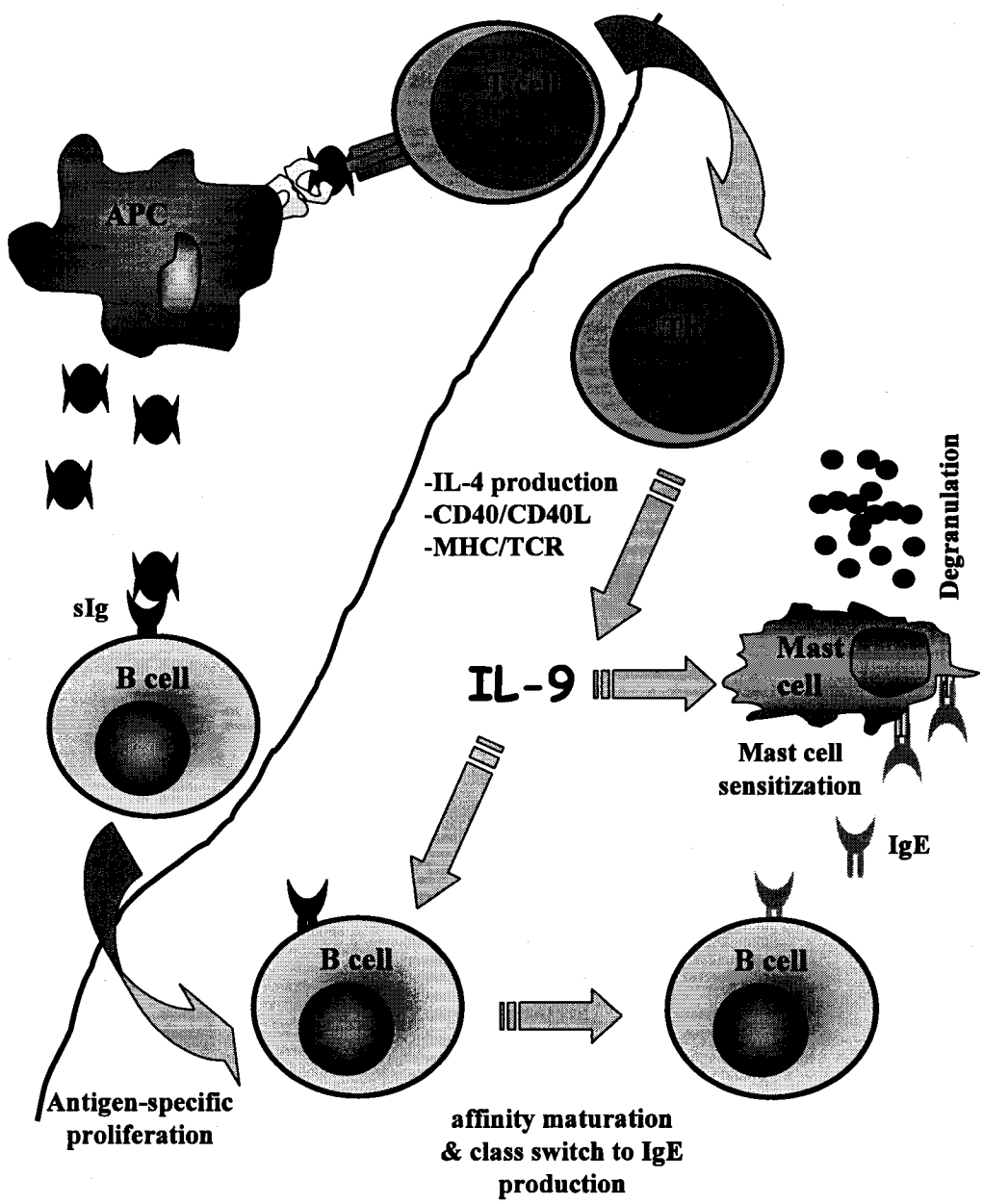

APC
-IL-4 production
-CD40/CD40L
-MHC/TCR
Degranulation
sIg
B cell
IL-9
Mast cell
Mast cell sensitization
IgE
B cell
B cell
Antigen-specific proliferation
affinity maturation & class switch to IgE production

Figure 11: Effect of IL-9 on B Cells

Based on studies performed on PBMCs, IL-9 may affect B cell differentiation and antibody production. While its role in the periphery has been approached, studying the effect of IL-9 in the context of B-T cell interaction in the lymphoid follicles with the potential modulation of immunoglobulin production will help elucidate its role in the late phase of the T-cell dependent immune response within immune synapses.

IV. F.5. Pleiotropic effects of IL-9

IL-9 overexpression studies amplified the direct effect of IL-9 and/or its synergistic effect with other cytokines resulting in its pleiotropic effects on different hematopoietic cell lineages leading to lymphomagenesis, increased immunoglobulin secretion, mastocytosis, eosinophilic infiltration, airway inflammation, and subepithelial collagen deposition [194,208]. In addition, IL-9 synergizes with TNF-α to induce IL-8 secretion, and synergizes with IL-13 to increase eotaxin release from airway smooth muscle cells in vitro [209]. Moreover, IL-9 synergizes with IL-5 in driving eosinophilic maturation of precursors (Figure 12) [190,198].

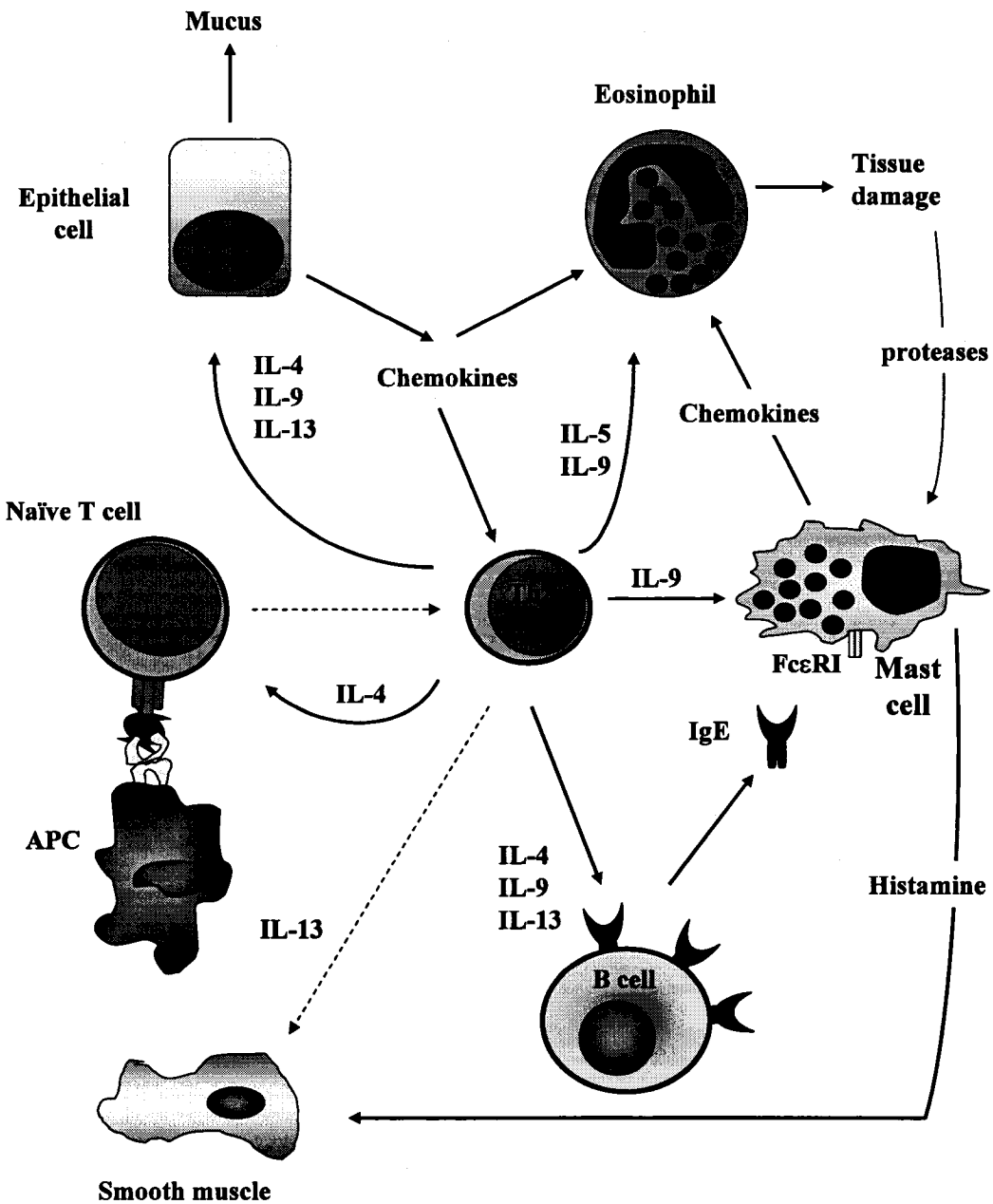

Mucus
Eosinophil
Epithelial cell
Tissue damage
IL-4
IL-9
IL-13
Chemokines
IL-5
IL-9
Chemokines
proteases
Naïve T cell
IL-9
FcεRI
Mast cell
IL-4
IgE
APC
IL-4
IL-9
IL-13
Histamine
IL-13
B cell
Smooth muscle

Figure 12: Pleiotropic Effects of IL-9: Potential Role in the Allergic Inflammatory Response.

IL-9 has been shown to exert an effect on a variety of immune and non immune cells. The effects range from modulation of T cell survival to induction of mucin expression from epithelial cells. IL-9's pleiotropic effects on key players of the allergic response may constitute a susceptibility factor for atopy and allergic inflammation. (depicted from Renauld, J. C. et al. (216))

IV.G. Role of IL-9 in the Allergic Inflammatory Response

Human and mouse genetic studies implicate IL-9R expression and IL-9 in the pathogenesis of asthma and allergic inflammation [154,162]. Indeed, overexpression of IL-9 in mice whether systemically [194], or under the control of a lung-specific promoter [208] results in a myriad of symptoms which resemble the phenotype of asthma including mucus overproduction, subepithelial fibrosis, increased intraepithelial mast cells, lung eosinophilia, airway hyperresponsiveness, and increased responsiveness to Ag stimulation [197,208]. Concomittant to IL-9's enhancement effect increased production of IL-4, IL-5, and IL-13 is observed. In a murine asthma model, IL-4 and IL-5 were necessary for IL-9-induced eosinophilia, and IL-13 was necessary for lung inflammation and mucus production [210].

The effect of IL-9 on different inflammatory and structural cell types has been studied in the context of asthma (Figure 12) [13]. Functional studies showed that stimulation of PMNs from asthmatics with IL-9 results in the release of IL-8 in a concentration-dependent manner. Anti-IL-9 neutralizing Abs suppressed this effect [175]. Airway epithelial cells are activated by IL-9 and produce several chemokines, proteases, ion channels and selective mucin genes [211,212]. Further, IL-9 induced mucin production in allergic airway disease both in vitro and in *in vivo* studies [213]. Airway smooth muscle cells are also a target for IL-9 which acts by amplifying the potential for these cells to recruit eosinophils and neutrophils into the airways through upregulation of the IL-5Rα receptor, the TNF-α induced secretion of IL-8 [209], and IL-13 induced eotaxin release [209]. Hence, IL-9 exerts a wide range of biological actions affecting not only inflammatory cells but also structural cells (Figure 12). IL-9 seems to be acting by synergizing with other inflammatory cytokines. Interestingly, the observed effects seem to be restricted or more pronounced in asthmatic human subjects which is consistent with the genetic studies performed in mice, whereby higher expression levels of IL-9 correlated with susceptibility to bronchial hyperresponsiveness [154], strongly suggesting a complex role for this cytokine in the pathogenesis of allergic inflammation and asthma. This has been substantiated by a series of studies in humans showing an increased expression of IL-9 in

bronchial biopsies from asthmatic patients when compared to control biopsies [151], and a role for IL-9 in airway hyperresponsiveness and mucus overproduction in asthmatics [212]. Specifically, an upregulation of the human calcium-activated chloride channel hCLCA1 in mucus producing epithelium of asthmatics compared to control subjects has been observed with a strong correlation between hCLCA1 and IL-9 found [214].

Moreover, IL-9 may promote systemic anaphylaxis reactions, acting at both the sensitization and effector stages, but is not absolutely required for this process as a murine passive model of systemic anaphylaxis shows. IL-9 expression is linked to increased susceptibility to fatal anaphylaxis when mice are sensitized by immunization against OVA or DNP-BSA [215].

The effect of blocking IL-9 activity in the allergic response is controversial. Neutralizing IL-9 with a mAb decreased the IgE response, pulmonary eosinophilia, goblet cell hyperplasia, airway epithelial hyperplasia, and airway hyper-responsiveness in DBA/2 x C57BL/6 F_1 mice [216]. In another study, anti-IL-9 Ab administration to previously sensitized mice was able to prevent airway hyperreactivity and lung eosinophilia upon aerosolized antigenic challenge [217]. By contrast, in a similar OVA-induced asthma model, IL-9-deficient mice showed no decrease in allergic pulmonary airway hyperactivity, eosinophilia, goblet cell hyperplasia, or specific IgE [199]. Finally, in a typical Th2 infection model, anti-IL-9 vaccination prevented *Trichuris muris* expulsion and blood eosinophilia, but not the IgE response [218].

IV. H. IL-9 and Disease

Besides its role during immune responses, its growth factor and antiapoptotic activities on multiple transformed cells suggest a potential role in tumorigenesis. Indeed, IL-9 overexpression induces thymic lymphomas in mice [191,194], and IL-9 release is associated with Hodgkin disease [219,220] and HTLV-I transformed T cells in humans [161,221,222]. Moreover, in vitro, dysregulated IL-9 response can result in autonomous cell

growth and malignant transformation of lymphoid cells associated with constitutive activation of the Jak/STAT pathway [183].

In addition to IL-9's described role in allergy and asthma, IL-9 was also shown to be a susceptibility factor in Leishmaniasis whereby IL-9 neutralization in L.major-infected nonhealer BALB/c mice doubled the time span until pathological disease progression occurred. This was translated in a reduction of detrimental Th2/type 2 responses with an observed shift toward protective Th1 immune responses [223].

V. Allergic Inflammation

The complex coexistence of three different features defines the pathophysiology of asthma, namely: 1) reversible intermittent airway obstruction 2) bronchoyyperresponsiveness (BHR), defined as an increased sensitivity of bronchial smooth muscle cells to bronchoconstrictors such as histamine or cholinergic agonists, and 3) chronic bronchial airway inflammation [224]. The inflammatory process of asthma is characterized by the accumulation of Th2 lymphocytes and eosinophils in the airways. The secretion of cytokines, particularly IL-4, IL-13, IL-5, and IL-9 is mediated by Th2 cells and contributes to the development of asthma by recruitment and accumulation of different inflammatory cellular types to the primary inflammatory lesion of asthma. In human asthma, expression of both IL-9 and its receptor was reported recently, and IL-9 mRNA expression correlated with the airway responsiveness to metacholine [151].

V.A. Cytokines in Allergic Inflammation

Once the existence of Th1 and Th2 cells have been established, accumulating evidence pointed to a role for Th2 cells in the incidence of atopic diseases based on experiments in mice and humans [225,226]. Conversely, Th1 cells preferentially produce IL-2 and interferon γ (IFN-γ), thereby promoting cellular immune responses including the activation of cytolytic T cells, and the killing of intracellular pathogens by macrophages. Indeed, it is the Th2 cells that recognize allergens via their T cell receptors (TCRs) and

release interleukins in their surrounding milieu that play a role in recruiting a variety of inflammatory cells such as IgE antibody-producing B cells (IL-4, IL-13), mast cells (IL-4, IL-10), and eosinophil granulocytes (IL-5) in allergic inflammation (reviewed in [226]). Experimental evidence obtained from murine models of allergy and asthma showed that transfer of Th2, but not of Th1, cells into recipient mice induces airway eosinophilia, mucus hyper-secretion, and airway hyperresponsiveness (AHR). Finally, transgenic mice that overexpress the Th2 cytokines in the airway epithelium exhibit airway eosinophilia, mucus hyperproduction, AHR, and airway remodeling [226]. In humans, $CD4^+$ T cells producing IL-4, IL-5, and IL-13 have been identified in BAL and airway biopsies from patients with asthma. These cytokines are also secreted in the airways of patients with mild or asymptomatic disease [140,227]. Moreover, the number of Th2 lymphocytes is augmented in the airways of patients with allergic asthma following antigen challenge [140,227,228]. Based on these data, it has been proposed that Th2 cells trigger an inflammatory response that results in asthma.

This proposal can be substantiated by a number of findings involving the Th2 cytokines IL-4, IL-5, IL-9, and IL-13 which can account directly or indirectly for the great majority of pathophysiological manifestations of allergic inflammation. IL-5 and chemokines attract eosinophils to target tissues, while IL-4 induces the rolling on, and adhesion to, endothelial cells of circulating eosinophils [229]. IL-13 is responsible for mucus hypersecretion by mucus cells, and induces metaplasia of mucus cells [230]. IL-4 and IL-13 stimulate fibroblast growth and chemotaxis, as well as the synthesis of extracellular matrix proteins [231]. In addition, IL-4 and IL-13 are key factors in immunoglobulin production by B lymphocytes particularly isotype switching to IgE.

V.B. Regulation of the IgE Response

As B cells are taught to fight infection by producing and refining weapons that are used for normal host immune reactions, disease emerges from their use against allergens present in the environment. This triggers a series of events that lead to atopic diseases namely allergic rhinitis, asthma, and atopic dermatitis.

Patients suffering from atopic diseases have elevated serum levels of antibodies of the IgE isotype, which is normally produced in response to parasitic infections. The clinical symptoms of atopy result from the release of inflammatory mediators (e.g histamine and leukotrienes), enzymes and cytokines subsequent to the crosslinking of IgE on tissue mast cells by its high-affinity Fc receptor, FcεRI [232]. The presence of FcεRI has been demonstrated on other cell types including Langerhans cells [233], a subset of monocytes [105], and dendritic cells [234]. Other cell types were shown to express FcεRI albeit with some controversy about their surface expression: eosinophils [235,236], neutrophils [175], and platelets [237]. Importantly, it was observed that the modulation of FcεRI on these cells depended on serum IgE levels, with important implications for the biology of atopy and the design of therapeutic intervention modules [238]. IgE plays also a role in antigen presentation by allergen uptake and binding through the low affinity Fc receptor, FcεRII, on B cells as well as other immune cells [232]. The low affinity IgE receptor (CD23) (FcεRII) has been identified on B cells, monocytes, follicular dendritic cells, Langerhan's cells, eosinophils, platelets, and airway smooth muscle cells [239,240]. FcεRII, (CD23) controls IgE secretion and transport [241]. Hence, understanding the regulation of IgE production is crucial to the elucidation of the mechanisms underlying IgE-related diseases such as asthma.

IgE production is controlled at the level of IgE isotype class switching which depends on the development of Th2 cells. As a number of cytokines and molecules discussed above influence this process (Figure 13), the Th2 cytokine IL-9 described thus far as an 'atopy' related gene may contribute to the regulation of IgE production alone or by synergizing with other Th2 cytokines.

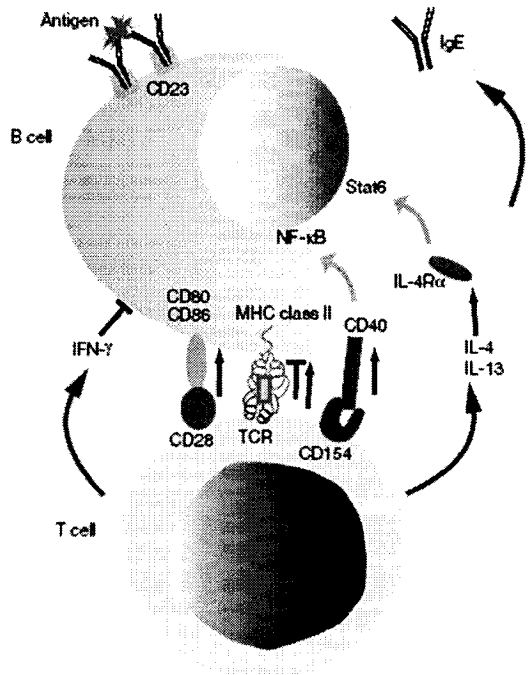


Figure 13: Molecular Control of the IgE Response

The established paradigms attribute induction of IgE production to IL-4 and IL-13 mainly, although other cytokines such as IL-9 may play a role in this regard. CD40-CD154, and CD86-CD28 interactions as well as other cognate interactions possibly through CD27-CD27L promote IgE production through direct effects on T cell and B cells. Transcription factors such as STAT-6, induced by IL-4Rα, and NF-κB, induced by CD40 promote IgE production by initiating epsilon transcript synthesis and class switching.

VI. IL-9 in the Immune Synapse

LD cells, representing mainly a population of GC cells, will be examined in our system for the expression of the IL-9Rα chain. It is actually at the level of the immune synapse in the GC of the secondary lymphoid follicles that B-T cell interaction occurs at the edge of their respective areas [242] with a variety of cognate molecules involved. At this stage, a role for CD40 in isotype switching is crucial for the development of the memory B cell response. Different key players have been documented: IL-4, a Th2 cytokine plays a major role in driving IgE production. The persistence of the memory B cell was shown to be independent of persisting immunizing antigen [243], suggesting that BCR-independent signals may operate at the immune synapse to sustain selected memory B cells. Is IL-9R expressed on GC cells, and if so what is the phenotype of IL-9R positive cells? Does IL-9 affect the survival of selected centrocytes thus increasing the number of memory B cells exiting the GC reaction? What effect does it exert in the presence of other cytokines such as IL-4 in the modulation of IgE production? Are there other molecules than IL-4 involved in IgE production that may interact with IL-9?

THESIS HYPOTHESIS

IL-9 plays a role in B-T cell interactions in the context of GC B cell maturation, and modulates memory B cell differentiation and IgE production in inflammatory responses.

THESIS OBJECTIVES

The major objectives of this work were to characterize the expression of the IL-9Rα chain on human tonsillar B cells at different maturational stages in the germinal center, and determine its effect on B cell differentiation and survival among high expressors.

With respect to the expression of the IL-9Rα chain, we were interested in investigating the expression levels on B cells of human tonsils following fractionation into different subpopulations. This was of interest as it shows the potential role of IL-9 in the maturation pathway of antigen-driven GC B cell differentiation. This is an important issue since this process leads ultimately to B cell differentiation into an immunoglobulin producing plasma effector cell or a memory B cell with the potential to isotype switching.

The human IL-9 gene has been mapped together with the other Th2-type cytokine genes IL-4, IL-5, and IL-13 in the 5q31-33 region of chromosome 5; this segment of the genome represents a candidate region for genes that determine susceptibility to bronchial hyperresponsiveness and atopy. Therefore, it was of interest to investigate the contribution of IL-9 to B-T cell interactions and its effect on IgE production in light of its potential role in health and disease as a switch factor in immunoglobulin production.

CHAPTER 2: MATERIALS AND METHODS

B-cell purification

Fresh B lymphocytes were isolated from human tonsils discarded following surgery. Hypertrophied tonsils from pediatric patients of variable ages were obtained after routine tonsillectomy. Tonsils were selected as a source of B cells for our study, because human tonsils receive repeated antigen stimulation, and display all stages of peripheral B cell development; hence, they constitute an ideal tissue to determine IL-9R expression at different stages of B cell maturation. The tonsils were thoroughly minced, resuspended in wash medium consisting of RPMI 1640 (Gibco, BRL Canada) supplemented with 2% FCS (Hyclone Laboratories Inc.), 50 U/ml penicillin, 50 μg/ml streptomycin, and amphotericin B (1/500 w/v) from Life Technologies (Burlington, ON, Canada); and then layered onto a Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) gradient. Tonsil lymphocytes were separated by rosetting with neuraminidase-treated sheep red blood cells and Ficoll-Paque density centrifugation. Monocytes were removed from the E-rosette-negative fraction by adherence depletion; the remaining B cells were routinely demonstrated to be > 98% pure on flow cytometry by CD20 staining, with <1% CD14+ and <1% CD3+.

Percoll gradient centrifugation

Adherence-depleted B cells were layered onto Percoll gradients (Pharmacia Biotech). A 90% Percoll solution was prepared by dilution with 10X PBS (Gibco, BRL). Dilutions of 60%, 50%, 30% and 20% were prepared by diluting the 90% solution with 1XPBS. Dilutions of 90%, 60%, 50% and 30% were layered in 15 ml Falcon tubes (Becton Dickinson, La Jolla, CA), according to the methods of Liu et al. [244] and Suzuki et al. [245]. The cell suspension ($25x10^6$ cells at $10x10^6$ cells/ml) was mixed 1:1 with 20% Percoll and applied to the top of the gradient. Following centrifugation (20 min, 4°C, 2400 rpm), the fractions were aspirated; cells recovered below the 60% layer were considered high density cells which are resting B cells; between the 50 and 60% layers, medium density or cycling centroblasts and centrocytes and low density cells GC cells were recovered from the 30-50% layers [244,246].

Anti-CD40 antibody purification

Culture supernatants of G28.5 hybridoma cell line were filtered on a 0.45 μm filter before passing on an anti-mouse IgG agarose column (Sigma, Saint Louis, USA). The column was washed with PBS (pH 7.3; no azide), and the flow adjusted to ~ 50-60 ml/hour. The filtered supernatant was passed twice over the column and the flow-through was collected. After washing the column with PBS until OD ≤ 0, bound antibody was eluted with a 15 ml of 100 mM glycine-HCl 0.15 nM NaCl pH 2.5. High OD280 tubes were then pooled, and neutralized with 1 M Tris pH 9.5 to pH 7.0. The elute was concentrated with Amicon Concentrator, filtered on a 0.22 μm membrane, and stored in aliquots at -80°C.

Cell Culture

Purified LD B cells were cultured in RPMI 1640 supplemented with 10% FCS, 5 mg/ml L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (Invitrogen). To assess IgE secretion, Percoll-fractionated tonsillar LD B lymphocytes were cultured in 24-well plates (1ml/well) at a concentration of $0.5x10^6$ cells/ml in the presence of anti-CD40 (1 μg/ml) and IL-4 (400 U/ml) alone or in the presence of IL-9 (10 ng/ml) in duplicates. Cell supernatants were harvested on the 7th and 14th day of culture and kept at -20°C until use. Alternatively cells were stained with different antibodies (anti-CD27, anti-CD38, anti-IgD, anti-IL-9Rα) and processed for flow cytometry at days 1, 6, and 10.

Immunocytochemistry

Cytospin slides were prepared from tonsillar B cells, fixed in 4% paraformaldehyde for 20 min at room temperature, and washed with 0.05 M Tris-HCl-buffered isotonic saline, pH 7.6 (TBS). After drying, the slides were stored at -20°C before immunocytochemistry.

The cytopreparations were washed with Tris-buffered saline (TBS) (pH 7.6). After saturation for 10 min with a Universal Blocking solution (DAKOCytomation Inc., Mississauga, Ontario, Canada), the cells were incubated with mouse anti-IL-9R-α (R&D Systems, Cedarlane Laboratories Ltd, Ontario Canada) or isotype-matched control each at 5 μg/ml overnight at 4°C. After washing, rabbit anti-mouse Ig (1:60) (DAKOCytomation) was added for 30 min at room temperature followed by alkaline phosphatase anti-alkaline phosphatase (1:60) (DAKOCytomation.) for 30 min at room temperature. After washing with TBS, the slides were developed using Fast Red (Sigma, Toronto, ON) and counterstained with hematoxylin Gill II (Surgipath Canada Inc.) and examined by microscopy.

Immunohistochemistry

Cryostat sections of human tonsillar tissue were fixed in acetone:methanol. Sections were blocked for 10 minutes with a Universal Blocking solution, briefly washed with TBS, and incubated in a humid chamber overnight at 4°C with a rabbit anti-human IL-9Rα antibody (1/100) (Santa Cruz Biotechnology, California USA), and a mouse anti-human CD20 (1/100) (DAKOCytomation, Inc. Canada). After washing in TBS, sections were incubated with a second layer antibody (horse anti-mouse IgG Biotin 1/100 (Vector Labs Canada Inc., Burlington, Ontario, Canada) for CD20 and swine anti-rabbit IgG biotin (DAKO) 1/100) for IL-9R. Following another wash in TBS, sections were incubated with StreptAvidin-alkaline phosphatase (1/200 in TBS) (DAKOCytomation Canada Inc.) for CD20 and Horse Radish Peroxidase complex for IL-9R for 30 min (DAKOCytomation Canada Inc.). This was followed by incubation in the alkaline phosphatase substrate Fast Red for 20 minutes, revealing bound mAb as a red deposit, and 5 min incubation with the chromagen solution 3,3'-diaminobenzidine (DAB) (DAKO) + H_2O_2 revealing the IL-9Rα chain. Slides were counterstained with hematoxylin, dehydrated, and mounted for viewing.

Immunofluorescence

Slides were processed for immunofluorescent labeling as described earlier for immunohistochemistry. Primary monoclonal antibodies directed against CD19 (1:10) (Pharmingen) were labeled with Alexa Fluor 568 using the Zenon Red kit from Molecular Probes (Portland, OR). Monoclonal Ab against IL-9Rα (1:50) (R&D) was followed by an FITC-conjugated goat anti-mouse IgG Ab (Pharmingen). Slides were mounted after washing in TBS in PermaFluor mounting medium from Immunon and viewed with a confocal fluorescence microscope.

Flow cytometric analysis

For flow cytometric analysis, B cells were incubated with saturating concentrations of surface marker staining antibodies. Three-color analysis was performed with unlabeled IL-9R mAb and FITC-goat anti-mouse Ab, directly PE-conjugated mouse anti-human IgD, and APC-conjugated mouse anti-human CD38 (BD Biosciences, Mississauga, Ontario, Canada). PE-conjugated IgG1 isotype was used as a control of specificity for IgD staining, APC-conjugated IgG1 isotype was used as a control for CD38, and mouse IgG1 followed by FITC-conjugated goat anti-mouse was used as a control for specificity for IL9R FITC staining. After staining, cells were resuspended in 2% paraformaldehyde and $5x10^5$ cells/sample were analyzed by FACSCalibur using Cell Quest software.

For flow cytometric analysis, B cells were incubated with saturating concentrations of surface marker staining antibodies. Two-color analysis was performed with unlabeled IL-9R mAb and FITC-goat anti-mouse Ab, and directly PE-conjugated mouse anti-human CD27 (BD biosciences). PE-conjugated IgG1 isotype was used as a control of specificity for CD27 staining, and mouse IgG1 followed by FITC-conjugated goat anti-mouse was used as a control for specificity for IL9R FITC staining. After staining, cells were resuspended in 2% paraformaldehyde and $5x10^5$ cells/sample were analysed by FACSCalibur using Cell Quest software.

STATs phosphorylation analysis

$1x10^6$ LD cells were stimulated with IL-9 (10 ng/ml) for 15 min. Cells were fixed at 37°C (for 10 min) by adding formaldehyde to a final concentration of 2%. After centrifugation, tubes were placed on ice for 2-3 min, then 1 ml 100% ice cold methanol was added. Cells were placed on ice for 30 min, then processed for antibody labeling. Phosphorylated STAT-5 was labeled with a phosphospecific antibody (rabbit polyclonal) that detects the phosphorylated tyrosine residue (Tyr 694) associated with activated STAT-5 (Cell Signaling Technology, Inc. Danvers, MA, USA), Phosphorylated STAT-3 with an antibody that detected phosphoTyr 705, and anti-phospho- STAT1 (Tyr 701). The antibodies were used at a 1/100 dilution. Cellular fixation and immunostaining were performed as described previously [247]. After staining, intracellular levels of phosphorylated STATs were measured by flow cytometry.

Immunoblotting

Total unfractionated tonsillar B cells as well as low density B cells were incubated with IL-9 (R&D) for 15 min. Following the reactions, cells destined for immunoblotting were washed once with ice-cold PBS and lysed in lysis buffer (1% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 25 mM Tris-HCl pH 7.5, 5 mM EDTA, 50 mM NaF, 0.1 mM Na_3VO_4). Whole cell lysates (15 μg total protein) boiled in 2 x Laemmli buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 25% glycerol, 10% 2-β-mercaptoethanol) were then resolved on 12% SDS-PAGE and immunoblotted with anti-phosphotyrosine monoclonal antibody 4G10 (Upstate Biotechnology, NY, USA), anti-phospho-STAT3 (Tyr 705), or anti-phospho-STAT5 (Tyr 694), anti-phospho-STAT1 (Tyr 701) (Cell Signaling Technology, Inc. Danvers, MA, USA).

Proliferation Assay

Low density B cell proliferation was assessed by thymidine incorporation. Cells were cultured for 72h in a 96-well plate in the presence of 10 ng/ml of rhIL-9 alone or in combination with an antibody to the BCR (aIgM 2 ug/ml). Alternatively rhIL-4 was added alone (400 U/ml) or in the presence of aIgM at a final cell density of 1 X 10^5 cells/ml in a volume of 200 µl/well in 96-well round-bottom plates for 3 days at 37°C in a humidified atmosphere with 5% CO_2. Then cells were pulsed with 1 uCi/well for 18h, thymidine incorporation levels were measured by liquid scintillation counting. Alternatively cells were cultured in the presence of IL-9, IL-4 and anti-CD40 mAb (1 µg/ml) in similar conditions.

Immunoglobulin assay by enzyme-linked immunosorbent assay

Purified LD tonsillar B cells were cultured with or without aCD40 (1µg/ml) and IL-4 (400 U/ml) in the presence or absence of IL-9 (10 ng/ml) at a final cell density of 5 X 10^5/ml in a volume of 1 ml/well for 14 days at 37°C in a humidified atmosphere with 5% CO_2. The cultured supernatants were harvested and added to Ninety-six-well plates (Costar, Corning Corp, Acton, Mass) coated overnight at 4°C with 5 µg/mL rat anti-human IgE (Biosource, Camarillo, Calif) in 0.05 mol/L carbonate-bicarbonate buffer, pH 9.6. After washing 3 times with PBS/0.1% Tween 20, the plates were blocked with PBS plus 0.5% gelatin (Sigma) for 2 hours at 37°C and then incubated for 2 hours at 37°C with cell culture supernatants or serial dilutions of human IgE standards. After washing, biotinylated goat anti-human IgE (Biosource) diluted 1:15,000 was added and incubated for 2 hours at 37°C. The plates were washed, and then streptavidin–horseradish peroxidase conjugate (Biosource) diluted 1:10,000 in blocking buffer was added. After incubation for 1 hour at 37°C, tetramethylbenzidine (Zymed Labs Inc, South San Francisco, Calif) was added, and the plate was incubated for 10 minutes at room temperature. The reaction was stopped with phosphoric acid (Sigma), and absorbance was measured with an ELISA reader at 450 nm. The limit of IgE detection was 50 pg/mL.

Apoptosis Assay

To determine the effect of IL-9 on tonsillar B cell survival, LD B cells were stimulated with immobilized CD40L monoclonal antibody for 72h alone or in the presence of IL-9, IL-4 or both cytokines for the last 24h of culture. Apoptosis was than induced with a monoclonal antibody (CH-11) to Fas receptor for 6h. After Fas-induced apoptosis, the cells were collected, washed twice with phosphate-buffered saline (PBS) and analyzed for the annexin V/PI double staining to measure phosphatidylserine translocation on cell membranes (Biosource International, California, USA). Levels of Fas receptors were determined by staining with anti-Fas FITC (R&D, Minneapolis) and analyzed by FACS.

CHAPTER 3: RESULTS

1) Tonsillar B cells' phenotype

In order to ascertain the purity and homogeneity of the B cell population studied, cells were purified from tonsils as described in materials and methods and stained for flow cytometry to detect the presence of monocytes (CD14), T cells (CD3), and assess the percentage of B cells (CD20) present as well as their maturation stage (sIgM). Results found indicate clearly that the majority of cells purified are $CD20^+$ $sIgM^+$ B cells, with a few cells $CD14^+$ and $CD3^+$ (Figure 1).

2) IL-9R is expressed on tonsillar B cells

The expression of the IL-9 receptor alpha chain (IL-9Rα) on B cells has not been well characterized. To address this[207,248], we assessed the expression of the human high affinity α chain of the IL-9R on freshly isolated purified human tonsillar B cells, because human tonsils receive repeated antigen stimulation, and display all stages of peripheral B cell development; hence, they constitute an ideal tissue to determine IL-9R expression at different stages of B cell maturation. Immunocytochemistry was performed on cytospins of unfractionated B cells purified from three independent tonsils, using an affinity-purified monoclonal antibody to the alpha chain of the IL-9R. Multiple tonsillar B cells stained positive for the IL-9Rα chain as shown in figure 2 in one representative sample (Figure 2A, b). Panel (a) of figure 2 shows the absence of specific staining when an isotype-matched control antibody is used. Moreover, flow cytometry analysis of total tonsillar B cells from different tonsils stained with a mAb to the IL-9Rα chain confirmed our microscopic observation, showing positive reactivity with a mean percentage positivity of 19% IL-9R positive cells amongst purified tonsillar B cells as depicted in Figure 2B. The result shown is representative of 3 different tonsils (mean 21.33±3.21SD)

3) Immunohistochemistry of IL-9R expression on tonsillar B cells

These findings prompted us to further investigate the precise localization of the IL-9R positive B cells within the lymphoid follicle. For this purpose, immunohistochemistry was performed on sections of human tonsils. Using double staining with anti-IL-9Rα (HRP staining developed with DAB, brown) and anti-CD20 (APAAP staining developed with Fast Red, red) (Figure 3,4) as well as with anti-IL-9Rα and anti-CD19 (immunofluorescence) (Figure 5), we found that CD20+/CD19+ B cells within the secondary lymphoid follicles (Figure 3A), and especially cells on the edge of these follicles (Figure 3A,b; Figure 4; Figure 5A), displayed IL-9Rα. This is clearly observed with the colocalization of IL-9R and CD20 (Figure 3A b brown), and CD19 and IL-9R (Figure 5A red and green) as detected by confocal microscopy for immunohistochemistry and fluorescent staining respectively. Comparing the intensities of expression of IL-9R inside the follicle versus the edge of the secondary lymphoid follicles, we observed that although IL-9R positive cells are found in both areas, cells on the edge of the follicles display a higher frequency of expression. Taken together, these results suggest that the IL-9R is principally expressed on follicular mantle B cells.

4) Percoll fractionation

B cells purified were layered onto a Percoll gradient and B cell subpopulations were isolated corresponding to Germinal center low density cells (LD) (between 30 and 50%), Centroblasts and Centrocytes medium density B cells (MD) (between 50 and 60%), as well as high density cells (HD) (above 60%) (Figure 6).

5) The distribution of IL-9R expression among B cell subsets

We then determined IL-9R expression on purified populations of tonsillar B cells at different stages of their maturation within lymphoid follicles. This was made possible by fractionating human tonsillar B lymphocytes using discontinuous Percoll density gradients. Using this method, we obtained three populations: low density (recovered in

the 30-50% Percoll fraction), which represents mainly germinal center B cells, medium density (50-60%), which are primarily centroblasts and centrocytes, and high density (>60%) or mature/resting B cells [246]. Using flow cytometry, IL-9Rα expression was higher in the low density or germinal center fraction of the B cells (39%), as compared to medium density (22%), and high density fractions (16%) (Figure 7A). Results presented are representative of three independent tonsils (with a mean and SD values for LD 36.1% ± 4.82, MD 17.5% ± 4.56, HD 18.4% ± 2.24).

6) Phenotype of GC cells expressing IL-9R

To further characterize the phenotype of germinal center cells expressing the IL-9R, three color flow cytometry was used to trace the IL-9R expression on human tonsillar B cells through their progression in the follicular germinal centers from naïve GC cells (F M B cells) ($CD38^-IgD^+$), to founder GC cells ($CD38^+IgD^+$) to memory B cells and plasma cells (CD38-IgD- and CD38+IgD- respectively) [54]. Upon analysis of CD38+ and IgD+ cells for IL-9R expression, we could clearly distinguish two distinct populations based on IgD expression: $IgD^-IL\text{-}9R^{lo}$ and $IgD^+IL\text{-}9R^{hi}$ cells (Figure 7B). IL-9R expression was highest within the IgD^+CD38^+ founder GC cells (57%) (Figure 7c), and the FM B cells which are IgD^+CD38^- (32.0%) (Figure 7a). Thus, IL-9R expression corresponds to the entry of naive FM B cells to the GC and their selection as founder cells. In contrast, GC cells that have lost IgD expression and are $CD38^+$, i.e centroblasts and centrocytes, show no significant expression of IL-9R (4.0%) (Figure 7b). Surprisingly, a population of memory B cells (IgD^-CD38^-) is positive for IL-9R (18.0%) (Figure 7d), indicating a possible role for IL-9 in secondary immune responses. The results shown are representative of three independent experiments with a mean value for IgD^+CD38^- (29.4% ± 6.22), IgD^+CD38^+ (54.3 ± 16.22), IgD^-CD38^- (19.7% ± 8.53), IgD^-CD38^+ (8.1% ± 4.82).

7) IL-9 induces tyrosine phosphorylation in LD cells

To ascertain the functionality of the IL-9R expressed on GC LD B cells, we assessed the total protein tyrosine phosphorylation on total unfractionated tonsillar B cells as well as on low density B cells, purified from tonsils, following activation with IL-9 for 15 min. As shown in figure 9A, specific upregulation of tyrosine phosphorylation is observed in IL-9 stimulated LD cells and not in unstimulated LD cells (lanes 6 and 5). This upregulation is diminished upon addition of a neutralizing antibody to IL-9 (lane 7). The Jurkat cell line constitutively activated was used as a positive control (shown in lane 2). Tonsillar B cells stimulated with IL-9 did not show any increase of tyrosine phosphorylation compared to untreated B cells. Amplification products specific for β-actin were of similar intensity between all samples, which suggests the equality of protein samples loaded. Results presented are representative of three independent experiments.

8) STAT activation following IL-9 stimulation of LD cells

In order to dissect further the signaling pathways involved in this activation, we sought to determine the activation of Signal Transducer and Activator of Transcription (STATs) following IL-9 stimulation of GC cells. STAT-5 belongs to the family of STAT transcription factors, which mediate the cellular response to a variety of cytokines including IL-9. Using specific antibodies that detect the phosphorylated tyrosine residue associated with activated STAT-5 (Tyr 694), we performed immunostaining of IL-9 stimulated LD cells and expression levels of phosphorylated STAT-5 by flow cytometry. The results show an upregulation of STAT-5 phosphorylation following IL-9 stimulation specifically in LD cells and not in total B cells, MD or HD cells as shown by flow cytometry (Figure 8A). Conversely, immunoblotting failed to show any significant upregulation of STAT5 phosphorylation in stimulated LD cells in comparison to unstimulated cells (Figure 9B), whereas in Jurkat T cell line, activation of STAT5 was visible (Figure 9B). Since flow cytometry amplifies the signal detected at the single cell level, it allows a higher sensitivity than immunoblotting.

Other STATs that have been linked to IL-9 signaling include STAT1 and STAT3. Staining for tyrosine phosphorylated residues in STAT1 (Tyr 701) and STAT3 (Tyr 705) in total tonsillar B cells and LD cells demonstrate a specific activation of STAT3 and not STAT1 in LD cells in the presence of IL-9 as shown by immunoblotting (Figure 9B), and flow cytometry data (Figure 8B). This is particularly interesting as STAT5 phosphorylation has been linked to cellular growth and proliferation [183], whereas STAT3 and STAT1 are upstream of differentiation genes [181] in IL-9 signaling events. In B cell responses, STAT5 has been reported recently to be phosphorylated in a subset of tonsillar B cells, its activation was linked to upregulation of BCL6, a transcription factor involved in memory B cell differentiation [249]. In mice, STAT3 has been shown to be required for T-dependent IgG plasma differentiation [250]. Taking into account the species difference, a similar role in human B cells seems likely.

These results demonstrate for the first time the expression of a functional IL-9R on GC B cells. Our findings demonstrate the presence of a functional IL-9R on primary human GC cells at physiologic levels of the receptor, in the absence of overexpression of the IL-9R as has been done in other studies to increase Stat activation signal and enhance its detection levels [179,251].

9) Synergistic effect of IL-9 on IL-4-induced IgE production

We have previously shown by immunohistochemistry and FACS analysis that tonsillar LD cells purified by Percoll fractionation have the highest expression of IL-9R, with a preferential expression on IgD+ and memory B cell populations in germinal centers.

To determine the effect of IL-9 on IgE production by LD cells, we cultured Percoll-fractionated LD cells in the presence or absence of anti-CD40 with or without IL-4, IL-9 or both cytokines. IL-9 alone in the presence of anti-CD40 does not induce IgE production as compared to treatment with anti-CD40 and IL-4 (Figure 10). However, addition of IL-9 to anti-CD40 and IL-4 potentiates IgE production with up to a five fold

increase in the amount of IgE produced as determined by ELISA on day 14 of cell culture (from 2972.4 ± 412.66 in the presence of anti-CD40 and IL-4 to 15253.5 ± 146.37 upon addition of IL-9) (Figure 10). The amount of IgE obtained is maximal at day 14 of cell culture, the synergistic effect of IL-9 is observed starting day 7 of the culture. Hence, although IL-9 is unable to drive B cell differentiation per se, it greatly potentiates IL-4's activity on B cells by upregulating IgE production.

10) IL-9 does not potentiate LD B cell proliferation

We asked whether this observed effect for IL-9 was due to an increase in cell number. To address this question cellular proliferation assays were set up to determine IL-9's role. Using incremental doses of IL-9: 10, 50, and 100 ng/ml, we show that IL-9 does not induce a significant LD B cell proliferation on its own in the presence of anti-CD40 as compared to cells cultured in the presence of anti-CD40 alone irrespective of the dose of the cytokine used (Figure 11A). However, these cells respond to IL-4 as shown by their incorporation of triutated thymidine readings. As shown in Fig 11 B, IL-9 had no effect on LD cellular proliferation whereas the presence of IL-4 induced proliferation. In order to verify the possibility that IL-9 may synergize with IL-4 in inducing LD B cell proliferation, we stimulated LD B cells with IL-9 in the presence of anti-CD40 and IL-4. There was no potentiation of the proliferative response in the presence of IL-9 compared to IL-4 alone (Figure 11B). To ascertain that IL-9's effect on LD cellular proliferation does not vary with different stimuli, we used a-IgM to induce cellular proliferation in LD cells. Results show (Figure 11C) that IL-9 does not affect cellular proliferation in the presence of anti-IgM, whereas IL-4 induces cellular proliferation in LD cells in these conditions. Hence, IL-9 does not seem to play a role in LD cellular proliferation irrespective of the stimulus.

11) IL-9 inhibits Fas-induced LD B cell apoptosis

LD cells were stained for Fas expression. The expression of the Fas receptor was stable during the culture period of 72h (Figure 12). To determine the effect of IL-9 on

tonsillar B cell survival, LD B cells were stimulated with immobilized anti-CD40 monoclonal antibody for 72h alone or in the presence of IL-9, IL-4 or both cytokines for the last 24h of culture. Apoptosis was than induced with a monoclonal antibody (CH-11) to Fas receptor for 6h. Assessment of apoptosis in B cell cultures was done by measuring phosphatidylserine translocation on cell membranes using Annexin V staining. As expected, CD40-activated LD B cells treated with anti-Fas antibody alone showed an induction of apoptosis. Treatment of equivalent cells with IL-9 or IL-4 resulted in a decrease in Fas-induced apoptosis; however, the induction of apoptosis was only completely abrogated when both IL-9 and IL-4 were present in culture (Figure 13). Therefore, data gathered from these experiments demonstrate for the first time that IL-9 not only rescues CD40-activated LD B cells from Fas-mediated apoptosis, but also potentiates IL-4's protective role with respect to counteracting induced apoptosis.

12) IL-9R and CD27 are upregulated on CD40-stimulated LD B ells

We sought to determine the phenotype of cells with increased IgE production. Are these cells IL-9 respondents throughout the cell culture period (14 days), do they differentiate into a memory or plasma cell phenotype? For this purpose, we stained for the IL-9R expression at different time points at Days 1, 6, and 10 of cell culture. Our results show that IL-9R expression is upregulated through CD40 stimulation and maintained throughout the culture period. However, the presence of IL-4 and not IL-9 appreciably contributed to an increase in IL-9R expression at Day 6 of cell culture (Figure 14A). Cells were also stained for CD27 expression which showed a pattern similar to the observed IL-9R upregulation whereby the observed increase correlated specifically with CD40 and IL-4 stimulation (Figure 14B). Interestingly, the increase in CD27 expression occurred nearly totally on IL-9Rα positive cells highly suggesting a link with augmented IgE production. Analysis of IL-9Rα expression on peripheral blood lymphocytes from normal donors is shown in Figure 15. Nearly the totality of IL-9R+ cells express CD27, this is of high interest in light of the described role of CD27 as a memory B cell marker.

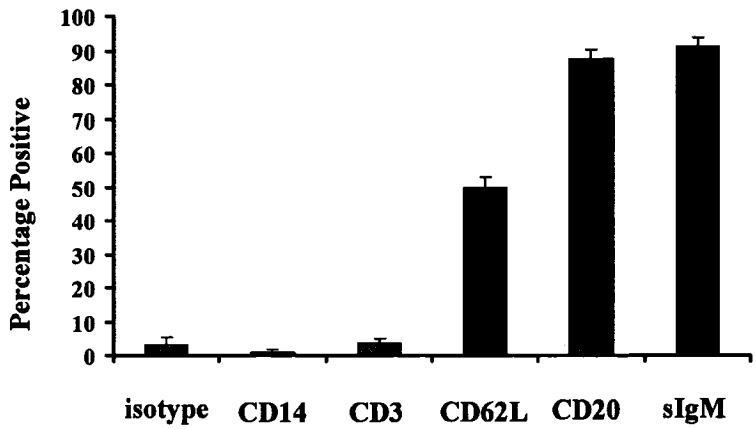

Percentage Positive
100
90
80
70
60
50
40
30
20
10
0
isotype
CD14
CD3
CD62L
CD20
sIgM

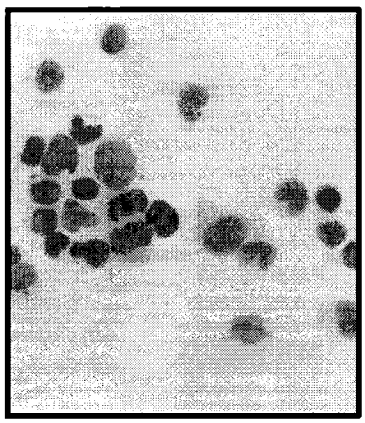
Figure 1

Figure 1: Tonsillar B Cells' Phenotype.

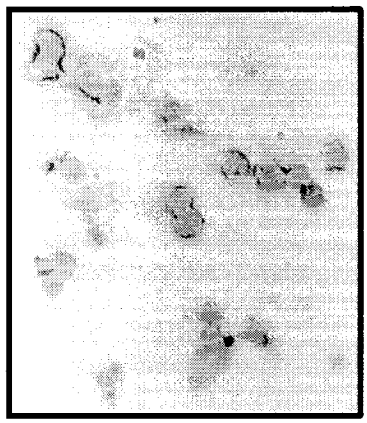
Tonsillar B cells were purified from freshly isolated tonsillar cells by rosetting using neuraminidase treatment. Purity of B cell population was assessed by flow cytometry by single staining for CD14, CD3, CD62L, and the B cell markers CD20 and sIgM. Cells obtained from three independent tonsils were assessed, and results shown represent their mean value with SD bars shown: for n=3, isotype (2.98±2.20%), CD14 (1.067±0.93%), CD3 (3.39±1.45%), CD62L (49.66±3.21%), CD20 (87.67±2.51%), sIgM (91.0±2.64%).

A)

a)



b)



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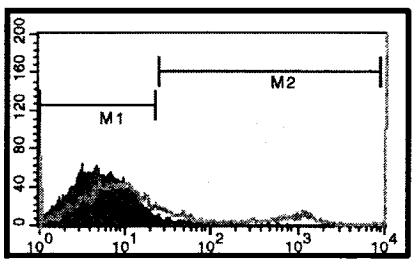


Figure 2

Figure 2: Detection of IL-9Rα on tonsillar B cells.

A) Immunocytochemistry for IL-9Rα on tonsillar B cells. IL-9Rα chain expression in unfractionated tonsillar B cells. a, absence of immunoreactivity signal when using an isotype-matched control antibody as the primary antibody b, immunocytochemical detection (red color) of IL-9Rα in a cytospin preparation of purified tonsillar B cells stained with a mouse affinity purified mAb antihuman IL-9R-α chain (isotype IgG1) followed by rabbit anti-mouse Ig (1:60) and alkaline phosphatase anti-alkaline phosphatase (1:60). The slides were developed using Fast Red and counterstained with hematoxylin Gill II. **B) FACS for IL-9Rα expression on tonsillar B cells using anti-IL-9Rα monoclonal antibody.** IL-9Rα cell surface expression on tonsillar B cells was assessed by flow cytometry. Results of analysis of $1x10^5$ cells are shown and are representative of 3 individual tonsillar B cells' fractions with a mean expression of 21.33% and SD of 3.21%. Percentages shown correspond to the proportion of cells that are IL-9Rα high.

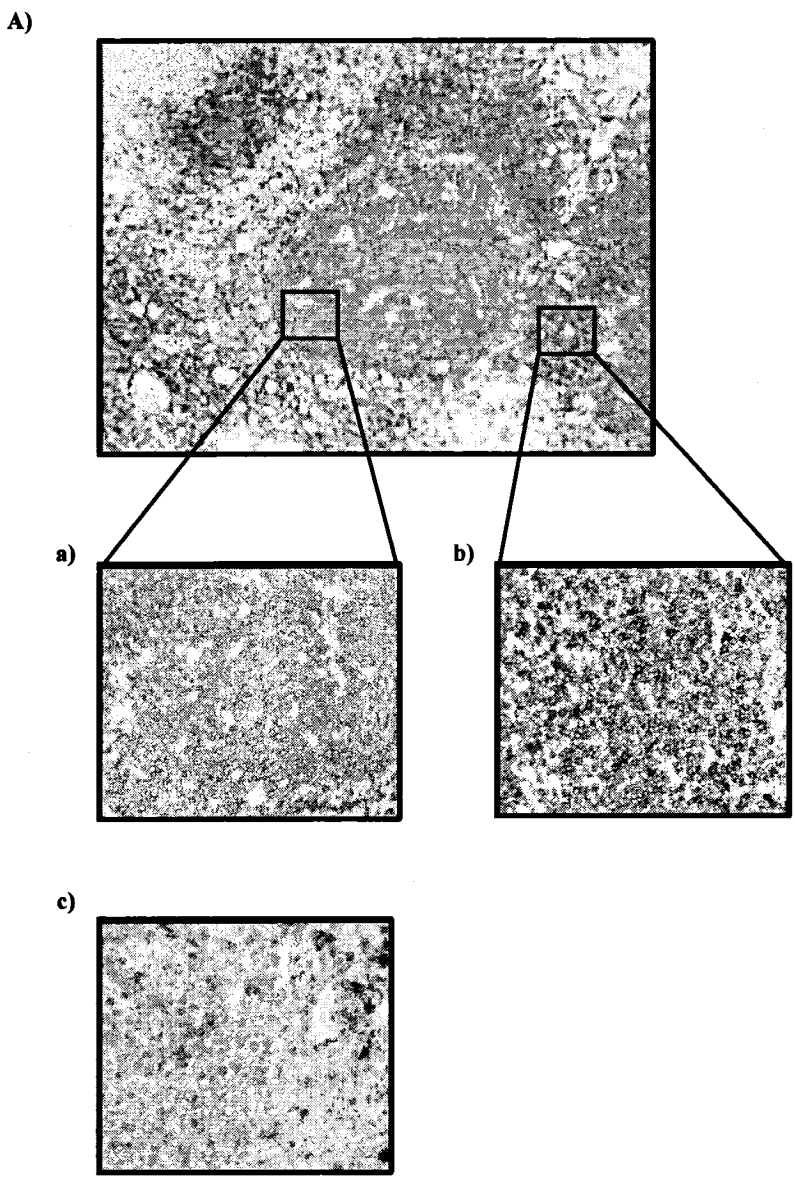


Figure 3

Figure 3: Immunohistochemistry for IL-9Rα on tonsillar tissue sections.

IL-9 receptor alpha chain expression and CD20/CD19 B cell markers on tonsillar tissue sections were assessed by immunohistochemistry. Alkaline phosphatase anti-alkaline phosphatase (APAAP) double immunostaining was used for IL-9Rα and CD20 expression. A) secondary follicle can be seen with double immunostaining(Magnification 20X); a- 40x magnification for inside the follicle; b-edge of the follicle (40x); c-Negative staining for CD20 and IL-9Rα as represented outside the follicles.

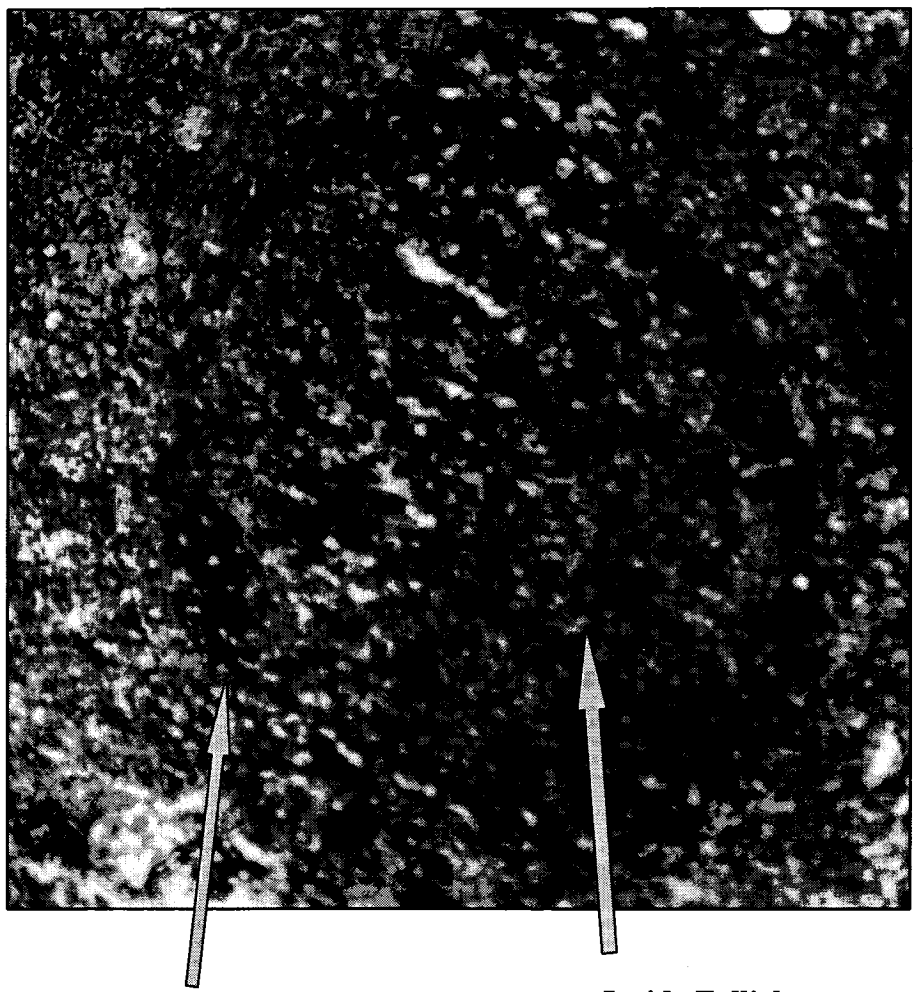


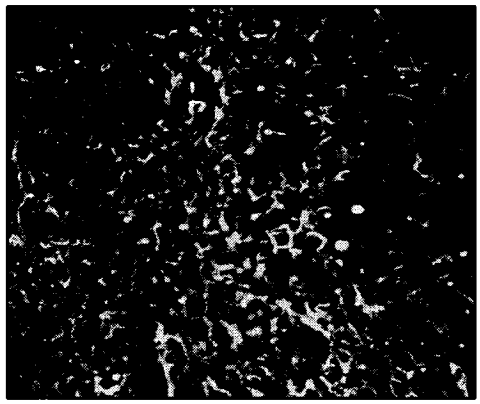
Figure 4

Figure 4: Secondary Follicle.

IL-9 receptor alpha chain expression and CD20 B cell markers on tonsillar tissue sections were assessed by immunohistochemistry. Alkaline phosphatase anti-alkaline phosphatase (APAAP) double immunostaining was used for IL-9Rα and CD20 expression. Secondary follicle can be seen with double immunostaining (Magnification 20X) with the hematoxylin blue counterstain delineating the edge of the follicle

A)

Follicle Rim



B)

Follicle

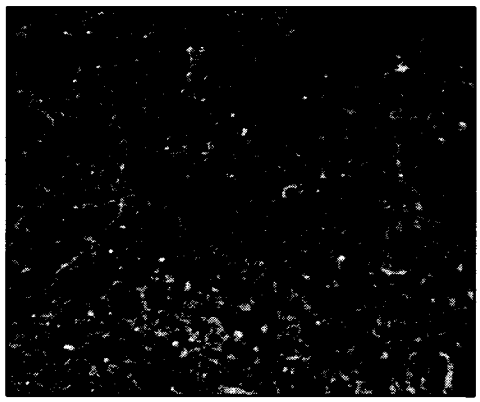


Figure 5

Figure 5: Immunohistochemistry for IL-9Rα on tonsillar tissue sections by confocal microscopy.

Tissue sections of tonsils were processed for immunohistochemistry as described in materials and methods. For Confocal microscopy, monoclonal antibodies for IL-9Rα and CD19 were used as primary antibodies, conjugated thereafter to goat anti-mouse FITC and zenon antibodies Alexa fluor 568 respectively. A) Follicle rim is shown with staining for IL-9Rα positive cells (green) and CD19 a B cell marker (red); B) Follicle staining shows scant IL-9Rα expression on B cells.

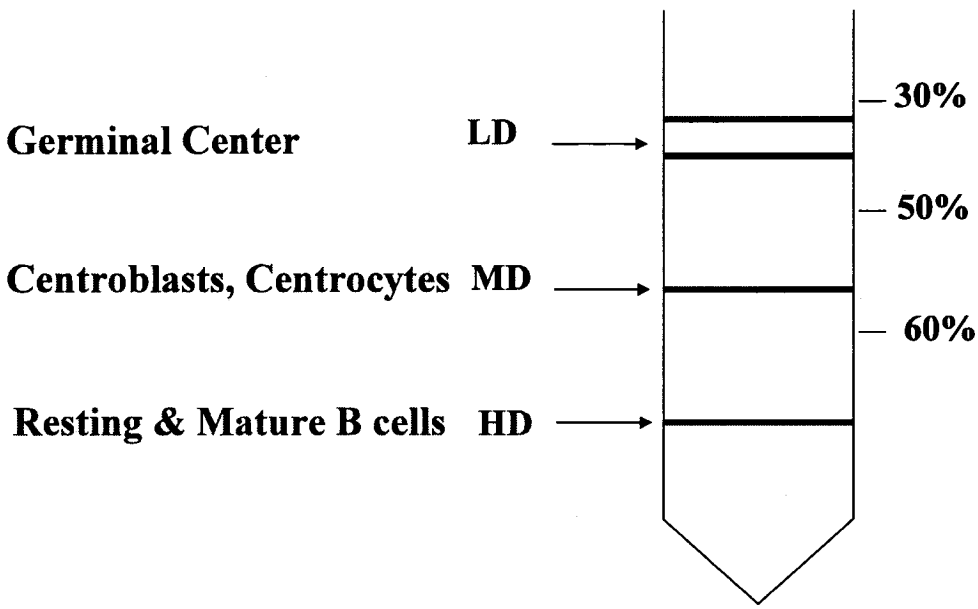

Germinal Center
LD
Centroblasts, Centrocytes
MD
Resting & Mature B cells
HD
30%
50%
60%

Figure 6

Figure 6: Percoll gradient.

Percoll density gradient centrifugation yields four layers leading to the isolation of three tonsillar B cell subtypes. The LD B cell subset (the fraction between 30-50%), MD (50-60%), and HD (> 60%).

A)

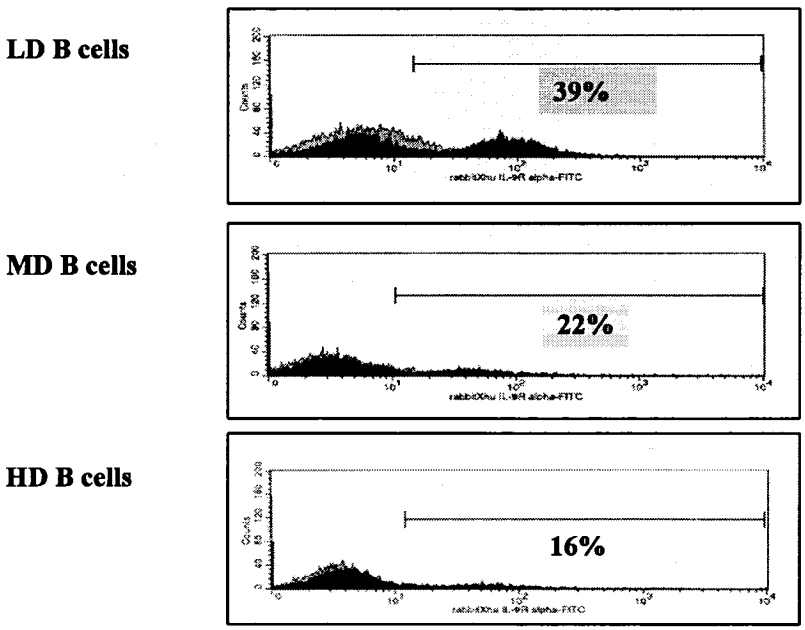


Figure 7

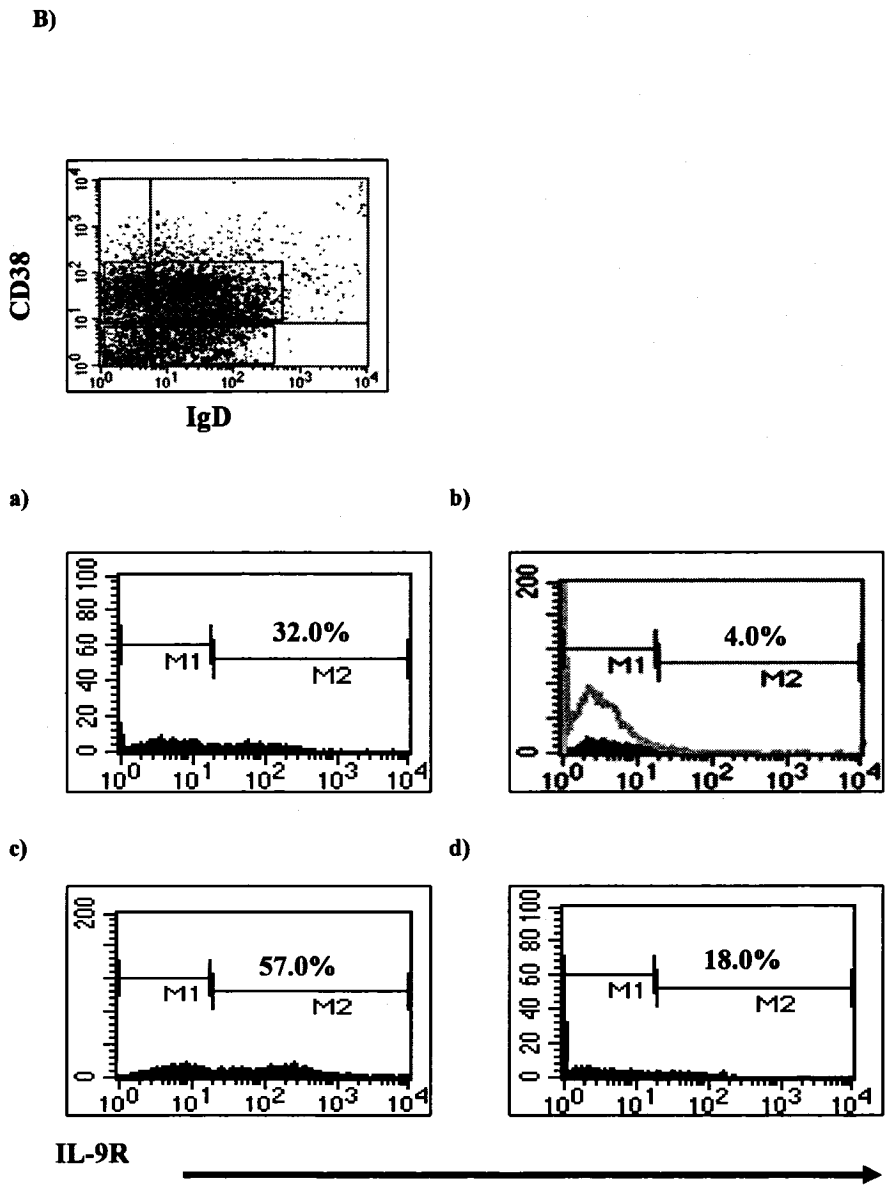


Figure 7

Figure 7: A) IL-9Rα expression on tonsillar B cell subsets.

Cell surface expression of IL-9Rα chain on tonsillar B cell subsets from Percoll gradient seperation. Percentages shown indicate the IL-9Rα high cells within the LD, MD, and HD B cells as obtained from fractionation of B cells using a discontinuous Percoll density gradient. This is representative of three distinct experiments with a mean of 36.1±4.8% for LD cells, MD (17.5±4.6%), and HD B cells (18.4±2.2%) **B) FACS for IL-9Rα/IgD and CD38 expression on LD B cells. a)**Three-color FACS staining of LD tonsillar B cells' fractions. LD cells were labeled as described in Materials and Methods with anti-IL-9 receptor alpha chain, anti-CD38 and anti-IgD. Results of analysis of $1x10^5$ cells are shown for one representative tonsil from 3 individual tonsillar B cells' fractions. A representative dot plot is shown for IgD and CD38 positive cells, and histograms for the selected gates are shown: a) FM: IgD^+CD38^- mean 29.4± SD 6.2% b) GC: IgD^-CD38^+ 8.1±4.8% c) Founder GC: IgD^+CD38^+ 54.3±16.2% d) Memory IgD^-CD38^- 19.7±8.5%. Percentages for each histogram correspond to the proportion of cells that are IL-9Rα positive amongst different gated populations as identified on the dot blot representing total triple stained LD B cells. **b)** IL-9Rα positive cells in IgD CD38 IL-9R stained LD cells are given as a percentage of positive cells within gated populations the results shown are representative of three individual tonsillar cells analyses.

A)

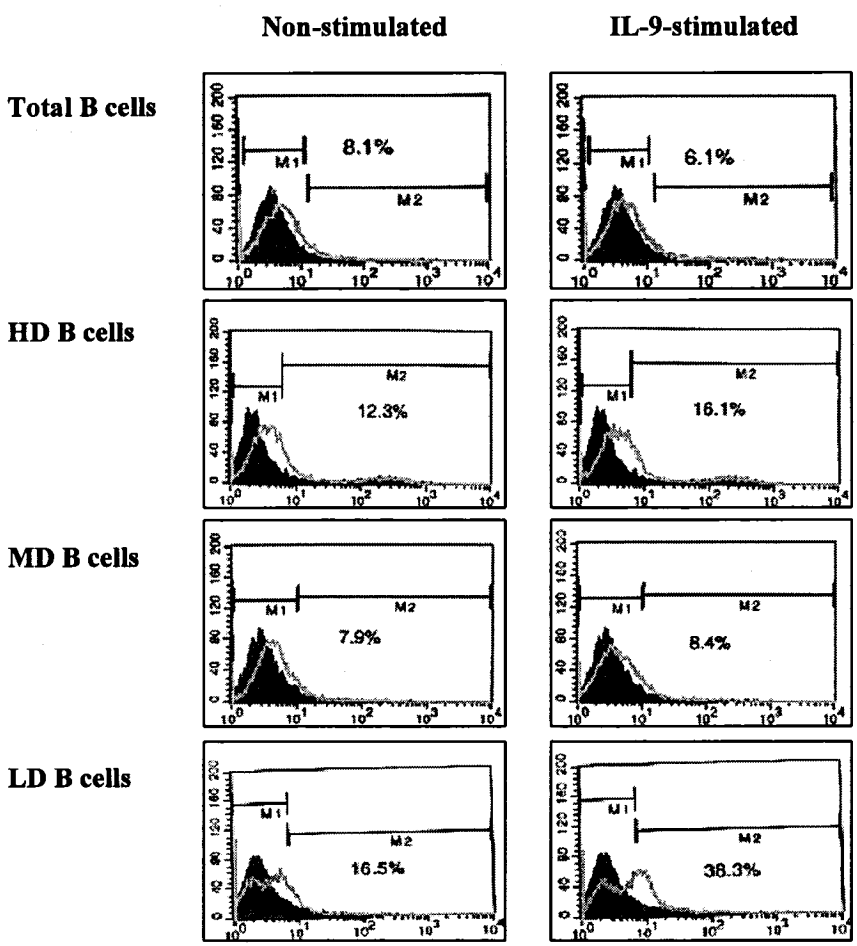

Non-stimulated
IL-9-stimulated
Total B cells
HD B cells
MD B cells
LD B cells
M1
M2
8.1%
6.1%
12.3%
16.1%
7.9%
8.4%
16.5%
38.3%

Figure 8

B)

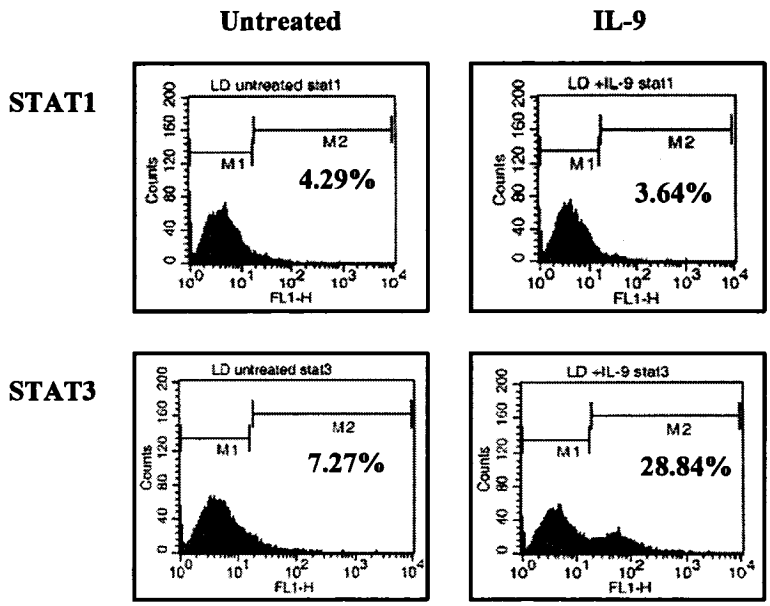

Untreated
IL-9
STAT1
STAT3
LD untreated stat1
LD +IL-9 stat1
LD untreated stat3
LD +IL-9 stat3
M1
M2
4.29%
3.64%
7.27%
28.84%
Counts
FL1-H

Figure 8

Figure 8: A) STAT5 phosphorylation.

Using a phosphospecific antibody that detects the phosphorylated tyrosine residue associated with activated STAT5, we performed an immunostaining of IL-9 stimulated or not tonsillar B cells, LD, MD and HD cells and analysed expression levels of phosphorylated STAT5 by flow cytometry. A polyclonal antibody conjugated to FITC was used to stain $1x10^6$ cells. Results shown are representative of three independent experiments (see table 2 attached). This demonstrates phosphorylation of STAT5 following addition of IL-9 to the LD B lymphocyte fraction. **B) STAT1 & STAT3 phosphorylation.** Staining for the phosphorylated tyrosine residues associated with activated STATs 1 & 3 was performed on LD cells unstimulated and stimulated with IL-9, expression levels were analysed by flow cytometry.

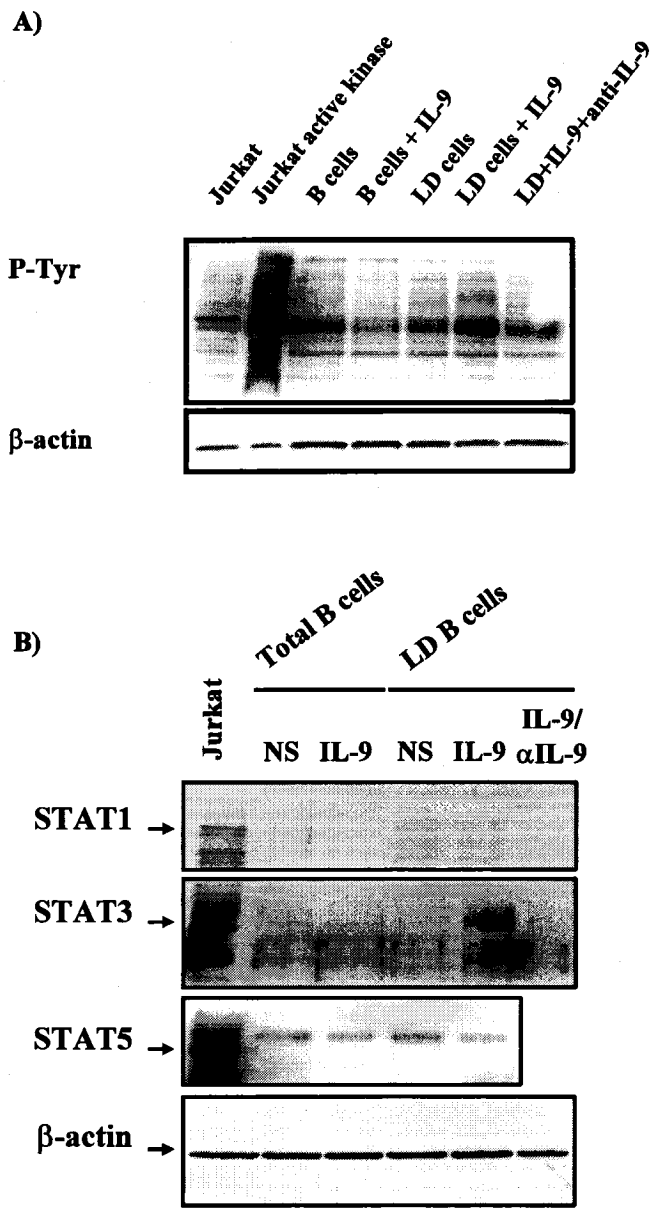

A)
Jurkat
Jurkat active kinase
B cells
B cells + IL-9
LD cells
LD cells + IL-9
LD+IL-9+anti-IL-9
P-Tyr
β-actin
B)
Total B cells
LD B cells
Jurkat
NS
IL-9
NS
IL-9
IL-9/ αIL-9
STAT1
STAT3
STAT5
β-actin

Figure 9

Fig 9: A) Immunoblot of tyrosine phosphorylation for IL-9 stimulated B cells.

Tyrosine phosphorylation of unfractionated tonsillar B cells as well as LD B cells both unstimulated and stimulated with IL-9 (10 ng/ml) for 15 minutes is shown. Cells were incubated with or without IL-9 and/or IL-9 plus anti-IL-9 for 15 minutes, washed with ice-cold PBS and cell pellets were lysed in lysis buffer for 20 mins on ice. 15 μg of protein was run on an SDS polyacrylamide gel then blotted for phosphorylated tyrosine using 4G10 or anti-actin antibodies. Figure is representative of three independent experiments, and demonstrates an increase in the total level of tyrosine phosphorylation in IL-9 stimulated LD B cells. **B) Immunoblot of STAT1, 3, and 5.** Immunoblotting for STATs 1, 3, and 5 is shown for total B cells as well as percoll-fractionated LD cells. Non-stimulated and IL-9 stimulated cells are lysed and immunoblotted with anti-phosphotyrosine antibodies for specific STATs. Jurkat cells are shown as positive controls and actin levels for equal samples loaded is shown.

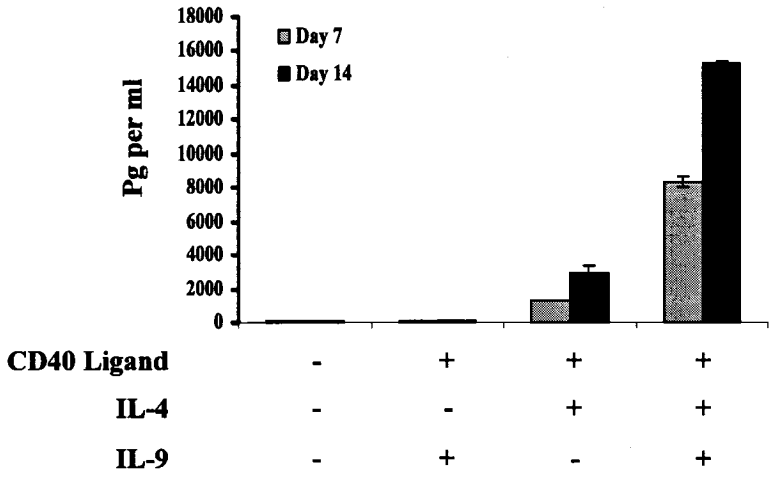

18000
16000
14000
12000
10000
8000
6000
4000
2000
0
Pg per ml
Day 7
Day 14
CD40 Ligand - + + +
IL-4 - - + +
IL-9 - + - +

Figure 10

Figure 10: Synergistic Effect of IL-9 on IL-4-Induced IgE Production

Percoll-fractionated tonsillar LD B lymphocytes were cultured in 24-well plates (1ml/well) at a concentration of $0.5x10^6$ cells/ml in the presence of anti-CD40 (1 μg/ml) and IL-4 (400 U/ml) alone or in the presence of IL-9 (10 ng/ml). Cell supernatants were harvested on the 7th and 14th day of culture and kept at -20°C until use. IgE was assayed by ELISA, values are given in pg/ml, SD bars are shown. Results of a representative experiment are shown (n=4)

A)

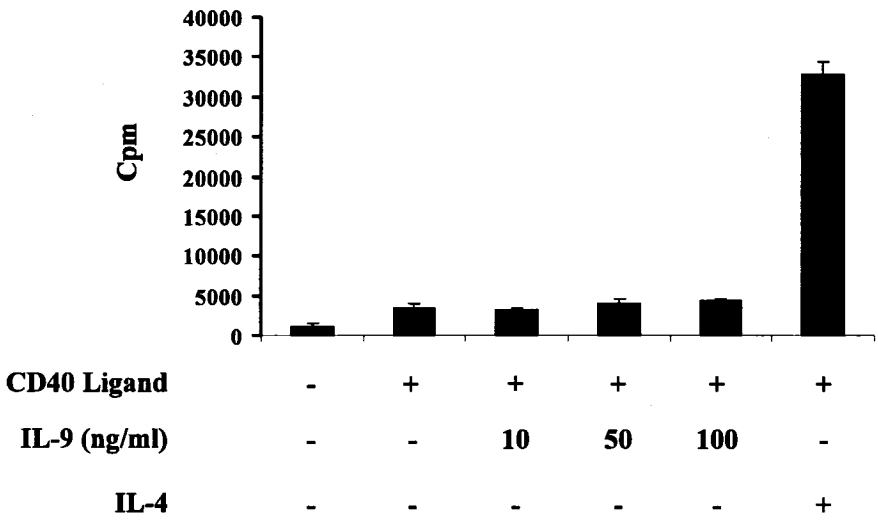

40000
35000
30000
25000
20000
15000
10000
5000
0
Cpm
CD40 Ligand - + + + + +
IL-9 (ng/ml) - - 10 50 100 -
IL-4 - - - - - +

Figure 11

Figure 11: A) IL-9 Does Not Potentiate CD40L-Activated LD B Cell Proliferation.

Low density B cell proliferation was assessed by thymidine incorporation. Cells were cultured for 72h in a 96-well plate in the presence of 10, 50 or 100 ng/ml of rhIL-9 alone or in combination with an antibody to CD40 (1 ug/ml). Alternatively rhIL-4 was added alone (400 U/ml) or in the presence of anti-CD40 at a final cell density of 1 X 10^5 cells/ml in a volume of 200 µl/well in 96-well round-bottom plates in triplicatas for 3 days at 37°C in a humidified atmosphere with 5% CO2. Then cells were pulsed with 1 uCi/well for 18h, thymidine incorporation levels were measured by liquid scintillation counting.

B)

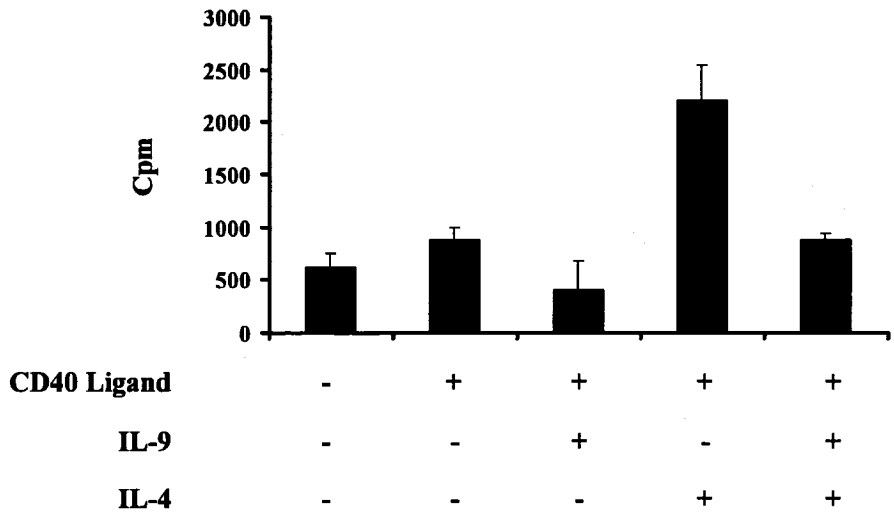


Figure 11

Figure 11: B) No Synergistic Effect for IL-9 on IL-4-Induced LD B Cell Proliferation

LD B cells were cultured as described above, LD cells were cultured alone in the presence of anti-CD40, IL-4 or in the presence of both IL-9 and IL-4 cytokines.

C)

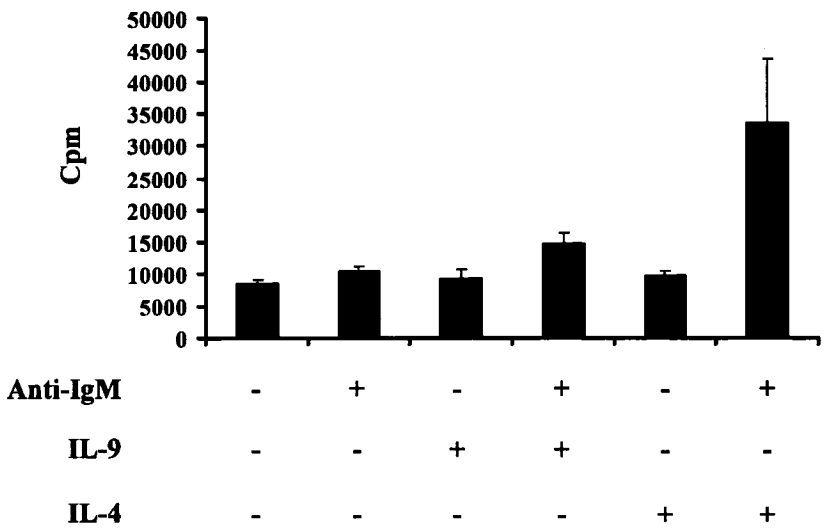

50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
Cpm
Anti-IgM - + - + - +
IL-9 - - + + - -
IL-4 - - - - + +

Figure 11

Figure 11: C) IL-9 Does Not Potentiate IgM-Activated LD B Cell Proliferation

Low density B cells were cultured for 72h in a 96-well plate in the presence of 10 ng/ml of rhIL-9 alone or in combination with an antibody to the BCR (anti-IgM 2 ug/ml). Alternatively rhIL-4 was added alone (400 U/ml) or in the presence of anti-IgM at a final cell density of 1 X 105 cells/ml in a volume of 200 μl/well in 96-well round-bottom plates for 3 days at 37°C in a humidified atmosphere with 5% CO2. Then cells were pulsed with 1 uCi/well for 18h, thymidine incorporation levels were measured by liquid scintillation counting.

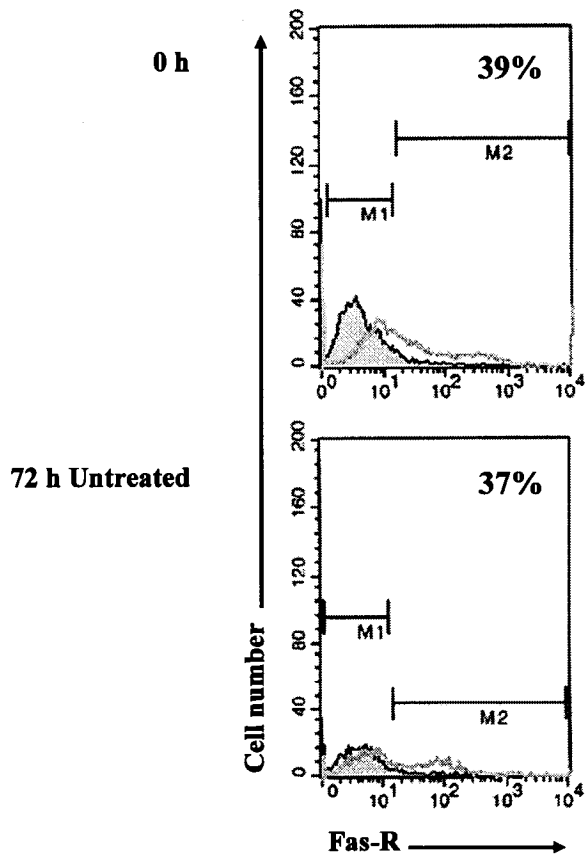


Figure 12

Figure 12: Fas expression on LD cells.

Fas expression is shown both at time 0 and at 72h of culture. LD cells were stained directly for Fas expression or cultured for 72h before labeling. Results are representive of two experiments with a mean expression level of 38.5 % ± 0.70 at time 0 and 38.5 % ± 2.12 at 72h.

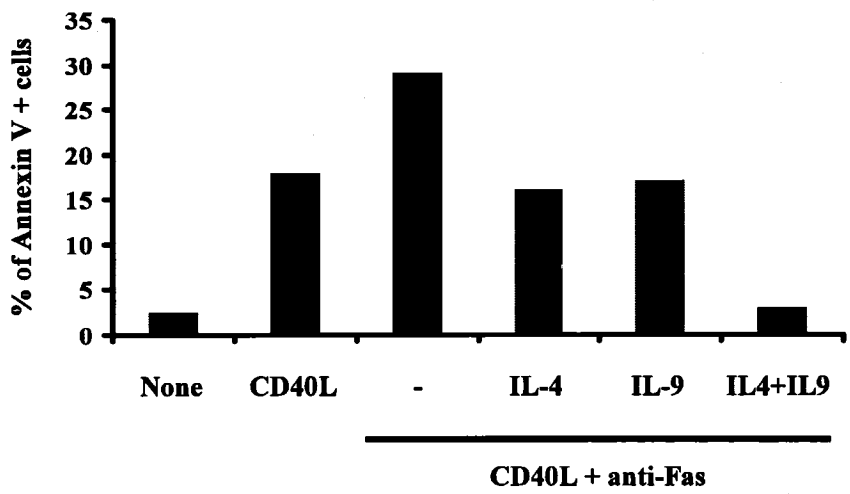

% of Annexin V + cells
35
30
25
20
15
10
5
0
None
CD40L
-
IL-4
IL-9
IL4+IL9
CD40L + anti-Fas

Figure 13

Figure 13: IL-9 Inhibits Fas-Induced LD B Cell Apoptosis

To determine the effect of IL-9 on tonsillar B cell survival, LD B cells were stimulated with immobilized CD40L monoclonal antibody for 72h alone or in the presence of IL-9, IL-4 or both cytokines for the last 24h of culture. Apoptosis was than induced with a monoclonal antibody (CH-11) to Fas receptor for 6h. Assessment of apoptosis in B cell cultures was done by measuring phosphatidylserine translocation on cell membranes using Annexin V staining.

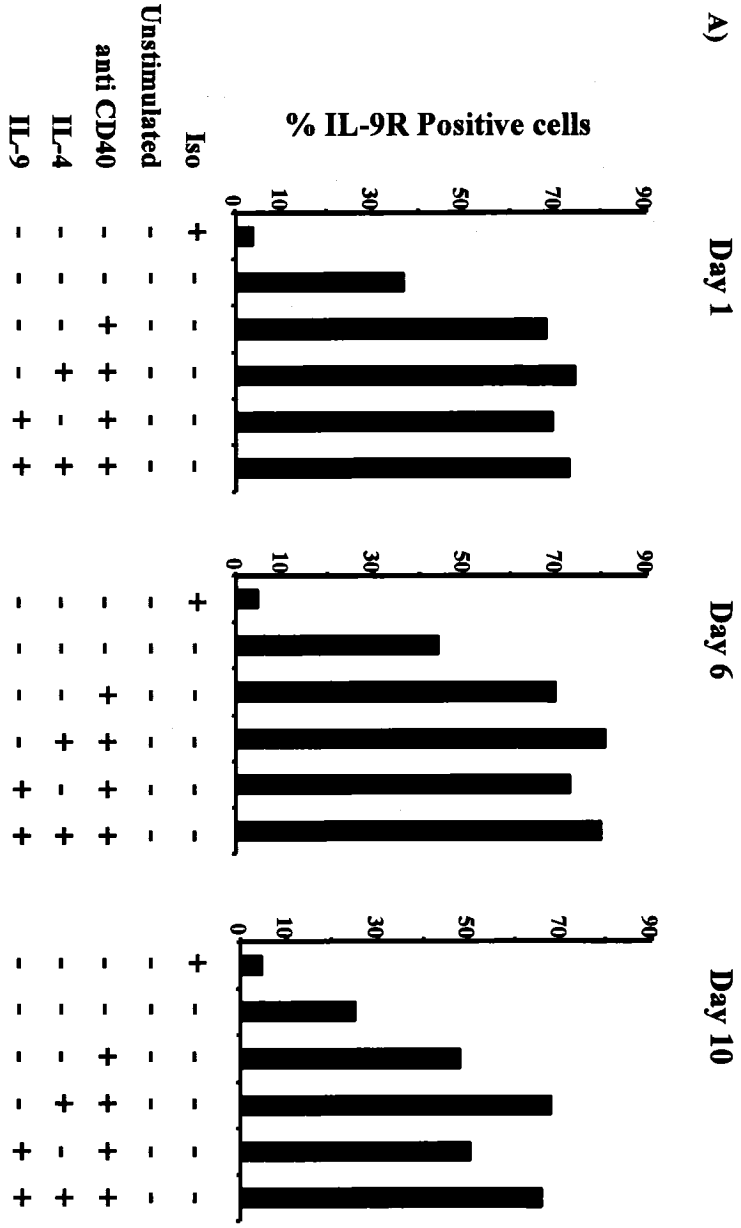

A)
% IL-9R Positive cells
Day 1
Day 6
Day 10
0
10
30
50
70
90
Iso
Unstimulated
anti CD40
IL-4
IL-9

Figure 14

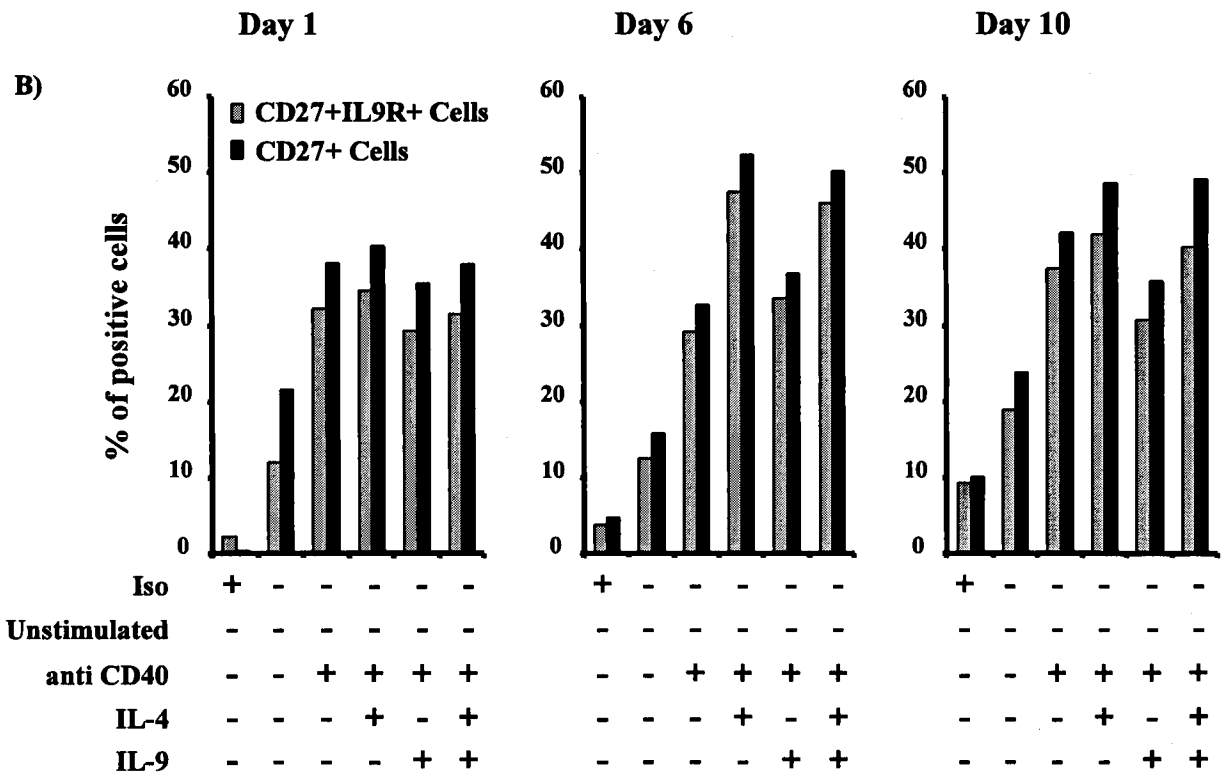

B)
Day 1
Day 6
Day 10
CD27+IL9R+ Cells
CD27+ Cells
% of positive cells
0
10
20
30
40
50
60
Iso
Unstimulated
anti CD40
IL-4
IL-9

Figure 14

Figure 14: A/ IL-9R Expression during 10 days culture

LD cells were stained with a monoclonal antibody to the IL-9Rα chain following isolation from tonsils, at days 1, 6, and 10. Cells were cultured in the presence or not of anti-CD40/IL-4 and/or IL-9 for the indicated periods. Results shown are representative of three independent experiments. **B/ IL-9Rα CD27 Expression.** Cells cultured were double stained for IL-9Rα and CD27.

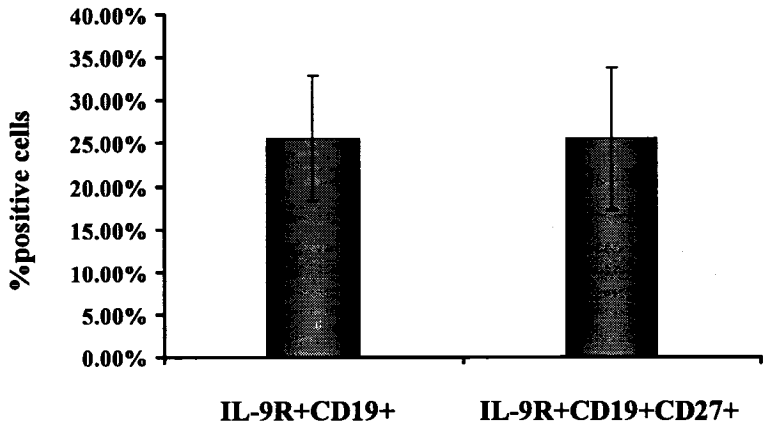

40.00%
35.00%
30.00%
25.00%
20.00%
15.00%
10.00%
5.00%
0.00%
%positive cells
IL-9R+CD19+
IL-9R+CD19+CD27+

Figure 15

Figure 15: PBMC staining for IL-9Rα.

Staining for IL-9Rα on PBMCs purified from freshly obtained blood from non-atopic donors. IL-9Rα+CD19+ were found to be 25.60% of total cells purified with a SD of 7.34%; IL-9Rα+CD19+CD27+ cells are 25.54% +/-SD 8.32%. Results represent the mean of three different experiments.

CHAPTER 3: DISCUSSION

We have examined the expression of IL-9Rα chain on tonsillar LD B cells. These mostly GC cells are implicated in B-T cell interactions occurring during the maturation process of memory B cells, with a variety of cognate molecules involved in the immune synapse. Data obtained in vitro from human PBMC as well as in vivo from mice indicate a possible effect for IL-9 and its receptor on B cell maturation. We therefore addressed IL-9Rα expression levels on human B lymphocyte subpopulations purified from tonsils, to better define IL-9's role in B cell differentiation in the germinal center response. In humans, to our knowledge, our findings are the first direct proof that IL-9Rα is expressed on follicular tonsillar B cells.

Studies performed to date in mice appear to indicate that IL-9Rα expression is mainly found on $CD5^+$ (B-1a) as well as $CD5^-$ (B-1b) cells in normal mice, [169] which are characterized by their peritoneal and pleuropericardial location and their ability to respond to T-independent antigens [178]. Expression of IL-9 has been shown to promote the expansion of B-1 cells in transgenic mice specifically B-1b $CD5^-$ lymphocytes [169]. In humans, although the function of $CD5^+$ B-1 cells is still not clear, the CD5 T cell marker was shown in human tonsillar B cells to be expressed on follicular mantle zone B cells $sIgD^+CD38^-$ mostly [252-254]. A small proportion of $CD5^+$ cells were found on $CD38^+/CD10^+$ medium-sized B cells [252]. Human follicular B cells have been documented to express the highest level of CD5 amongst human B cell populations [255]. .We have analysed IL-9Rα expression on human tonsillar B cells and shown that IL-9Rα expression is specifically enriched on LD cells, with a high level expressed on IgD^+ cells which are reported to express the CD5 marker, and include FM B cells ($CD38^-IgD^+$) and founder GC cells ($CD38^+IgD^+$). This is in line with previous animal data if one is to compare rodent and human phenotype of cells. In the absence of human studies describing the expression of IL-9Rα, our results provide unique and novel data in that respect. Moreover, our findings delineate a potential role for IL-9 in B cell maturation.

We have specifically identified the low density (LD) percoll-fractionated subset of cells which is mainly naive and antigen-activated follicular mantle zone B cells as well as

germinal center B cells (GC) [246], as the major tonsillar B cell population expressing the IL-9Rα chain (Figure 7a). In addition, we characterize the phenotype of LD B cells expressing the IL-9Rα by using three color flow cytometry and tracing two B cell maturation markers: IgD and CD38. Studies have shown that IgD is expressed on human peripheral blood memory B cells characterized by the presence of V region genes somatic mutation and CD27 [107]. This was also observed in tonsillar subpopulations whereby memory IgD^+ B cells were identified carrying somatic mutations and expressing CD27 in the presence or absence of CD38 [256]. CD27, a member of the tumour necrosis factor-receptor family, has been characterized as an antigen on human T cells, natural killer cells, plasma cells and memory B cells [257]. Hence IgD^+CD38^- cells may be considered as recirculating early memory B cells, activated B cells, or naïve B cells depending on their antigenic profile (CD5, CD23, CD27 expression) and the presence of genetic mutation. Data presented in this study show that a significant proportion of IL-9Rα positive cells expresses IgD (Figure 7B), the majority being on $CD38^+IgD^+$ (57%) cells and $CD38^-IgD^+$ (32%) cells. Moreover, IL-9Rα+ cells are found to express CD27 as well (Figure 14B), implicating IL-9 in the maturation of a memory subtype of tonsillar B cells. In support of these data, immunohistochemistry and confocal microscopy studies demonstrate the colocalization of the IL-9Rα and CD19/CD20 B cell specific markers within secondary lymphoid follicles, specifically on the edge of these follicles, coinciding with the presence of follicular mantle naïve and memory B cells as well as founder GC cells in this area (Figures 3, 4, 5). GC cells enter the follicles and form the dark zone through the edge of the follicle. In this vicinity, founder GC cells are selected and proceed to cell division thus forming the highly dense dark zone containing centroblasts. The recirculation of memory B cells to reenter the GC and undergo a second round of selection has been also proposed, thus widening the phenotype of B cells in the FM zone of the secondary lymphoid organs [38].

STATs that have been linked to IL-9 signaling include STAT1, STAT3, and STAT5. We demonstrate that the expressed IL-9Rα chain mediates its signal mainly through the induction of STAT3 and to a lesser extent STAT5 phosphorylation. Staining for tyrosine phosphorylated residues in STAT1, STAT3, and STAT5 in tonsillar B cells

and LD cells demonstrates specific activation of STAT3 and not STAT1 in LD cells in the presence of IL-9 as shown by immunoblotting (Figure 9B), and flow cytometry data (Figure 8B). This is particularly interesting as STAT3 induce the expression of differentiation genes such as granzyme A, Ly-6A/E, and L-selectin [181] in IL-9 signaling events; whereas STAT5 phosphorylation has been linked to cellular growth and proliferation [183]. Our data also show that STAT5 phosphorylation is upregulated following IL-9 stimulation of human tonsillar primary LD B cells, and this effect is specifically associated with IL-9Rα expression on these cells. This is the first study to show STAT5 upregulation in primary cells in the absence of overexpression of the receptor chain to augment the transduced signals (Figures 8, 9). However, this upregulation is not observed by immunoblotting, possibly due to its localization to a small percentage of IL-9R$^+$ cells. Our findings are in agreement with a study undertaken on tonsillar B cells whereby phosphorylated STAT5 was only detected following enrichment of the cellular population, and the signal obtained by FACS was weak [249]. Our findings demonstrate the presence of a functional IL-9R on primary human GC cells under normal physiologic conditions, in the absence of overexpression of IL-9Rα chain, as has been done in other studies to increase Stat activation signal and enhance its detection levels [179,251].

Our experiments therefore point to a role for IL-9 in B cell differentiation rather than proliferation. A recent study implicated STAT3 in plasma cell differentiation [250]. This may occur by binding to repressor elements in DNA and competing for these binding sites with Bcl-6, a transcriptional repressor of Blimp-1. Release from Bcl-6 repression is required for differentiation into plasma cells. Since IL-9 was shown in our experiments to activate Stat3, IL-9 may be amplifying the synergistic effect of IL-4 which signals through Stat-6, and CD40 that activates NF-κB on IgE transcription at the molecular level. Although it may be simplistic to directly attribute plasma cell differentiation to IL-9, it is worth contemplating the potential molecular players activated downstream of IL-9 and how they may synergize with other signals induced in the immune synapse by the different cognate molecules such as IL-4 to induce IgE transcription.

It is worth noting that the use of primary human cells in our system engendered a few difficulties: A higher number of human tonsillar LD B cells was needed relative to Jurkat cells to obtain a comparable amount of protein to be analysed. This is one facet of difficulty encountered with the use of primary cells as compared to established or transfected cell lines. Another facet of difficulty in manipulating LD cells is due to their increased propensity to apoptosis. Hence, obtaining a substantially high apoptotic background when measuring Annexin V staining decreased the sensitivity of our system to detect subtle changes in apoptosis induction within differentially stimulated populations (± IL-9), and demonstrated the fragility of the cells used.

Previous reports have shown that IL-9 potentiated IL-4-induced IgG, IgM and IgE production from normal human B lymphocytes [206,207,248]. Our data demonstrate the expression of the IL-9Rα mainly on IgD^+ human tonsillar B cells, and further examines the effect of IL-9 on IgE production from LD cells in the presence of another inflammatory cytokine: IL-4. LD cells were cultured in the presence of a monoclonal antibody to CD40, and IL-4 in the presence or absence of IL-9. We have found that IL-9 synergizes with IL-4 to augment IgE production in low density tonsillar B cells (Figure 10), but could not induce IgE secretion on its own. This is highly interesting in the light of the published animal models data whereby IL-9's role in hypersensitivity and allergic inflammation has been documented [169,196,197]. It further highlights the importance of considering the role of IL-9's gene as a susceptibility factor in asthma. Evidence gathered thus far in humans, also points to an important role for this cytokine in allergic inflammation [258,259]. Interestingly, the observed effects seem to be restricted or more pronounced in asthmatic human subjects with a predominant Th2 phenotype [175,209,211-213].

A recent study demonstrated the coexpression of IgD and CD27 on memory B cells and described the presence of CD27 in superficial anatomic areas of B cell follicles of lymphatic organs namely the spleen, reactive lymph nodes, inflamed appendices, tonsils and terminal ilea [260]. The report also shows that $CD27^+$ B cells may also occupy the entire follicular periphery around the germinal centre, hence implying the presence of

recirculating memory B cells in addition to naïve cells in that area as the authors conclude [260]. Thus, assessing CD27 expression allows a better characterization of the phenotype of IL-9Rα$^+$IgD$^+$ cells and their maturation pathway in culture in response to stimulation with IL-9 and anti-CD40.

Data presented here shows a specific upregulation for IL-9Rα and CD27 following CD40 and IL-4 stimulation after one week of cell culture. The observed upregulation of the IL-9Rα and CD27 on LD B cells suggests a linkage in the role of these two molecules in IgE production downstream of CD40 and IL-4 stimulation. This evidence in primary human B cells delineates the specific stage at which IL-9 acts during B cell differentiation and antibody production. CD27, a member of the TNF-R family, has been proposed as a marker for memory B cells [104]. Flow cytometric analysis revealed that CD27 molecules were expressed on GC B and memory B cells but not on naïve B cells [105-109]. Interestingly, upregulation of CD27 on IL-4 treated LD B cells occurs almost entirely on IL-9Rα positive cells. Moreover, IL-9Rα$^+$ PBMC express almost entirely CD27 (Figure 15) indicating their memory phenotype. This suggests the possibility of differentiating to an IgE secreting plasma cell in the presence of IL-4 with an exacerbated production. How does CD27 and IL-9 interact and to what extent they are complementary remains to be addressed.

Our findings constitute a new paradigm showing that memory B (IL-9Rα$^+$ CD27$^+$) cells within this subpopulation of GC B cells may be driving IgE production in the context of inflammatory responses. This is of great significance in light of the pleiotropic effects described for IL-9 in health and in the context of asthmatic inflammatory responses.

There is no potentiation effect for IL-9 on LD B cell proliferation observed in our system. This is true irrespective of the ligand used to induce B cell proliferation (Figure 11). In comparison, IL-4 sustained B cell proliferation in these different conditions. In agreement with our results, in humans, another study reported that IL-9 was not found to enhance cellular proliferation of PBMCs in the presence or absence of IL-4 [248]. Indeed,

strong proliferative effects of IL-9 have been only observed in mast cells and activated T-cells [182]. Other systems involving transfected cell lines have shown a proliferative response [186], however, relevance to primary cells and in vivo situations remains to be verified. At the molecular level, different signaling molecules, namely STATs-1,3 and 5, seem to be involved in the proliferative response [183]. Our data show activation of STAT3 and STAT5 in our system. This might account for the protective anti-apoptotic effect observed with IL-9 in the presence of fas receptor stimulation on CD40-stimulated LD cells (Figure 13) which could not be attributed to a decrease in fas receptor expression (Figure 12). This is consistent with the published role of STAT5 [183]. Data obtained using transgenic mice indicate that IL-9 overexpression increases susceptibility to T-cell lymphoma development following irradiation or administration of low doses of mutagens [191,194] whereas in vitro, only murine T lymphoma and myeloid leukemia cells proliferate in response to IL-9 and not normal cells [191,195]. This evidence points to a role for IL-9 in T cell survival and tumor formation. Recently, a subpopulation of the human GC B cells has been described with an activated STAT-5 phenotype. These cells were able to self-renew in vitro, and differentiate to a memory B cell phenotype [273]. Hence, IL-9 might play a role in B cell survival.

Our findings (flow cytometry and IHC) indicate the presence of the IL-9Rα chain on FM B cells as well as memory B cells. However, the two populations are not distinct. In fact, FM B cells encompass different maturational stages of B cells: naïve, activated, and memory cells. The characterization of the memory B cell subset remains a challenge as these may be recirculating B cells that have been described as 'early memory' vs 'late memory'. Some can readily differentiate to become ASCs, or migrate to the BM where they can permanently reside to constitute long-term memory B cells. The coexpression of CD27 on IL-9Rα$^+$ cells signifies that a proportion of these cells are memory B cells. How can we reconcile this finding with the observed flow cytometry data whereby IgD expression was highly found on IL-9Rα$^+$ cells. In fact, reports have shown that a fraction of IgD$^+$ cells are memory B cells , thus refuting the old concept that IgD$^+$ cells are naïve or immature cells.

Taken together, our data show a synergistic role for IL-9 in IgE production from human primary germinal center cells with IL-4 in the CD40/l culture system. This is particularly relevant to in vivo situations where IL-4 and IL-9 may affect B cell differentiation by their presence at the immune synapse, especially in the light of genetic evidence linking IL-9's gene to increased susceptibility to asthma. Furthermore, our data shows that the observed increase in IgE is paralleled by an upregulation of IL-9Rα and CD27, the latter being implicated in increased IgE secretion and described previously as a memory B cell marker. Interestingly, CD27 is only upregulated on IL-9R positive LD B cells pointing to a role for IL-9 in memory B cell differentiation downstream of IL-4, whereby a cascade of cytokines may be involved in the production of IgE. As no strong proliferative response for IL-9 has been observed in our experiments, IL-9's effect seems to be linked to its ability to promote B cell survival as shown by its anti-apoptotic role in the context of fas-induced apoptosis. One may envision a role for IL-9 at a later stage of the immune response following IL-4. Our data show an induction of IL-9Rα and CD27 on LD cells following stimulation with anti-CD40 and IL-4. The majority of the IL-9R+ cells coexpress CD27, these cells respond to IL-9 by an increase in IgE production. Thus, IL-9 may play a role in the late phase of the response, with a potential synergism with other molecules such as CD27, supporting B cell survival and optimizing conditions for IgE secretion in the germinal centers.

CONCLUSION

The results presented in this study demonstrate clearly the expression of IL-9Rα on human LD tonsillar B cells, and the ability of this receptor to transduce signals through activation of STAT-3 and STAT5 transcription factors. Although IL-9 was unable to induce IgE secretion by itself, it potentiated IgE production from LD cells mediated by IL-4. Thus, IL-9 exerts a synergistic effect with IL-4 to modulate IgE secretion.

Our data show that the observed increase in IgE is paralleled by an upregulation of IL-9Rα and CD27, the latter being implicated in increased IgE secretion and described previously as a memory B cell marker. Our findings highlight a new role for IL-9 in memory B cell differentiation.

Our results establish a new paradigm for understanding the stimulatory role of Th2 cytokines in the context of B-T cell interactions in the human GC, and their modulatory effect on B cell differentiation and IgE production.

FUTURE PERSPECTIVES

The specific signals that drive the differentiation of human naïve B cells into memory cells and plasma cells, have not been completely defined. Several reports demonstrated an involvement for cytokines in human plasma cell differentiation [102,118,261-265]. Our experiments lead to the identification of the IL-9Rα chain on FM and GC cells raising many interesting questions that remain to be answered. These include the precise mechanism by which IL-9 enhances IgE production from CD40 and IL-4 stimulated LD B cells, and whether CD27 synergizes with IL-9 in that respect. Therefore, it will be of a great interest to identify the molecular components induced by IL-9 such as possibly Akt, Bcl-2 or Bcl-xl to enhance survival of B cells. Determining the expression of B cell transcription factors activated by IL-9 such as BLIMP-1, which is required for plasma cell differentiation [266], AID with its role in CSR [267], and Bcl-6, which is involved in GC reactions [268] would elucidate the precise contribution of IL-9 to IgE production.

On the other hand, to examine the role of CD27, CD27+ cells could be purified and their IgE production assessed in the presence or absence of IL-9 with CD70 transfectants. A dose increase of IL-9 would allow a dose-dependent effect to be observed. Neutralization of CD70-CD27 interaction by the use of anti-CD70 MAb would confirm the role of CD27. CD40 antibodies may be added to check if IgE secretion can be induced in the presence or absence of CD40 stimulation. Next, studying this interaction in asthmatic subjects would prove helpful in confirming IL-9's role in vivo in memory B cell differentiation.

Interestingly, we have identified the expression of the IL-9Rα on human tonsillar dendritic cells by immunohistochemistry. Double expression of DC-LAMP, a member of the LAMP family of associated glycoproteins, and IL-9Rα was demonstrated by confocal microscopy using a Mab to DC-LAMP which is specifically expressed by mature interdigitating DC (IDC) [84], on human tonsillar sections (Figure 1). These cells (IDC) are mature DC located in T cell areas of lymphoid tissues [269,270]. The implications of this expression merits further investigation especially in light of the role of DC in driving Th1-Th2 differentiation.

Ultimately, the researcher's objective is to bridge bench to clinic. IL-9's role in allergic inflammation and asthma has been documented with pleiotropic effects on the differentiation of major key players of the asthmatic immune response namely mast cells and eosinophils. An immunotherapeutic approach targeting this cytokine may help alleviate the exacerbated responses observed.

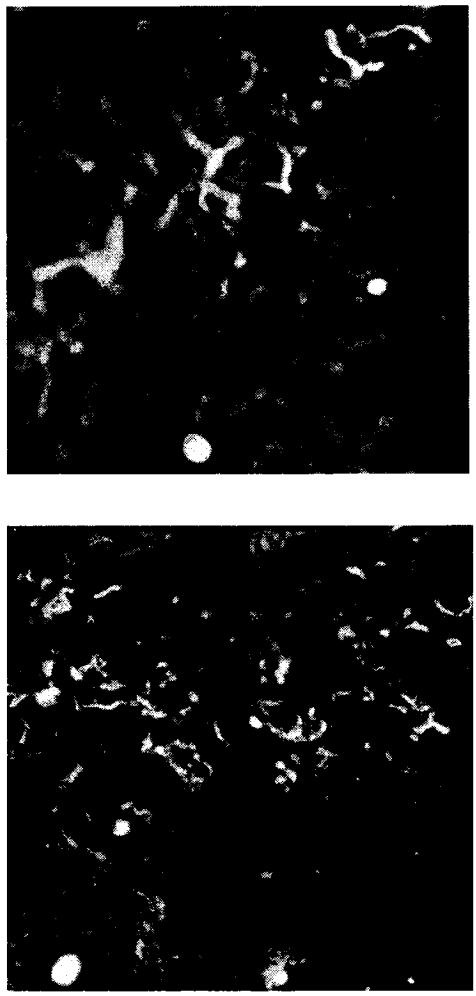


Figure 1: Expression of IL-9Ralpha on IDC of human tonsils. Tonsillar tissue sections were prepared and stained as described in materials and methods. A primary monoclonal antibody directed against DC LAMP was labeled with Alexa Fluor 568 using the Zenon Red kit from Molecular Probes. Monoclonal Ab against IL-9Rα was followed by an FITC-conjugated goat anti-mouse IgG Ab. Double positive cells show merged red and green colors (yellow). Single positive areason cells are also discernable.

Reference List

(1) Fearon DT, Locksley RM. The instructive role of innate immunity in the acquired immune response. Science. 1996;272:50-53.

(2) Manz RA, Hauser AE, Hiepe F, Radbruch A. Maintenance of serum antibody levels. Annu Rev Immunol. 2005;23:367-386.

(3) Malissen B. An evolutionary and structural perspective on T cell antigen receptor function. Immunol Rev. 2003;191:7-27.

(4) Mellman I, Turley SJ, Steinman RM. Antigen processing for amateurs and professionals. Trends Cell Biol. 1998;8:231-237.

(5) Crawford A, Macleod M, Schumacher T, Corlett L, Gray D. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. J Immunol. 2006;176:3498-3506.

(6) NOSSAL GJ, LEDERBERG J. Antibody production by single cells. Nature. 1958;181:1419-1420.

(7) Bretscher P, Cohn M. A theory of self-nonself discrimination. Science. 1970;169:1042-1049.

(8) Lafferty KJ, Cunningham AJ. A new analysis of allogeneic interactions. Aust J Exp Biol Med Sci. 1975;53:27-42.

(9) Janeway CA, Jr. Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol. 1989;54 Pt 1:1-13.

(10) Matzinger P. An innate sense of danger. Semin Immunol. 1998;10:399-415.

(11) Matzinger P. The danger model: a renewed sense of self. Science. 2002;296:301-305.

(12) Jack HM. The origin of signals predicating life and development of B cell precursors: Inside out or outside in? Semin Immunol. 2006;18:1.

(13) Busslinger M. Transcriptional control of early B cell development. Annu Rev Immunol. 2004;22:55-79.

(14) Espeli M, Rossi B, Mancini SJ et al. Initiation of pre-B cell receptor signaling: Common and distinctive features in human and mouse. Semin Immunol. 2006;18:56-66.

(15) Hardy RR, Hayakawa K. B cell development pathways. Annu Rev Immunol. 2001;19:595-621.

(16) Melchers F, ten Boekel E, Seidl T et al. Repertoire selection by pre-B-cell receptors and B-cell receptors, and genetic control of B-cell development from immature to mature B cells. Immunol Rev. 2000;175:33-46.

(17) Schiff C, Lemmers B, Deville A, Fougereau M, Meffre E. Autosomal primary immunodeficiencies affecting human bone marrow B-cell differentiation. Immunol Rev. 2000;178:91-98.

(18) Ghia P, ten Boekel E, Rolink AG, Melchers F. B-cell development: a comparison between mouse and man. Immunol Today. 1998;19:480-485.

(19) Hendriks RW, Middendorp S. The pre-BCR checkpoint as a cell-autonomous proliferation switch. Trends Immunol. 2004;25:249-256.

(20) Loder F, Mutschler B, Ray RJ et al. B cell development in the spleen takes place in discrete steps and is determined by the quality of B cell receptor-derived signals. J Exp Med. 1999;190:75-89.

(21) Chung JB, Silverman M, Monroe JG. Transitional B cells: step by step towards immune competence. Trends Immunol. 2003;24:343-349.

(22) Allman D, Lindsley RC, DeMuth W et al. Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. J Immunol. 2001;167:6834-6840.

(23) Su TT, Rawlings DJ. Transitional B lymphocyte subsets operate as distinct checkpoints in murine splenic B cell development. J Immunol. 2002;168:2101-2110.

(24) Shapiro-Shelef M, Calame K. Regulation of plasma-cell development. Nat Rev Immunol. 2005;5:230-242.

(25) Benschop RJ, Cambier JC. B cell development: signal transduction by antigen receptors and their surrogates. Curr Opin Immunol. 1999;11:143-151.

(26) Reth M, Wienands J, Schamel WW. An unsolved problem of the clonal selection theory and the model of an oligomeric B-cell antigen receptor. Immunol Rev. 2000;176:10-18.

(27) Lanzavecchia A. Antigen-specific interaction between T and B cells. Nature. 1985;314:537-539.

(28) Rock KL, Benacerraf B, Abbas AK. Antigen presentation by hapten-specific B lymphocytes. I. Role of surface immunoglobulin receptors. J Exp Med. 1984;160:1102-1113.

(29) Carrasco YR, Fleire SJ, Cameron T, Dustin ML, Batista FD. LFA-1/ICAM-1 interaction lowers the threshold of B cell activation by facilitating B cell adhesion and synapse formation. Immunity. 2004;20:589-599.

(30) Goodnow CC, Crosbie J, Adelstein S et al. Altered immunoglobulin expression and functional silencing of self-reactive B lymphocytes in transgenic mice. Nature. 1988;334:676-682.

(31) Lang J, Jackson M, Teyton L et al. B cells are exquisitely sensitive to central tolerance and receptor editing induced by ultralow affinity, membrane-bound antigen. J Exp Med. 1996;184:1685-1697.

(32) Haberman AM, Shlomchik MJ. Reassessing the function of immune-complex retention by follicular dendritic cells. Nat Rev Immunol. 2003;3:757-764.

(33) Wykes M, Pombo A, Jenkins C, MacPherson GG. Dendritic cells interact directly with naive B lymphocytes to transfer antigen and initiate class switching in a primary T-dependent response. J Immunol. 1998;161:1313-1319.

(34) Szakal AK, Kosco MH, Tew JG. FDC-iccosome mediated antigen delivery to germinal center B cells, antigen processing and presentation to T cells. Adv Exp Med Biol. 1988;237:197-202.

(35) Batista FD, Iber D, Neuberger MS. B cells acquire antigen from target cells after synapse formation. Nature. 2001;411:489-494.

(36) Dustin ML, Dustin LB. The immunological relay race: B cells take antigen by synapse. Nat Immunol. 2001;2:480-482.

(37) MacLennan IC, Gulbranson-Judge A, Toellner KM et al. The changing preference of T and B cells for partners as T-dependent antibody responses develop. Immunol Rev. 1997;156:53-66.

(38) McHeyzer-Williams LJ, McHeyzer-Williams MG. Antigen-specific memory B cell development. Annu Rev Immunol. 2005;23:487-513.

(39) Kroese FG, Wubbena AS, Seijen HG, Nieuwenhuis P. Germinal centers develop oligoclonally. Eur J Immunol. 1987;17:1069-1072.

(40) Liu YJ, Zhang J, Lane PJ, Chan EY, MacLennan IC. Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens. Eur J Immunol. 1991;21:2951-2962.

(41) Jacob J, Kelsoe G, Rajewsky K, Weiss U. Intraclonal generation of antibody mutants in germinal centres. Nature. 1991;354:389-392.

(42) Kuppers R, Zhao M, Hansmann ML, Rajewsky K. Tracing B cell development in human germinal centres by molecular analysis of single cells picked from histological sections. EMBO J. 1993;12:4955-4967.

(43) Pascual V, Liu YJ, Magalski A et al. Analysis of somatic mutation in five B cell subsets of human tonsil. J Exp Med. 1994;180:329-339.

(44) Klein U, Kuppers R, Rajewsky K. Variable region gene analysis of B cell subsets derived from a 4-year-old child: somatically mutated memory B cells accumulate in the peripheral blood already at young age. J Exp Med. 1994;180:1383-1393.

(45) Maizels N. Immunoglobulin gene diversification. Annu Rev Genet. 2005;39:23-46.

(46) Tarlinton D. Germinal centers: form and function. Curr Opin Immunol. 1998;10:245-251.

(47) Liu YJ, Johnson GD, Gordon J, MacLennan IC. Germinal centres in T-cell-dependent antibody responses. Immunol Today. 1992;13:17-21.

(48) MacLennan IC. Germinal centers. Annu Rev Immunol. 1994;12:117-139.

(49) Wolniak KL, Shinall SM, Waldschmidt TJ. The germinal center response. Crit Rev Immunol. 2004;24:39-65.

(50) Lanzavecchia A, Sallusto F. Progressive differentiation and selection of the fittest in the immune response. Nat Rev Immunol. 2002;2:982-987.

(51) Arpin C, Dechanet J, Van Kooten C et al. Generation of memory B cells and plasma cells in vitro. Science. 1995;268:720-722.

(52) Muramatsu M, Kinoshita K, Fagarasan S et al. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 2000;102:553-563.

(53) Cattoretti G, Buettner M, Shaknovich R et al. Nuclear and cytoplasmic AID in extrafollicular and Germinal Center B cells. Blood. 2006;.

(54) Liu YJ, Arpin C. Germinal center development. Immunol Rev. 1997;156:111-126.

(55) Lebecque S, de Bouteiller O, Arpin C, Banchereau J, Liu YJ. Germinal center founder cells display propensity for apoptosis before onset of somatic mutation. J Exp Med. 1997;185:563-571.

(56) Weiss U, Zoebelein R, Rajewsky K. Accumulation of somatic mutants in the B cell compartment after primary immunization with a T cell-dependent antigen. Eur J Immunol. 1992;22:511-517.

(57) Coffman RL, Lebman DA, Rothman P. Mechanism and regulation of immunoglobulin isotype switching. Adv Immunol. 1993;54:229-270.

(58) Sideras P, Mizuta TR, Kanamori H et al. Production of sterile transcripts of C gamma genes in an IgM-producing human neoplastic B cell line that switches to IgG-producing cells. Int Immunol. 1989;1:631-642.

(59) Zhang J, Bottaro A, Li S, Stewart V, Alt FW. A selective defect in IgG2b switching as a result of targeted mutation of the I gamma 2b promoter and exon. EMBO J. 1993;12:3529-3537.

(60) Lederman S, Yellin MJ, Inghirami G et al. Molecular interactions mediating T-B lymphocyte collaboration in human lymphoid follicles. Roles of T cell-B-cell-activating molecule (5c8 antigen) and CD40 in contact-dependent help. J Immunol. 1992;149:3817-3826.

(61) Casamayor-Palleja M, Khan M, MacLennan IC. A subset of CD4+ memory T cells contains preformed CD40 ligand that is rapidly but transiently expressed on their surface after activation through the T cell receptor complex. J Exp Med. 1995;181:1293-1301.

(62) Butch AW, Chung GH, Hoffmann JW, Nahm MH. Cytokine expression by germinal center cells. J Immunol. 1993;150:39-47.

(63) Kosco MH, Szakal AK, Tew JG. In vivo obtained antigen presented by germinal center B cells to T cells in vitro. J Immunol. 1988;140:354-360.

(64) Banchereau J, de Paoli P, Valle A, Garcia E, Rousset F. Long-term human B cell lines dependent on interleukin-4 and antibody to CD40. Science. 1991;251:70-72.

(65) Lane P, Burdet C, McConnell F, Lanzavecchia A, Padovan E. CD40 ligand-independent B cell activation revealed by CD40 ligand-deficient T cell clones: evidence for distinct activation requirements for antibody formation and B cell proliferation. Eur J Immunol. 1995;25:1788-1793.

(66) Callard RE, Herbert J, Smith SH, Armitage RJ, Costelloe KE. CD40 cross-linking inhibits specific antibody production by human B cells. Int Immunol. 1995;7:1809-1815.

(67) Lin Y, Wong K, Calame K. Repression of c-myc transcription by Blimp-1, an inducer of terminal B cell differentiation. Science. 1997;276:596-599.

(68) Chi N, Epstein JA. Getting your Pax straight: Pax proteins in development and disease. Trends Genet. 2002;18:41-47.

(69) Shaffer AL, Yu X, He Y et al. BCL-6 represses genes that function in lymphocyte differentiation, inflammation, and cell cycle control. Immunity. 2000;13:199-212.

(70) Reimold AM, Iwakoshi NN, Manis J et al. Plasma cell differentiation requires the transcription factor XBP-1. Nature. 2001;412:300-307.

(71) Quezada SA, Jarvinen LZ, Lind EF, Noelle RJ. CD40/CD154 interactions at the interface of tolerance and immunity. Annu Rev Immunol. 2004;22:307-328.

(72) Lin KI, Tunyaplin C, Calame K. Transcriptional regulatory cascades controlling plasma cell differentiation. Immunol Rev. 2003;194:19-28.

(73) Kuppers R, Dalla-Favera R. Mechanisms of chromosomal translocations in B cell lymphomas. Oncogene. 2001;20:5580-5594.

(74) Cambier JC, Ransom JT, Harris LK et al. Coupling of B cell surface Ig, Ia and BSF1 receptors to intracellular "second messengers". Adv Exp Med Biol. 1987;213:195-205.

(75) Cambier JC, Newell MK, Justement LB et al. Ia binding ligands and cAMP stimulate nuclear translocation of PKC in B lymphocytes. Nature. 1987;327:629-632.

(76) Newell MK, VanderWall J, Beard KS, Freed JH. Ligation of major histocompatibility complex class II molecules mediates apoptotic cell death in resting B lymphocytes. Proc Natl Acad Sci U S A. 1993;90:10459-10463.

(77) Lang P, Stolpa JC, Freiberg BA et al. TCR-induced transmembrane signaling by peptide/MHC class II via associated Ig-alpha/beta dimers. Science. 2001;291:1537-1540.

(78) Bishop GA, Hostager BS. B lymphocyte activation by contact-mediated interactions with T lymphocytes. Curr Opin Immunol. 2001;13:278-285.

(79) Moy VT, Brian AA. Signaling by lymphocyte function-associated antigen 1 (LFA-1) in B cells: enhanced antigen presentation after stimulation through LFA-1. J Exp Med. 1992;175:1-7.

(80) Poudrier J, Owens T. CD54/intercellular adhesion molecule 1 and major histocompatibility complex II signaling induces B cells to express interleukin 2 receptors and complements help provided through CD40 ligation. J Exp Med. 1994;179:1417-1427.

(81) Warnatz K, Bossaller L, Salzer U et al. Human ICOS-deficiency abrogates the germinal center reaction and provides a monogenic model for common variable immunodeficiency. Blood. 2005;.

(82) Arch RH, Gedrich RW, Thompson CB. Tumor necrosis factor receptor-associated factors (TRAFs)--a family of adapter proteins that regulates life and death. Genes Dev. 1998;12:2821-2830.

(83) Mills DM, Cambier JC. B lymphocyte activation during cognate interactions with CD4+ T lymphocytes: molecular dynamics and immunologic consequences. Semin Immunol. 2003;15:325-329.

(84) DiSanto JP, Bonnefoy JY, Gauchat JF, Fischer A, de Saint BG. CD40 ligand mutations in x-linked immunodeficiency with hyper-IgM. Nature. 1993;361:541-543.

(85) Xu J, Foy TM, Laman JD et al. Mice deficient for the CD40 ligand. Immunity. 1994;1:423-431.

(86) Kawabe T, Naka T, Yoshida K et al. The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation. Immunity. 1994;1:167-178.

(87) Van Kooten C, Banchereau J. CD40-CD40 ligand. J Leukoc Biol. 2000;67:2-17.

(88) Calderhead DM, Kosaka Y, Manning EM, Noelle RJ. CD40-CD154 interactions in B-cell signaling. Curr Top Microbiol Immunol. 2000;245:73-99.

(89) Schonbeck U, Libby P. The CD40/CD154 receptor/ligand dyad. Cell Mol Life Sci. 2001;58:4-43.

(90) Mauri C, Mars LT, Londei M. Therapeutic activity of agonistic monoclonal antibodies against CD40 in a chronic autoimmune inflammatory process. Nat Med. 2000;6:673-679.

(91) Town T, Tan J, Mullan M. CD40 signaling and Alzheimer's disease pathogenesis. Neurochem Int. 2001;39:371-380.

(92) Tamada K, Tamura H, Flies D et al. Blockade of LIGHT/LTbeta and CD40 signaling induces allospecific T cell anergy, preventing graft-versus-host disease. J Clin Invest. 2002;109:549-557.

(93) Lutgens E, Gorelik L, Daemen MJ et al. Requirement for CD154 in the progression of atherosclerosis. Nat Med. 1999;5:1313-1316.

(94) Dallman C, Johnson PW, Packham G. Differential regulation of cell survival by CD40. Apoptosis. 2003;8:45-53.

(95) Renshaw BR, Fanslow WC, III, Armitage RJ et al. Humoral immune responses in CD40 ligand-deficient mice. J Exp Med. 1994;180:1889-1900.

(96) Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. Annu Rev Immunol. 1998;16:111-135.

(97) Foy TM, Aruffo A, Bajorath J, Buhlmann JE, Noelle RJ. Immune regulation by CD40 and its ligand GP39. Annu Rev Immunol. 1996;14:591-617.

(98) Fuleihan RL. The X-linked hyperimmunoglobulin M syndrome. Semin Hematol. 1998;35:321-331.

(99) Fecteau JF, Neron S. CD40 stimulation of human peripheral B lymphocytes: distinct response from naive and memory cells. J Immunol. 2003;171:4621-4629.

(100) Banchereau J, Bazan F, Blanchard D et al. The CD40 antigen and its ligand. Annu Rev Immunol. 1994;12:881-922.

(101) Gascan H, Gauchat JF, Aversa G, Van Vlasselaer P, de Vries JE. Anti-CD40 monoclonal antibodies or CD4+ T cell clones and IL-4 induce IgG4 and IgE switching in purified human B cells via different signaling pathways. J Immunol. 1991;147:8-13.

(102) Briere F, Servet-Delprat C, Bridon JM, Saint-Remy JM, Banchereau J. Human interleukin 10 induces naive surface immunoglobulin D+ (sIgD+) B cells to secrete IgG1 and IgG3. J Exp Med. 1994;179:757-762.

(103) Rousset F, Peyrol S, Garcia E et al. Long-term cultured CD40-activated B lymphocytes differentiate into plasma cells in response to IL-10 but not IL-4. Int Immunol. 1995;7:1243-1253.

(104) Agematsu K, Hokibara S, Nagumo H, Komiyama A. CD27: a memory B-cell marker. Immunol Today. 2000;21:204-206.

(105) Maurer D, Fischer GF, Fae I et al. IgM and IgG but not cytokine secretion is restricted to the CD27+ B lymphocyte subset. J Immunol. 1992;148:3700-3705.

(106) Agematsu K, Nagumo H, Yang FC et al. B cell subpopulations separated by CD27 and crucial collaboration of CD27+ B cells and helper T cells in immunoglobulin production. Eur J Immunol. 1997;27:2073-2079.

(107) Klein U, Rajewsky K, Kuppers R. Human immunoglobulin (Ig)M+IgD+ peripheral blood B cells expressing the CD27 cell surface antigen carry somatically mutated variable region genes: CD27 as a general marker for somatically mutated (memory) B cells. J Exp Med. 1998;188:1679-1689.

(108) Tangye SG, Liu YJ, Aversa G, Phillips JH, de Vries JE. Identification of functional human splenic memory B cells by expression of CD148 and CD27. J Exp Med. 1998;188:1691-1703.

(109) van Oers MH, Pals ST, Evers LM et al. Expression and release of CD27 in human B-cell malignancies. Blood. 1993;82:3430-3436.

(110) Agematsu K, Nagumo H, Oguchi Y et al. Generation of plasma cells from peripheral blood memory B cells: synergistic effect of interleukin-10 and CD27/CD70 interaction. Blood. 1998;91:173-180.

(111) Jacquot S, Kobata T, Iwata S, Morimoto C, Schlossman SF. CD154/CD40 and CD70/CD27 interactions have different and sequential functions in T cell-dependent B cell responses: enhancement of plasma cell differentiation by CD27 signaling. J Immunol. 1997;159:2652-2657.

(112) Nagumo H, Agematsu K. Synergistic augmentative effect of interleukin-10 and CD27/CD70 interactions on B-cell immunoglobulin synthesis. Immunology. 1998;94:388-394.

(113) Nagumo H, Agematsu K, Shinozaki K et al. CD27/CD70 interaction augments IgE secretion by promoting the differentiation of memory B cells into plasma cells. J Immunol. 1998;161:6496-6502.

(114) Akiba H, Nakano H, Nishinaka S et al. CD27, a member of the tumor necrosis factor receptor superfamily, activates NF-kappaB and stress-activated protein kinase/c-Jun N-terminal kinase via TRAF2, TRAF5, and NF-kappaB-inducing kinase. J Biol Chem. 1998;273:13353-13358.

(115) Gravestein LA, Amsen D, Boes M et al. The TNF receptor family member CD27 signals to Jun N-terminal kinase via Traf-2. Eur J Immunol. 1998;28:2208-2216.

(116) Klein U, Goossens T, Fischer M et al. Somatic hypermutation in normal and transformed human B cells. Immunol Rev. 1998;162:261-280.

(117) Agematsu K, Kobata T, Yang FC et al. CD27/CD70 interaction directly drives B cell IgG and IgM synthesis. Eur J Immunol. 1995;25:2825-2829.

(118) Nagumo H, Agematsu K, Kobayashi N et al. The different process of class switching and somatic hypermutation; a novel analysis by CD27(-) naive B cells. Blood. 2002;99:567-575.

(119) Swallow MM, Wallin JJ, Sha WC. B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNFalpha. Immunity. 1999;11:423-432.

(120) Liu YJ, Banchereau J. Regulation of B-cell commitment to plasma cells or to memory B cells. Semin Immunol. 1997;9:235-240.

(121) Harris DP, Haynes L, Sayles PC et al. Reciprocal regulation of polarized cytokine production by effector B and T cells. Nat Immunol. 2000;1:475-482.

(122) Duddy ME, Alter A, Bar-Or A. Distinct profiles of human B cell effector cytokines: a role in immune regulation? J Immunol. 2004;172:3422-3427.

(123) Hajoui O, Janani R, Tulic M et al. Synthesis of IL-13 by human B lymphocytes: regulation and role in IgE production. J Allergy Clin Immunol. 2004;114:657-663.

(124) Huse M, Lillemeier BF, Kuhns MS, Chen DS, Davis MM. T cells use two directionally distinct pathways for cytokine secretion. Nat Immunol. 2006;7:247-255.

(125) Nelms K, Keegan AD, Zamorano J, Ryan JJ, Paul WE. The IL-4 receptor: signaling mechanisms and biologic functions. Annu Rev Immunol. 1999;17:701-738.

(126) Kupfer H, Monks CR, Kupfer A. Small splenic B cells that bind to antigen-specific T helper (Th) cells and face the site of cytokine production in the Th cells selectively proliferate: immunofluorescence microscopic studies of Th-B antigen-presenting cell interactions. J Exp Med. 1994;179:1507-1515.

(127) Cambier JC, Lehmann KR. Ia-mediated signal transduction leads to proliferation of primed B lymphocytes. J Exp Med. 1989;170:877-886.

(128) Defrance T, Carayon P, Billian G et al. Interleukin 13 is a B cell stimulating factor. J Exp Med. 1994;179:135-143.

(129) Roldan E, Brieva JA. Terminal differentiation of human bone marrow cells capable of spontaneous and high-rate immunoglobulin secretion: role of bone marrow stromal cells and interleukin 6. Eur J Immunol. 1991;21:2671-2677.

(130) Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. J Immunol. 1986;136:2348-2357.

(131) Coffman RL, Seymour BW, Lebman DA et al. The role of helper T cell products in mouse B cell differentiation and isotype regulation. Immunol Rev. 1988;102:5-28.

(132) Gajewski TF, Fitch FW. Anti-proliferative effect of IFN-gamma in immune regulation. I. IFN-gamma inhibits the proliferation of Th2 but not Th1 murine helper T lymphocyte clones. J Immunol. 1988;140:4245-4252.

(133) Fernandez-Botran R, Sanders VM, Mosmann TR, Vitetta ES. Lymphokine-mediated regulation of the proliferative response of clones of T helper 1 and T helper 2 cells. J Exp Med. 1988;168:543-558.

(134) Randolph DA, Huang G, Carruthers CJ, Bromley LE, Chaplin DD. The role of CCR7 in TH1 and TH2 cell localization and delivery of B cell help in vivo. Science. 1999;286:2159-2162.

(135) Smith KM, Brewer JM, Rush CM, Riley J, Garside P. In vivo generated Th1 cells can migrate to B cell follicles to support B cell responses. J Immunol. 2004;173:1640-1646.

(136) Smith KM, Pottage L, Thomas ER et al. Th1 and Th2 CD4+ T cells provide help for B cell clonal expansion and antibody synthesis in a similar manner in vivo. J Immunol. 2000;165:3136-3144.

(137) Pape KA, Kouskoff V, Nemazee D et al. Visualization of the genesis and fate of isotype-switched B cells during a primary immune response. J Exp Med. 2003;197:1677-1687.

(138) Toellner KM, Luther SA, Sze DM et al. T helper 1 (Th1) and Th2 characteristics start to develop during T cell priming and are associated with an immediate ability to induce immunoglobulin class switching. J Exp Med. 1998;187:1193-1204.

(139) Paul WE, Seder RA. Lymphocyte responses and cytokines. Cell. 1994;76:241-251.

(140) Robinson DS, Hamid Q, Ying S et al. Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med. 1992;326:298-304.

(141) Kon OM, Kay AB. T cells and chronic asthma. Int Arch Allergy Immunol. 1999;118:133-135.

(142) Yssel H, Groux H. Characterization of T cell subpopulations involved in the pathogenesis of asthma and allergic diseases. Int Arch Allergy Immunol. 2000;121:10-18.

(143) Zhou Y, McLane M, Levitt RC. Th2 cytokines and asthma. Interleukin-9 as a therapeutic target for asthma. Respir Res. 2001;2:80-84.

(144) Van Snick J, Goethals A, Renauld JC et al. Cloning and characterization of a cDNA for a new mouse T cell growth factor (P40). J Exp Med. 1989;169:363-368.

(145) Uyttenhove C, Simpson RJ, Van Snick J. Functional and structural characterization of P40, a mouse glycoprotein with T-cell growth factor activity. Proc Natl Acad Sci U S A. 1988;85:6934-6938.

(146) Simpson RJ, Moritz RL, Rubira MR, Gorman JJ, Van Snick J. Complete amino acid sequence of a new murine T-cell growth factor P40. Eur J Biochem. 1989;183:715-722.

(147) Hultner L, Druez C, Moeller J et al. Mast cell growth-enhancing activity (MEA) is structurally related and functionally identical to the novel mouse T cell growth factor P40/TCGFIII (interleukin 9). Eur J Immunol. 1990;20:1413-1416.

(148) Stassen M, Arnold M, Hultner L et al. Murine bone marrow-derived mast cells as potent producers of IL-9: costimulatory function of IL-10 and kit ligand in the presence of IL-1. J Immunol. 2000;164:5549-5555.

(149) Houssiau FA, Renauld JC, Fibbe WE, Van Snick J. IL-2 dependence of IL-9 expression in human T lymphocytes. J Immunol. 1992;148:3147-3151.

(150) Soussi-Gounni A, Kontolemos M, Hamid Q. Role of IL-9 in the pathophysiology of allergic diseases. J Allergy Clin Immunol. 2001;107:575-582.

(151) Shimbara A, Christodoulopoulos P, Soussi-Gounni A et al. IL-9 and its receptor in allergic and nonallergic lung disease: increased expression in asthma. J Allergy Clin Immunol. 2000;105:108-115.

(152) Hauber HP, Bergeron C, Hamid Q. IL-9 in allergic inflammation. Int Arch Allergy Immunol. 2004;134:79-87.

(153) Modi WS, Pollock DD, Mock BA et al. Regional localization of the human glutaminase (GLS) and interleukin-9 (IL9) genes by in situ hybridization. Cytogenet Cell Genet. 1991;57:114-116.

(154) Nicolaides NC, Holroyd KJ, Ewart SL et al. Interleukin 9: a candidate gene for asthma. Proc Natl Acad Sci U S A. 1997;94:13175-13180.

(155) McKenzie AN, Li X, Largaespada DA et al. Structural comparison and chromosomal localization of the human and mouse IL-13 genes. J Immunol. 1993;150:5436-5444.

(156) Frazer KA, Ueda Y, Zhu Y et al. Computational and biological analysis of 680 kb of DNA sequence from the human 5q31 cytokine gene cluster region. Genome Res. 1997;7:495-512.

(157) Postma DS, Bleecker ER, Amelung PJ et al. Genetic susceptibility to asthma--bronchial hyperresponsiveness coinherited with a major gene for atopy. N Engl J Med. 1995;333:894-900.

(158) van Leeuwen BH, Martinson ME, Webb GC, Young IG. Molecular organization of the cytokine gene cluster, involving the human IL-3, IL-4, IL-5, and GM-CSF genes, on human chromosome 5. Blood. 1989;73:1142-1148.

(159) Mock BA, Krall M, Kozak CA et al. IL9 maps to mouse chromosome 13 and human chromosome 5. Immunogenetics. 1990;31:265-270.

(160) Renauld JC, Goethals A, Houssiau F et al. Human P40/IL-9. Expression in activated CD4+ T cells, genomic organization, and comparison with the mouse gene. J Immunol. 1990;144:4235-4241.

(161) Kelleher K, Bean K, Clark SC et al. Human interleukin-9: genomic sequence, chromosomal location, and sequences essential for its expression in human T-cell leukemia virus (HTLV)-I-transformed human T cells. Blood. 1991;77:1436-1441.

(162) Marsh DG, Neely JD, Breazeale DR et al. Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. Science. 1994;264:1152-1156.

(163) Zhu YX, Kang LY, Luo W et al. Multiple transcription factors are required for activation of human interleukin 9 gene in T cells. J Biol Chem. 1996;271:15815-15822.

(164) Houssiau FA, Schandene L, Stevens M et al. A cascade of cytokines is responsible for IL-9 expression in human T cells. Involvement of IL-2, IL-4, and IL-10. J Immunol. 1995;154:2624-2630.

(165) Okada S, Inoue H, Yamauchi K et al. Potential role of interleukin-1 in allergen-induced late asthmatic reactions in guinea pigs: suppressive effect of interleukin-1 receptor antagonist on late asthmatic reaction. J Allergy Clin Immunol. 1995;95:1236-1245.

(166) Hultner L, Kolsch S, Stassen M et al. In activated mast cells, IL-1 up-regulates the production of several Th2-related cytokines including IL-9. J Immunol. 2000;164:5556-5563.

(167) Erpenbeck VJ, Hohlfeld JM, Volkmann B et al. Segmental allergen challenge in patients with atopic asthma leads to increased IL-9 expression in bronchoalveolar lavage fluid lymphocytes. J Allergy Clin Immunol. 2003;111:1319-1327.

(168) Schindler C, Darnell JE, Jr. Transcriptional responses to polypeptide ligands: the JAK-STAT pathway. Annu Rev Biochem. 1995;64:621-651.

(169) Vink A, Warnier G, Brombacher F, Renauld JC. Interleukin 9-induced in vivo expansion of the B-1 lymphocyte population. J Exp Med. 1999;189:1413-1423.

(170) Holroyd KJ, Martinati LC, Trabetti E et al. Asthma and bronchial hyperresponsiveness linked to the XY long arm pseudoautosomal region. Genomics. 1998;52:233-235.

(171) Gounni AS, Hamid Q, Rahman SM et al. IL-9-mediated induction of eotaxin1/CCL11 in human airway smooth muscle cells. J Immunol. 2004;173:2771-2779.

(172) Giannella-Neto A, Paoletti P, Fornai E, Giuntini C. Estimates of mean alveolar gas in patients with chronic airways obstruction. Eur Respir J. 1989;2:451-460.

(173) Fabbri LM, Boschetto P, Zocca E et al. Bronchoalveolar neutrophilia during late asthmatic reactions induced by toluene diisocyanate. Am Rev Respir Dis. 1987;136:36-42.

(174) Metzger WJ, Richerson HB, Worden K, Monick M, Hunninghake GW. Bronchoalveolar lavage of allergic asthmatic patients following allergen bronchoprovocation. Chest. 1986;89:477-483.

(175) Gounni AS, Lamkhioued B, Koussih L et al. Human neutrophils express the high-affinity receptor for immunoglobulin E (Fc epsilon RI): role in asthma. FASEB J. 2001;15:940-949.

(176) Hauber HP, Tsicopoulos A, Wallaert B et al. Expression of HCLCA1 in cystic fibrosis lungs is associated with mucus overproduction. Eur Respir J. 2004;23:846-850.

(177) Druez C, Coulie P, Uyttenhove C, Van Snick J. Functional and biochemical characterization of mouse P40/IL-9 receptors. J Immunol. 1990;145:2494-2499.

(178) Hayakawa K, Hardy RR, Herzenberg LA, Herzenberg LA. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. J Exp Med. 1985;161:1554-1568.

(179) Demoulin JB, Uyttenhove C, Van Roost E et al. A single tyrosine of the interleukin-9 (IL-9) receptor is required for STAT activation, antiapoptotic activity, and growth regulation by IL-9. Mol Cell Biol. 1996;16:4710-4716.

(180) Zhu YX, Sun HB, Tsang ML et al. Critical cytoplasmic domains of human interleukin-9 receptor alpha chain in interleukin-9-mediated cell proliferation and signal transduction. J Biol Chem. 1997;272:21334-21340.

(181) Demoulin JB, Van Roost E, Stevens M, Groner B, Renauld JC. Distinct roles for STAT1, STAT3, and STAT5 in differentiation gene induction and apoptosis inhibition by interleukin-9. J Biol Chem. 1999;274:25855-25861.

(182) Bauer JH, Liu KD, You Y, Lai SY, Goldsmith MA. Heteromerization of the gammac chain with the interleukin-9 receptor alpha subunit leads to STAT activation and prevention of apoptosis. J Biol Chem. 1998;273:9255-9260.

(183) Demoulin JB, Uyttenhove C, Lejeune D et al. STAT5 activation is required for interleukin-9-dependent growth and transformation of lymphoid cells. Cancer Res. 2000;60:3971-3977.

(184) Keegan AD, Nelms K, White M et al. An IL-4 receptor region containing an insulin receptor motif is important for IL-4-mediated IRS-1 phosphorylation and cell growth. Cell. 1994;76:811-820.

(185) Yin T, Keller SR, Quelle FW et al. Interleukin-9 induces tyrosine phosphorylation of insulin receptor substrate-1 via JAK tyrosine kinases. J Biol Chem. 1995;270:20497-20502.

(186) Demoulin JP, Grasso L, Atkins JM et al. Role of insulin receptor substrate-2 in interleukin-9-dependent proliferation. FEBS Lett. 2000;482:200-204.

(187) Xiao H, Yin T, Wang XY et al. Specificity of interleukin-2 receptor gamma chain superfamily cytokines is mediated by insulin receptor substrate-dependent pathway. J Biol Chem. 2002;277:8091-8098.

(188) Richard M, Louahed J, Demoulin JB, Renauld JC. Interleukin-9 regulates NF-kappaB activity through BCL3 gene induction. Blood. 1999;93:4318-4327.

(189) Lejeune D, Demoulin JB, Renauld JC. Interleukin 9 induces expression of three cytokine signal inhibitors: cytokine-inducible SH2-containing protein, suppressor of cytokine signalling (SOCS)-2 and SOCS-3, but only SOCS-3 overexpression suppresses interleukin 9 signalling. Biochem J. 2001;353:109-116.

(190) Gounni AS, Gregory B, Nutku E et al. Interleukin-9 enhances interleukin-5 receptor expression, differentiation, and survival of human eosinophils. Blood. 2000;96:2163-2171.

(191) Renauld JC, Kermouni A, Vink A, Louahed J, Van Snick J. Interleukin-9 and its receptor: involvement in mast cell differentiation and T cell oncogenesis. J Leukoc Biol. 1995;57:353-360.

(192) De Smedt M, Verhasselt B, Kerre T et al. Signals from the IL-9 receptor are critical for the early stages of human intrathymic T cell development. J Immunol. 2000;164:1761-1767.

(193) Townsend JM, Fallon GP, Matthews JD et al. IL-9-deficient mice establish fundamental roles for IL-9 in pulmonary mastocytosis and goblet cell hyperplasia but not T cell development. Immunity. 2000;13:573-583.

(194) Renauld JC, van der LN, Vink A et al. Thymic lymphomas in interleukin 9 transgenic mice. Oncogene. 1994;9:1327-1332.

(195) Lemoli RM, Fortuna A, Tafuri A et al. Interleukin-9 stimulates the proliferation of human myeloid leukemic cells. Blood. 1996;87:3852-3859.

(196) Levitt RC, McLane MP, MacDonald D et al. IL-9 pathway in asthma: new therapeutic targets for allergic inflammatory disorders. J Allergy Clin Immunol. 1999;103:S485-S491.

(197) McLane MP, Haczku A, van de RM et al. Interleukin-9 promotes allergen-induced eosinophilic inflammation and airway hyperresponsiveness in transgenic mice. Am J Respir Cell Mol Biol. 1998;19:713-720.

(198) Louahed J, Zhou Y, Maloy WL et al. Interleukin 9 promotes influx and local maturation of eosinophils. Blood. 2001;97:1035-1042.

(199) McMillan SJ, Bishop B, Townsend MJ, McKenzie AN, Lloyd CM. The absence of interleukin 9 does not affect the development of allergen-induced pulmonary inflammation nor airway hyperreactivity. J Exp Med. 2002;195:51-57.

(200) Gounni AS, Nutku E, Koussih L et al. IL-9 expression by human eosinophils: regulation by IL-1beta and TNF-alpha. J Allergy Clin Immunol. 2000;106:460-466.

(201) Godfraind C, Louahed J, Faulkner H et al. Intraepithelial infiltration by mast cells with both connective tissue-type and mucosal-type characteristics in gut, trachea, and kidneys of IL-9 transgenic mice. J Immunol. 1998;160:3989-3996.

(202) Elias JA, Lee CG, Zheng T et al. New insights into the pathogenesis of asthma. J Clin Invest. 2003;111:291-297.

(203) Eklund KK, Ghildyal N, Austen KF, Stevens RL. Induction by IL-9 and suppression by IL-3 and IL-4 of the levels of chromosome 14-derived transcripts that encode late-expressed mouse mast cell proteases. J Immunol. 1993;151:4266-4273.

(204) Louahed J, Kermouni A, Van Snick J, Renauld JC. IL-9 induces expression of granzymes and high-affinity IgE receptor in murine T helper clones. J Immunol. 1995;154:5061-5070.

(205) Faulkner H, Humphreys N, Renauld JC, Van Snick J, Grencis R. Interleukin-9 is involved in host protective immunity to intestinal nematode infection. Eur J Immunol. 1997;27:2536-2540.

(206) Dugas B, Renauld JC, Pene J et al. Interleukin-9 potentiates the interleukin-4-induced immunoglobulin (IgG, IgM and IgE) production by normal human B lymphocytes. Eur J Immunol. 1993;23:1687-1692.

(207) Petit-Frere C, Dugas B, Braquet P, Mencia-Huerta JM. Interleukin-9 potentiates the interleukin-4-induced IgE and IgG1 release from murine B lymphocytes. Immunology. 1993;79:146-151.

(208) Temann UA, Geba GP, Rankin JA, Flavell RA. Expression of interleukin 9 in the lungs of transgenic mice causes airway inflammation, mast cell hyperplasia, and bronchial hyperresponsiveness. J Exp Med. 1998;188:1307-1320.

(209) Baraldo S, Faffe DS, Moore PE et al. Interleukin-9 influences chemokine release in airway smooth muscle: role of ERK. Am J Physiol Lung Cell Mol Physiol. 2003;284:L1093-L1102.

(210) Temann UA, Ray P, Flavell RA. Pulmonary overexpression of IL-9 induces Th2 cytokine expression, leading to immune pathology. J Clin Invest. 2002;109:29-39.

(211) Dong Q, Louahed J, Vink A et al. IL-9 induces chemokine expression in lung epithelial cells and baseline airway eosinophilia in transgenic mice. Eur J Immunol. 1999;29:2130-2139.

(212) Louahed J, Toda M, Jen J et al. Interleukin-9 upregulates mucus expression in the airways. Am J Respir Cell Mol Biol. 2000;22:649-656.

(213) Longphre M, Li D, Gallup M et al. Allergen-induced IL-9 directly stimulates mucin transcription in respiratory epithelial cells. J Clin Invest. 1999;104:1375-1382.

(214) Toda M, Tulic MK, Levitt RC, Hamid Q. A calcium-activated chloride channel (HCLCA1) is strongly related to IL-9 expression and mucus production in bronchial epithelium of patients with asthma. J Allergy Clin Immunol. 2002;109:246-250.

(215) Knoops L, Louahed J, Van Snick J, Renauld JC. IL-9 promotes but is not necessary for systemic anaphylaxis. J Immunol. 2005;175:335-341.

(216) Kung TT, Luo B, Crawley Y et al. Effect of anti-mIL-9 antibody on the development of pulmonary inflammation and airway hyperresponsiveness in allergic mice. Am J Respir Cell Mol Biol. 2001;25:600-605.

(217) Cheng G, Arima M, Honda K et al. Anti-interleukin-9 antibody treatment inhibits airway inflammation and hyperreactivity in mouse asthma model. Am J Respir Crit Care Med. 2002;166:409-416.

(218) Richard M, Grencis RK, Humphreys NE, Renauld JC, Van Snick J. Anti-IL-9 vaccination prevents worm expulsion and blood eosinophilia in Trichuris muris-infected mice. Proc Natl Acad Sci U S A. 2000;97:767-772.

(219) Fischer M, Bijman M, Molin D et al. Increased serum levels of interleukin-9 correlate to negative prognostic factors in Hodgkin's lymphoma. Leukemia. 2003;17:2513-2516.

(220) Merz H, Houssiau FA, Orscheschek K et al. Interleukin-9 expression in human malignant lymphomas: unique association with Hodgkin's disease and large cell anaplastic lymphoma. Blood. 1991;78:1311-1317.

(221) Matsushita K, Arima N, Ohtsubo H et al. Frequent expression of interleukin-9 mRNA and infrequent involvement of interleukin-9 in proliferation of primary adult T-cell leukemia cells and HTLV-I infected T-cell lines. Leuk Res. 1997;21:211-216.

(222) Kubota S, Siomi H, Hatanaka M, Pomerantz RJ. Cis/trans-activation of the interleukin-9 receptor gene in an HTLV-I-transformed human lymphocytic cell. Oncogene. 1996;12:1441-1447.

(223) Arendse B, Van Snick J, Brombacher F. IL-9 is a susceptibility factor in Leishmania major infection by promoting detrimental Th2/type 2 responses. J Immunol. 2005;174:2205-2211.

(224) Renauld JC. New insights into the role of cytokines in asthma. J Clin Pathol. 2001;54:577-589.

(225) Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol. 1989;7:145-73.:145-173.

(226) Romagnani S. The role of lymphocytes in allergic disease. J Allergy Clin Immunol. 2000;105:399-408.

(227) Walker C, Bode E, Boer L et al. Allergic and nonallergic asthmatics have distinct patterns of T-cell activation and cytokine production in peripheral blood and bronchoalveolar lavage. Am Rev Respir Dis. 1992;146:109-115.

(228) Wills-Karp M. Immunologic basis of antigen-induced airway hyperresponsiveness. Annu Rev Immunol. 1999;17:255-81.:255-281.

(229) Bochner BS, Schleimer RP. The role of adhesion molecules in human eosinophil and basophil recruitment. J Allergy Clin Immunol. 1994;94:427-438.

(230) Zhu Z, Homer RJ, Wang Z et al. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. J Clin Invest. 1999;103:779-788.

(231) Postlethwaite AE, Holness MA, Katai H, Raghow R. Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. J Clin Invest. 1992;90:1479-1485.

(232) Geha RS, Jabara HH, Brodeur SR. The regulation of immunoglobulin E class-switch recombination. Nat Rev Immunol. 2003;3:721-732.

(233) Wang B, Rieger A, Kilgus O et al. Epidermal Langerhans cells from normal human skin bind monomeric IgE via Fc epsilon RI. J Exp Med. 1992;175:1353-1365.

(234) Maurer D, Fiebiger S, Ebner C et al. Peripheral blood dendritic cells express Fc epsilon RI as a complex composed of Fc epsilon RI alpha- and Fc epsilon RI gamma-chains and can use this receptor for IgE-mediated allergen presentation. J Immunol. 1996;157:607-616.

(235) Kita H, Kaneko M, Bartemes KR et al. Does IgE bind to and activate eosinophils from patients with allergy? J Immunol. 1999;162:6901-6911.

(236) Gounni AS, Lamkhioued B, Ochiai K et al. High-affinity IgE receptor on eosinophils is involved in defence against parasites. Nature. 1994;367:183-186.

(237) Joseph M, Gounni AS, Kusnierz JP et al. Expression and functions of the high-affinity IgE receptor on human platelets and megakaryocyte precursors. Eur J Immunol. 1997;27:2212-2218.

(238) Macglashan D, Jr. IgE and Fc{epsilon}RI regulation. Ann N Y Acad Sci. 2005;1050:73-88.

(239) Matz J, Williams J, Rosenwasser LJ, Borish LC. Granulocyte-macrophage colony-stimulating factor stimulates macrophages to respond to IgE via the low affinity IgE receptor (CD23). J Allergy Clin Immunol. 1994;93:650-657.

(240) Belleau JT, Gandhi RK, McPherson HM, Lew DB. Research upregulation of CD23 (FcepsilonRII) expression in human airway smooth muscle cells (huASMC) in response to IL-4, GM-CSF, and IL-4/GM-CSF. Clin Mol Allergy. 2005;3:6.

(241) Bruhns P, Fremont S, Daeron M. Regulation of allergy by Fc receptors. Curr Opin Immunol. 2005;17:662-669.

(242) Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. Annu Rev Immunol. 2005;23:127-159.

(243) Maruyama M, Lam KP, Rajewsky K. Memory B-cell persistence is independent of persisting immunizing antigen. Nature. 2000;407:636-642.

(244) Liu YJ, Joshua DE, Williams GT et al. Mechanism of antigen-driven selection in germinal centres. Nature. 1989;342:929-931.

(245) Suzuki N, Ueda Y, Sakane T. Differential mechanism for differentiation into immunoglobulin-secreting cells in human resting B lymphocyte subsets isolated on the basis of cell density. J Clin Invest. 1988;81:261-269.

(246) Bastien Y, Toledano BJ, Mehio N et al. Detection of functional platelet-activating factor receptors on human tonsillar B lymphocytes. J Immunol. 1999;162:5498-5505.

(247) Chow S, Patel H, Hedley DW. Measurement of MAP kinase activation by flow cytometry using phospho-specific antibodies to MEK and ERK: potential for

pharmacodynamic monitoring of signal transduction inhibitors. Cytometry. 2001;46:72-78.

(248) Jeannin P, Delneste Y, Lecoanet-Henchoz S, Gretener D, Bonnefoy JY. Interleukin-7 (IL-7) enhances class switching to IgE and IgG4 in the presence of T cells via IL-9 and sCD23. Blood. 1998;91:1355-1361.

(249) Scheeren FA, Naspetti M, Diehl S et al. STAT5 regulates the self-renewal capacity and differentiation of human memory B cells and controls Bcl-6 expression. Nat Immunol. 2005;6:303-313.

(250) Fornek JL, Tygrett LT, Waldschmidt TJ et al. Critical role for Stat3 in T-dependent terminal differentiation of IgG B cells. Blood. 2006;107:1085-1091.

(251) Demoulin JB, Van Snick J, Renauld JC. Interleukin-9 (IL-9) induces cell growth arrest associated with sustained signal transducer and activator of transcription activation in lymphoma cells overexpressing the IL-9 receptor. Cell Growth Differ. 2001;12:169-174.

(252) Dono M, Burgio VL, Tacchetti C et al. Subepithelial B cells in the human palatine tonsil. I. Morphologic, cytochemical and phenotypic characterization. Eur J Immunol. 1996;26:2035-2042.

(253) Billian G, Bella C, Mondiere P, Defrance T. Identification of a tonsil IgD+ B cell subset with phenotypical and functional characteristics of germinal center B cells. Eur J Immunol. 1996;26:1712-1719.

(254) Abe M, Tominaga K, Wakasa H. Phenotypic characterization of human B-lymphocyte subpopulations, particularly human CD5+ B-lymphocyte subpopulation within the mantle zones of secondary follicles. Leukemia. 1994;8:1039-1044.

(255) Dono M, Zupo S, Colombo M et al. The human marginal zone B cell. Ann N Y Acad Sci. 2003;987:117-124.

(256) Bohnhorst JO, Bjorgan MB, Thoen JE, Natvig JB, Thompson KM. Bm1-Bm5 classification of peripheral blood B cells reveals circulating germinal center founder cells in healthy individuals and disturbance in the B cell subpopulations in patients with primary Sjogren's syndrome. J Immunol. 2001;167:3610-3618.

(257) Agematsu K. Memory B cells and CD27. Histol Histopathol. 2000;15:573-576.

(258) Nouri-Aria KT, Pilette C, Jacobson MR, Watanabe H, Durham SR. IL-9 and c-Kit+ mast cells in allergic rhinitis during seasonal allergen exposure: effect of immunotherapy. J Allergy Clin Immunol. 2005;116:73-79.

(259) Sitkauskiene B, Radinger M, Bossios A et al. Airway allergen exposure stimulates bone marrow eosinophilia partly via IL-9. Respir Res. 2005;6:33.

(260) Steiniger B, Timphus EM, Jacob R, Barth PJ. CD27+ B cells in human lymphatic organs: re-evaluating the splenic marginal zone. Immunology. 2005;116:429-442.

(261) Kindler V, Zubler RH. Memory, but not naive, peripheral blood B lymphocytes differentiate into Ig-secreting cells after CD40 ligation and costimulation with IL-4 and the differentiation factors IL-2, IL-10, and IL-3. J Immunol. 1997;159:2085-2090.

(262) Arpin C, Banchereau J, Liu YJ. Memory B cells are biased towards terminal differentiation: a strategy that may prevent repertoire freezing. J Exp Med. 1997;186:931-940.

(263) Jego G, Bataille R, Pellat-Deceunynck C. Interleukin-6 is a growth factor for nonmalignant human plasmablasts. Blood. 2001;97:1817-1822.

(264) Burdin N, Van Kooten C, Galibert L et al. Endogenous IL-6 and IL-10 contribute to the differentiation of CD40-activated human B lymphocytes. J Immunol. 1995;154:2533-2544.

(265) Rousset F, Garcia E, Defrance T et al. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc Natl Acad Sci U S A. 1992;89:1890-1893.

(266) Turner CA, Jr., Mack DH, Davis MM. Blimp-1, a novel zinc finger-containing protein that can drive the maturation of B lymphocytes into immunoglobulin-secreting cells. Cell. 1994;77:297-306.

(267) Honjo T, Muramatsu M, Fagarasan S. AID: how does it aid antibody diversity? Immunity. 2004;20:659-668.

(268) Ye BH, Cattoretti G, Shen Q et al. The BCL-6 proto-oncogene controls germinal-centre formation and Th2-type inflammation. Nat Genet. 1997;16:161-170.

(269) Steinman RM. The dendritic cell system and its role in immunogenicity. Annu Rev Immunol. 1991;9:271-96.:271-296.

(270) Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. Nat Rev Immunol. 2002;2:151-161.

APPENDIX

1. SHOLARSHIPS

1.1 Montreal Children's Hospital Doctoral research award, 2000-2001

1.2 CIHR Doctoral research award, 2001-2004

2. ABSTRACTS

IL-9 Upregulates IgE Production in IL-4 Stimulated Human Follicular Germinal Center B Cells.

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Rationale: IL-9 is a pleiotropic cytokine secreted by activated Th2 cells. Interleukin-9 and the α-chain of the IL-9 receptor (IL-9Rα) have been shown to affect the differentiation pathway of different cell types including human T cells, eosinophils, and mast cells. In vivo and in vitro studies have linked IL-9 to increased immunoglobulin production, specifically IgE. We have recently characterized the expression of the IL-9R on human tonsillar B cells at different stages of their maturation. Our findings show for the first time that a functional IL-9Rα chain is expressed preferentially on human follicular germinal center B cells, suggesting a potential role for IL-9 in B cell differentiation and IgE production. ***Methods:*** In order to assess directly the effect of IL-9 on IgE production from human B cells. Freshly purified human tonsillar B cells were fractionated, using a discontinuous percoll density gradient, into three populations representing different stages of B lymphocyte maturation: low density (LD), medium density (MD), and high density (HD) cells. LD cells, which are germinal center cells expressing the highest level of IL-9Rα, were stimulated with CD40L and IL-9 in the presence or absence of IL-4 and IgE production was measured by ELISA. ***Results:*** Although IL-9 was unable to induce IgE secretion by itself, it potentiated IgE production mediated by IL-4. Thus, IL-9 exerts a synergistic effect with IL-4 to modulate IgE production. This is of great interest in the light of the linkage of IL-9 and the IL-9R genes to increased susceptibility to allergy and asthma. Further characterization of the phenotypic changes resulting from IL-9 stimulation leading to the upregulation of IgE production are underway.

Demonstration of Interleukin-9 Receptor Function in Human Germinal Center B Cells

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Rationale: IL-9 is a pleiotropic cytokine secreted by activated Th2 cells, which has been linked to bronchial hyper-responsiveness and asthma. We have shown that human tonsillar B cells express IL-9R predominantly in the germinal center (GC). ***Methods***: To ascertain the functionality of the IL-9R expressed on GC B cells, we activated low density (LD) B cells with IL-9 for 15 min, and assessed their protein phosphorylation status. Human tonsillar B cells were fractionated into low, medium and high density fractions, using discontinuous percoll density gradients. The low density fraction includes sIgD- GC B cells. ***Results***: Immunoblotting demonstrated an increase in total phosphotyrosine levels in LD cells stimulated with IL-9 compared to non stimulated cells or IL-9 treated total B cells. Because STAT-5 (Signal transducer and activator of transcription 5) belongs to the family of transcription factors which mediate the cellular response to a variety of cytokines including IL-9, we sought to determine STAT-5 phosphorylation status following IL-9 stimulation of GC cells. Using a phosphospecific antibody that detects the phosphorylated tyrosine residue associated with activated STAT-5 (Tyr 694), we analyzed expression of phosphorylated STAT-5 by flow cytometry. The results show an upregulation of STAT-5 phosphorylation following IL-9 stimulation specifically in LD cells and not in total B cells, MD or HD cells. ***Conclusions***: These results demonstrate for the first time the expression of a functional IL-9R on GC B cells. Further studies will be done to define the activation status of other described IL-9 induced STATs namely STAT-1 and 3.

Expression of Interleukin-9 Receptor Alpha on Germinal Center B Cells: Potential Role for Interleukin-9 in B Cell Maturation

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IL-9 is a pleiotropic cytokine secreted by activated Th2 cells. Recently, the human IL-9 receptor (IL-9R) gene locus and IL-9 have been linked to bronchial hyperresponsiveness and asthma. In vitro studies have pointed to a role for IL-9 in enhancing the release of IgE from B cells. Although IL-9 may play a role in B cell differentiation, the direct effect of IL-9 on B cell survival and differentiation has not been addressed. Furthermore, the expression of the IL-9R on B cells at different stages of their development has not been characterized. We therefore determined the expression of IL-9R using human tonsillar B cells. Human tonsillar B cells were fractionated, using a discontinuous percoll density gradient, into three populations representing different stages of B lymphocyte maturation: low density (recovered in the 30-50% Percoll fraction), which represents sIgD- germinal center B cells, medium density (50-60%), which are primarily centroblasts and centrocytes, and high density (> 60%) or more mature B cells. We have determined the expression of the IL-9Rα chain by immunocytochemistry, FACS analysis and immunohistochemistry. Immunocytochemistry was performed on freshly isolated purified human tonsillar B cells using a monoclonal antibody to the alpha chain of the IL-9R. This clearly demonstrated the presence of the IL-9Rα protein on tonsillar B cells. Using FACS analysis, unfractionated as well as fractionated B cells showed expression of IL-9Rα. However, IL-9Rα expression was predominantly higher in the low density or germinal center fraction of the B cells (38.7%), as compared to the medium density (21.78%), and high density fractions (16.22%). These findings prompted us to further investigate the precise localization of the IL-9Rα positive B cells within the germinal centers. For this purpose, immunohistochemistry was performed on sections of human tonsils. Using double staining with anti-IL-9Rα and anti-CD20, we found that CD20+ B cells within the secondary lymphoid follicles, as well as cells on the edge of these follicles displayed IL-9Rα. Analysis of CD38 and lgD expression on IL-9Rα

positive cells by three color flow cytometry revealed that IL-9Rα was expressed on naive follicular mantle zone B cells (CD38-IgD+) and germinal center cells (CD38+IgD+). Taken together, these results confirm that IL-9Rcα is principally expressed on naive B lymphocytes and early germinal center cells. These data show for the first time that the IL-9Rα chain is expressed on human follicular germinal center B cells, and suggest a potential role for IL-9 in B cell differentiation. Further studies will be done to define the effect of IL-9 on follicular B cells.We hypothesize that IL-9 is an early signal that could promote isotype switching and consequently affect IgE production.

A Comparative Study of the Effects of Intact and Fragmented Intravenous Immunoglobulin (IVIG) on In Vitro IgE Production by Human B Lymphocytes

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Background: Intravenous immunoglobulin (IVIG) has been employed extensively in the treatment of autoimmune and allergic disorders, which may be mediated by the overproduction of pathogenic antibodies. Numerous mechanisms have been proposed to explain its beneficial actions, yet none satisfactorily explain all clinical situations. We have previously demonstrated that IVIG inhibits IgE production in highly purified human tonsillar B cells stimulated with IL-4 and anti-CD40 antibody (anti-CD40). ***Objectives***: To further clarify the mechanism underlying the inhibition of cell proliferation and IgE synthesis by IVIG, we have investigated the effects of intact whole molecule IVIG as well as the F(ab')2 and Fc fragments of IVIG. ***Methods:*** Human tonsillar B-lymphocytes were purified by Ficoll-Hypaque density centrifugation and T cells were removed by E-rosetting. Cell proliferation was measured by [3H] thymidine incorporation and supernatant IgE was determined by ELISA. Cell phenotyping and cell cycle analysis was performed by flow cytometry. Fractionated IVIG was kindly provided by Centeon Corp. ***Results***: Whole molecular IVIG inhibited anti-CD40/IL-4 stimulated IgE production in a dose-dependent manner (I-20mg/ml). Similarly the Fc fragment also blocked the production of IgE in stimulated tonsillar B cells at equimolar doses ranging from 0.4-8mg/ml. The F(ab')2 portion significantly decreased the IgE production in anti-CD40/IL4 stimulated B lymphocytes (dose range 0.6-12 mglml); however, this fragment increased the baseline IgE level in the supematant of unstimulated B lymphocytes. Similar to intact IVIG, the inhibition of IgE production by fragmented IVIG was associated with inhibition of B cell proliferation, as both the F(ab')2 and Fc fragments significantly decreased [3H]-thymidine incorporation into B cells. The F(ab')2 fragment was more potent than the Fc fragment at the above equal molar concentrations. The inhibitory effects of all IVIG preparations were not due to the induction of apoptosis as neither the intact nor the fragmented IVIG had any significant effect on cell cycle or programmed cell death at the concentrations used. ***Conclusion***: These data show that both F(ab')2, and Fc portions

contribute to the effect of IVIG, indicating multiple mechanisms of IVIG immunomodulatory activity.

Expression of IL13 Receptor Alpha 1 in Human B Cells Committed to Produce IgE

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Rationale: Our previous studies have shown that human B lymphocytes, stimulated to synthesize IgE, produce IL-13 as an autocrine growth factor. Little is known about the expression of IL-13 receptor alpha 1 (IL-13Rα1) during the development of IgE secreting B cells. ***Methods***: Purified B cells were isolated from human tonsils and cultured with or without anti-CD40 antibody (anti-CD40, 1 μg/ml), recombinant human IL4 (400 /ml), IL13 neutralizing antibody (3 μg/ml). Cells were serially harvested during 14 day culture. IL-13Rα mRNA expression was evaluated by real time-PCR, and IL-13Rα1, IL-4Rα; and CD23 surface expression was examined by flow cytometry and laser scanning microscopy.***Results***: IL13Rα1 mRNA was expressed at very low levels prior to stimulation, but increased following addition of anti-CD40. Over a 14 day culture period, surface IL13Rα1 was detected at low levels (MFI 65.4 ± 29.8) in unstimulated B cells, but increased significantly at 1, 5, 10, and 14 days after anti-CD40/IL4 stimulation (MFI 249.6 ± 56.5 at day 10), $P<0.01$ for all time points). In contrast, IL4Rα was highly expressed in unstimulated B cells, and did not significantly increase after stimulation. CD23 expression increased following CD40/IL4 stimulation ($P<0.01$) and was co-expressed on cells that expressed IL-13Rα1. Anti-IL13 treatment had no effect on either IL13Rα1 or CD23 expression following stimulation. ***Conclusions***: IL13Rα1 and IL4Rα are not equally expressed in resting B lymphocytes. IL13Rα1 and CD23 are highly inducible following B cell activation. These data for the first time demonstrate the kinetics of IL-13Rα1 expression on human B cells.

B-Lymphocyte-Derived IL-4 Contributes to Th2 Differentiation of T Lymphocytes

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Background: IL-4 is the crucial cytokine that causes T helper lymphocytes to differentiate into Th2 cells. The cells that supply IL-4 for Th2 differentiation are not well defined. Data from several laboratories, as well as work that has been recently performed in our laboratory suggests that B cells can be a source of IL-4. Platelet-activating factor (PAF), a potent lipid mediator released during inflammatory and immune responses, has a wide range of physiologic actions. Previous work in our laboratory has detected functional PAF receptors on human tonsillar B cells, which upon stimulation with PAF induce the B lymphocytes to produce IL-4. PAF induced IL-4 mRNA expression is present on approximately 17% of CD20+ cells in tonsils, and IL-4 was detected in supernatants of tonsillar B cell cultures after 48-72 h. We wished to determine if B cell derived IL-4 was capable of directing Th2 differentiation. ***Methods***: B cells were stimulated by PAF (10^7 M) and cultured with mixed tonsillar T lymphocytes. The T lymphocytes were either unstimulated or cultured with anti-CD3 and anti-CD28 antibodies for 2 days before exposure to B cells. After 5 days of coculture, detection of intracellular interferon-γ (INF-γ) and IL-4 was assessed by flow cytometry gating on CD4+ T lymphocytes. ***Results***: The tonsils used in these experiments were obtained from random pediatric patients, and were subject to some variability. We observed 2 patterns of cytokine production by CD4+ T lymphocytes stimulated with anti-CD3/CD28 antibodies for 5 days. A number of specimens produced INF-γ with little IL-4, and an equal number produced primarily IL-4. In tonsils where the CD4+ population was predisposed to INF-γ production, coculture of PAF-stimulated B-cells with T lymphocytes for 5 days lead to abrogation of INF-γ synthesis (Control: 61% vs 11% IFN-γ secreting cells in T:B co-culture), suggesting that PAF-stimulated B cells inhibited INF-γ production by the T cells. In tonsils where the CD4+ population was predisposed to IL-4 production upon stimulation with anti-CD3/CD28 antibodies, co-culture of PAF-stimulated B with T lymphocytes increased the production of IL-4 (19.3% increase in IL-4 secreting cells after 5 days of co-culture compared to 9% in the control). ***Conclusion***:

These preliminary results suggest that IL-4 from human B cells may regulate the differentiation of Th0 cells. PAF stimulated B cells, because of their ability to produce IL-4, can provide both a negative and positive signal for Thl and Th2 cells.