IN VIVO T-B CELL INTERACTIONS AND CYTOKINE PRODUCTION IN THE MURINE SPLEEN

Cover: *In vivo* T-B cell interactions and cytokine production in the murine spleen. Spleen section of mouse 5 days after immunization with TNP-Ficoll. Brown: TNP-Ficoll localized in marginal zone and follicle; Red: TNP-specific antibody-forming B cells in periarteriolar lymphocyte sheath / around terminal arterioles and TNP-specific antibodies in the follicle; and violet: IFN-γ-producing T cells in close conjunction to TNP-specific antibody-forming B cells.

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IN VIVO T-B CEL INTERACTIES EN CYTOKINE PRODUKTIE IN DE MILT VAN DE MUIS

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Labor improbus omnia vincit
Vergilius (70 v.C. - 19 v.C.)

Ter nagedachtenis aan mijn vader

Aan mijn moeder

Aan Esther en Sebastiaan

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CHAPTER 1

GENERAL INTRODUCTION

1.1 IMMUNE CELLS AND CYTOKINES IN ANTIBODY RESPONSES

During life, man encounters a large variety of infectious agents such as bacteriae, fungi, viruses and parasites. The cytopathogenic effects of these micro-organisms may have detrimental consequences on body functions, and may eventually lead to severe damages or even the death of the infected host. The fact that most of the time these organisms do not get the opportunity to harm our body, is largely due to our immune system. The immune system consists of a specific and a non-specific part. The latter, also called innate immune system, is the primary defense against micro-organisms and consist of a.o. the skin, shedding of epithelial cells, cilia lining the trachea, mucus, acid in the stomach, cytolytic enzymes in secretions, phagocytes, acute phase proteins, etc.

The second line of defense is formed by the specific (adaptive) immune system, which can be divided into a cellular and a humoral part. Cellular immunity is primarily exerted by T cells, consisting of cytotoxic T cells, which kill virus-infected cells and tumor cells, or helper T cells which regulate the function of other cells via secreted cytokines.

In humoral immunity the immunoglobulin producing B cell plays a central role. B cells, derived from bone marrow, produce antibodies, proteins that are secreted in body-fluids and can bind to antigens with high specificity. Specificity of the antibody molecule resides in two identical antigen-binding sites located on the two tops of the Y-shaped molecule. Effector functions of antibodies include neutralization, complement activation, opsonization and antibody-dependent cell mediated cytotoxicity. Most of these effector functions are dependent on the structures on the tail of the Y-shaped molecule, the Fc-part, which varies between the (sub)classes of antibodies. Five distinct classes of immunoglobulin molecules (isotypes) are recognized in most higher mammals, namely IgM, IgD, IgG, IgA and IgE. These differ from each other in size, charge, amino acid composition and carbohydrate content. In the mouse, the IgG class is subdivided into four subclasses (isotypes), called IgG1, IgG2a, IgG2b and IgG3. The diversity of the structure of these subclasses determines the variety of functions of these effector molecules in immunity.

B cells differentiate in the bone marrow where they are equipped with antigen-specific receptors, membrane immunoglobulin, and subsequently migrate to the peripheral lymphoid tissue where they can differentiate into antibody-forming cells (AFC), provided that they encounter the right antigen in the appropriate microenvironment. The interaction of antigen with membrane immunoglobulin on B cells is the first phase of all antibody responses. Most antigens cannot activate B cells directly, and require the help of T cells and macrophages or

dendritic cells to generate an effective antibody response. Macrophages and dendritic cells are potent antigen-presenting cells and have a role in the initial activation and expansion of antigen-specific T cells. Upon activation, T cells express new surface antigens (a.o. gp39) and produce cytokines which enable them to activate and regulate B cell differentiation. The binding of a 39 kD protein (gp39) on activated T cells to the CD40 molecule on B cells provides the initial B cell activating signal, while the T helper cell derived cytokines regulate the differentiation of B cells into antibody-forming cells. Cytokines are glycosylated polypeptides, produced by a variety of cell types of both haematopoietic and non-haematopoietic origin. Originally, these proteins were called lymphokines, monokines, interleukins based on their source or their role in the communication between leucocytes. However, detailed investigations revealed that most of these polypeptides were produced by more than one cell and exerted their regulatory role on various cell types. Therefore, the term cytokines is now generally used. In general, cytokine-producing cells are paracrine cells, meaning that their regulatory activity is directed to target cells which are localized in close conjunction. Evidence accumulates which suggests that the T cell derived cytokines interleukin 2 (IL-2), IL-4, IL-5, IL-6 and interferon-γ (IFN-γ) have an important role in B cell proliferation, differentiation and isotype switching.

To facilitate and regulate the cell-cell interactions, immune cells are organized in tissues and organs called the lymphoid system. The primary lymphoid organs are the major sites of lymphopoiesis: the thymus produces T cells, and the fetal liver and bone marrow B cells. The secondary lymphoid organs include lymph nodes, spleen, bronchus associated lymphoid tissue and gut associated lymphoid tissue, which provide an environment for cells to develop an appropriate immune response against various antigens. The lymph nodes and the spleen can be seen as immunological filters of the lymph or blood, respectively. Experimentally administered antigen is processed depending on the route of entry, e.g. subcutaneous administration predominantly stimulates the draining lymph node, and intravenously injected antigen is processed mainly by the spleen.

As already stated, the local microenvironment in secondary lymphoid tissues is important in the regulation of antibody production, and consists of the extracellular matrix, non-lymphoid cells and lymphoid cells. The non-lymphoid cells can be classified into two major groups. The first group are macrophages and interdigitating cells and the second group are stromal cells, consisting of reticular cells, follicular dendritic cells and endothelial cells. The lymphoid cells, producing antibodies and/or cytokines, contribute to the microenvironment in an antigen-specific way.

All these locally active cells and factors have a steering role in antibody responses. Therefore, detailed knowledge of the microenvironment of lymphoid organs is required to improve our insight in the mechanisms involved into the generation and regulation of the antibody response.

1.2 AIM OF THE STUDY

The aim of the studies presented in this thesis was to investigate *in vivo* the immunological events taking place during the development of various types of antibody responses. Special emphasis was put on time-course, *in situ* localization and *in vivo* function of the different immunological events, e.g. antigen-presentation, T cell activation, cytokine-production, cell-cell interactions and antibody-formation in the spleen, as these processes are thought to have a decisive role in the development of *in vivo* antibody responses.

1.3 THE IN VIVO APPROACH

The responses of lymphocytes to antigens and pathogens have mainly been analyzed *in vitro*. However, whereas cells can interact relatively randomly in cell suspensions, peripheral lymphoid tissues like lymph nodes and spleen, are compartmentalized into distinct B and T cell zones. Cells are exposed to the influence of locally present immune cells, stromal cells, cytokines and the extracellular matrix. *In vitro* experiments necessarily disregard this complex organization of lymphoid organs. Still, they are powerful tools in the delineation of essential events during cell-cell interactions and may give an indication which cells and cytokines are active during immune responses. The *in vitro* approach is valuable for the investigation of the actual effects of specific cytokines on specific cells. As a consequence, lymphoid cell functions and interactions seen *in vitro* reflect the full potential and repertoire of such cells, but the events actually occurring *in vivo* are dictated and restricted by the lymphoid microenvironment. Therefore, we developed *in vivo* techniques and applied these in *in vivo* models.

In vivo research is roughly performed in two ways. One approach is the utilization of *in situ* techniques, such as immunohistochemistry and *in situ* hybridization, which allow the *in vivo* study of the localization, activity and interactions of various cells and cytokines in relation to their direct microenvironment in all types of tissue at any given moment. The second *in vivo* approach is the use of modulating agents, e.g. cytokines, neutralizing-, blocking-, immune

cell-depleting antibodies, complement-, macrophage-depletion etc., in intact animals, which effects can be examined on the level of body fluids (serum) and/or tissue sections. The combination of these techniques are powerful tools to delineate the role of cytokines and cell-cell interactions during *in vivo* antibody responses. In the studies presented in this thesis, both immunohistochemical and *in vivo* modulating techniques are used to investigate the immunological events in mice mounting antibody responses against various antigens.

1.4 INTRODUCTION TO THE CHAPTERS

The aim of this thesis was to investigate *in vivo* the immunological events taking place during the development of various types of antibody responses. The following chapters describe the time-course and localization of the distinct immunological events, such as antigen-handling, T cell activation, cytokine-production and antibody-formation in the spleen during thymus independent type 2 (TI-2) and thymus dependent (TD) antibody responses.

Chapter 2 describes the complex organization of the spleen, consisting of specialized cell types localized in distinct splenic compartments. In view of all the cell-cell interactions that can occur in the spleen, the migration patterns of T cells, B cells and dendritic cells are discussed. The experimentally used antigens are characterized. Furthermore, the vast amount of *in vitro* data dealing with antigen-presentation, cell-cell interactions, T and B cell activation, and the immunoregulatory role of cytokines is evaluated. Finally, the experimental questions addressed in this thesis are presented.

In chapter 3 the localization of TI-2 antigens in lymphoid tissue is investigated. Previous reports demonstrated that fluoresceinated TI-2 antigens were taken up by marginal zone macrophages, suggesting that these cells played a role in the presentation of TI-2 antigens. However, *in vivo* elimination or blocking of marginal zone macrophages did not affect the antibody response against TI-2 antigens, indicating that other cells are involved in the induction of TI-2 antibody responses. Therefore, a new and more sensitive technique was developed for the detection of administered haptenated antigens. Chapter 3 describes a TNP-specific monoclonal antibody (mAb) conjugated to β-galactosidase, which is used for the detection of trinitrophenylated antigens. This highly sensitive approach enables the detection of TI-2 antigens in the marginal zone and also in the follicles of the spleen, a hitherto undescribed phenomenon. The localization of administered TI-2 antigens in the spleen and

lymph nodes is described and is compared with the localization of particulate TI-1, soluble TI-1 and soluble TD antigens. Furthermore, the follicular localization of TNP-Ficoll is characterized and its role in humoral immunity is investigated. The implications of these new findings with regard to the putative role of marginal zone macrophages in TI-2 antibody responses is discussed.

Chapter 4 describes the development of a method for the immunohistochemical detection of IFN-γ-producing cells (IFN-γ-PC) in murine lymphoid tissue. Immunization with Bacille Calmette Guèrin (BCG) is used as model immune response, because *in vitro* experiments indicated that such a protocol results in high levels of IFN-γ production. For the detection of IFN-γ-PC, spleen sections of BCG immunized mice are incubated with an IFN-γ-specific mAb conjugated to alkaline phosphatase. For the characterization of IFN-γ-PC spleen sections are simultaneously incubated with IFN-γ-specific immuno-conjugate and mAb specific for membrane markers (CD4, CD8, Asialo-GM1), followed by a secondary conjugate. Furthermore, it is investigated whether the local splenic IFN-γ production is also detectable in the serum, and if systemic application of a T cell mitogen (Concanavalin A) increases the local and systemic IFN-γ production.

Chapter 5 investigates *in vivo* the activity of T cells and IFN-γ in TI-2 antibody responses. The *in vivo* development and localization of IFN-γ-PC is investigated during antibody responses against various TI-2 antigens. An IFN-γ-specific ELISPOT assay is used to substantiate the *in vivo* results. Furthermore, IFN-γ serum levels are measured. Double staining techniques are applied to determine whether IFN-γ is produced by $CD4^+$, $CD8^+$ or NK cells. The localization of IFN-γ-PC and TNP-specific antibody forming cells (TNP-AFC) is examined. Since *in vitro* experiments suggested that IFN-γ has a regulatory role in TI-2 antibody responses, it is investigated whether IFN-γ-PC and TNP-AFC are co-localizing in the spleen.

In chapter 6 the putative defect of peripheral lymph nodes to mount an antibody response against TI-2 antigens is investigated. Therefore, the TNP-specific antibody response is determined in draining popliteal lymph nodes and spleens of mice after s.c. immunization with TNP-Ficoll. Analysis is performed by means of immunohistochemical techniques and ELISPOT assays. Furthermore, the cytokine-production in the (high responding) spleen and the (low responding) lymph node are compared after immunization with TNP-Ficoll. To increase the TNP-specific antibody response in lymph nodes, mice are immunized with TNP-Ficoll

supplemented with *Brucella abortus*, a particulate TI-1 antigen. The effect of this immunization protocol on the cytokine-production in popliteal lymph nodes is assessed. Evidence is provided that the local microenvironment of lymphoid tissue is important for the maturation of activated B cells during TI-2 antibody responses. Finally, by intraperitoneal implantàtion of alginate encapsulated IL-4 and/or IL-5-producing cell lines it is tried to overcome *in vivo* the maturation defect of TNP-Ficoll activated lymph node B cells.

In vitro studies revealed that upon activation (by ConA or anti-CD3 mAb) T helper cells produce cytokines and express a 39 kD protein. Binding of gp39 to the CD40 molecule on B cells was found to result in the activation of B cells, while added cytokines regulated the B cell differentiation into AFC. *In chapter 7* we tried to validate these *in vitro* observations in *in vivo* model antibody responses. The detection and activity of $gp39^+$ cells and cytokine-PC in the antibody response against a TD antigen (KLH) and a TI-2 antigen (TNP-Ficoll) is described. $Gp39^+$ cells are detected by using a specific mAb or a fusion protein of the receptor of gp39, CD40-IgG1. Double staining techniques are used to determine whether gp39 is expressed by $CD4^+$ cells *in vivo*, as was demonstrated *in vitro*. The cytokine-PC are detected with specific mAb conjugated to alkaline phosphatase, horse radish peroxidase or β-galactosidase. It is investigated whether $gp39^+$ cells produce cytokines. Furthermore, the localization of $gp39^+$ cells and cytokine-PC in the spleen is determined. It is tried to visualize the *in vivo* interactions between antigen-specific AFC and cytokine-PC or $gp39^+$ cells. Finally, we investigated the *in vivo* function of $gp39^+$ cells in germinal center formation and in antibody responses against TI-2 and TD antigens.

The injection of goat anti-mouse IgD antibodies results in high increases of serum IgG1 and IgE. The high degree of T cell activation and cytokine-production, which is critical to the observed large increases of serum IgG1 and IgE, makes this model ideal for analysis of *in vivo* TD antibody responses. *In chapter 8* the immunological events, such as antigen-localization, gp39 expression, cytokine-production and antibody formation, are described after injection of rabbit anti-mouse IgD antibodies (RαIgD). The localization of injected RαIgD is monitored in the spleen and related to the *in vivo* localization and development of cytokine-PC and $gp39^+$ cells. Because IL-4 and IFN-γ are suggested to be important for the regulation of IgE production, the localization, development and frequencies of IL-4-PC and IFN-γ-PC are investigated. Furthermore, the cytokine profile (IL-2, IL-4 and IFN-γ) of cytokine-PC is assessed. The localization and kinetics of development of cytokine-PC and $gp39^+$ cells is

related to that of activated B cells and isotype-switched AFC. Finally, the functional role of gp39 is assessed in the (non-)specific antibody response observed after injection of RαIgD.

In chapter 9 the main points emerging from the experimental studies are discussed. These results will be related to the *in vitro* and *in vivo* data dealing with antigen-presentation, localization/migration of immune cells, cell-cell interactions and the immunoregulatory role of cytokines, as discussed in chapter 2. Finally, we try to bring order to the sequence of events that occur in the spleen after antigenic challenge, by presenting *in vivo* models for the cell-cell interactions that occur in the spleen during TD and TI-2 antibody responses.

CHAPTER 2

Immune cells in the spleen: localization, migration and cell-cell interactions

A great part of this chapter has been published in:

Immunological functions and *in vivo* cell-cell interactions of T lymphocytes in the spleen

Alfons J.M. Van den Eertwegh, Wim J.A. Boersma, and Eric Claassen
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Immunological Functions and *In Vivo* Cell-Cell Interactions of T Cells in the Spleen

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ABSTRACT: The spleen is an important lymphoid organ, involved in immune responses against all types of antigen that appear in the circulation. Its complex anatomical organization, with distinct compartments containing specialized cell types, provides a microenvironment which allows different cell-cell interactions and determines the direction of developing immune responses. In this review we evaluate the vast amount of *in vitro* data dealing with antigen presentation, cell-cell interactions, T and B cell activation, and the immunoregulatory role of cytokines, as suggested to be involved in immune responses. As a basis for understanding of *in vivo* processes, these *in vitro* data will be related to discrete phenomena of *in vivo* immune responses, such as antigen localization/trapping, cell migration patterns of immunocompetent cells, cytokine production, and antibody formation in the different compartments of the spleen. Finally, we try to bring order to the sequence of events that occur in the spleen after antigenic challenge by presenting an *in vivo* model for T cell dependent and T cell independent immune responses.

KEY WORDS: T cells, B cells, accessory cells, antibody-forming cells, cytokines, antigen, localization, migration, cell-cell interactions.

I. INTRODUCTION*

Because of its central position in the bloodstream and the large amount of migrating lymphocytes, the spleen plays a central role in the primary defense against bloodstream infections. Two major critical functions of the spleen can be recognized: it serves as a large phagocytic filter and it is a major antibody-producing organ.[15] Although the liver appears to remove the majority of well-opsonized bacteria from the bloodstream, the spleen has a principal function in the nonimmune host because of its ability to sequester bacteria that are not well opsonized. Although the spleen participates significantly in host defense mechanisms, it is not essential to life. Nevertheless, its removal increases the risk of overwhelming infections by bacteria with polysaccharide capsules, e.g., *Streptococcus pneumoniae*, *Neisseria meningitidis*, or *Haemophilus influenza*.[4,5,35,36]

The spleen plays a central role in the antibody response both to intravenous antigens and potentially to polysaccharides, regardless of the route of inoculation. After primary i.v. immunization, the spleen is, for most antigens, the major site of antibody production.[114–116] Since eight times more lymphocytes recirculate via the spleen than via all lymph nodes together,[170–172] it is most likely that the entire antigen-specific B/T cell repertoire is available in the spleen. The complex anatomical organization of the spleen, with distinct compartments containing specialized cell types, provides a unique microenvironment allowing cell-cell interactions which are essential for the initiation and continuation of various immune responses.[38,224]

* A list of abbreviations used throughout the text appears after the acknowledgments section and before the reference list.

The approaches to increase our knowledge about immune responses can be divided in roughly two ways, *in vitro* and *in vivo*. *In vitro* experiments are tools for the determination of which individual factors and cells may be required for cell activation, proliferation, and differentiation. However, they necessarily disregard the complex organization of lymphoid organs and the obtained results reflect the full potential of factors and cells *ex vivo*. *In vivo* experiments, on the contrary, are tools to gain insight into the lymphoid microenvironment, which is determined by the localization and timing of activity of immune cells in the specific lymphoid compartments. Thus, it is reasonable to assume that a combination of both approaches would markedly increase our knowledge of immune responses.

In this review we show the potential of such a combined approach. We discuss the architecture of the spleen and the major cell types present. Special emphasis is placed on the localization of sessile and mobile immune cells within the specific splenic compartments, since the microenvironment dictates which cell-cell interactions are allowed and which are impossible. Furthermore, we discuss both *in vitro* and *in vivo* data dealing with the sequence of events during a variety of immune responses with respect to antigen routing/handling, cell-cell interactions, cytokine production, and antibody production. Mechanisms leading to T cell activation and the regulatory role of T cells in immune responses receive particular attention.

II. ANATOMY AND FUNCTIONAL ARCHITECTURE OF THE SPLEEN

Morphologically, the spleen can be divided into red and white pulp (Figure 1). The red pulp consists of erythrocytes and nucleated cells like lymphocytes, megakaryocytes, and macrophages.[217,224] In the white pulp three compartments can be distinguished: the periarteriolar lymphocyte sheath (PALS); the follicles; and the marginal zone. The PALS surrounds the central artery and the inner part consists mainly of T cells. The outer part of the PALS is populated by T cells, B cells, and macrophages. The follicles are globular structures attached to the PALS, and are populated mainly by B cells. The follicle consists of a mantle zone, the lymphocyte corona, with small and medium-sized lymphocytes and a follicle center, the germinal center,[81] with large lymphocytes. The marginal zone surrounds PALS and follicles and contains predominantly B cells, macrophages, and relatively few T cells.

Blood enters the spleen at the hilus through the splenic artery, which ramifies into trabecular arteries (Figure 1). Small arterial vessels leave the trabeculae as so-called center arterioles, which become gradually surrounded by lymphatic tissue called the PALS. Small terminal arterioles traverse the white pulp and open into the red pulp, where they are surrounded by a small sheath of lymphoid cells. Blood flows here in small channels, formed by reticular cells, and is subsequently collected by venous sinuses. Another part of the terminal arterioles opens into the marginal zone where, due to the reduced flow, the first cell-cell interactions between the immunocompetent cells like T cells, B cells, and macrophages might occur. A third type of terminal arterioles shunts the arterial blood flow directly through arterioles into the efferent venules. This latter type forms the closed circulation of the spleen and probably has relatively low importance with respect to immune function.

The splenic architecture is ideal for two of the principal immunological functions of the spleen: phagocytosis and antibody production. The large volume of blood which passes the macrophages of the spleen allows it to filter soluble or particulate foreign material from the bloodstream.[39,181] Furthermore, the close proximity of large numbers of lymphocytes allows efficient initiation and expansion of the specific immune response to this filtered material.

III. LOCALIZATION OF IMMUNE CELLS IN THE SPLEEN

A. T Cells

T cells differentiate and develop in the thymus and subsequently home into specific parts of peripheral lymphoid organs, the so-called thymus-dependent areas.[87] Flow cytofluorometric analysis of splenic T cell subpopulations of vari-

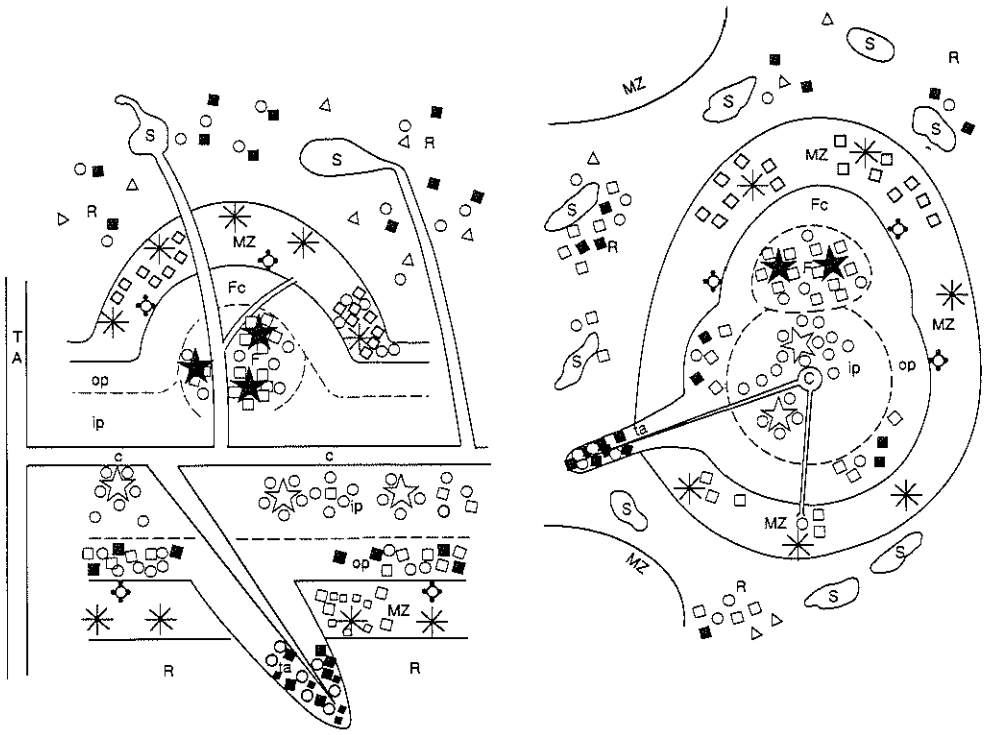


FIGURE 1. Schematic representation of the localization of immune cells in the spleen. C, central arteriole; ip, inner PALS; F, follicle; Fc, follicle corona; MZ, marginal zone; op, outer PALS; R, red pulp; S, sinus; TA, trabecular artery; ta, lymphocyte sheath around terminal arteriole. (□) B Cell; (■) B cell with high IgM expression; (■) antibody-forming B cell; (○) T cell; (★) FDC; (☆) IDC; (✳) marginal zone macrophage; (◇) marginal metallophilic macrophage; (△) red pulp macrophage.

ous strains of mice demonstrates that ±26% of the nucleated cells are $CD4^+$ cells and 8% $CD8^+$ cells.[68,221] Studies on the localization of these T cell subpopulations reveal that $CD4^+$ and $CD8^+$ cells are mainly localized in the PALS (Figure 2).[217,221,222] Within the PALS, distribution of the two T cell subsets was found to be random with no special organization (Figure 2b, c). In this compartment, the majority of T cells are $CD4^+$ (Figure 2b). In B cell areas like follicles, $CD4^+$ cells are found, but in much lower frequencies than in the PALS.[182] A role for these cells in the activation of B memory cells which gives rise to the local plasma-cell reaction in the germinal center has been suggested.[135,159] Virtually no $CD8^+$ cells are found in the follicles (Figure 2c), although in some pathological conditions high numbers can be found. Both subpopulations of $CD4^+$ and $CD8^+$ cells can be found scattered throughout the marginal zone. This is also the case in the red pulp where a substantial number of T cells can be found. It is not clear whether these red pulp T cells are actively involved in various local immune functions or merely migrating through the spleen. It has been suggested, on the basis of fluorescence intensity of Thy-1 staining, that T cells in the marginal zone and red pulp represent an immature population as compared to the T lymphocytes in the PALS.[221]

B. B Cells

The B lymphocytes in the spleen are mainly found in three compartments, i.e., the marginal

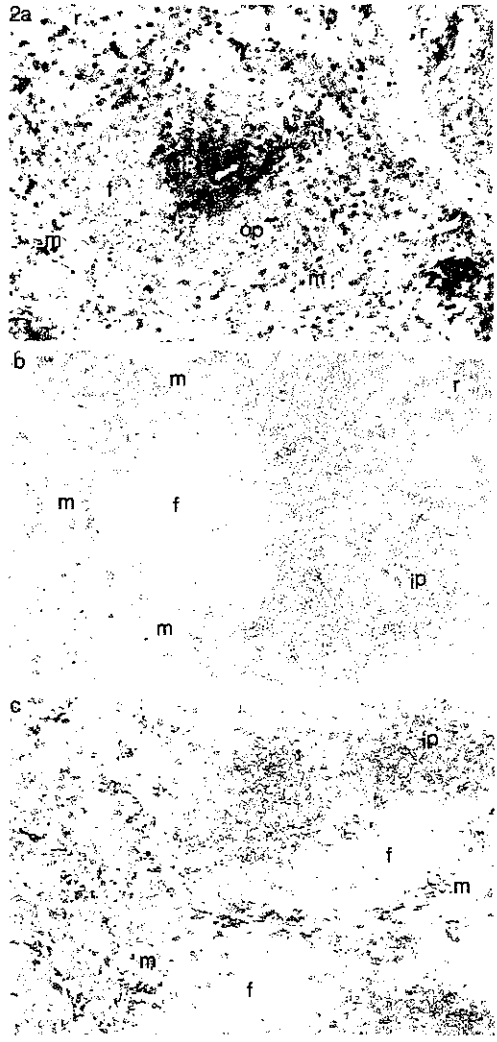


FIGURE 2. Localization of B cells, $CD4^+$, and $CD8^+$ T cells in the spleen. (a) T lymphocytes (Thy1.2, dark membrane staining) and IgM-positive B cells (light membrane staining); almost all cells in the inner part of the PALS (iP) are T cells. In the outer PALS (oP), follicle (f), marginal zone (m), and red pulp (r), relatively few T cells cells are present; almost all cells in follicle (f) and marginal zone (m) are B cells. (b) $CD4^+$ cells, recognized by monoclonal MT4; the majority of the cells in the inner PALS (iP) are $CD4^+$ cells, and relatively few cells in follicle (f), marginal zone (m), and red pulp (r) are $CD4^+$. (c) $CD8^+$ cells, recognized by monoclonal Lyt2+; inner PALS (iP) is populated by the majority of $CD8^+$ cells, and relatively few $CD8^+$ cells are found in marginal zone (m) and red pulp. Hardly any $CD8^+$ cells are found in the follicles (f).

zone, the follicles, and the outer PALS. The typical medium-sized B lymphocytes in the marginal zone have a strong membrane staining for IgM, whereas the small follicular lymphocytes show a moderate membrane staining for IgM (Plate 1b*).[51] Gray et al.[78] demonstrated that marginal zone B cells exhibit a predominant $\mu+\delta-$ (or weak positive) surface phenotype, while follicular B cells mainly express both μ and δ on their surface (Plate 1a, b). The percentage of IgM^+ nucleated cells in the spleen is strain dependent and varies between 40 and 60%.[68]

In both rats and man, B cells with the phenotype of the marginal zone cells comprise about one third of the splenic B cells,[78] whereas in mice only 15% of the splenic B cells have this phenotype.[3] The marginal zone B cells are sessile cells[125] unless they are activated by antigen. After LPS injection, a dramatic decrease in marginal zone B cells and an increase in IgM^+IgD^- (marginal zone phenotype) in the splenic follicles was observed, suggesting that after activation by antigens, marginal zone B cells migrate into the follicles of the spleen.[86] Marginal zone B cells express interleukin (IL)-2[95] and complement receptors[83] and are therefore well equipped for mounting humoral immune responses.[136,137,218]

Primary nonantigen-stimulated follicles mainly consist of small resting IgM^+IgD^+ B cells. Within 4 to 8 days of antigen administration secondary follicles call "germinal centers" are formed, which are associated with an exponential growth of B cell blasts.[124] Recently, Liu et al. suggested a role for germinal centers in antigen-driven B cell proliferation, somatic mutation, and memory cell and plasma cell development during TD immune responses.[135]

After activation, B cell proliferate or develop into antibody-forming cells. We demonstrated that antigen-specific antibody-forming cells which develop during primary thymus-dependent (TD) or thymus-independent (TI) immune responses are predominantly found in the outer PALS and in the sheaths of lymphoid tissue surrounding the terminal arterioles in the spleen.[33,34,40,223] Only a relatively low number of antigen-specific antibody-forming cells are localized in the red pulp, representing the antibody-forming cells which leave the spleen.

* Plate 1 follows page 348.

C. Macrophages

In the spleen, three major groups of acid phosphatase-positive macrophages can be distinguished (Plate 1c). When functional and phenotypic differences are taken into account, over 11 subsets can be distinguished.[39] The most studied subsets are marginal zone macrophages, marginal metallophilics, and red pulp macrophages. Marginal zone macrophages have been identified as a distinct subset of spleen macrophages[97] because they almost exclusively take up neutral polysaccharides[98,141,218] (Plate 1c, e, Figure 6). In the mouse, marginal zone macrophages can be specifically stained by monoclonal antibody ERTR9[52] (Plate 1f, g) and appear as large cells with extensive cell processes. They are positioned throughout the marginal zone, from the marginal sinus to the border area with the red pulp. They are highly phagocytic and do not express major histocompatibility complex (MHC) class II antigens.[97] Snook[191] described a rim of cells blackened by silver impregnation at the periphery of the white pulp along the inner border of the marginal zone and called these cells acid phosphatase-positive macrophages, marginal metallophils.[191] Marginal metallophils in mice show very strong nonspecific esterase activity and can be specifically stained by the monoclonal antibody MOMA-1[121] (Plate 1f, g). Their function is still unclear, but some studies suggest a role in antigen processing by these cells in particular immune responses.[122]

D. Follicular Dendritic Cells (FDC)

Weakly acid phosphatase-positive, nonphagocytic cells are also found in the follicles of the spleen. These cells are linked with the presence of immune complexes and complement-opsonized antigen (bacteria/viruses/model antigens) (Plate 1c, e, Figure 6).[112,118,164–167,218] FDC with their long cell processes, which ramify extensively among the B cells localized in the follicle, are responsible for retention of these complexes and can be recognized by a recently developed monoclonal antibody.[236] Immune-complex trapping is thought to play a role in the induction, maintenance, regulation, and memory of humoral immune responses.[112,118,135,218]

E. Interdigitating Cells (IDC)

IDC were first described in the T cell area of lymph nodes[227] and were also found in the main T cell area of the spleen, the inner PALS,[226] IDC are recognized by the antibodies NLDC-145,[120] MIDC-8,[18] or M1-8.[138] They constitutively express MHC class II molecules, in contrast to macrophages where class II expression is an inducible phenomenon.[194] *In vitro* experiments have suggested that IDC are essential for the induction of primary immune responses.[100,101] Because of their specific localization surrounding the central arterioles, a role for IDC in the homing of T cells has also been postulated.[96] However, migration studies with labeled dendritic cells (DC) revealed that DC did not home to the splenic PALS of nude mice unless they were reconstituted with T cells,[127] suggesting that migration of DC was regulated by T cells.

F. Natural Killer Cells (NK Cells)

NK cells form a subpopulation of non-T cell, CD3-negative, large granular lymphocytes which are capable of lysing neoplastic or virally infected target cells, as well as certain microorganisms without previous sensitization or MHC restriction. Information regarding the *in situ* localization of NK cells in the spleen is rather limited.[211] Recently, Brink et al. described the localization of NK cells in the spleens of rats, using a NK specific monoclonal antibody (designated 3.2.3.).[20] They observed that most NK cells were localized, sometimes in aggregates, in the red pulp (±12% of all nucleated cells in the red pulp), while relatively few were noted in the white pulp (±0.2%). Fewer (as compared to the frequency in the red pulp) but still significant numbers of NK cells were detected in the marginal zone (±2%). The localization pattern of NK cells in the rat spleen, as described by Van den Brink, is in agreement with our observations in murine spleens, after staining for asialo-GM_1, a marker which is highly expressed on NK cells.[216]

G. MHC Class II Distribution in the Spleen

MHC class II (Ia) antigens are involved in cellular interactions in the immune system, most notably in the presentation of antigens to T cells by antigen-presenting cells (APC). Therefore, *in situ* expression of Ia antigens may indicate in which compartments of the spleen antigen presentation occurs. Several groups investigated the Ia expression in spleens of rats and mice.[13,150,188] A strong staining in the inner PALS was demonstrated and most likely represents the MHC class II expression on IDC.[194] Since the majority of cells in the inner PALS are T cells, these historical data are very suggestive for antigen presentation and T cell activation in this area. The follicle was also found to be a compartment with an intense Ia staining.[13,150,188] Because of the close contact between B cells and FDC in the follicle, it is difficult to establish to which individual cells the observed Ia expression belong. Gray et al.[82] investigated mouse chimeras in which FDC were bearing $H\text{-}2^d$ of the host and B cells expressed $H\text{-}2^k$ from the donor, providing evidence that in the follicles B cells were the principal cells with high Ia expression. Like follicular B cells, the marginal zone B cells showed a high expression of Ia, suggesting that these cells are also important APC in the spleen. In contrast to marginal zone macrophages, the red pulp macrophages expressed MHC class II antigens, indicating that these macrophages may have different functions in immune responses.

All these studies were performed in nonimmunized animals. In some studies the effect was investigated of IFN-γ injection on the Ia expression of cells in different organs.[150,188] However, to our knowledge, no studies describe Ia expression in the spleen during immune responses against antigens, bacteria, viruses, or other organisms. Such studies could provide valuable information about which cells and compartments are involved in antigen presentation and in the initiation of immune responses.

IV. MIGRATION PATTERNS OF T, B, AND DENDRITIC CELLS IN THE SPLEEN

The continuous recirculation of lymphocytes is the basis for their contact with antigens at all sites of the body and for interaction with other immune cells in the appropriate microenvironment of lymphoid organs. Therefore, knowledge of the routing of lymphocyte subsets in lymphoid

organs is important for the understanding of immune responses (Figures 3 to 5).

A. Migration of T Cells in the Spleen

Lymphocyte migration through the spleen was studied by autoradiography after the injection of *in vitro* radioactively labeled thoracic duct lymphocytes.[158,222] These experiments suggested that T cells entered the spleen via the marginal zone. Subsequently, T cells were found to leave this area rapidly and concentrate around the terminal arterioles and at the periphery of the PALS. After 1 h, T cells were already penetrating towards the central region of the PALS. At later stages (3 to 6 h), T cells were still present in large numbers in the central areas of the PALS. In these migration studies, T cells were not found to enter the follicles in the first 48 h.

Willführ et al. labeled thoracic duct lymphocytes with fluorescein isothiocyanate (FITC) and detected these cells after injection, using an antibody against FITC.[232] Double staining for FITC and surface markers enabled the investigation of the migration pathways of the different lymphocyte subsets. They showed that 15 min after injection half of the immigrant cells were found in compartments other than the marginal zone, especially in the red pulp, indicating that the marginal zone is a main entry site but not the only place where lymphocytes can enter the spleen (Figures 3 to 5). T Lymphocytes, most notably $CD4^+$ cells, enter the spleen through two compartments (red pulp and marginal zone) and the majority of these cells are found in only one compartment, the PALS, after 24 h (Figure 3). In contrast to Van Ewijk and Nieuwenhuis,[222] Willführ and coworkers[232] demonstrated that both T cell subsets ($CD4^+$ and $CD8^+$) migrated into the follicles. In addition, a significantly higher percentage of $CD4^+$ than $CD8^+$ cells immigrated into the PALS, whereas $CD8^+$ cells localized in higher frequencies into the marginal zone and red pulp than $CD4^+$ lymphocytes (Figure 4). From 6 h on after injection of thoracic duct lymphocytes, the PALS contained more immigrant T and B lymphocytes than any other single organ of the body, showing the important role of the spleen in both B and T lymphocyte recirculation.[232] Michie and Rouse investigated T lymphocyte homing in lymph nodes, by using the Thy-1 alloantigen as a T cell marker.[145] They observed a higher amount of

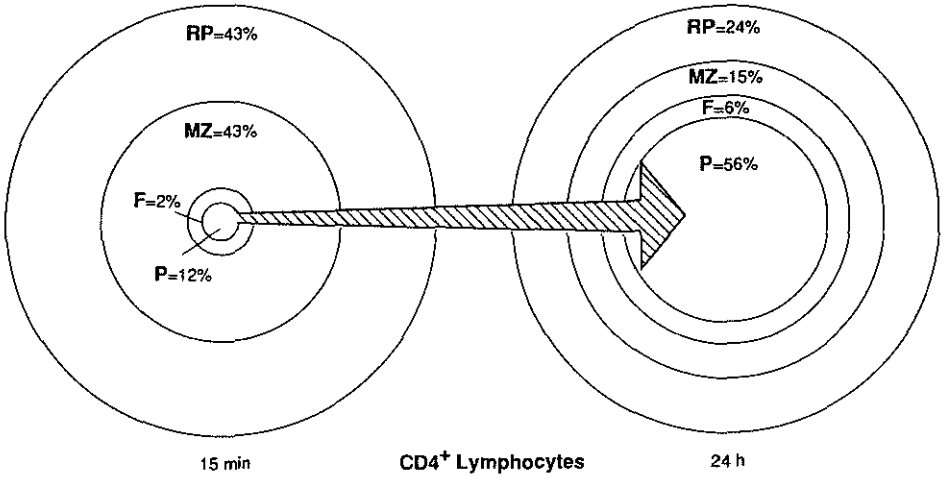


FIGURE 3. Distribution of $CD4^+$ cells in the different compartments of the spleen 15 min and 24 h after injection. RP, red pulp; MZ, marginal zone; F, follicle; P, PALS. Numbers represent the percentage of $CD4^+$ cells localized in specific compartments of the spleen at 15 min or 24 h after injection. Arrow indicates the increase of $CD4^+$ cells in the PALS. (The drawing is based on data of Willführ, K. U. et al., *Eur. J. Immunol.*, 20, 903, 1990.)

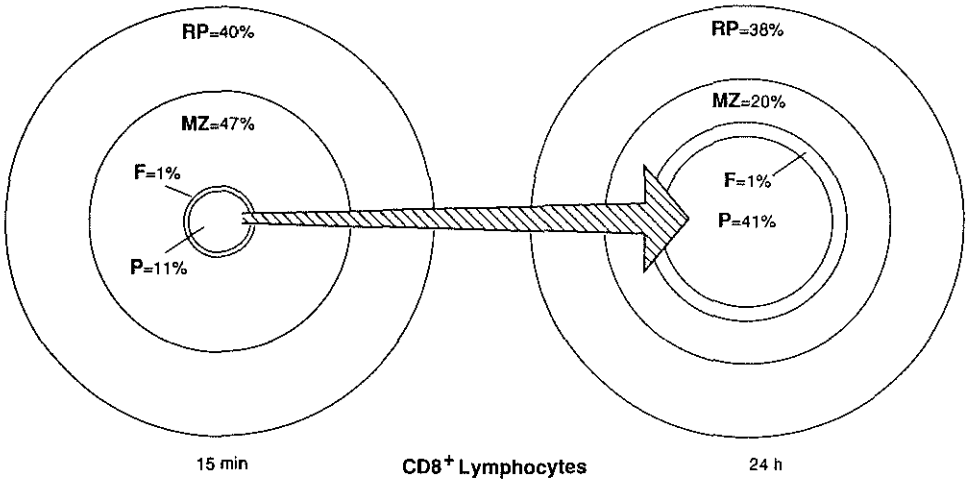


FIGURE 4. Distribution of $CD8^+$ cells in the different compartments of the spleen 15 min and 24 h after injection. RP, red pulp; MZ, marginal zone; F, follicle; P, PALS. Numbers represent the percentage of $CD8^+$ cells localized in specific compartment of the spleen at 15 min or 24 h after injection. Arrow indicates the increase of $CD8^+$ cells in the PALS. (The drawing is based on data of Willführ, K. U. et al., *Eur. J. Immunol.*, 20, 903, 1990.)

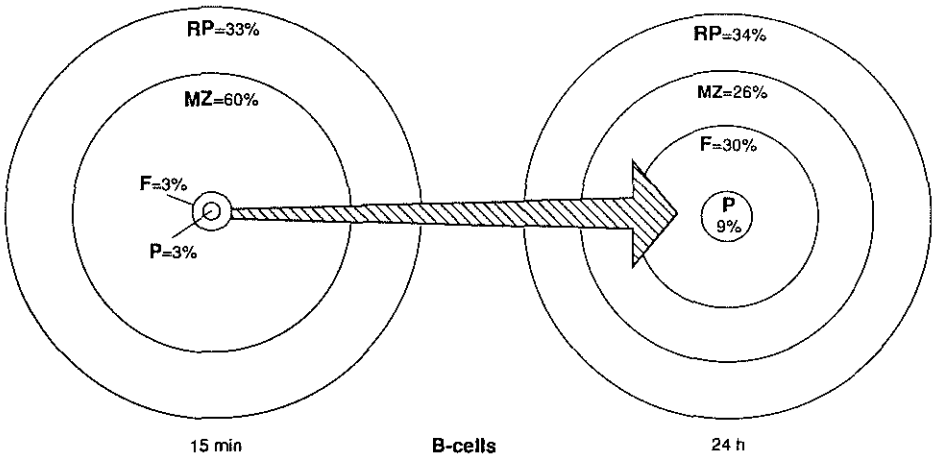


FIGURE 5. Distribution of B cells in the different compartments of the spleen 15 min and 24 h after injection. RP, red pulp; MZ, marginal zone; F, follicle; P, PALS. Numbers represent the percentage of B cells localized in specific compartments of the spleen at 15 min or 24 h after injection. Arrow indicates the increase of B cells in follicles. (The drawing is based on data of Willführ, K. U. et al., *Eur. J. Immunol.*, 20, 903, 1990.)

keyhole limpet hemocyanin (KLH)-primed Thy-1 positive cells in the follicles of lymph nodes draining the site of antigen-challenge than in follicles of lymph nodes in the same mouse draining the site of challenge with control antigen. This experiment suggested that antigen-specific T cells preferentially localize in antigen-primed follicles and may activate antigen-specific B memory cells in follicles during secondary immune responses. Furthermore, it shows the differ-

ences in migration patterns of T cells in immunized mice compared to nontreated mice, which is highly relevant for the interpretation of the migration studies of Van Ewijk and Nieuwenhuis,[222] and Willführ et al.[232]

B. Migration of B Cells in the Spleen

Van Ewijk and Nieuwenhuis,[222] demonstrated that B cells, like T cells, entered the spleen via the marginal zone, where they were especially associated with follicular structures. Subsequently, B cells concentrated in the outer PALS with a tendency to accumulate towards the junctional area at the base of the follicles. By 6 h after injection, most of the labeled cells were at the junctional region between the PALS and the lymphocyte corona. After 18 h, most labeled cells were evenly distributed throughout the lymphocyte corona and only a few labeled cells could be detected in the PALS or marginal zone. This picture persisted for at least 48 h after injection of labeled cells. Willführ et al. found that B cells predominantly enter the spleen through one compartment: the marginal zone. Subsequently they are equally distributed over three compartments: red pulp, marginal zone, and follicles (Figure 5).[232] Vonderheide and Hunt investigated migration of antigen-primed B cells in lymph nodes of immunized rats.[230] They found that antigen-primed B cells immigrated much better into germinal centers than nonprimed B cells, an observation that might be related to the follicular role in memory formation.

C. Migration of DC in the Spleen

Migration patterns of DC were described by Austyn et al.[10] They labeled purified splenic DC with a fluorochrome, injected the cells intravenously, and demonstrated that the labeled cells first localized in the marginal zone before entering the inner PALS of the spleen. The migration of i.v.-injected labeled DC into the splenic PALS was confirmed by Fossum.[69] In addition, electron microscopic studies showed that the labeled cells found in the PALS had acquired the ultrastructural characteristics of IDC, indicating that injected cells and IDC are closely related. Larsen and co-workers investigated the migration of dendritic leukocytes from cardiac allografts into host spleens.[133] They found that donor-derived MHC class II-positive DC were demonstrable in the inner PALS of the spleen, associated predominantly with $CD4^+$ T lymphocytes, as early as 1 day after transplantation. This supported the postulate of Austyn,[12] who suggested that DC in the blood originate from peripheral nonlymphoid tissues where they take up antigens. From there they migrate to lymphoid organs like spleen and lymph nodes, where they have the greatest chance to encounter and activate specific T cells, ensuring the initiation of immune responses. In contrast to T and B cells, the migration pathways of DC seem to be entirely afferent and not recirculating, suggesting a final effector function of these cells in the spleen.[144]

D. General Remarks

The homing of lymphocytes to the spleen does not involve high endothelial venules such as in the lymph nodes, and their migration into the spleen appears to be nonselective. To investigate the role of membrane-associated structures on routing in lymphocyte recirculation, lymphocytes have been treated *in vitro* with a whole range of substances to modify their surfaces.[19,94,170,171] Several *in vitro* treatments of lymphocytes revealed major differences in homing to the spleen and lymph nodes; many reduced homing to lymph nodes without having an effect on splenic homing (e.g., trypsin, phospholipases, dextran sulfate, α/β-interferon, low temperature *in vitro*, blocking of homing receptors), whereas other treatments reduced lymphocyte homing both to the spleen and lymph nodes (neuramidase, concanavalin A, corticosteroids). The high rate of nonspecific recirculation of blood-borne lymphocytes, which comprises most likely the entire T/B cell repertoire, through the spleen may be the reason for the spleen not requiring the ability to select lymphocytes.

When interpreting the migration studies discussed, care should be taken with regard to the method of cell preparation, the route of administration (e.g., intravenous in the blood vs. thoracic

duct in the lymph), and the rate of lymphocyte infusion; variation in these factors could very well explain differences found by several authors.[89] Moreover, these studies were performed with normal nonantigen-primed animals, not immunized animals, which might make an essential difference.

V. CLASSIFICATION OF ANTIGENS

Immunological investigations revealed that some antigens can induce humoral immune responses without the obvious cooperation of T lymphocytes, while many other antigens require the helper function of T cells for the successful generation of an antibody response.[105,149] The former class of antigens is termed thymus independent (TI) and the latter thymus dependent (TD), and this operational definition has been useful even though improved techniques for cell separation have indicated that some antigens may belong in an intermediate category.[21,148,176] In this review we will basically divide antigens into TD or TI and subcategorize them as particulate and soluble. In Table 1, frequently used model antigens are listed with some of their properties (see also Laman et al.[131]).

A. Thymus-Dependent Antigens

By definition, B cells cannot respond to TD antigens without T cell help. As a consequence, these antigens do not induce a humoral immune response in nude mice because of the lack of a functional thymus. The first evidence for thymus dependency of particular immune responses was found in the sheep red blood cell (SRBC) system.[155] It was demonstrated that spleen cells from adult thymectomized, lethally irradiated mice reconstituted with syngeneic bone marrow gave no primary antibody response to SRBC. However, when such mice were reconstituted with a thymus graft, the *in vitro* response was normal. Subsequent T cell depletion studies have clearly documented that both primary and secondary anti-SRBC humoral immune responses were T cell dependent. Shortly after these initial studies, analogous T cell dependency was demonstrated in a variety of protein antigens such as keyhole limpet hemocyanin (KLH), bovine γ-globulin (BGG), ovalbumin (OVA), bovine serum albumin (BSA) and, perhaps more importantly, to hapten carrier conjugates (4-hydroxy-5-iodo-3-nitrophenyl hapten (NIP), 2,4-dinitrophenyl hapten (DNP), and 2,4,6-trinitrobenzenesulfonic acid hapten (TNP).[106]

B. Thymus-Independent Antigens

By definition, TI antigens can directly stimulate B cells to proliferate and differentiate into plasma cells in the absence of T cell help. Direct activation can be achieved either by a mitogenic component (e.g., lipid-A in lipopolysaccharide [LPS]), or by crosslinking of antigen-specific membrane immunoglobulins (mIg) on B cells.[156] This is due to the repetitive structural character of the antigen, as in the case of polysaccharides of bacterial capsules. The different modes of direct activation are reflected in the subdivision of TI antigens into types 1 and 2. Bacterial capsules, consisting of polysaccharide and lipid-A, are typical TI-1 antigens. TI-2 antigens are model antigens obtained by detoxifying TI-1 antigens by removing the lipid-A component. Detoxified TI antigens are not able to mount an immune response in deficient CBA/N and neonatal mice, and hence they are designated as TI-2 antigens.[186] The most common and extensively studied TI-2 antigen is TNP-Ficoll, a synthetic neutral polysaccharide.

VI. *IN VIVO* ANTIGEN ROUTING INTO THE SPLEEN

Antigens in the body may reach the spleen directly via the circulation, e.g., from an infectious infiltrate or after i.v. injection. Antigens may also reach the spleen indirectly, i.e., they may gain access to the lymph flow anywhere in the body and subsequently reach the circulation via the ductus thoracicus, after having migrated through one or more lymph nodes. When antigens

TABLE 1
Properties of Some Experimental Antigens

	Type of antigen	Uptake by	Presentation by	Polyclonal activation
Thymus dependent				
Soluble	Myoglobin, OVA, BSA, KLH	RPM, IDC	IDC, B cells	–
	RαIgD, GαIgD	B cells	B cell	+
Particulate	Liposomes with protein	MZM (RPM)	IDC, B cells	–
	SRBC	MZM (RPM)	IDC, B cells	–
	TNP-Lactobacillus	MZM (RPM), FDC	FDC, IDC, B cells	–
Thymus independent type 1				
Soluble	LPS	MZM, RPM	B cells direct	+
	Dextran-sulfate	?	B cells direct	+
Particulate	*Brucella abortus*	MZM, F	MZM, B cells	+
	Streptococci	MZM, F	MZM, B cells	+
Thymus independent type 2				
Soluble	Dextran	MZM, B cells, FDC	FDC, B cells (MZ/F)	–
	TNP-Ficoll/HES	MZM, B cells, FDC	FDC, B cells (MZ/F)	–
	Detoxified LPS	MZM, B cells, FDC	FDC, B cells (MZ/F)	–
Particulate	Liposomes (no protein)	MZM	B cells (MZ/F), FDC	–
	Haptenated liposomes MZM	B cells (MZ/F), FDC	–	

Note: F, Follicle; RP, red pulp; MZ, marginal zone; M, macrophage; RPM, red pulp macrophages (see also general abbreviations).

arrive in the circulation they may remain unbound, or they can be bound by antibody/complement and/or be captured by granulocytes, macrophages, precursors of DC or other phagocytes. Subsequently, they are carried into the spleen by the splenic arteries and are finally discharged from the terminal arterioles into the marginal zone. The fate of the antigen is largely dependent on its characteristics, i.e., soluble vs. particulate, protein vs. polysaccharide, complement-activating capability, and complex formation with antibody.

A. Localization of Particulate Antigens in the Spleen

Kotani et al.[119] and Miyakawa et al.[146] investigated which cells could be involved in the transport of antigens from marginal zone to white pulp. They demonstrated that i.v. injected carbon particles localized first in the marginal zone, where they were taken up by macrophages. Moreover, they found evidence that these macrophages transported the antigen to the germinal centers and inner PALS and suggested that these cells might play a role in the initiation of immune responses. However, they did not provide evidence for Ia expression by these cells and did not test the antigen-presenting capacity of these cells. Studies by Van Ewijk et al. showed that SRBC i.v. injected in mice are trapped in the red pulp and marginal zone.[220] After 48 h, SRBC-containing macrophages were found within the T cell-dependent areas of the white pulp, in the same areas where IDC are found and where T cell activation is observed after SRBC immunization. Recently, we investigated the localization pattern in the spleen of various i.v. injected particulate antigens (TD: TNP-SRBC, BSA-liposomes; TI: TNP-liposomes, *Brucella abortus*).[23,39] Two hours after injection of particulate antigen, irrespective of its nature, the majority of antigen in the spleen was found at sites where blood flows into the organ: the marginal zone, taken up by marginal zone macrophages and marginal metallophilics (Plate 1d). Twenty-four hours after injection some particulate antigen was also ob-

served in the macrophages of white and red pulp. Although immune responses against particulate antigens show large differences (Table 1), we found a similar localization pattern for all particulate antigens. These data suggest that the localization pattern of particulate antigens does not necessarily play a crucial role in directing the type of immune response.

In vitro studies demonstrated that preprocessing of particulate antigens by macrophages was crucial for the generation of immune responses.[102,234] Such a critical role for marginal zone macrophages in the generation of a humoral immune response was also suggested by the experiments of Van Rooijen and co-workers. They observed a marked decrease of the immune response against liposome-associated human serum albumin[199] and against TNP-SRBC[49] after *in vivo* elimination of marginal zone macrophages. Therefore, it was suggested that marginal zone macrophages were required for the preprocessing of particulate antigens, which subsequently passed over processed antigens to other APC. Marginal zone B cells, with high MHC expression[95] and located between the cytoplasmic processes of marginal zone macrophages,[52] were suggested to be the most likely candidates for the acceptance of processed antigens.

The data discussed here clearly show that the initial localization of all types of particulate antigens in the spleen is similar. Further investigation should aim at the eventual localization of fragments of these particulate antigens after they are processed and transported.

B. Localization of TD Antigens in the Spleen

Several investigations have demonstrated that intravenously injected soluble antigens are removed from the bloodstream in the marginal zone of the spleen and then move toward the follicles in the white pulp to remain there for a long period of time.[164,165] We observed that haptenated TD antigen, like TNP-KLH and TNP-BSA, localized exclusively in the red pulp macrophages on the first days after injection.[218] The localization in red pulp macrophages could very well represent the scavenging of bulk antigen, whereas the antigen which is involved in the induction of the humoral immune responses may be localized in other compartments. The impossibility to detect these immunologically relevant antigens *in situ* could be due to the very low amount of antigen in APC or due to processing by APC which may change their antigenic structure.

After i.v. administration of horseradish peroxidase into mice, Chen et al. detected the antigen on FDC and macrophages, but not in the T cell area.[30] Clearance of antigen by macrophages which reside in sites at which antigens first enter the spleen may leave only small (undetectable) amounts of antigen with access to the T cell area itself. Although antigen localization studies for soluble TD antigens were not able to localize these antigens in the inner PALS, experiments of Kyewski et al. suggested that TD antigens were indeed presented by splenic DC.[129] In their study, they found that after i.v. injection of myoglobin, splenic DC were the major cell types that could stimulate myoglobin-specific T cell clones, although the antigen could not be detected in splenic tissue. Mathur et al.[139] examined, using immunohistochemical techniques, the localization of live replicating virus in murine spleens during Japanese encephalitis virus infection. They found that i.p. injected virus first replicated in the peritoneal macrophages and subsequently entered the spleen via the circulation, spreading to the marginal zone of the spleen by day 3 and then extending into PALS and perifollicular areas on day 5. The virus localization in the PALS was accompanied by an increased proliferation of T cells in this area. These results suggest that antigen presentation and T cell activation occur in the inner PALS during Japanese encephalitis virus infection.

In the primary immune response, immune complexes will be formed as soon as antigen-specific antibodies appear in the circulation[112] (Plate 1e). Immune complexes are also formed if specific antibodies against the antigen are already present in the circulation when the antigen is administered, e.g., during secondary immune responses. Antibody-complexed antigens can be digested by macrophages as discussed for nonantibody-complexed antigens. Phagocytosis of small and soluble antigens in antigen-antibody complexes will be facilitated especially in comparison with their nonopsonized counterparts. A

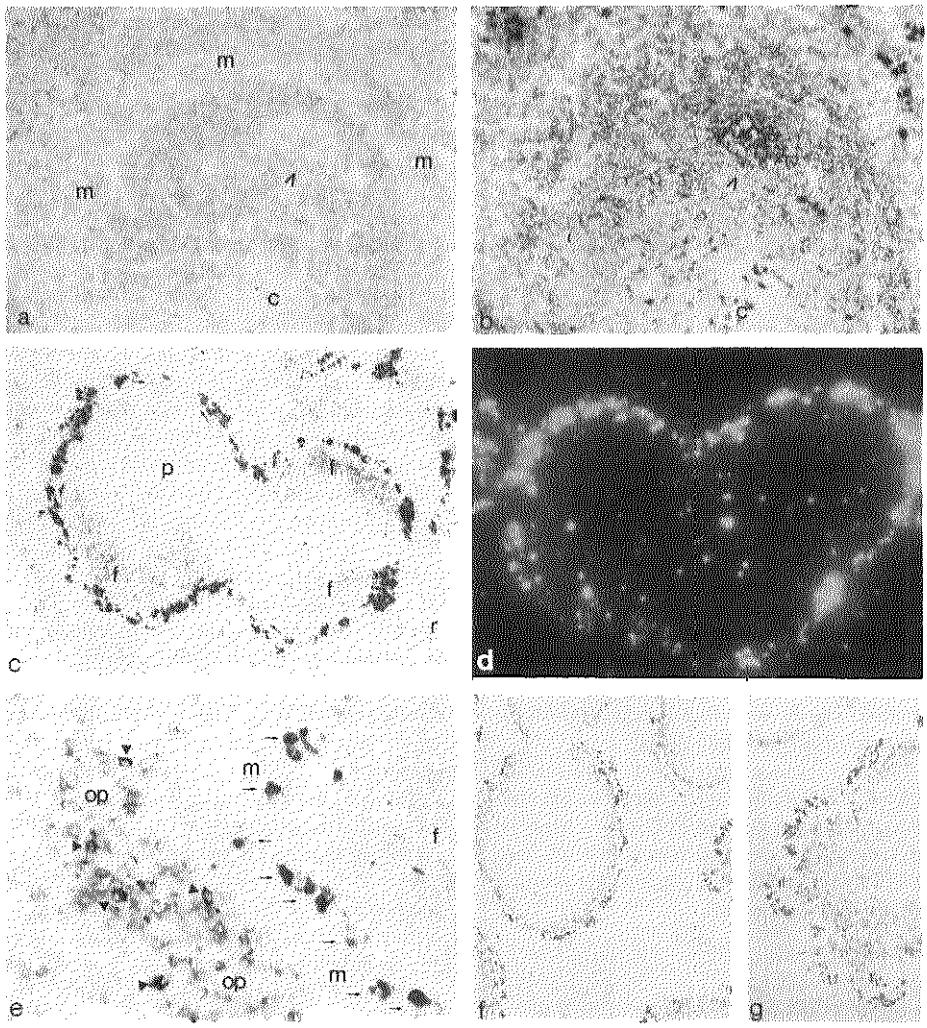
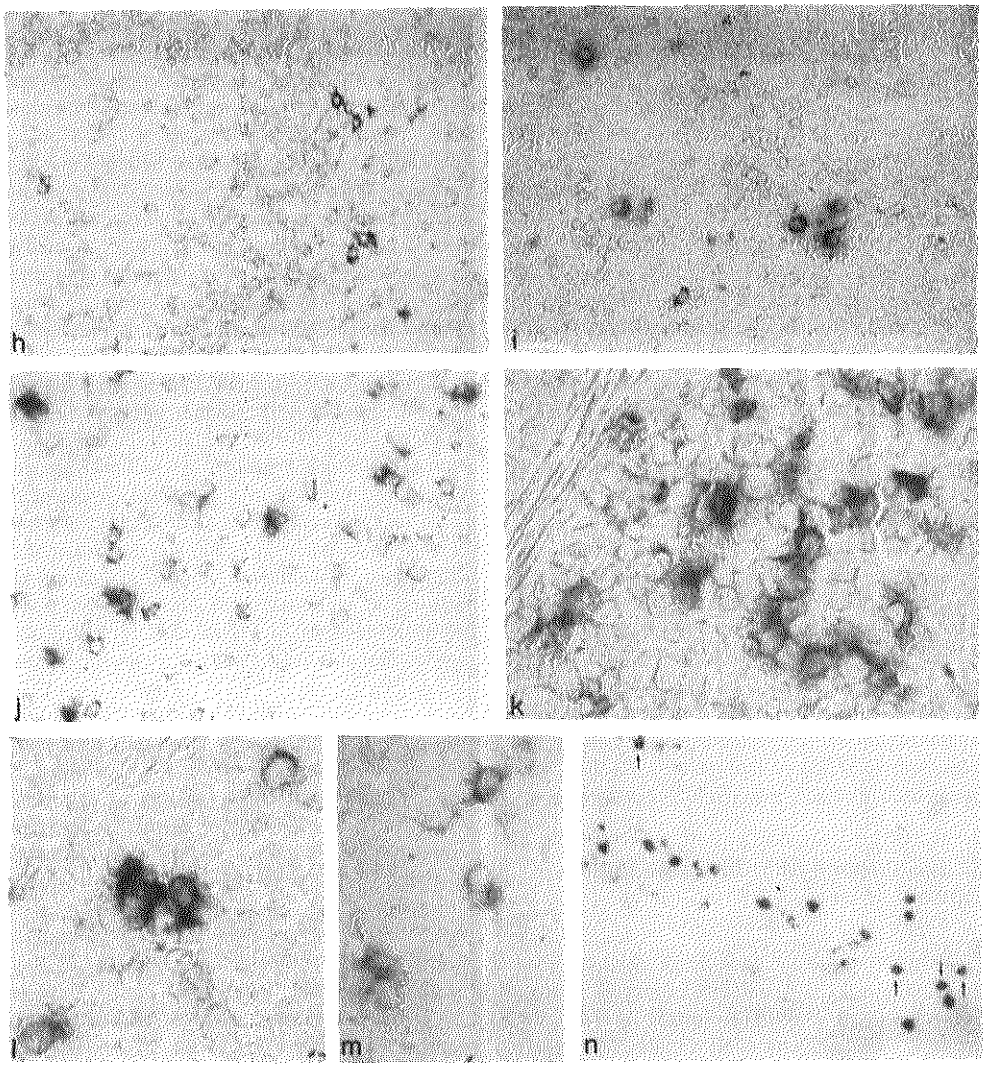


PLATE 1. Localization of immune cells in different compartments of the spleen. (a) IgD$^+$ B cells are predominantly localized in the follicle, whereas marginal zone (M) B cells and germinal center B cells (arrow) express hardly any mIgD; (b) IgM is highly expressed on marginal zone and germinal center (arrow) B cells, whereas the expression on follicular B cells is relatively low [compare with serial section-a]; (c) cryostat section of spleen 1 d after TNP-Ficoll injection; acid-phosphatase positive macrophages (red) are localized in red pulp (r), marginal zone, and PALS (P); TNP-Ficoll (turquoise) is localized in marginal zone macrophages (double stained cells in the marginal zone), but not in the marginal metallophilics (arrows); in addition TNP-Ficoll is localized in follicles in association with FDC and B cells; (d) 4 h after i.v. injection DiI labeled group B. *Streptococci* are predominantly localized in the marginal zone of the spleen; (e) 5 d after injection TNP-Ficoll is localized in the marginal zone, whereas TNP-specific antibody-forming cells (red) and IFN-γ-producing cells (violet) are found in juxtaposition in the outer-PALS and around the terminal arterioles (note TNP-specific antibodies (red) in follicle); (f) marginal zone macrophages (red) stained with the monoclonal antibody ERTR9 are forming the outer ring, whereas marginal metallophilics (brown) are detected with MOMA-1 in the inner ring of the marginal zone; (g) high power magnification of figure f; (h) double-staining for CD4 (red)



and IFN-γ (turquoise) in cryostat section of spleen of mice 5 d after TNP-Ficoll immunization; violet (double staining) cells in the PALS are $CD4^-$ cells producing IFN-c; (i) double staining for CD8 (brown) and IL-4 (turquoise) in cryostat section of the spleen 3 d after TNP-KLH injection; green (double staining) cells are $CD8^-$ cells producing IL-4; (j) IgE positive (red) antibody-forming B cells are localized in same compartment as IL-4 producing cells (turquoise) 4 d after TNP-KLH immunization; (k) TNP-specific antibody-forming B cells (red) are localized in the same compartment (around terminal arterioles) as IL-2 producing (turquoise) cells 4 d after TNP-KLH immunization; (l) IL-2-producing cells (turquoise), IFN-γ-producing cells (red) and double staining (violet) cells producing both IL-2 and IFN-γ (Th1 cells) are localized around terminal arterioles 3 d after TNP-KLH immunization; (m) IL-2-producing cells (brown), IL-4-producing cells (turquoise) and double staining (green) cells producing both IL-2 and IL-4 (Th0 cells) in the spleen 4 d after RαIgD injection; (n) IL-4-producing cells (turquoise; Th2), IFN-γ-producing cells (red) and double staining (violet) cells producing both IL-4 and IFN-γ (Th0 cells) in the spleen 3 d after TNP-KLH immunization.

small proportion of the antibody-complexed antigens is trapped in the follicle centers by the cell processes of the FDC and can be retained there for long periods of time[112,113,130,164,207] via Fc receptors or complement receptors. The trapped complexed antigen plays a crucial role in B cell memory and regulation of humoral immune responses.[80,112] Recently, Gray and Matzinger demonstrated that T cell memory for the soluble protein KLH was short-lived in the absence of the antigen.[83] FDC are the only cells known which can preserve native antigens for months to years. Therefore, it was suggested that T cell memory was dependent on antigen presentation by memory B cells, indicating that follicular trapped antigen may play a role in the maintenance of T cell memory.

In conclusion, the discussed data suggest that antigen localization for soluble TD antigens is not identical for primary and secondary immune responses. In both responses macrophages take up the bulk of antigen. However, after the first antigenic challenge, antigen localizes in the inner PALS containing DC and T cells, and play a role in the induction of primary immune responses; whereas after a secondary challenge the follicle, containing FDC, B, and T memory cells, is the important compartment where antigen localizes and which is crucial for induction and maintenance of secondary immune responses.

C. Localization of TI Antigens in the Spleen

Recently, the localization of thymus-independent type 2 (TI-2) antigens in murine spleens was monitored.[218] Within minutes after i.v. injection, TI-2 antigens (TNP-Ficoll, TNP-HES) localized in the marginal zone attached to marginal zone macrophages and B cells. Twenty minutes after injection the antigen was also detected in the follicles and gradually accumulated there up until 7 h after injection. Thereafter, the amount of follicular antigen gradually decreased but was still detectable up to 14 d after immunization (Figure 6). The immediate follicular localization of TNP-Ficoll was complement dependent and not antibody mediated. In addition, in the case of administration of high doses, a relatively large amount of TI-2 antigens was observed in marginal zone macrophages, the cells which are the predominant phagocytes of capsular bacteria (Plate 1d). Double staining revealed that TNP-Ficoll was bound to B cells, FDC, and macrophages. *In vivo* macrophage elimination drastically increased the amount of TNP-Ficoll in the follicles, and enhanced the humoral immune response at low doses of antigen.[37] In contrast, complement depletion of mice abrogated the localization of TI antigens in the follicles, and led to a decreased humoral TI-2 immune response. These results suggested that follicular localization of antigen is a prerequisite for the induction of humoral TI-2 immune responses.

Injection of particulate TI-1 antigens showed a similar antigen distribution in the spleen as was observed for TNP-Ficoll (Plate 1d). After injection of DiI-labeled group B streptococci, the encapsulated bacteria was localized in marginal zone macrophages and in the follicles, although the follicular localization was less as compared to haptenated TI-2 antigens (Plate 1e, f). This finding was in agreement with experiments of Chao and MacPherson[29] and Kraal et al.,[123] which suggested that the main function of marginal zone macrophages was to clear encapsulated pathogens from the circulation via the TI-2 antigen receptor. The observed localization pattern also supports the hypothesis of Van Rooijen who postulated that marginal zone macrophages are involved in the processing of particulate antigens and subsequently transfer the processed antigens to B cells in the marginal zone.[225] These B cells are thought to present the antigens, such as soluble antigens, to T cells in the PALS and differentiate into antibody-forming cells after receiving appropriate T cell help.

In conclusion, it is clear that TD and TI antigens localize differently in the spleen; the fact that TI antigens are directly trapped in the follicles correlates well with their ability to directly stimulate B cells and their independence of (pre-)processing.

VII. CELLS INVOLVED IN IMMUNE RESPONSES

There is general agreement that at least three different cell types are involved in TD immune

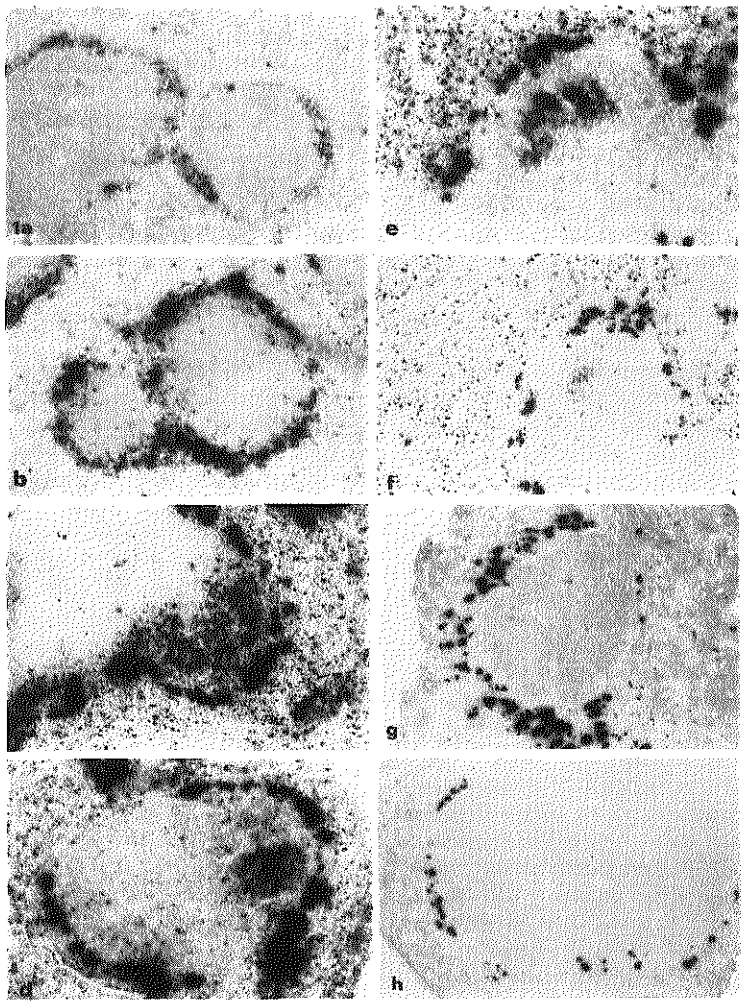


FIGURE 6. Localization of TNP-Ficoll in representative spleen sections of mice at various time points after injection. Cryostat sections were stained with a β-galactosidase conjugated monoclonal antibody directed to TNP: (a) 5 min; (b) 20 min; (c) 2 h; (d) 2 d; (e) 7 d; (f) 10 d; (g) 14 d; and (h) 21 d after injection of 100 μg TNP-Ficoll. Note both uptake of TNP-Ficoll by marginal zone macrophages and trapping of TNP-Ficoll in follicles.

responses:[47] B Cells, which produce specific antibodies and are regulated by T cells, which in turn require the appropriate signals provided by the processed antigen and the APC, such as macrophages, IDC, or B cells.

A. Antigen Presentation to T Cells

APC like B cells, macrophages, and DC, are able to present the appropriate antigen fragment and MHC protein to a T cell in such a way that a

stable interaction is formed and the T cell is activated. The two major APC in the spleen are DC and B cells.[111,144,195] T Cells recognize, via their T cell receptor, proteolytic degraded fragments of antigenic proteins that are presented at the cell surface by either MHC class I or class II molecules. Each type of presentation is recognized by a specific T cell subpopulation: $CD8^+$ T cells recognize peptides presented by MHC class I molecules and $CD4^+$ T cells recognize peptides presented by MHC class II molecules. Activated $CD8^+$ T cells are usually cytolytic *in vitro* and are therefore referred to a cytolytic T cells (CTL) despite their capacity to produce cytokines. $CD4^+$ T cells produce various cytokines required for the synthesis of antibodies and for the proliferation of $CD8^+$ T cells, and are therefore referred to as T helper (Th) cells, but they also include CTL.

In contrast to macrophages, DC or B cells do not take up large amounts of model antigens *in vitro* as well as *in vivo*. This suggests that the study of antigen presentation in these APC might seem misplaced, but the opposite view could also be argued. The bulk of an internalized load in an actively endocytic cell such as the macrophage may be degraded down to the level of amino acids since these cells are specialized for scavenging and microbicidal activity.[39] This function can be enhanced by the presentation of processed antigen to T cells, activating these cells to exert their effector functions, e.g., cytolysis or secretion of cytokines, such as macrophage-activating factors. In contrast, B cells and especially DC, because they show so little bulk endocytic activity, seem to utilize endocytosis primarily as a presentation mechanism and are, accordingly, the specialized APC in immune responses.

1. Antigen Presentation by DC

DC are highly effective in presentation of antigen in the context of class I or class II MHC molecules, which in general is attributed to both qualitatively and quantitatively superior MHC expression.[143] One distinctive feature of DC, which may influence their capacity to find infrequent clones of antigen-specific T cells, is their ability to form and retract cell processes, particularly broad veils which are 10 mm or more in extension. In primary *in vitro* responses, it has been observed that DC physically associate or cluster with T cells and that DC T cell clustering is essential for T cell activation.[11] DC and T cells can cluster in a special way independent of antigen and MHC.[99] It has, therefore, been suggested that DC initially cluster with resting T cells in an antigen-independent manner. In the presence of antigen, DC will present antigen and activate the antigen-specific T cells. The irrelevant T cells will leave the clusters after a short time[9] and the activated T cell will proliferate. Antigen presentation by DC provides an efficient pathway for inducing the large numbers of active and specific lymphoblasts, while presentation by B cells is essential for helper T-cell-dependent antibody formation. DC were found to be required *in vitro* for the initial priming of T cells,[193] which in turn could regulate the differentiation/proliferation of antigen-specific B cells.[99] The latter could be demonstrated in the apparent absence of DC in an antigen-specific, MHC-restricted fashion. *In vitro,* DC do not make Ig or cytokines and are not phagocytic. Therefore, when DC present antigen, the effect seems to be unidirectional on the T cell, generating large numbers of antigen-specific lymphoblasts.

So far, no soluble TD antigen has been demonstrated in the T cell area of the spleen, the compartment where DC are localized. It is not unlikely that antigens can be bound and processed in other compartments or organs and arrive in the T cell area ready to be presented. This was indeed observed by Kyewski et al.,[129] who observed after i.v. injection of myoglobin that splenic DC were the major cell types that could stimulate myoglobin-specific T cell clones, although they could not be detected in splenic tissue. Crowley et al. extended these results by comparing the antigen-presenting capacity of isolated splenic macrophages and DC after i.v. injection of myoglobin.[46] This study provided evidence that DC were the main cell type in the spleen which carries myoglobin in a form that is immunogenic for antigen-specific T cells in the spleen. The capacity of DC to take up antigen and present it in the spleen was also supported by experiments of Larsen et al.[133] They demonstrated that donor-derived MHC class II-positive DC from a cardiac allograft were mi-

grated into the inner PALS of the host spleen, associated predominantly with $CD4^+$ T lymphocytes, 1 d after transplantation.[133] Sornasse and co-workers pulsed isolated dendritic cells with the TD antigens: myoglobin and gamma-globulin, and injected them i.v. into syngeneic mice.[192] After 5 d, mice were injected with the same TD antigen and a strong humoral immune response was observed, whereas control mice produced only a low specific antibody response. A similar approach was used by Francotte and Urbain,[70] who demonstrated that purified DC and macrophages pulsed *in vitro* with tobacco mosaic virus were potent APC in TD antibody responses *in vivo*, although macrophages were found to be about 100 times less efficient. All these studies suggest a role for DC in initiating and augmenting primary immune responses against TD antigens.

Thus, DC appear to acquire antigen from nonlymphoid organs or circulation, and present processed antigen in the T cell areas of the spleen, in the path of the recirculating T cell pool, and are thereby in a very strategic position to select and expand antigen-specific T cell clones.

2. B Cells as Antigen-Presenting Cells

In contrast to DC, B cells are not able to activate resting T cells, as was demonstrated by Lassila et al.[134] They showed that for the initiation of *in vivo* humoral immune responses, T cells must recognize antigen presented by different types of cells, first a non-B cell and then the antigen-specific B cell which is also the recipient of the regulatory factors released by the T cell. It was not clear whether the requirement of a non-B cell for the first antigen-presentation step reflects an actual inability of B cells to activate resting T cells or whether the initial step leads to expansion of the resting T cells, thereby increasing the probability of finding the appropriate B cell. This study indicated a more prominent role for B cells in T cell activation during secondary immune responses.

The fact that B cells are clonal and capable of relatively high-affinity and specific interactions with one vs. many antigens is a striking distinction from DC, which lack antigen specificity. B cells are specialized to pick up antigens in the bloodstream by their antigen-specific receptor (mIg). In addition, later in primary or during secondary immune responses B cells may encounter antigen in the germinal center, where there is a depot of extracellular antigen on follicular cells.[205,207] It has been demonstrated *in vitro* that antigen-specific B cells specifically capture antigen via their antigen-specific receptor and present it more than a thousand times more efficiently as compared to control B cells.[132,179] Sanders et al. demonstrated that when the affinity of the B cell antigen receptor increased, as in memory cells, the efficacy of the B cell as an APC increases too.[185] In addition, the efficacy of antigen presentation by B cells can also be improved by clonal expansion and antigen-dependent selection of B cells, mechanisms which are part of the memory response or secondary response. Antigen-presenting properties of B cells have drawn a lot of attention.[7] *In vitro* studies revealed that B cells are generally less potent as low-density APC. However, resting B cells still may be effective APC *in vivo*, where their large number and strategic location may compensate for lower capability for antigen presentation per cell. Moreover, *in vitro* studies showed that antigens bound to B cell surface was 100 to 1000 times more efficient than unbound antigen, implying that antigen-specific antigen presentation by B cells might have a significant role in *in vivo* immune responses.[132,179] Ron and Sprent showed that *in vivo* B cell-depleted mice failed to mount a proliferative response to KLH, whereas in the presence of exogenous B cells the response reached control values.[180] Moreover, Kurt-Jones et al. demonstrated *in vivo* that mice reconstituted with FITC-specific B cells responded to FITC-OVA much better than to TNP-OVA, although both antigens were equally immunogenic in normal mice.[128] On the whole, these studies provide consistent and convincing arguments that antigen-specific B cells play a central role in presentation of protein antigens to T lymphocytes *in vivo*.

Antigen presentation by B cells is bidirectional in its consequences. B Cells activate T cells, which in their turn stimulate B cells by cell-cell interaction and cytokines to proliferate and/or differentiate into antibody-forming cells.

It is not clear whether B cells play a role in presenting antigens to those T cells which become CTL or T cells which mediate macrophage activation and delayed-type hypersensitivity (DTH). The role of B cells in the presentation of particulate, viral, or microbial antigen *in vivo* is not clear yet, as these antigens may first require phagocytic uptake and subsequent extensive degradation for presentation. Such a mechanism for viral antigen handling was suggested by experiments of Rizvi et al. who showed that preprocessing of dengue type 2 virus proteins by macrophages was required before an immune response by B cells with the degraded form of viral proteins was possible.[178]

The data discussed suggest that during primary humoral immune responses T cells are activated by non-B cell APC, prior to their encounter with antigen-specific B cells. This in contrast to secondary immune responses where the antigen is preferentially captured by high-affinity memory B cells and presented to memory T cells, both of which cell types are present in relatively large numbers. In primary as well as in secondary immune responses, antigen-presenting B cells will activate T cells, the latter cells in turn will regulate the differentiation of B cells into antibody-forming cells.

B. Antigen Presentation to B Cells

After immunization, immune complexes are formed as soon as antibodies appear in the circulation.[112] A small proportion of these antibody-antigen complexes are trapped in the follicles on the cell processes of the FDC and retained for long periods of time[207] via Fc receptors[164] and/or complement receptors.[113,117] It has been demonstrated that antigen which is preserved extracellularly in an undegraded form as immune complex associated with FDC has a crucial role in the generation of B memory cells[112] and is required for the maintenance of B cell memory.[80] In addition, it has been suggested that the exposure and obscuring of epitopes present in immune complexes may fine-tune the titer of circulating antibodies.[207] Recent electron microscopic and *in vitro* evidence[206] indicates that FDC may release immune complex-coated beads termed "iccosomes". These iccosomes are ingested by antigen-specific germinal center B cells, processed, and subsequently presented to T cells,[117] which are activated to initiate an immune response.

The antigen presentation of FDC to B cells and the presentation of dengue virus by macrophages to B cells, discussed in the previous section, are examples of antigen presentation to B cells. The relative importance of such mechanisms of antigen presentation *in vivo* is not yet known, but the system described may provide sophisticated processes for the enhancement of the ability of the immune system to deal with a pathogen.

C. T and B Cell Activation in TD Immune Responses

The activation of B cells is a complex mechanism and has been excellently discussed by several groups[160–162,175] (Figure 7). Briefly, TD antigens bind to B cells expressing complementary mIg receptors. The crosslinking of the mIg does not result in B cell activation, but may be a signal for the B cell to process the selected antigen and present it to T cells. Indeed, Casten and Pierce observed up to 100–1000 times increase in the capacity of B cells to present cytochrome c to a T cell hybridoma when the antigen was covalently coupled to anti-Ig.[28] The potent activity of the cytochrome c-antibody conjugates appeared to be due to the B cell targeting features of the conjugated Ig, because cytochrome c was not more effectively presented in the presence of unconjugated antibodies as compared with cytochrome c alone. The recognition of complexes of processed antigens and MHC on the B cell by T cells is responsible for the initial class II-restricted, antigen-specific, physical interaction between T and B cells. These T and B cells form conjugates which are stabilized by the binding of ICAM-1 to LFA-1 and $CD4^+$ to monomorphic domains of the class II proteins. This interaction induces T cell activation, resulting in the expression of novel surface proteins on the membranes. These newly expressed membrane proteins on activated Th cells together with resident Th membrane proteins form the major growth stimulus

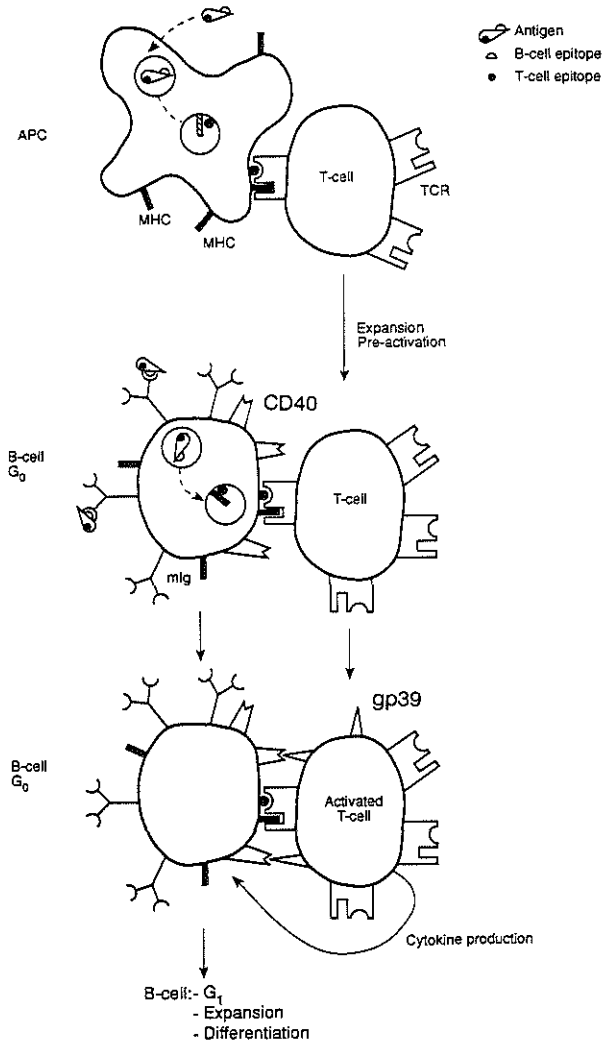


FIGURE 7. Cell-cell interactions during a TD immune response (see text for explanation).

for B cells. Recently, Noelle et al. demonstrated that blocking of the prominent Th surface molecules, LFA-1, CD4, ICAM-1, CD3, or the T cell receptor (TCR) did not inhibit the capacity of activated Th cells to induce B cell cycle entry.[163] In contrast, CD40-Ig fusion proteins, or antibodies specific to the CD40 ligand, blocked B cell activation by activated Th cell, suggesting that the CD40 molecule expressed by B cells is the receptor of the Th cell activation antigen. This activation antigen was identified as a 39-kDa protein that was selectively expressed on activated Th cells.[163] The initial B cell activation by activated Th cells requires no soluble factors and

induces enhanced B cell RNA synthesis ($G_0 \rightarrow G_1$). After activation, B cells develop the competence to respond to cytokines, which are produced by activated T cells. These cytokines act solely or predominantly at different steps in the B cell activation pathway. Cytokines, like IL-1, IL-2, and IL-4, support the growth of activated B cells, while others such as IL-4, IL-5, IL-6, and IFN-γ participate as differentiation factors.[1,2]

The experiments described above imply that T-B cell conjugate formation is an important event for the induction of TD immune responses. This interaction is not a short event but requires contact for a least 48 h *in vitro* to obtain the maximum proliferative response of B cells.[106] In addition, cytokine production by T cells was found to require the continuous presence of the stimulating signal *in vitro*,[204] suggesting that for the generation of an effective immune response, T-B cell conjugate formation is required for at least 24 to 48 h. Such a T-B cell interaction was recently demonstrated by Kupfer et al *in vitro*.[126] They showed that after *in vitro* mixing of antigen-specific T-helper, B cells, and antigen, T-helper–B cell couples were formed up to 29 h later. This interaction resulted in cytokine production by these T cells near the antigen-specific B cells. The cytokines were not seen in the T cells prior to their interaction with the B cells and their production was strictly antigen-specific. They proposed that T cells were in this way able to induce selective and specific B cell responses.[126,173] The *in vitro* mixing of T and B cells resulted in formation of 1:1 T-B couples, dependent on the initial T/B ratio. In 60% of the more complex conjugates, which had three T helper cells simultaneously bound to one B cell, not all the cytokine production was found toward the bound B cell. Also in the case of very low concentrations of antigen, no polar cytokine production at the T-B contact site was observed.[126]

All these *in vitro* experiments demonstrate that for both T cell as well as B cell activation a close physical T-B cell interaction is required. It is most likely that such an interaction also occurs *in vivo*. The duration of such an interaction and the T-B cell ratio required for the generation of an immune response is still controversial. Studies on the existence and characteristics of T-B cell conjugates *in vivo* are now underway in our laboratory.

D. T and B Cell Activation during TI Immune Responses

The role of T cells, accessory cells, and cytokines in TI immune responses is still under discussion (Figure 8). The original definition of TI antigens reflects its ability to stimulate humoral responses in nude mice *in vivo* or in T cell-depleted cultures *in vitro*. These criteria are relatively nonstringent, insofar as nude mice and the T cell-depleted cultures have been shown to possess both small numbers of T cells as well as large numbers of NK cells, which are both able to secrete cytokines.[103,175] Rigorous elimination of T cells consistently and significantly reduces the *in vitro* immune response to TI antigens, indicating that these antigens are T cell dependent to a significant degree.[21,53,148,176] Thompson et al. demonstrated that the degree of T cell dependency of these antigens *in vitro* depends on the state of activation of B cells.[209] TI Antigens were extremely efficient in eliciting immune responses in large-sized B cells compared to small nonactivated B cells (cells in G_0). Responsiveness could be enhanced by the addition of T cell-derived cytokines like IL-2.[53,149] The mechanism of T cell activation by TNP-Ficoll was investigated by Dekruyff et al., who demonstrated that TNP-Ficoll-activated B cells were able to stimulate directly T cells to proliferate and secrete cytokines.[48] They suggested that these T cell factors activated accessory cells to supply the additional factors required for TI immune responses. Accessory cells were found to be essential for an optimal TI immune response. In addition, it was demonstrated *in vitro* that, in contrast to T cell activation, accessory cell function in TI B cell activation did not involve presentation of processed antigen in the context of Ia molecules and could be entirely replaced by recombinant IL-1.[77,187] The Ia independency of TI-1 immune responses was confirmed *in vivo* by Fultz et al. who observed no changes in the humoral immune against TNP-Ficoll in mice suppressed from birth with anti-Ia antibody.[71] All these data point to B cells as the principal cells in the induction of humoral TI immune responses. B Cell activation studies with haptenated antigens revealed that TNP-*Brucella abortus* and TNP-Ficoll were able to activate TNP-binding cells, via mIgM, to enter the cell cycle.[24] No activation was detected after

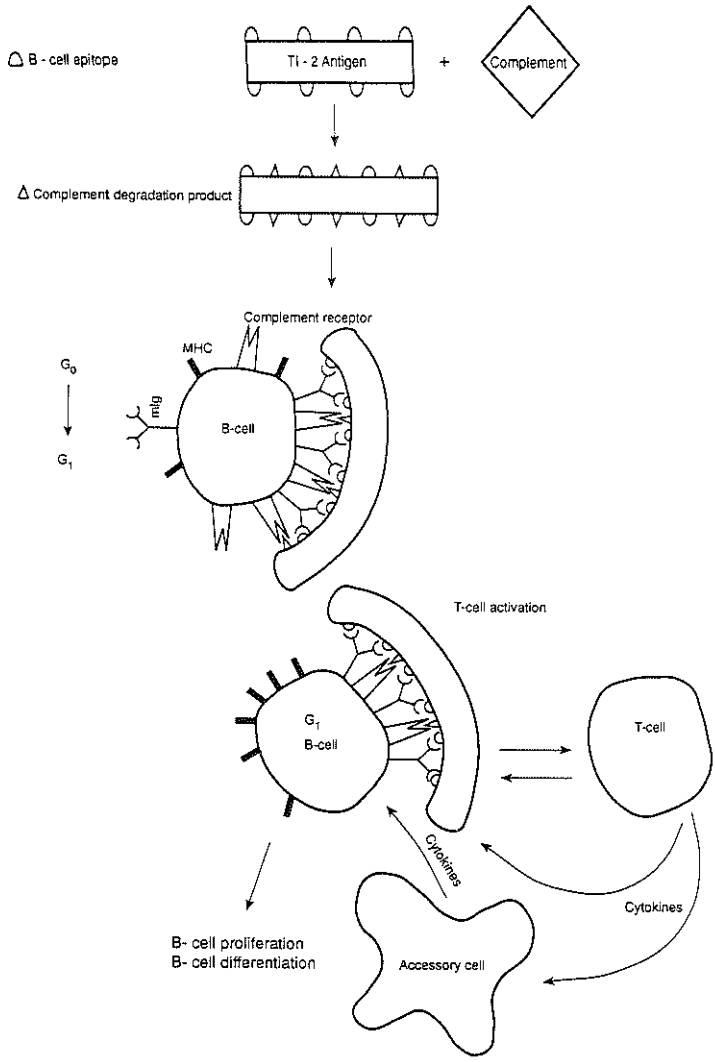


FIGURE 8. Cell-cell interactions during a TI-2 immune response (see text for explanation).

challenge with the haptenated TD antigens TNP-SRBC and TNP-OVA. Carter et al.[26,27] demonstrated synergistic signaling of complement receptor and mIg, as measured by intracellular calcium mobilization, indicating that complement could provide an additional activation signal for B cells. This is in agreement with observations by Griffioen et al., who showed that polysaccharides, TI-2 antigens, were able to activate complement via the alternative pathway.[84] Moreover, they demonstrated that polysaccharides complexed with a degradation product of complement (C3d) were able to bind to the complement receptors of B cells. These data suggest that TI-2

antigens complexed with C3d are able to activate B cells directly, thereby providing an activation pathway in TI-2 immune responses. Several *in vivo* studies indicated that complement was only synergistic after immunization with relatively low doses of TI antigens. In the case of high-dose immunizations, complement was not found to be obligatory for the generation of maximal humoral immune responses.[140,210,231]

In conclusion, B cell activation by soluble TI-2 antigens *in vitro* occurs directly via the antigen-specific mIg without requiring antigen processing by accessory cells and is enhanced by complement. Cytokines produced by T cells and accessory cells seem to be required for the clonal expansion and further differentiation of activated B cells (Figure 8).

E. Cytokines in *In Vitro* Immune Responses

1. *Cytokine Profile of Th Subsets* In Vitro

Mosmann et al. studied a large panel of antigen-specific Th cell clones.[151,152] Virtually all clones fell into one of two groups, depending on the pattern of cytokines secreted: the first type of T cell clone, designated Th1, synthesized IL-2 and IFN-γ but not IL-4, whereas the second type of clone (Th2) produced IL-4 and IL-5,[32] but not IL-2 and IFN-γ (Table 2). All Th1 clones, in contrast to the Th2 clones, were able to mediate DTH reactions.[31] The functional capabilities of murine Th1 and Th2 cells correlate well with the cytokines they secrete such as IFN-γ, lymphotoxin, and granulocyte macrophage colony-stimulating factor (GM-CSF).[66] The signals necessary to induce the differentiation of Th cells into Th1 or Th2 are not well defined.[45] One explanation could be the different specificity repertoires of Th1 and Th2 cells. However, at the moment there is not much evidence for a relation between T cell repertoire and Th1/Th2 cell development.

DTH responses mediated by Th1 cells and antibody responses predominantly stimulated by Th2 cells are mutually exclusive reactions, suggesting that these cells are reciprocally regulated *in vivo*. IL-4, a Th2 product, was found to support the growth of Th2 cells,[202] whereas IFN-γ, a Th1 product, inhibited the proliferation of Th2 cells.[72,73] The search for cytokines that were produced by Th2 cells and inhibited either proliferation or cytokine production by Th1 cells resulted in the characterization of a new cytokine, cytokine synthesis inhibitory factor (CSIF, IL-10),[63,208] that inhibits the production of cytokines by Th1 cells responding to antigen and APC. However, several lines of evidence suggest that IL-10 acts via the APC rather than directly on the Th1 cell.[64]

The establishment of Th1/Th2 cells was largely derived from clones which were selected for other purposes and had been maintained *in vitro* for long periods of time. Cytokine profile analysis of $CD4^+$ clones shortly after isolation demonstrated that a high proportion of these clones secreted a broad spectrum of cytokines, including IL-2, IL-3, IL-4, IFN-γ, and tumor necrosis factor (TNF).[65] It was suggested that these cells, termed Th0 cells, were either precursor subsets of Th1/Th2 cells or transition cells in a pathway leading from one subset to the other. Also, studies of Kelso and Gough revealed the existence of clones coexpressing the cytokines of both subsets.[108] The lack of dissociation of IL-4 and IFN-γ production by $CD4^+$ cells was attributed to the short-term *in vitro* activation of these cells. It is nevertheless clear that *in vitro* culture conditions and *in vivo* priming can influence the relative frequencies of clones expressing different cytokine profiles. Analysis of whole-tissue cytokine gene expression showed that this was dependent on the priming protocol.[110] Graft-versus-host reaction showed a high IFN-γ and a low IL-4 gene expression, while KLH immunization led to high IL-4 and low IFN-γ expression. The upregulation of Th2 cells by IL-4, and the cross-downregulation by IFN-γ and IL-10 of Th2 and Th1 cells, respectively, could explain the Th subset obtained after *in vitro* cloning. Dominance of IL-4 would lead to a preferential *in vitro* development of Th2 clones, whereas an IFN-γ-dominated immune response would result in Th1 clones.[197,203] From the data discussed it is clear that the subdivision of $CD4^+$ T cell clones into Th1 and Th2 subsets, as originally described by Mosman and Coffman[152] does not represent the

TABLE 2
***In Vitro* Cytokine Profiles of Murine T Cells**

	CD4+: TH0	TH1	TH2	CD8+
IL-2	++[a]	++	–	++
IL-3	++	++	++	+
IL-4	++	–	++	+
IL-5	++	–	++	+
IL-6	++	–	++	+
IL-10	++	–	++	–
IFN-γ	++	++	–	++
TNF-α	++	++	+	+

[a] Relative cytokine production.

full range of phenotypes that can be expressed by murine T cells.

2. B Cell Regulation by Th Cell Subsets

One major function of Th cells is to provide the signals necessary for activation, proliferation, and differentiation of B cells that have been exposed to antigen. The ability of Th cell clones to provide B cell help *in vitro* depends on the concentration and type of cytokines being secreted by the T cell clones at the time of T-B cell interaction (Table 3). If the levels of IFN-γ are low, Th1 clones are able to provide help to B cells in the production of antibody, primarily of the IgG2a isotype.[43] If high levels of IFN-γ are present during a strong Th1 response, these cytokines have an inhibitory effect on B cell responses. Thus, Th1 cells can both help and suppress antibody production. Conversely, Th2 clones effectively help B cells in the production of IGM, IgG1, IgA, and IgE, due to their ability to secrete the B cell growth and differentiation factors IL-4, IL-5, and IL-6. Moreover, it was demonstrated *in vitro* that IL-4 was an essential maturing factor for immature resting B cells, with a low Ia expression, to secrete IgG2a upon IFN-γ stimulation.[196] The IgE response is mainly controlled by the cytokines IL-4 and IFN-γ. IL-4 induces a switch to IgE in preactivated B cells, whereas IFN-γ inhibits this response.[42]

The capacity of Th1 and Th2 cells in the induction of antibody responses was recently described.[93,162] Noelle et al. showed that B cells stimulated by isolated membranes of an activated Th1 clone or a Th2 clone plus IL-4 and IL-5 produced similar levels of IgM, IgG1, and IgG2a.[162] Hodgkin et al. extended these results by testing more Th1 or Th2 clones in their ability to stimulate B cells in the secretion of the whole range of isotypes.[93] Though the mechanism is still not completely resolved, the results of both groups provide strong evidence that the actual cytokine repertoire, and not the cell contact-delivered signals of Th1 cells, are responsible for their inability to provide stimulation of B cell differentiation. The fact that Th1 and Th2 cells can only be characterized by the pattern of cytokine secretion might be the reason that no cell surface marker has been described yet that exclusively distinguishes Th1 from Th2 cells.

Bradley et al. examined the kinetics of *in vivo* development of antigen-specific CD4+ T cells which arose in the primary immunization with KLH and characterized the cytokines that were produced.[17] In this study, mice were immunized subcutaneously with KLH in Freund's complete adjuvant and draining lymph nodes were removed at various time points. CD4 positive cells were isolated and restimulated *in vitro* with KLH and 24 h later supernatants were assayed for IL-2, IL-3, IL-4, IL-5, and IFN-γ. All the cytokines were detectable as early as 3 d after immunization and peaked at day 5 to 7. Similar kinetics were observed for the development of all cytokine-producing CD4+ T cells, indicating that T cell activation for all cytokine-producing T cells (Th0, Th1, and Th2) follows a similar mechanism. However, the amount of cytokine produced differed for each cytokine. The effector T cells produced relatively high levels of IL-2 and IL-4, and little IFN-γ or IL-5, as far as they are comparable. Mice thymectomized 6 months before showed a reduced capacity to generate cytokine-producing CD4+ cells compared to control mice, indicating that the majority of cytokine-secreting cells were derived from short-lived, presumably naive precursor cells. The effector T cells provided excellent helper activity for *in vitro* antibody production of B cells, which produced principally the immunoglobulins IgG1 and IgM. The required *in vitro* restimulation of CD4+ cells with KLH suggested that these antigen-specific T cells *in vivo* only produce cytokines when they en-

TABLE 3
***In Vitro* Effects of Cytokines on Immune Cells**

	B cells	Isotypes	T cells	Accessory cells
IL-1	Proliferation (↑)		Costimulatory	Chemotactic, IL-1(↑) Acute-phase proteins(↑)
IL-2	Proliferation	IgM (↑)	Proliferation	
IL-4	Ia-Expression (↑) Activation, FcεR (↑)	IgG1 and IgE (↑) IgG2a, Ig2b, and IgG3 (↓)	TH2 proliferation (↑) IL-2R (↑)	Macrophage Ia (↑) Mast cells (↑)
IL-5	Proliferation IL-2R Expression (↑)	IgA, IgM, and IgG (↑)		Proliferation and differentiation of eosinophils
IL-6	Proliferation	Ig secretion (↑)	Activation factor	
IFN-γ	Differentiation factor Antagonist of IL-4	IgG2a (↑) IgG1 and IgE (↓)	TH2 proliferation (↓)	Ia, FcγR expression (↑) Antiviral/microbial

For References see Vitetta, E. S. et al., *Adv. Immunol.*, 45, 1, 1989.

counter the appropriate antigen presented by B lymphocytes or other APC. In this study the cytokine production by individual cells was not assessed and therefore this study did not provide information about the activity of Th subsets in this immune response.

In conclusion, it is clear that Th subsets or function *in vivo* can only be determined by determining the actual cytokine profile *in vivo* or *in situ*.

3. Th Cell Subsets in Infectious Diseases

Th2 Clones are considered to be important regulating cells in allergic or immediate hypersensitivity responses. IgE is bound by mast cells and eosinophils and is the antigen-specific receptor for induction of hypersensitivity reactions. IL-4 stimulates IgE production by B cells. In addition, the growth and differentiation of mast cells and eosinophils is also stimulated by the Th2 cytokines IL-3, IL-4, and IL-5. Animals infected with many parasites display high IgE levels,[57] eosinophilia, and mast cell hyperplasia.[107] Numerous other features of immune responses to helminth parasites suggest a preferential involvement of Th2 cells. Street and Mosmann found that spleen cells isolated from *Nippostrongylus brasiliensis*–infected mice secrete dramatically increased levels of IL-4, IL-5, and IL-10, but reduced levels of IL-2 and IFN-γ after *in vitro* mitogen stimulation.[198] Coffman et al. investigated the *in vivo* role of IL-4 and IL-5 in parasitized mice.[44] They demonstrated that injection of a neutralizing monoclonal antibody to IL-5 completely suppressed the blood eosinophilia and the infiltration of eosinophils in the lungs of *Nippostrongylus brasiliensis*–infected mice, whereas serum IgE was not affected. In contrast, injection of an IL-4-neutralizing antibody inhibited the parasite-induced IgE serum levels but not the eosinophilia, indicating that IgE and eosinophil production *in vivo* are regulated by different cytokines produced by Th2 subsets. A similar outcome was observed in mice infected with *Heligmosomoides polygyrus*.[213]

In contrast, the Th1 subset is probably the most capable of helping against intracellular parasites such as viruses, intracellular bacteria, and protozoans. IFN-γ stimulates IgG2a production, which is a complement-activating/cytotoxic isotype,[169] and has a high affinity for Fc receptors on macrophages.[212] Also, the ability to induce DTH reactions contributes to the defense against intracellular parasites, as this reaction leads to the recruitment of macrophages and granulocytes to the area of infection. Furthermore, Th1 cells activate macrophages, by secreting IFN-γ and lymphotoxin, to capture and kill infected cells.[66]

The cross-inhibitory effects of Th1 and Th2 cytokines appear to determine the predominance of either of the two subsets during *in vitro* immune responses against microorganisms, e.g., Th2 cells in parasitic diseases[91,198] and Th1 cells during mycobacteria infections.[88,184,233]

Not only the nature of antigens, but also the genetic make-up of inbred mice can be an important factor in the development of Th1 or Th2 cells. Murray et al.[157] demonstrated that human collagen type IV or even a single peptide of this protein activated CD4 cells from H-2^5 mice to secrete Th1 cytokines (IL-2 and IFN-γ), whereas CD4 cells from H-$2^{b,d}$ were stimulated to produce Th2 cytokines (IL-4 and IL-5). In particular cases such a differential activation of Th1 or Th2 cells can have dramatic consequences, which is illustrated in a mouse model of *Leishmania major* infection. *Leishmania major* is an obligate intracellular parasite that causes death when injected into susceptible mice, i.e., BALB/c (H-2^d), but causes only limited disease in resistant mice, i.e., C57BL/6 (H-2^b). During infection BALB/c mice exhibit a Th2-like response (high IL-4 and IgE), whereas C57BL/6 mice produce a Th1-like response (high IFN-γ and strong DTH).[90] Treatment of BALB/c mice with IL-4-neutralizing antibodies beginning at the time of infection allowed these mice to heal and develop effective immunity.[183] In contrast, injection of anti-IFN-γ antibodies in resistant mice abrogated the natural resistance to infection with *L. major*.[14] These studies indicated that IL-4 (Th2 cells) and IFN-γ (Th1 cells) are crucial cytokines (Th subsets) in the defense against *L. major*.

4. Cytokine Profile of $CD8^+$ T Cells

Most of the data discussed above deal with the cytokine profile of $CD4^+$ cells. However, $CD8^+$ cells are also capable of secretion of a variety of cytokines (Table 2). Kelso et al. described $CD8^+$ clones which transcribed and/or secreted any of the cytokines assayed (IL-2, IL-3, IL-4, IL-5, IFN-γ, GM-CSF).[110] However, alloreactive $CD4^+$ T cell clones on average produced higher levels of cytokine mRNA and secreted more protein than $CD8^+$ clones. Fong and Mosmann[67] analyzed a panel of alloreactive $CD8^+$ clones and observed that they all expressed a Th1 cytokine profile and did not secrete IL-4 and IL-5. Most of the $CD8^+$ clones of Kelso et al. secreted a Th1-like profile, but in addition some did transcribe and/or produce IL-4, IL-5, and IL-6. The amount of (Th2) cytokines produced was lower for $CD8^+$ than for $CD4^+$ clones. It was suggested that these observations support the interpretation that the difference between the two types of clones is quantitative rather than qualitative.[109] Experiments of Cardell et al.[25] confirmed this suggestion by demonstrating that $CD8^+$ cells were able to produce the cytokines IL-2, IL-4, and IL-5 in a lower amount as compared to $CD4^+$ cells.

It is clear from the data presented above that both $CD4^+$ and $CD8^+$ cells can have the same cytokine profile. The quantitative differences in cytokine production between $CD4^+$ and $CD8^+$ cells found by several groups could very well be attributed to *in vitro* culture conditions and may not reflect the actual cytokine production of these cells *in vivo*.

5. Type 1/2 Pathway-Regulated Immune Responses

The data discussed above, concerning the Th1/Th2 cell subsets in *in vitro*/*in vivo* immune responses, indicate that such a sharp dichotomy does not exist *in vivo*. It appears that $CD4^+$ and $CD8^+$ cells have in potential an unrestricted cytokine profile. Which cytokines are produced in immune responses is dependent on a variety of factors: genetic make-up of animal; type, dose, route, and nature of antigen; and activity of cytokine-producing cells prior to immunization. In some immune responses IL-4 is the predominant cytokine, such as in helminthic infections in BALB/c mice, and in other immune responses IFN-γ, e.g., *L. major* infection in C57BL/6 mice. In both immune responses the cytokine profiles of T cells can have all possible combinations and the high frequency of either of the two Th subsets found *in vitro* is most likely due to artefacts. The differential secretion of cytokines by $CD4^+$ or $CD8^+$ cells most likely determines their regula-

tory function *in vivo*. Since IFN-γ and IL-4 are representing the cytokines of Th1 and Th2, respectively, and have important antagonistic functions *in vitro* as well as *in vivo*, this distinction is valuable for immunological research. However, since $CD8^+$ cells can have similar cytokine profiles as Th subsets, it is more realistic to speak of type 1 or type 2 pathway-regulated immune responses. Furthermore, the experiments discussed show clearly the necessity of *in vivo* research on the activity and profiles of cytokine-producing cells in immune responses.

VIII. *IN VIVO* IMMUNE RESPONSES IN THE SPLEEN

The responses of lymphocytes to antigen and pathogens have mainly been analyzed *in vitro*. However, whereas cells can interact relatively randomly in cell suspensions, peripheral lymphoid tissues like lymph nodes and spleen are compartmentalized into distinct B and T cell zones. Cells are exposed to the influence of locally present accessory cells, cytokines, and the extracellular matrix. *In vitro* experiments necessarily disregard this complex organization of lymphoid organs. They can provide information about essential events during cell-cell interactions and immune cell activation. They give us an idea about which cells and cytokines might be active during immune responses. The *in vitro* approach can be valuable for the investigation of the effects of specific cytokines on specific cells. As a consequence, lymphoid cell functions and interactions seen *in vitro* reflect the full potential of such cells, but the events actually occurring *in vivo* are dictated and restricted by the lymphoid microenvironment. Therefore, techniques were developed to investigate the localization and activity of antigen, immunocompetent cells, and cytokine-genes/proteins during *in vivo* immune responses. The determination of whole-tissue RNA is a method to demonstrate which cytokine-genes are activated in a particular organ during various immune responses and in this respect will probably reveal data which lie close to those obtained by bulk *in vitro* data. Immunohistochemistry or *in situ* hybridization allows the *in vivo* study of the specific localization and activity of various cells and cytokines in relation to their direct environment or cell-cell interactions in all types of tissue at any given moment.

A. *In Vivo* Cytokine Gene Expression

Although it is not certain that cytokine mRNA levels are directly correlated with rates of cytokine secretion, because of possible variations in the rates of cytokine mRNA translation, they give an indication about the timing and intensity of cytokine gene expression in immune responses. Gauchat et al.[74] found a good correlation between the percentage of cells containing mRNA for a cytokine and the steady-state level of the same mRNA measured on the whole population of peripheral blood mononuclear cells, suggesting that changes in the production of cytokines mainly reflects differences in the frequency of cells that are recruited for synthesis of these cytokines. Kelso et al. found that protein production and mRNA of IFN-γ or IL-4 of stimulated T cell clones was not strongly correlated.[110] The lack of correlation could be due to differences in translational or secretory regulation of protein production, of which the latter was observed in alveolar macrophages.[8] Resting alveolar macrophages and activated macrophages were found to have similar β-transforming growth factor gene expression, while the activated macrophages produced significantly higher amounts of this cytokine.[8] Moreover, lack of correlation could also be attributed to clonal heterogeneity in the kinetic relationship between mRNA accumulation and protein secretion. Thus, data concerning cytokine-gene expression should be interpreted carefully and require additional experiments for the determination of actual protein activity.

1. Cytokine Gene Expression during Model Immune Responses

Using the reverse transcriptase polymerase chain reaction, Svetic et al. examined the gene expression of seven cytokines in spleens of mice

injected with goat antibodies to mouse IgD (GαIgD).[200] This treatment gives an *in vivo* polyclonal activation stimulus, leading to the production of large amounts of IgG1 and IgE,[57] Ig isotypes whose production depends largely upon the presence of IL-4.[42,228] The injected GαIgD antibody binds to and crosslinks mouse B cell membrane IgD, activating the B cells.[55] GαIgD is internalized and processed, and presented to goat-specific T cells, leading to a massive T cell activation. At day 2 an increase was observed of the gene expression of IL-2 and IL-9, which reached maximum values 3 d after GαIgD injection; IL-4 and IL-6 gene expression peaked between days 3 and 6 at values more than 100-fold over baseline. IL-10 Gene expression showed a small peak at day 1 and subsequently followed a similar pattern as observed for IL-4. IFN-γ Increased at day 3 and reached peak levels 5 to 7 days after GαIgD injection. The sequential production of cytokine mRNA of IL-2 and IL-4 demonstrated in the GαIgD model was also observed by Mohler and Butler in lymph nodes after sensitization with picril chloride.[147] The observed early IL-2 activity in both systems and the fact that IL-2 is a T cell growth factor,[214] suggest that IL-2 stimulates the proliferation of T cells producing cytokines like IL-4 and IFN-γ which in their turn act as differentiation factors in these model immune responses. Cytokine gene expression analysis of isolated $CD4^+$ cells from GαIgD-treated mice revealed that $CD4^+$ cells were the main source of most of the cytokines examined. In contrast, only the gene expression of IL-6 and IFN-γ was detected in the CD4-negative spleen cell population. One of the limitations of this approach is its inability to determine cytokine profiles of individual cells. Nevertheless, as the genes of the IL-2 and IFN-γ, which are both Th1 cytokines, were found to be expressed at different time points, and the gene expression of Th1 (IFN-γ) and Th2 (IL-4, IL-10) were elevated at about the same time, it is evident that the observed cytokine gene expression patterns were not consistent with the Th1/Th2 dichotomy.[154] These results confirm recent findings which showed that different T cell clones isolated relatively early in an immune response produce every possible combination of cytokines.[65,110]

2. Cytokine Gene Expression during Infectious Diseases

Poston and Kurlander investigated the activity of IFN-γ in murine spleens during primary infection with *Listeria monocytogenes*.[177] Analysis of IFN-γ mRNA revealed high expression 1 d after infection, followed by a gradual decline. IFN-γ serum levels developed according to a similar pattern as observed for the IFN-γ gene expression. The spontaneous IFN-γ release by isolated splenocytes showed high levels only at day 1. However, when cells were cultured for 48 h with heat-killed *Listeria monocytogenes*, a strikingly different release pattern of IFN-γ was observed *in vitro*, with maximum levels at 6 d after infection. This peak probably reflects the clonal expanded *Listeria monocytogenes*–specific memory T cells which produce high levels of IFN-γ upon stimulation with antigen, but does not demonstrate the actual frequency of active IFN-γ-producing cells. *In vivo*, these potential IFN-γ-producing cells will only be active after encountering *Listeria monocytogenes*-infected macrophages. These findings demonstrate clearly the potential limitations of *in vitro* assays as tools for the determination of *in vivo* cytokine production. The role of IFN-γ in *L. monocytogenes* infection is probably the recruitment and activation of macrophages early during listeriosis. As maximal bacterial destruction is observed 5 to 7 d after infection, these data strongly suggest that IFN-γ in itself cannot be the crucial factor during the T cell-dependent resolution of infection. Alternatively, the *in vivo* localization of these potential IFN-γ-producing cells in relation to *L. monocytogenes*–infected macrophages might be more important than their quantity. In that case, IFN-γ could still be crucial for the reduction of bacterial load, as suggested by *in vivo* modulation studies.[22] Poston and Kurlander[177] did not determine the source of IFN-γ, but based on the observed early IFN-γ activity the authors suggested that NK-like lymphocytes could be the early producers of IFN-γ during listeriosis. Recently, Hiromatsu et al. demonstrated that γ/δ T cells, producing IFN-γ, appeared at a relatively early stage of listeriosis.[92] Depletion studies revealed that γ/δ T cells were the principal cells in

host defense at the early stage of infection, whereas the α/β T cells contributed to the host protection at the stage after *L. monocytogenes* infection. All together, these data suggest that the early-appearing γ/δ T cells play a role, at least in part by secreting IFN-γ, in the primary host defense against *L. monocytogenes*.

Heinzel et al. determined mRNA levels of various cytokines from lymphoid organs of susceptible BALB/c and resistant C57BL/6 mice after *Leishmania major* infection.[90] IL-2 mRNA levels in infected organs of BALB/c and C57BL/6 mice were similar after infection, but IFN-γ and IL-4 mRNA levels were reciprocally expressed. IL-4 mRNA levels in BALB/c mice were high and possibly the cause of susceptibility of these mice to *L. major*. Treatment of BALB/c mice with anti-IL-4 antibodies significantly attenuated the progression of leishmaniasis. Therefore, it was postulated that IL-4 might inhibit the development of IFN-γ-producing cells (Th1 cells) which are crucial in the defense against *L. major*. IFN-γ mRNA level was high in C57BL/6 mice and was in agreement with *in vivo* studies which demonstrated that IFN-γ treatment improves the course of *L. major* infection in mice. Finally, T cell subset (CD4/CD8) depletion studies suggested that $CD4^+$ cells were the principal producers of IFN-γ and IL-4 in C57BL/6 and BALB/c mice, respectively. This *in vivo* study demonstrated the existence of an IFN-γ/IL-4 dichotomy rather than a Th1/Th2 dichotomy in chronically infected mice, as IL-2 mRNA showed the same level of activity in both mice. Type 1-pathway regulation of *L. major* infection prevented the progression of disease, whereas type 2-pathway regulation led to dissemination of the parasite and eventually to death.

Kasaian and Biron investigated the activity of IL-2 mRNA in splenocytes during lymphocytic choriomeningitis virus (LCMV) infection.[104] They demonstrated that the time course, peaking at day 7, of IL-2 transcription corresponded to CTL activation, CD4/CD8 proliferation, and to the production of IL-2 by splenocytes. It was demonstrated that IL-2 was essential for the expansion of CD4- and CD8-positive cells. Furthermore, $CD4^+$ and $CD8^+$ cells were both producing IL-2 during this virus infection, indicating that cytotoxic $CD8^+$ cells can produce their own helper factors. This study demonstrated that IL-2 is active *in vivo* during LCMV infection and supports the suggestion that IL-2 mediates CTL proliferation.

B. *In Vivo* Isotype Switching

Several different *in vitro* models have been used to study the effects of cytokines on proliferation and differentiation of B cells[190] (Table 3). Snapper and Paul demonstrated a central role for IL-4 in the regulation of Ig isotype secretion by LPS-stimulated B cells *in vitro*;[189] IgG1 and IgE was induced by IL-4 in a concentration-dependent manner, while IgM, IgG2b, and IgG3 secretion was decreased. In contrast, IFN-γ stimulated the expression of IgG2a and inhibited the production of IgG3, IgG1, IgG2b, and IgE.[189] IL-5 was found to enhance proliferation/differentiation of B cells into IgM- and IgG1-secreting cells.[16] IL-2 was also found to enhance B cell proliferation following LPS and anti-Ig treatment *in vitro*.[235] McHeyzer-Williams demonstrated that the isotypes secreted by LPS-stimulated B cells could be regulated using combinations of IL-2, IL-4, and IL-5 that were acting in synergy.[142] All these *in vitro* observations so far do not prove that these cytokines were active or had similar effects during *in vivo* antibody responses. In *in vitro* experiments cell numbers, cell types, cytokines, and LPS are all chosen arbitrarily. Possibly, a cytokine found to have a particular effect *in vitro* might not be active or present in the necessary quantity at the required place at the right time to have the same effect in an *in vivo* immune response. Moreover, *in vitro* experiments may have omitted stimulating or inhibiting factors that might be simultaneously active *in vivo*. Therefore, *in vivo* validation of *in vitro* observations is required to delineate the precise role of cytokines in immune responses. Such *in vivo* validation studies have been performed for IL-4 and IFN-γ in several models.[60] The investigation of the actual *in vivo* participation of cytokines in immune responses has been discussed in the previous section. Below we will discuss some *in vivo* modulation studies that are dealing with the *in vivo* role of cytokines in isotype selection.

Eight days after injection of anti-IgD antibodies as many as one third of spleen cells secrete

IgG1 and a 100-fold increase of serum IgG1 and IgE levels is observed.[55] These *in vivo* data suggest that IL-4 may be a crucial cytokine in the upregulation of IgG1 and IgE antibody responses. When mice were injected with GαIgD and simultaneously received anti-IL-4[56] or anti-IL-4 receptor antibodies,[62] an almost complete inhibition of IgE production was observed. Furthermore, it was demonstrated that anti-IL-4 antibodies also inhibited primary *in vivo* polyclonal IgE responses by 99% in mice infected with *Nippostrongylus brasiliensis*, indicating that in these models IgE production is also IL-4 dependent *in vivo*. However, neither anti-IL-4 nor anti-IL-4 receptor significantly inhibited the IgG1 responses generated 8 d after GαIgD injection. These observations indicate that IgG1 production in GαIgD-stimulated mice, unlike IgG1 production in some *in vitro* experiments, must be largely independent of IL-4 *in vivo*. *In vivo* modulation studies of Finkelman et al. revealed that between day 3 and day 5 after immunization T cell help, in general, and IL-4 in particular, was required for the generation of an IgE response.[59] This was in agreement with our *in vivo* study, in which the presence of high numbers of IL-4-producing cells in the spleen was demonstrated 3 and 4 d after injection of RαIgD.[219]

In vitro studies have demonstrated that IFN-γ can inhibit IgE and IgG1 responses and promote IgG2a responses. Finkelman et al. tried to verify these *in vitro* observations *in vivo*.[58] GαIgD-treated mice were injected with high doses of IFN-γ, which led to a significant decrease of IgE and IgG1 serum levels, whereas IgG2a serum levels were increased. Injection of anti IFN-γ-antibodies in GαIgD-treated mice led to an increase of IgG1 and IgE serum levels, indicating that IFN-γ was produced endogenously. This was in accordance with our experiments, in which we demonstrated immunohistochemically that IFN-γ was active, though to a lesser degree than IL-4, in the immune response to RαIgD. Killed and fixed *Brucella abortus* stimulate a polyclonal IgG2a response, but relatively little or no IgE or IgG1. Treatment of mice with anti-IFN-γ antibody considerably, but not completely, inhibited the *B. abortus*-induced IgG2a response.[58] This *in vivo* modulation study indicated that IFN-γ is one of the principal cytokines that regulate IgG2a production by B cells in the response to *B. abortus*.

From the above data, the following conclusions can be drawn. First, IL-4 and IFN-γ are the principal cytokines that regulate *in vivo* IgE production by B cells. Second, *in vitro* data can be obtained under more controlled conditions but may not always reflect the actual *in vivo* situation.

IX. In vivo cell-cell interactions and cytokine production in the spleen

In this review, the immune cells and cytokines that are assumed to be involved in immune responses were discussed. Furthermore, the characteristic localization of T cells, B cells, macrophages, DC, antibody-forming cells and other immune cells was described. However, data presented do not show whether, when and where these immune cells are active during *in vivo* antibody responses.
These questions are addressed in this thesis. The development, localization and role of immune cells during various types of antibody responses are investigated *in vivo*. The localization of injected antigens is related to the type of antigen and to the cells in the spleen involved in the handling and processing of antigens.
It is investigated whether and where activated T cells, expressing gp39, are found during TI-2 or TD antibody responses. The localization and kinetics of development of $gp39^+$ cells is related to that of antigen, resting/activated B cells and antigen-specific B cells. The cells expressing gp39 are characterized *in vivo*, and the role of gp39 in *in vivo* TI-2 and TD antibody responses is assessed. Furthermore, it is investigated whether $gp39^+$ cells are able to produce cytokines *in vivo*. Methods are developed for the *in situ* detection and characterization of cytokine-producing cells (IL-2-, IL-4-, IL-5-, and IFN-γ-PC), which are suggested to have an important regulatory role in B cell differentiation. The localization and development of these cytokine-PC is related to that of antigen, $gp39^+$ cells, resting-, activated-, antigen-specific B cells and to the isotypes produced by these B cells. Finally, functional studies are performed to the role of cytokines during the development of *in vivo* antibody responses.
The main points emerging from the experimental studies will be related to the *in vitro* and *in vivo* data dealing with antigen-presentation, localization/migration of immune cells, cell-cell interactions and the immunoregulatory role of cytokines, as was discussed in this chapter. Finally, *in vivo* models are presented which show the cell-cell interactions that occur in the spleen during TD and TI-2 antibody responses.

RαIgD	Rabbit antibodies anti-IgD
SRBC	Sheep red blood cell
Th	T Helper
TD	Thymus dependent
TI-1 (2)	Thymus-independent type 1 (2)
TNP	2,4,6-trinitrobenzenesulfonic acid
TNP-HES	TNP-Hydroxyethyl starch

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ABBREVIATIONS

APC	Antigen-presenting cells
BCG	Bacille Calmette Guèrin
BSA	Bovine serum albumin
BGG	Bovine γ-globulin
CTL	Cytolytic T cell
DC	Dendritic cell
DNP	2,4-Dinitrophenyl
DTH	Delayed-type hypersensitivity
FDC	Follicular dendritic cells
FITC	Fluorescein isothiocyanate
GαIgD	Goat antibodies anti-IgD
h	Hour
HSA	Human serum albumin
Ia	Major histocompatibility complex class II
IDC	Interdigitating cells
Ig	Immunoglobulin
IL	Interleukin
IFN	Interferon
KLH	Keyhole limpet hemocyanin
LCMV	Lymphocytic choriomeningitis virus
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
min	Minutes
mIg	Membrane immunoglobulin
NK	Natural killer cell
NIP	4-Hydroxy-5-iodo-3-nitrophenyl
OVA	Ovalbumin
PALS	Periarteriolar lymphocyte sheath

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CHAPTER 3

Localization of thymus-independent antigens in the spleen

3.1 Complement mediated follicular localization of T-independent type 2 antigens: the role of marginal zone macrophages revisited

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3.2 Differential uptake and trapping of TI-2 antigens: Unexpected role for follicular dendritic cells in the induction of TI-2 immune responses

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Complement-mediated follicular localization of T-independent type-2 antigens: the role of marginal zone macrophages revisited

In this study we demonstrate a hitherto undescribed phenomenon, namely that thymus-independent type-2 antigens (TI-2 Ag) localize in splenic follicles within 1 h after administration. The follicular localization of 2,4,6-trinitrophenyl (TNP)-Ficoll was not antibody mediated. In addition in case of high-dose administration we observed a relatively large amount of TI-2 Ag in marginal zone macrophages. However, after low-dose administration we observed a preferential localization of TNP-Ficoll in the splenic follicles. Detection of TNP-haptenated Ag in cryostat sections of murine spleens was performed with a high-affinity TNP-specific monoclonal antibody conjugated to β-galactosidase. Within minutes after injection the TI-2 Ag localized in the marginal zone, attached to marginal zone macrophages and B cells. Twenty minutes after injection the Ag was also detected in the follicles and gradually accumulated there until 7 h after injection. Thereafter, the amount of follicular Ag gradually decreased but was still detectable up to 14 days after immunization. The follicular localization of TNP-Ficoll was complement dependent in contrast to the binding to and uptake by marginal zone macrophages. Double staining revealed that Ag was bound by macrophages, B cells and follicular dendritic cells. Haptenated thymus-dependent (TD) Ag localized exclusively in the red pulp macrophages. *In vivo* macrophage elimination drastically increased the amount of TNP-Ficoll in the follicles, and enhanced the humoral immune response at low doses of Ag. Moreover, complement deprivation of mice abrogated the localization of TI-2 Ag in the follicles, and led to a decreased humoral TI-2 immune response. In conclusion, we demonstrate for the first time that TI-2 Ag localize in follicles. Moreover, the presented results provide further evidence that B cells and follicular localized Ag play an important role in the induction of humoral TI-2 immune responses.

1 Introduction

The spleen plays an important role in the protection against, *e.g. Streptococcus pneumoniae, Neisseria meningitides* and *Haemophilus influenzae* [1]. Following splenectomy patients are at high risk for fulminant infections due to these bacteria. The protective immune response against these micro-organisms is directed mainly against the polysaccharide component of the bacterial capsule, classified as a thymus-independent type-2 (TI-2) Ag [2]. The presence of the spleen seems to be important in the primary encounter of the Ag whereas secondary humoral immune responses can also be evoked at sites outside the spleen [3]. This has led to the suggestion that in the spleen, specific subsets of B cells may be present or that the splenic microenvironment as such may be crucial for the response of B cells to TI-2 Ag. In particular, the marginal zone macrophages of the spleen have received much interest in this respect. These macrophages, which take up and retain carbohydrate macromolecules such as FITC/TNP-Ficoll (model TI-2 Ag), have been suggested to play a role in the processing and presentation of TI-2 Ag [4–7]. However, after *in vivo* elimination of marginal zone macrophages we observed no change in the humoral immune response to TI-2 Ag [8–11]. In spleen autotransplantation studies we found that the humoral immune response was already restored after 4 weeks, despite the fact that the marginal zone macrophages did not return until 10 weeks after transplantation, indicating that these cells are not obligatory in the humoral TI-2 immune response [8]. Furthermore, blocking of polysaccharide uptake by marginal zone macrophages did not affect TI-2 immune responses [9]. These studies demonstrated that marginal zone macrophages do not play a major role in humoral immune responses against TI-2 Ag. In case of low-dosage immunization with TNP-Ficoll we observed an unexpected relative increase of the immune response after elimination of marginal zone macrophages, suggesting that the role of these macrophages was Ag removal rather than Ag presentation [12]. These results led to the suggestion that other cell types then the marginal zone macrophages were involved in the presentation of TI-2 Ag. In a parallel line of research we developed a high-affinity mAb directed to TNP. This mAb conjugated to β-galactosidase was found to be extremely suitable for the immunohistochemical detection of very low amounts (3 μg/mouse) of injected trinitrophenylated Ag as compared to fluoresceinated Ag [4–7, 9]. This conjugate enabled us to re-

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Abbreviations: **CVF:** Cobra venom factor **FDC:** Follicular dendritic cells **HRP:** Horseradish peroxidase **PAP:** Peroxidase anti-peroxidase **TD:** Thymus dependent **TI-2:** Thymus-independent type 2

evaluate the localization of TI-2 and consequently to identify other cell types which might be involved in the presentation of TI-2 Ag.

2 Materials and methods

2.1 Animals

BALB/c and BCBA.F_1 mice were bred at TNO, Rijswijk, The Netherlands, and were used at 12–16 weeks of age. CBA/N mice were obtained from Bomholtgård, Ry, Denmark. Rabbits (Flemish Giant) were randomly bred at TNO and were used as rabbit RBC donors.

2.2 Chemicals

Alkaline phosphatase (P-6774, type VII-T, 1020 U/mg protein), 3-amino-9-ethylcarbazole (A-5754), Fast blue BB Base (F-0125), levamisole, MHS (maleimidohexanoyl-n-hydroxysuccinimide ester, Pierce, Rockford, IL), naphthol AS MX phosphate (3-hydroxy-2-naphtoic acid, 2,4-dimethyl-anilide), TNP sulfonic acid (TNBS, grade I) were obtained from Sigma, St. Louis, MO. β-Galactosidase (*E. coli*-derived β-D-galactoside galactohydrolase, molecular weight 540 000) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were obtained from Boehringer-Mannheim, Mannheim, FRG. All reagents used were analytical grade or better.

2.3 Ag

TNP-Ficoll, TNP-KLH, TNP-BSA, TNP-chicken gammaglobulin and TNP-lysine were prepared as previously described [13]. TNP-hydroxyethyl starch (TNP-HES) was a kind gift from the late Prof. Dr. J. H. Humphrey, Royal Post-graduate Medical School, London, GB [7].

2.4 Reagents

The murine mAb SH4.1C9, directed against TNP, has been described before [14]. The rat mAb MOMA-2, which recognizes monocytes and macrophages [15] was a kind gift of Dr. G. Kraal, Free University, Amsterdam, The Netherlands. Rabbit anti-rat Ig conjugated to horseradish peroxidase (HRP), rabbit anti-mouse Ig conjugated to HRP, and rabbit Ab against peroxidase complexed with peroxidase (PAP) were obtained from Dakopatts, Copenhagen, Denmark. The affinity chromatography-purified anti-TNP mAb (SH4.1C9) was conjugated to β-galactosidase according to the procedure described by Deelder and De Water [16].

2.5 Experimental design

To study the localization of TNP-Ficoll in murine spleens, BALB/c mice were immunized i.v. with 100 μg TNP-Ficoll in 200 μl PBS. Mice were anesthetized and killed at various time intervals: immediately and 5, 10, 20 and 40 min, 2 and 7 h, 1, 2, 3, 4, 5, 6, 7, 10, 14 and 21 days after injection of Ag. To study the detection limit of injected TNP-Ficoll, BALB/c mice were injected i.v. with 0, 1, 3, 6, 13, 25, 50 and 100 μg TNP-Ficoll and killed after 7 h. To investigate localization of injected TNP-Ficoll in CBA/N and BCBA.F_1 mice, mice were injected i.v. with 100 μg TNP-Ficoll and killed 7 h after injection. To study the role of route of administration on TNP-Ficoll localization, BALB/c mice were injected i.p. or s.c. with 100 μg TNP-Ficoll and killed after 7 h. To study the localization of various TI and TD Ag, BALB/c mice were injected i.v. with either 100 μg TNP-HES, TNP-KLH, TNP-BSA, TNP-chicken gammaglobulin or TNP-lysine and killed after 7 h. To rule out that TNP-cross-reacting Ab were mediating TNP-Ficoll localization, BALB/c mice were injected i.v. with either 2 mg TNP-lysine, TNP-BSA or PBS, 3 h before i.v. injection of 50 μg TNP-Ficoll. Mice were killed 4 h later.

BALB/c mice were injected i.v. with 1 mg PAP. After 3 h mice were injected with 50 μg TNP-Ficoll and subsequently killed 7 h later. Spleens and liver were removed and immediately frozen in liquid nitrogen and stored at −70 °C if not used immediately.

2.6 C depletion and immunization

To investigte the involvement of C in the localization of TNP-Ficoll in murine spleens, BALB/c mice were injected i.v. twice with 2 μg cobra venom factor (CVF; Sigma) in PBS with an 8-h interval [17]. TNP-Ficoll (10 μg or 100 μg) was administered 16 h after the last CVF injection. Control mice received heat-inactivated CVF (65 °C, 60 min). Seven hours after TNP-Ficoll injection mice were bled and killed. Spleen and liver were removed and immediately frozen in liquid nitrogen and stored at −70 °C. After clotting of the blood at 20 °C for 1.5 h, serum was separated by centrifugation and subsquently stored at −70 °C until use. Determination of the alternative C pathway activity was performed according to the procedure described by Van Dijk et al. [18], using rabbit RBC as indicator cells. Alternative C pathway activity was expressed in AP_{50} U/ml, in which 1 AP_{50} U causes 50% lysis of the rabbit RBC.

2.7 Macrophage elimination with dichloromethylene-phosphonate (Cl_2MDP)-containing liposomes

Cl_2MDP-containing liposomes were used to eliminate macrophages *in vivo* and were prepared as described earlier [11, 19]. Two days after injection of (empty or Cl_2MDP) liposomes, 50 μg TNP-Ficoll was injected i.v. and mice were killed 7 h later. Macrophage elimination in the spleen was confirmed by acid phosphatase staining and with the mAb MOMA-2, as described previously [20].

2.8 Immunohistochemistry

Cryostat sections (−20 °C, 8 μm) of spleens, one of every mouse, were picked up on the same glass slide and kept overnight under high humidity at room temperature. Slides were air dried and stored in air-tight boxes at 4 °C until use. Cryostat sections were fixed for 10 min in acetone (p.a.) containig 0.02% H_2O_2 (freshly prepared). Subsequently slides were rinsed with PBS and incubated horizontally overnight at 4 °C with the anti-TNP mAb conjugated to

β-galactosidase. For double staining for macrophages and Ag, acid phosphatase activity was revealed, prior to incubation with the anti-TNP-β-galactosidase conjugate. Acid phosphatase activity was demonstrated by incubation with naphthol AS-BI phosphate and p-rosaniline for 30–45 min at 37 °C [10]. Staining with the mAb MOMA-2 was performed as previously described [20]. For double staining for B cells and Ag, sections were incubated overnight with HRP-rabbit anti-mouse Ig, followed by HRP histochemistry. Thereafter, detection of Ag was performed. To detect PAP complexes [21], sections were fixed in acetone without H_2O_2 followed by peroxidase staining. Detection of TNP-specific antibody-forming cells (AFC) was performed with TNP-alkaline phosphatase conjugates according to Claassen et al. [13]. All reagents were diluted in PBS containing 0.1% BSA and titrated to obtain optimal results. Histochemical revelation of the HRP activity was demonstrated as previously described [22]. β-Galactosidase activity was demonstrated according to the procedure described in Bondi et al. [23]. Sections were rinsed with PBS, counterstained with hematoxylin and mounted in glycerin-gelatin.

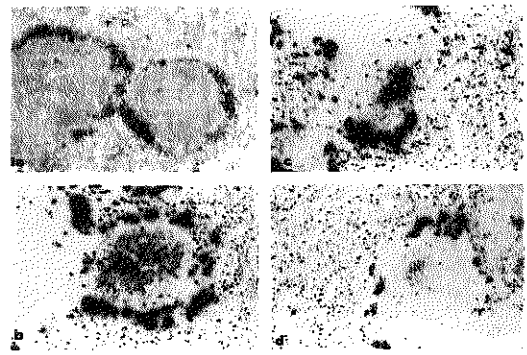

Figure 1. Localization of TNP-Ficoll in representative spleen sections of mice at various time points after i.v. injection of 100 μg TNP-Ficoll. Detection of TNP-Ficoll was performed with TNP-specific mAb conjugated to β-galactosidase. (a) 5 min, TNP-Ficoll is only located in marginal zone, (b) 7 h, TNP-Ficoll is located in marginal zone, follicle (maximal amount) and red pulp, (c) 3 days, and (d) 10 days after injection of TNP-Ficoll (see also Table 1).

2.9 Statistical evaluation

Results were analyzed by the two-sample Student's *t*-test for comparison of two empirical means in a normally distributed population.

3 Results

3.1 Localization and kinetics of TNP-Ficoll

We studied the localization of TNP-Ficoll in spleens of BALB/c mice from 5 min until 21 days after i.v. injection of 100 μg TNP-Ficoll (Fig. 1 and Table 1). For a detailed schematic representation of compartments and cell types in the spleen see [24]. Already 5 min after injection, a small amount of Ag was detectable in the marginal zone (Fig. 1a). The amount of TNP-Ficoll increased gradually in the marginal zone and spreaded slowly to the follicular areas. At 2 h after TNP-Ficoll injection all the splenic follicles were heavily loaded with TNP-Ficoll and this remained so for the next 7 h (Fig. 1b). Thereafter, the amount of follicular TNP-Ficoll decreased gradually but was still detectable up to 14 days after injection. Throughout the experimental period (0–21 days) we observed a relatively large amount of TNP-Ficoll in the macrophages of the marginal zone. Up to 40 min after TNP-Ficoll injection, hardly any Ag was detected in the red pulp (Fig. 1a). Subsequently, the amount of Ag in the red pulp increased gradually up to 2 days after injection (Fig. 1b). Thereafter, the localization of TNP-Ficoll in the red pulp decreased gradually and 21 days after injection no Ag was observed in the red pulp.

Table 1. Localization and kinetics of i.v. injected TNP-Ficoll in different compartments of murine spleens

Time[a]	TNP-Ficoll localization			TNP-specific antibodies	
	Marginal zone	Follicle	Red pulp	Follicular complexes	TNP-AFC
5 min	++[b]	–	–	–	–
10 min	+++	–	–	–	–
20 min	+++	±	–	–	–
40 min	+++	+	±	–	–
2 h	++++	+++	++	–	–
7 h	++++	++++	+++	–	–
1 day	++++	+++	++++	–	–
2 days	++++	++	++++	±	+
3 days	++++	++	+++	+	++
4 days	+++	++	+++	+++	++++
5 days	+++	++	+++	++++	++++
6 days	+++	++	+++	++++	++++
7 days	++	++	+++	+++	+++
10 days	++	+	++	++	++
14 days	++	±	+	+	++
21 days	+	–	–	±	++

a) Time after injection of 100 μg TNP-Ficoll (each time point represents three to five mice).

b) Subjective scale for the amount of TNP-Ficoll localization: –, 0%, ±, 0%–5%; +, 5%–25%; ++, 25%–50%; +++, 50%–75%; ++++, 75%–100% of maximal observed amount of TNP-Ficoll in the different splenic compartments.

Table 2. Localization of TNP-Ficoll after injection of different doses in different compartments of murine spleens after various treatments

Treatment[a]	Control		CVF		Cl_2MDP	
Dose TNP-Ficoll (μg)	100	10	100	10	50	5
Marginal zone macrophages	++++[b]	±	++++	±	−	−
B cells	+	±	−	−	−	−
Follicles FDC	++++	++	±	−	+++++	+++
B cells	++++	++	±	−	+++++	+++
Red pulp	+++	−	+++	−	±	−
PALS	−	−	−	−	−	−

a) Treatment of mice: CVF represents C-depleted mice and Cl_2MDP represents macrophage-depleted mice.

b) Subjective scale for the amount of TNP-Ficoll localization: −, 0%; ±, 0%–5%; +, 5%–25%; ++, 25%–50%; +++, 50%–75%; ++++, 75%–100%; +++++, 100%–125% of maximal observed amount of TNP-Ficoll in the different splenic compartments.

To test the detection limit of the anti-TNP conjugate we injected various doses (1–100 μg) of TNP-Ficoll and killed the mice 7 h later. We observed after injection of 3–12 μg TNP-Ficoll a preferential localization of Ag in the follicular areas of the spleen (Table 2). After administration of low doses of TNP-Ficoll we could hardly detect any TNP-Ficoll in the marginal zone and red pulp macrophages. In contrast, after injection of high doses (50–100 μg) of TNP-Ficoll we observed, besides the follicular localization, a high amount of TNP-Ficoll in the marginal zone and red pulp.

Seven hours after i.p. or s.c. injection of 100 μg of TNP-Ficoll we observed a low amount of TNP-Ficoll in the red pulp, while the localization of TNP-Ficoll in the marginal zone and follicles was similar to what we found for i.v. injected TNP-Ficoll. The localization of TNP-Ficoll in spleens of BCBA.F_1 and CBA/N mice was similar to that described for BALB/c mice.

3.2 Characterization of TNP-Ficoll-binding cells

To detemine which cells were binding TNP-Ficoll, we performed double-staining experiments for TNP-Ficoll and B cells or macrophages (Table 2). Double staining for B cells, using HRP-conjugated Ab against mouse Ig(G + M), and TNP revealed that almost all the Ag was localized in the same areas where B cells were found. The staining of Ig(G + M) in the follicular areas of mice 7 h after TNP-Ficoll injection was of the same intensity as in the follicular areas of PBS-treated mice. However, from 3 to 14 days after injection we observed a more intensive Ig(M + G) staining in the splenic follicles of TNP-Ficoll-treated mice than in control mice (data not shown). The observed staining pattern was typical for immune complexes trapped in the follicles and was most likely caused by anti-TNP Ab bound by TNP-Ficoll localized in the follicles. These results suggest that the follicular localization in the beginning of the immune response is not mediated by TNP-cross-reacting Ab. As soon as TNP-specific Ab are formed they will bind to follicular localized Ag, as we observed 3 days after immunization. In the red pulp and marginal zone we observed TNP-Ficoll-binding cells double staining with anti-Ig(G + M), suggesting that in these compartments B cells were binding TNP-Ficoll.

Double staining for TNP-Ficoll and macrophages, using acid phosphatase or the mAb MOMA-2, revealed that marginal zone macrophages were heavily loaded with TNP-Ficoll. In the white pulp we observed no TNP-Ficoll-binding macrophages at all. This was in contrast to the red pulp where we observed a high frequency of macrophages having TNP-Ficoll in their cytoplasm.

Follicular dendritic cells (FDC) are characterized by their ability to bind immune complexes. This capability can be demonstrated by peroxidase histochemistry of spleens of mice, after injection of PAP complexes [21]. To investigate whether localization of TNP-Ficoll in the follicular areas was similar to the localization of PAP complexes in the spleen, we performed double-staining experiments on spleen sections of mice injected with PAP complexes and TNP-Ficoll for both Ag. We observed a more intense staining of TNP-Ficoll than of PAP complexes in the follicles, but both Ag were confined to the same follicular areas.

3.3 Localization of haptenated TD and TI Ag

To investigate whether the localization pattern of TNP-Ficoll was typical for this TI-2 Ag only, we tested several other (haptenated) TD and TI-2 Ag. Injection of another TI-2 Ag, TNP-HES, resulted in a localization pattern of the Ag similar to that found for TNP-Ficoll. Seven hours after injection of TD Ag like TNP-BSA, TNP-KLH and TNP-chicken gammaglobulin the Ag were mainly found in the red pulp. No haptenated TD Ag was localized in the follicular areas, in contrast to our observations with TNP-Ficoll and TNP-HES. Double staining revealed that the haptenated TD Ag were mainly captured by red pulp macrophages. For TNP-lysine we found a staining pattern comparable to that of haptenated TD Ag. These results demonstrate that the localization pattern of TI-2 Ag in marginal zone, follicles and red pulp of the spleen is typical for this kind of Ag.

3.4 Effect of pre-injection of high doses haptenated (non-TI) Ag on localization of TNP-Ficoll

In non-immunized mice a proportion of the circulating Ab can cross-react with the hapten TNP [25]. To determine

whether the observed localization pattern of TNP-Ficoll was due to trapping of complexes formed by these TNP-cross-reacting Ab and TNP-Ficoll, we injected mice i.v. with a high dose (2 mg) of TNP-BSA or TNP-lysine, which will bind the majority of TNP-cross-reacting Ab, or with PBS as control. After 3 h we injected i.v. 50 μg of TNP-Ficoll in each pre-injected mouse, which we killed after another 4 h. Staining for TNP revealed that the characteristic staining of TNP-Ficoll localization in splenic follicular areas was similar in TNP-BSA/TNP-lysine pre-injected mice and in control mice. The staining observed had the same intensity, suggesting that circulating TNP-cross-reacting Ab were not mediating the specific localization of TNP-Ficoll in the murine spleen.

3.5 Localization and kinetics of TNP-specific Ab and TNP-AFC

To investigate whether TNP-Ficoll localization was mediated by (circulating or localized) TNP-cross-reacting Ab, we incubated spleen sections of mice from day 0–21 with TNP-alkaline phosphatase conjugates. Up to 2 days after TNP-Ficoll injection we observed no TNP-binding Ab, indicating that no TNP-specific binding sites were available (Figs. 1b and 2a). This provided additional evidence against the assumption that TNP-cross-reacting Ab might mediate the follicular localization of TNP-Ficoll. After 3 days the TNP-binding Ab in the splenic follicle increased gradually together with the number of TNP-AFC in outer periarteriolar lymphocyte sheath (PALS) around the terminal arterioles and in the red pulp (Table 1) and reached maximum levels 4 days after immunization. In serial spleen sections of mice 3–10 days after TNP-Ficoll injection a similar localization of TNP-Ficoll and TNP-specific Ab was observed, indicting that after 3 days complexes (TNP-Ficoll anti-TNP) were present in the follicles (Figs. 1c and 2b). The follicular localization of TNP-specific Ab decreased gradually from 6 days after immunization, but was still demonstrable up to 21 days after injection.

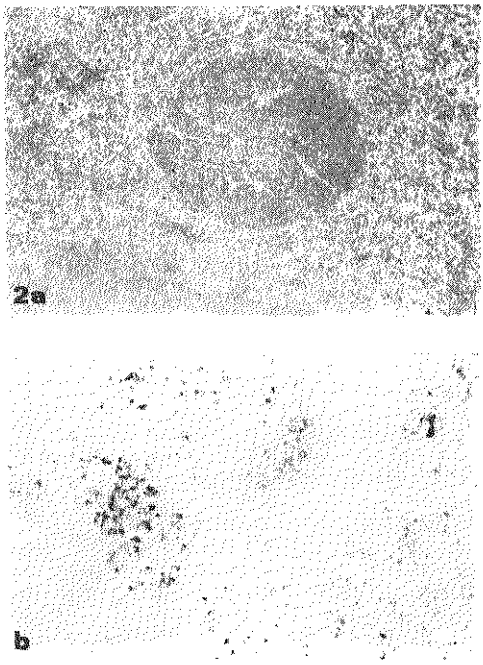

Figure 2. Localization of TNP-specific Ab in representative cryostat sections of murine spleens after injection of TNP-Ficoll. Sections were stained with a TNP-alkaline phosphatase conjugate. (A) No TNP-specific Ab 7 h after TNP-Ficoll injection, whereas in the alternate section (Fig. 1B) the follicle is heavily loaded with TNP-Ficoll. (B) Ab of Ab-Ag complexes are detected in follicle of spleen 3 days after immunization, whereas in Fig. 1C the Ag of Ab-Ag complexes are detected in the same follicle of an alternate section. Note TNP-AFC in outer-PALS, terminal arterioles and red pulp (see also Table 1).

3.6 Effect of C depletion on localization of TNP-Ficoll

Studies with Ag-Ab complexes have already demonstrated that C mediates the follicular localization of such complexes [26]. Moreover, several studies have demonstrated that TI-2 Ag, like polysaccharides, are able to activate the alternative C pathway [27–30]. Consequently, it is conceivable that C mediates the follicular localization of TNP-Ficoll, by forming polysaccharide-C3d complexes. Therefore, we investigated the effect of C depletion, by CVF treatment, on localization of TNP-Ficoll in murine spleens. After injection of 10 μg TNP-Ficoll the typical follicular localization of TNP-Ficoll in spleen sections of CVF-treated mice was totally abrogated. After injection of a high dose of TNP-Ficoll (100 μg) we observed in CVF-treated mice a substantial reduction of the typical follicular localization of TNP-Ficoll in the splenic compartments (compare Figs. 1b and 3, Table 2). C depletion did not affect the capacity of marginal zone macrophages to bind and retain TNP-Ficoll (Fig. 3).

The sera of CVF-treated mice had AP_{50} values below measurable levels (< 3 U/ml, data not shown). The AP_{50} of heat-inactivated CVF-treated mice after injection of 100 μg

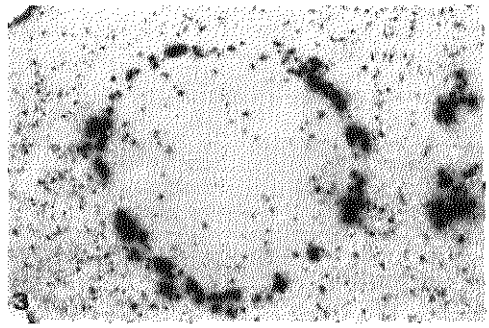

Figure 3. TNP-Ficoll localization in complement depleted mice. TNP-Ficoll localization, detected with TNP-specific mAb conjugated to β-galactosidase, in a representative cryostat section of a spleen of CVF-treated mice 7 h after injection of 100 μg TNP-Ficoll. Note the relatively low amount of follicular TNP-Ficoll as compared to untreated mice (Fig. 1b, see also Table 2).

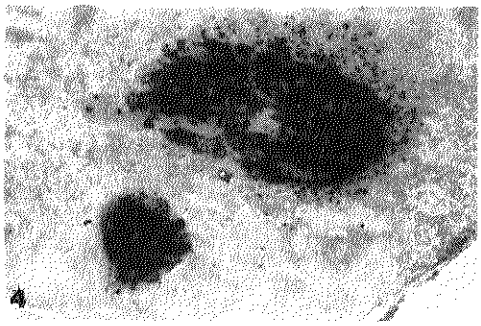

Figure 4. TNP-Ficoll localization in mice depleted of macrophages by Cl_2MDP containing liposomes. 7 h after TNP-Ficoll injection. Detection of TNP-Ficoll was performed with TNP-specific mAb conjugated to β-galactosidase. Note the absence of TNP-Ficoll in the marginal zone and the relatively large amount of TNP-Ficoll in the follicles as compared to untreated mice (Fig. 1b, see also Table 2).

TNP-Ficoll was significantly lower than after injection of 0 or 10 μg TNP-Ficoll, indicating that TNP-Ficoll activates C (Fig. 5). These experiments demonstrate that the follicular localization of TNP-Ficoll in murine spleens is C3 dependent.

3.7 Effect of macrophage elimination on follicular localization of TNP-Ficoll

To investigate whether the presence of macrophages influences the localization of TNP-Ficoll, we treated mice with Cl_2MDP-containing liposomes to eliminate the red pulp and marginal zone macrophages *in vivo*, prior to TNP-Ficoll administration [11]. As demonstrated in Fig. 4 and Table 2, no TNP-Ficoll was present in the marginal zone after elimination of macrophages. Moreover, the amount of TNP-Ficoll in the red pulp was markedly reduced, when compared to control mice. This was in contrast to the follicular localization of TNP-Ficoll, which increased markedly after macrophage elimination. These results

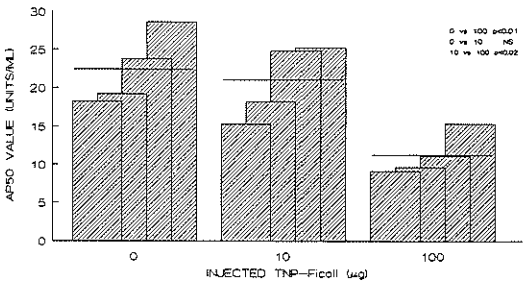

Figure 5. TNP-Ficoll activates C *in vivo*. Alternative C pathway activity of sera of heat-inactivated CVF-treated mice 7 h after injection of 0, 10 or 100 μg TNP-Ficoll. Alternative C pathway activity is expressed as AP_{50} U/ml, in which 1 AP_{50} U causes 50% lysis of the rabbit RBC. Horizontal lines in gate represent means.

suggest that red pulp and marginal zone macrophages form a physiological boundary, which bind part of the injected TNP-Ficoll preventing it to localize in the follicles.

4 Discussion

4.1 General remarks

The major novel finding of this study is that TI-2 Ag localize in splenic follicles within 1 h after administration. After low-dose administration (3–12 μg) TNP-Ficoll localizes preferentially in the follicles, whereas in the marginal zone macrophages hardly any Ag can be detected. We demonstrate that the follicular localization of TI-2 Ag is C mediated and different from the Ab-mediated trapping of Ag, which is observed during the peak of every (TI and TD) humoral immune response. In addition to this new phenomenon we confirmed earlier reports that TI-2 Ag are taken up by marginal zone macrophages, albeit in a C-independent way as could be expected in view of studies on the putative TI-2 Ag receptor [9, 31].

4.2 Follicular localization of TI-2 Ag

This study demonstrates for the first time that TI-2 Ag localize in splenic follicles. Previous studies investigated the localization of TI-2 Ag using fluorescein isothiocyanate conjugates of HES and Ficoll Ag (100 μg or more/mouse) [4–7, 9]. The high affinity for TNP of the mAb we used and the high sensitivity of immunohistochemical techniques [32] allowed us to detect TNP-Ficoll in follicular areas of murine spleens after injection of dosages as low as 3 μg/mouse of this TI-2 Ag.

This early follicular localization of TI-2 Ag can be explained by two mechanisms. First, TI-2 Ag may activate C directly and bind to CR of follicular dendritic cells (FDC) and B cells. Second, TI-2 Ag may bind to cross-reacting Ab and subsequently bind to FcR or after C activation to CR of FDC and B cells. Nossal et al. [33] demonstrated that TD Ag localize in follicles of lymph nodes bound to FDC, by an Ab-dependent mechanism, within minutes after injection. We also demonstrated that at the moment of TNP-specific Ab production these Ab where involved in the follicular localization of TI-2 Ag. However, the early observed follicular localization of TI-2 Ag was found to be C mediated. We demonstrated that a role of TNP-cross-reacting Ab in the early follicular localization of TI-2 Ag was highly unlikely.

Double-staining experiments indicated that part of the injected TNP-Ficoll was bound by B cells in the marginal zone, follicles and later also in the red pulp of the spleen. However, the double staining for B cells and TNP-Ficoll in the follicles could also indicate that TNP-Ficoll was bound to FDC. Double-staining for injeced PAP complexes [21], which bind to FDC, and TNP-Ficoll revealed an identical staining pattern, suggesting that TNP-Ficoll indeed may also be bound by FDC. The C dependency of the follicular localization of TNP-Ficoll indicates that both B cells and FDC may be the TI-2 Ag-binding cells in the follicles, since both cell types have CR [34]. The dendrites of the FDC form an intricate network, called the follicular web, where

follicular B cells lie in close association with FDC. This close contact hampers the immunohistochemical identification of which cells bind Ag in the follicles [33, 35].

The population of CR-positive B cells in CBA/N mice is significantly smaller than in other strains of mice [36]. Injection of TNP-Ficoll in CBA/N mice resulted in a splenic localization of TNP-Ficoll similar to that found in spleens of other strains of mice. If B cells were the principal TNP-Ficoll-binding cells in follicles, we would expect to see no, or at least a lower amount of TNP-Ficoll in splenic follicles of CBA/N mice. Thus, these results are more supportive for FDC rather than B cells as TNP-Ficoll-binding cells in splenic follicles.

4.3 Role of marginal zone macrophages in handling of TI-2 Ag

Double-staining for macrophages and TNP revealed that a part of the injected TNP-Ficoll was detectable in macrophages of marginal zone and red pulp. Macrophage elimination, using Cl_2MDP-containing liposomes, resulted in abrogation of the TNP-Ficoll localization in the marginal zone and a substantial reduction of TNP-Ficoll localization in the red pulp, indicating that macrophages both in the marginal zone and in the red pulp retail TNP-Ficoll. However, the follicular localization of TNP-Ficoll increased markedly after macrophage elimination, indicating that these macrophages prevent TNP-Ficoll to localize in the follicular areas and that they may have a negative influence on the induction of TI-2 immune responses. This supports previous *in vivo* experiments in which after low dose immunization with TNP-Ficoll a relative increase of the humoral TI-2 immune response was observed when the splenic marginal zone macrophages of mice were eliminated [12]. In bacterial infections a primary requirement for protection of the individual is reduction of the number of micro-organisms, *i.e.* Ag load. Elimination may be the task of marginal zone macrophages, while the specific immune response may be initiated by antigen-presenting cells, like FDC and B cells.

Seven hours after injection of high doses of TNP-Ficoll, we observed an intensive staining for TNP-Ficoll in the red pulp, whereas after lower doses of TNP-Ficoll hardly any TNP-Ficoll could be detected in the red pulp macrophages (Table 2). In case of high doses, either administration i.p. or s.c. we also observed a relatively low amount of TNP-Ficoll in the red pulp. This is probably due to the slow release of Ag into the circulation. We conclude that TNP-Ficoll is primarily taken up and retained by marginal zone macrophages, FDC and B cells, while in case of high doses this primary Ag handling system is overloaded, leading to additional TNP-Ficoll uptake by red pulp macrophages.

4.4 Immune response against TI-2 Ag

Matsuda et al. [37] found in CVF-treated mice after the administration of low doses (1–10 μg) Ag an impaired humoral immune response against haptenated TI-2 Ag. However, the immune response against high doses (100 μg) was not affected by CVF treatment, suggesting that C plays only a role in immune responses against low doses TNP-Ficoll. Our C depletion studies revealed that the *in vivo* localization of TNP-Ficoll was mediated by C3. In addition to this, we found that after C depletion and administration of a high dose of TNP-Ficoll (100 μg) a small amount of TNP-Ficoll still localized in the follicles (Figs. 1b and 3, Table 2), whereas C depletion in case of a lower dose (10 μg) of TNP-Ficoll completely abrogated the localization of TNP-Ficoll in the splenic follicles. The lack of follicular Ag localization in CVF-treated animals injected with low doses of TNP-Ficoll thereby may explain the difference in antibody responses after high- and low-dose administration of Ag, as reported by Matsuda et al. [37]. The low amount of TNP-Ficoll found in splenic follicles of CVF-treated mice after a high dose of Ag could be due to binding of TNP-Ficoll to TNP-specific B cells which pass the follicles during their migration throught he spleen [38], or to residual C-binding activity remaining after CVF treatment. Furthermore, C-independent localization of TNP-Ficoll after high-dose injection could also be due to the mechanical trapping by the follicular web formed by dendritic cell processes, as discussed previously for immune complexes [20].

In vivo CR blocking studies demonstrated a marked decrease of the humoral immune response particularly after administration of suboptimal doses (1–10 μg FITC-Ficoll) of TI-2 Ag [39, 40]. These studies indicated that CR of B cells and FDC are important for the generation of an Ab response against TI-2 Ag. A role of C in TI B cell stimulation was already suggested by Pryjma in 1974 [30]. Moreover, several reports have demonstrated that polysaccharides and bacteria are able to activate the C alternative pathway [27–30] and that C3 subsequently is able to activate B cells [41–43]. Our experiments, in which we found that follicular localization of TNP-Ficoll is C dependent and that injection of 100 μg TNP-Ficoll resulted in a significant decrease of the alternative C activity in sera of these mice, also show that C is active during TI-2 immune responses.

These results together with data obtained earlier [8–12] suggest the following sequence of events in the generation of Ab responses against TI-2 Ag. After injection, TI-2 Ag will activate C and subsequently bind to B cells, in marginal zone and follicles, and to FDC. The TI-2 Ag will activate these B cells directly, without requiring a processing step by marginal zone macrophages. Apart from direct B cell activation in the marginal zone as suggested by MacLennan et al. [44, 45], TI-2 Ag will be exposed by FDC to B cells which migrate through the follicles, resulting in activation of part of these B cells. TNP-Ficoll drives these resting TNP-Ficoll binding B cells into the G_1 stage of the cell cycle [41, 42]. These activated B cells will eventually differentiate into TNP-AFC.

In conclusion, the role of marginal zone macrophages is not primarily Ag presentation but rather removal of (soluble) Ag, killing and processing of particulate Ag/bacteria as already has been suggested [46], whereas B cells and FDC are most likely involved in the actual induction of immune responses to TI-2 Ag.

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DIFFERENTIAL UPTAKE AND TRAPPING OF TI-2 ANTIGENS: AN UNEXPECTED ROLE FOR FOLLICULAR DENDRITIC CELLS IN THE INDUCTION OF TI-2 IMMUNE RESPONSES

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INTRODUCTION

The spleen plays an important role in the protection against capsulated micro-organisms e.g. *Streptococcus pneumoniae, Neisseria meningitides and Haemophilus influenzae*[1]. The protective immune response against these micro-organisms is directed mainly against the polysaccharide component of the bacterial capsule, classified as a TI-2 antigen[2]. Following splenectomy patients are at high risk for fulminant infections due to these bacteria. The presence of the spleen seems to be important in the primary encounter of the antigen, whereas secondary humoral immune responses can also be evoked at sites outside the spleen[3]. This has led to the suggestion that in the spleen, specific subsets of B cells may be present or that the splenic micro-environment as such may be crucial for the response of B cells to TI-2 antigens. In particular the marginal zone macrophages of the spleen have received much interest in this respect. These macrophages, which take up and retain carbohydrate macromolecules such as TNP-Ficoll/FITC-Ficoll (model TI-2 antigens), have been suggested to play a role in the processing and presentation of TI-2 antigens[4-7]. However, recent *in vivo* modulation studies demonstrated that marginal zone macrophages are not required in humoral immune responses against TI-2 antigens[8-10].

Evidence for an important role of B cells in TI-2 immune responses was found in experiments with neonatal mice and CBA/N mice, which carry an X-linked immuno-deficiency, which were both unable to mount a humoral response against TI-2 antigens[11]. The B cells of these mice have many properties in common with immature B lymphocytes so that their defect is probably due to immaturity, in case of neonatal mice, or due to a maturation arrest in case of the CBA/N mice. Griffioen et al.[12] showed that polysaccharides, TI-2 antigens, were able to activate complement via the alternative pathway. Moreover, they demonstrated that polysaccharides complexed with C3d, a degradation product of complement, were able to bind to CR2 of B cells. In addition, Carter et al.[13,14] demonstrated synergistic signaling of CR2 and membrane immunoglobulin, as measured by intracellular calcium mobilization. These data provide an alternative activation pathway of B cells in TI-2 immune responses. However, the localization studies for FITC-Ficoll indicated the marginal zone macrophages as the principal TI-2 binding cells in the spleen, which is not consistent with the above discussed (direct) B cell activation models. Therefore, we re-evaluated the localization of TI-2 antigens using a highly sensitive immuno-histochemical approach.

Materials and Methods

Animals BALB/c and BCBA.F1 mice were bred at TNO, Rijswijk, The Netherlands and were used at 12-16 weeks of age. CBA/N mice were obtained from Bomholgård, Rye, Denmark.

Chemicals 3-amino-9-ethylcarbazole (A-5754), TNP sulfonic acid (TNBS, grade I) were obtained from Sigma, St. Louis, MO, USA. MHS (maleimidohexanoyl-n-hydroxysuccinimide ester, Pierce, Rockford, IL, USA); β-galactosidase (E. Coli-derived β-D-galactoside galactohydrolase, MW 540 KD) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were obtained from Boehringer, Mannheim, FRG).

Antigens and bacteria TNP-Ficoll was prepared as previously described[15]. TNP-hydroxyethyl starch (TNP-HES) was a kind gift from the late Prof. Dr. J.H. Humphrey, Royal Post-graduate Medical School, London[7]. Group B streptoccoci (Dr. G. Teti, Istituta di Microbiologia, Messina, Italy) were labeled with 1,1,-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI), a fluorescent lipophylic dye which diffuses into cell membranes[16].

Reagents The murine monoclonal antibody SH4.1C9, directed against TNP, has been described before[17]. The rat mAb MOMA-2, which recognizes monocytes and macrophages was a kind gift of Dr. G. Kraal, Free University, Amsterdam, The Netherlands[18]. Rabbit-horseradish-peroxidase (HRP) anti-rat Ig, rabbit-horse-radish-peroxidase anti-mouse Ig was obtained from Dakopatts, Copenhagen, Denmark. The affinity-chromotography purified anti-TNP mAb (SH4.1C9) was conjugated to β-galactosidase according to the procedure described by Deelder and De Water with some modifications[19,20].

Experimental design BALB/c mice were immunized i.v. with 100 μg TNP-Ficoll in 200 μl PBS. Mice were anaesthetized and killed at various time intervals: immediately and 5 min, 10 min, 20 min, 40 min, 2 hr, 7 hr, 1, 2, 3, 4, 5, 6, 7, 10, 14, 21 days after injection of the antigen.
CBA/N and BCBA.F1 mice were injected i.v. with 100 μg TNP-Ficoll and killed 7 hr after injection.
BALB/c mice were injected i.p./f.p. with 100 μg TNP-Ficoll and killed after 5 hr.
BALB/c mice were injected i.v. with 500 μg DiI labeled Group B streptococci and killed 7 hr after injection. Spleens and lymph nodes (i.c. of footpad immunization) were removed and immediately frozen in liquid nitrogen and stored at -70°C if not used immediately.

Complement depletion and immunization Complement depletion was performed according to Van de Berg et al.[21]. Complement depleted mice were i.v. injected with 10 μg or 100 μg TNP-Ficoll and were bled and killed 7 hr later. Spleens were removed and immediately frozen in liquid nitrogen and stored at -70°C. Complement depletion of serum was confirmed according to the procedure described by Van Dijk et al.[22].

Macrophage elimination with dichloromethylene-phosphonate (Cl_2MDP) containing liposomes Cl_2MDP containing liposomes were used to eliminate *in vivo* macrophages and were prepared as described earlier[23]. Macrophage depleted mice were i.v. injected with 5 or 50 μg TNP-Ficoll and killed 7 hr later. Macrophage elimination in the spleen was confirmed, as described previously[8].

Immunohistochemistry Immunohistochemistry was performed as earlier described[20,24].

TNP-Ficoll serum levels TNP-Ficoll serum levels were determined by ELISA. 96 well plate were coated with purified SH4.1C9 (5 $\mu g.ml^{-1}$) in PBS; then blocked with 0.1% gelatin. Seriel dilutions of mouse sera were added to wells, followed sequentially by Rabbit anti-TNP, Goat anti-Rabbit alkaline phosphatase, and substrate (p-nitrophenyl phosphate). Standard curves were prepared from TNP-Ficoll diluted in normal mouse serum.

RESULTS AND DISCUSSION

We studied the localization and kinetics of TNP-Ficoll in spleens of BALB/c mice from 5 min until 21 days after injection[20] (Fig. 1,2). Already 5 min after injection of 100 μg TNP-Ficoll there was a small amount of antigen detectable in the marginal zone. The amount of TNP-Ficoll gradually increased in the marginal zone and was slowly spreading to the follicular areas. At 2 hr after TNP-Ficoll injection all the splenic follicles were heavily loaded with TNP-Ficoll and this remained so for the next 5 hr. Thereafter, the amount of follicular TNP-Ficoll decreased gradually, but was still detectable 14 days after injection. During the whole experimental period (0 - 21 days) we observed a large amount of TNP-Ficoll in the macrophages of the marginal zone. From 40 min up to 2 days after TNP-Ficoll injection the amount of TNP-Ficoll in the red pulp gradually increased. Thereafter, the localization of TNP-Ficoll in the red pulp decreased gradually and at 21 days after injection no antigen was observed in the red pulp.

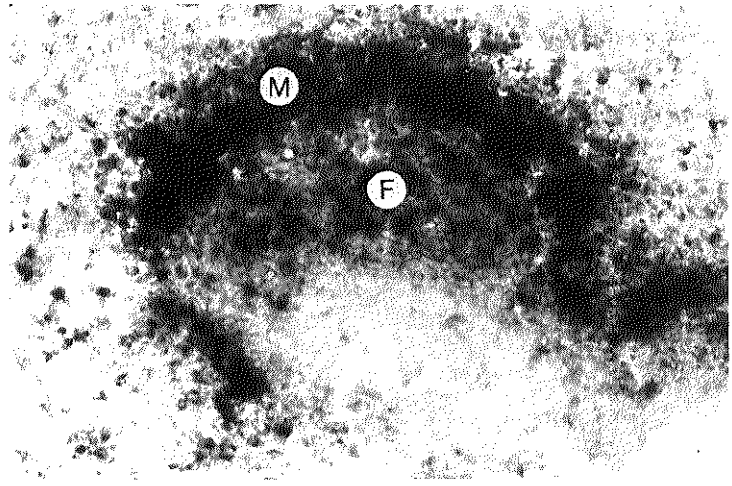

Fig. 1 TNP-Ficoll localization in the spleen 7 hours after i.v. injection. F, follicle; M, marginal zone, P, periarteriolar sheath, R, red pulp.

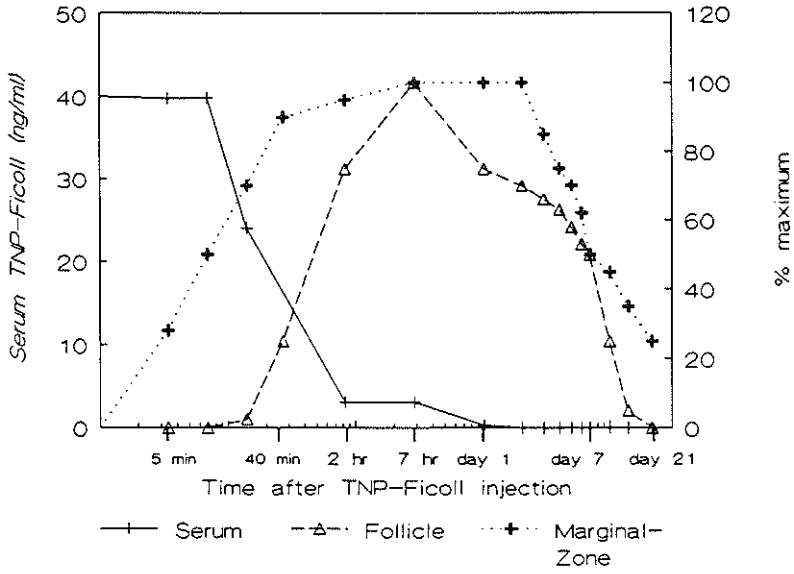

Fig. 2 Kinetics of i.v. injected TNP-Ficoll (100 μg) in sera and in splenic compartments of mice. TNP-Ficoll concentrations of serum were determined by ELISA. The amount of TNP-Ficoll was related to the maximal observed amount of TNP-Ficoll in follicle or marginal zone.

Two hours after injection we observed a striking decrease of TNP-Ficoll serum levels, simultaneously with a marked uptake of TNP-Ficoll by splenic cells (Fig. 2). We found in all tested strains of mice a similar localization pattern as we found in BALB/c mice. In a previous report we ruled out that antibodies were mediating the follicular localization of TNP-Ficoll[20].

Double staining for B cells and TNP-Ficoll revealed that a part of the injected TNP-Ficoll was bound by B cells in the marginal zone, in the follicles and later also in the red pulp of the spleen. However, the follicular localization pattern of TNP-Ficoll could also represent binding of TNP-Ficoll to follicular dendritic cells, as was observed for immune complexes later in the immune response. The close contact of B cells and follicular dendritic cells hampers the immunohistochemical identification of which cells bind TNP-Ficoll in the follicle.

Complement depletion resulted in a total abrogation of follicular TNP-Ficoll after injection of 10 μg of TNP-Ficoll (table 1). After injection of a higher dose of TNP-Ficoll (100 μg) we observed a substantial reduction of follicular TNP-Ficoll. As the population of complement receptor positive B cells in CBA/N mice is markedly lower than in BALB/c mice[25], the similar observed follicular localization pattern after TNP-Ficoll injection together with the complement dependency of this localization are more supportive for follicular dendritic cells than B cells as the TNP-Ficoll binding cells in the follicles.

Double staining revealed that TNP-Ficoll was taken up by the macrophages of marginal zone and red pulp. Macrophage elimination resulted in a drastic decrease of TNP-Ficoll in the marginal zone and red pulp, whereas follicular TNP-Ficoll increased markedly(table 1).

Table 1. Follicular TNP-Ficoll is correlated with humoral TI-2 immune response in case of low dose immunizations

Treatment[a]	CVF		CL_2MDP	
Dose of TNP-Ficoll (μg)	100	10	50	5
Follicular TNP-Ficoll[b]	↓	↓	↑	↑
Humoral immune response	=[26]	↓[26]	=[27]	↑[27]

[a] Treatment of mice: CVF represents complement-depleted mice and CL_2MDP represents macrophage-depleted mice

[b] Follicular localization is related to staining patterns of control

The above described complement depletion and macrophage elimination techniques enabled us to study the role of follicular TNP-Ficoll in the humoral TI-2 immune response (table 1). Matsuda et al.[26] observed in complement depleted mice, corresponding with a decrease in follicular TNP-Ficoll, a significant decrease of the antibody response against TNP-Ficoll in case of immunization with 10 μg of TNP-Ficoll. In addition, we observed in macrophage depleted mice, corresponding with an increase in follicular TNP-Ficoll, a significant increase of the humoral immune response against TNP-Ficoll[27]. These results indicate that the amount of follicular TNP-Ficoll is correlated with the titer of the antibody response against relatively low doses of TNP-Ficoll, suggesting a role for follicular TNP-Ficoll in the induction of TI-2 immune responses.

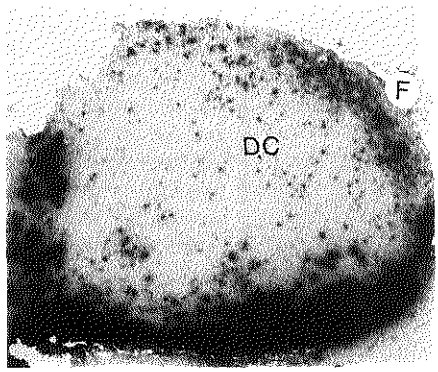

Fig. 3 Localization of TNP-Ficoll (100 μg) in a popliteal lymph node 7 hours after footpad injection. DC, deep cortex; F, follicle.

We also investigated the localization of TNP-Ficoll in popliteal lymph nodes after footpad immunization (Fig. 3). We observed the presence of a large amount of TNP-Ficoll in the subcapsular macrophages and in the macrophages of the medulla. Like in the spleen, we found a high amount of TNP-Ficoll in the follicles of the lymph nodes. Goud et al.[28] demonstrated that antibody responses against TI-2 antigens did not occur in lymph nodes. Since we found that both macrophages and follicular dendritic cells were binding and/or taking up TNP-Ficoll in the lymph node, our results indicate that other factors in the lymph node microenvironment, such as a deficiency of specific cytokine-producing cells and/or specific subsets of B cells/macrophages, may be the cause of this inability.

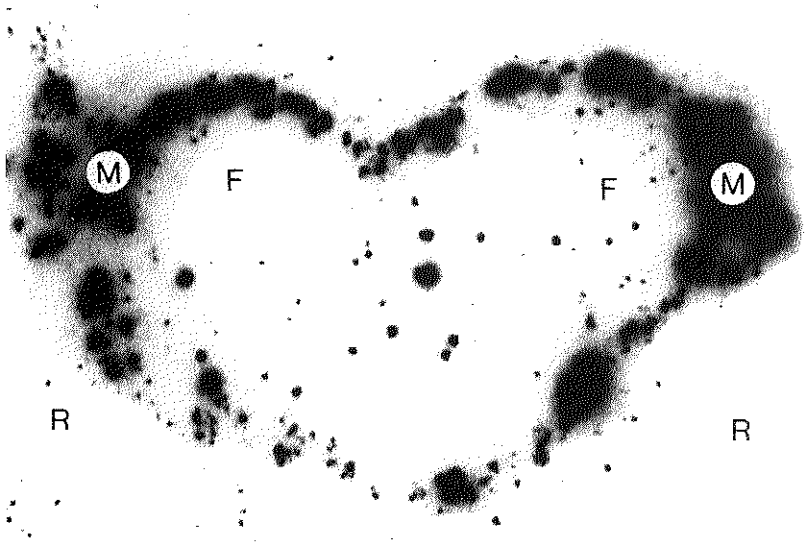

Fig. 4 Localization of Dye I labeled Group B streptococci in the spleen 5 hours after i.v. injection. F, follicle; M, marginal zone; R, red pulp.

After injection of DiI labeled Group B streptococci, a particulate TI-1 antigen, we found that the bacteria localized, like TI-2 antigens, in marginal zone macrophages and to a lower degree in red pulp macrophages (Fig. 4). This is in agreement with experiments of Chao and MacPherson[29], which suggested that the main function of marginal zone macrophages was to clear encapsulated pathogens from the circulation via the TI-2 antigen receptor. We observed hardly any antigen in the follicles, suggesting that only after pre-processing by macrophages fragments of the bacterial capsules may be bound by B cells and follicular dendritic cells[30].

Based on the available data concerning TI-2 immune responses we have postulated a model for TI-2 humoral immune responses[30]. We hypothesize that the role of marginal zone macrophages is not primarily found in antigen-presentation but rather in removal (i.e. of TI-2 antigens), killing and pre-processing of particulate antigen/bacteria, whereas follicular dendritic cells and B cells are most likely involved in the induction of immune responses to TI-2 antigens.

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CHAPTER 4

In vivo detection, kinetics and characterization of IFN-γ-producing cells producing cells during a thymus-dependent immune response: an immunohistochemical study

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In Vivo Detection, Kinetics, and Characterization of Interferon-γ–Producing Cells During a Thymus-Dependent Immune Response

An Immunohistochemical Study

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INTRODUCTION

Interferon-γ (IFN-γ) is a cytokine of central importance in the regulation of the immune system that also displays nonimmune effects (1). The knowledge concerning the mechanisms of production and action of IFN-γ is mainly based on in vitro experiments. This is somewhat surprising considering the fact that in vitro manipulations like cell separation and culture may introduce artefacts and essentially change the microenvironment of the cells. Immunohistochemical techniques, on the contrary, enable the study of the cells in their unchanged original microenvironment, which is of great importance, since regulation of immune responses by cytokines is most likely a local and strongly compartmentalized process.

There are several reports which describe the detection of cytokine-producing cells at the single cell level (2), in human tissues (3–5), and in tissues of mice (6,7) with cytokine-specific antibodies. In the present study, we investigate the potentials of immunohistochemical techniques in the in vivo analysis of a cytokine-mediated immune response. Therefore, we conducted an experiment analogous to the in vitro experiments performed by Wada et al. (8) and in particular with reference to the in vivo activity and cellular source of IFN-γ.

MATERIALS AND METHODS

Animals

Male BALB/c mice were bred at T.N.O., Rijswijk, The Netherlands, and were used at the age of 20–26 weeks.

Bacteria and Mitogen

Live lyophilized BCG (bacille Calmette-Guérin), a kind gift of Dr. I. Claassen (RIVM, Bilthoven, The Netherlands), were killed by exposure to 60°C for 90 min and were suspended in phosphate-buffered saline (PBS). Concanavalin A (Con A) was purchased from Pharmacia, Fine Chemicals Inc., Uppsala, Sweden.

Treatment of Mice

Three groups of mice were injected i.p. with heat-killed BCG (3.3×10^7) in 400 μl PBS, and stimulated 3 weeks later with 200 μg Con A i.v. Subsequently, mice were bled and sacrificed 0, 1.5, 3.0, 4.5, 6.0 hr after Con A stimulation. Three mice were injected i.p. with 400 μl PBS. Subsequently, these mice were stimulated with 200 μg Con A i.v. Control mice were treated with PBS. Mice were bled and sacrificed after 3 hr.

Reagents

DB-1, a mouse antirat monoclonal antibody (MAb) with a high specificity for mouse IFN-γ, was a kind gift of Dr. P. H. van der Meide (9). DB-1 was conjugated to alkaline phosphatase (AP) according to the procedure described by Claassen et al. (10). The rat MAb Lyt-2^+ (CD8) (clone 53.6.7.2) (11), L3T4 (CD4) (clone GK-1.5) (12), and MOMA2 (panmacrophage marker) (13), kindly provided by Dr. G. Kraal (Amsterdam, The Netherlands), were used as cell markers. Rabbit antiasialo GM_1 serum (natural killer [NK] cell) (14) was obtained from WAKO Chemicals GmBh, West Germany. As control antibodies, the mouse MAb directed to human immunoglobulin (Ig) G_1 (clone 3-1.1) (15) and IgG3 (clone 330-2.2) (16) conjugated to alkaline phosphatase (AP) were used. Second-step conjugates were rabbit antirat conjugated to horseradish peroxidase (HRP) (Dakopatts, Copenhagen, Denmark) and goat antirabbit-HRP (Miles-Yeda, Ltd., Rehovot, Israel).

Immunocytochemistry

Spleens were removed and snap-frozen in liquid nitrogen. Cryostat sections (8 μm) were fixed for 10 min in fresh acetone, containing 0.02% H_2O_2. Slides were rinsed with PBS and incubated overnight at 4°C with Db-1-AP. For the double-staining experiments, Db-1-AP was simultaneously incubated with one of the above-described reagents. Slides were again washed with PBS and incubated for 30 min at room temperature with second-step conjugates. Sections were washed three times with PBS and histochemical revelation of AP was performed prior to the histochemical revelation of HRP, as described previously (10). Slides were counterstained with hematoxylin for 15 sec, and mounted in glycerol/gelatin.

IFN-γ ELISA

Serum samples were assayed for IFN-γ according to the procedure described by Cherwinski et al. (17).

RESULTS AND DISCUSSION

We investigated cryostat sections of spleens of three groups of BALB/c mice after priming with heat-killed BCG and stimulation with Con A. After incubation with DB-1-AP and histochemical revelation the IFN-γ–producing cells characterized by a blue cytoplasm, were detected (Fig. 1a). No staining was seen in control slides from any specimen treated by omission or substitution of primary antibody. Preincubation of DB-1-AP with IFN-γ completely abolished the cytoplasmic staining. The IFN-γ–producing cells were found in small clusters of two to five and were located in the T-cell–dependent areas like the periarteriolar lymphocyte sheaths and the coaxial sheaths of lymphoid tissue surrounding the terminal arteriolar branches (Fig. 1b). Analysis of the kinetics of the IFN-γ–producing cells showed that the maximum number of IFN-γ–producing cells was detected in spleens of BCG-primed mice that were not stimulated with Con A (Fig. 2). The IFN-γ–producing cells in the Con A–stimulated and BCG-primed mice had an enlarged cytoplasm, staining more intensively than in the unstimulated BCG-primed mice, suggesting an increased IFN-γ production. In unprimed mice there were hardly any IFN-γ-PC detectable at 0 and 3 hr after Con A stimulation. The kinetics of the IFN-γ serum levels and of the IFN-γ–producing cells developed according a similar pattern after the first 4.5 hr of Con A stimulation (Fig. 3). However, the IFN-γ serum levels of mice 6 hr after Con A stimulation showed a striking increase, whereas the number of IFN-γ–producing cells remained at the same level.

Possibly the BCG sensitizes a part of the spleen cells to produce and secrete IFN-γ in low concentrations. Con A may stimulate these cells to assemble and secrete IFN-γ in larger quantities, causing an increase of the IFN-γ serum levels after 6 hr. Double staining of the spleen sections for IFN-γ and surface antigens revealed that ± 60% of the IFN-γ–producing cells were expressing the CD4 antigen (see Fig. 1c) and ± 30% the CD8 antigen. NK cells and macrophages producing IFN-γ were not demonstrated. These results are in agreement with the experiments of Wada et al. (8), who found in BCG-primed and Con A–stimulated mice, after in vivo T-cell depletion with MAb, that T cells were the principal cells directly or indirectly responsible for the IFN-γ production, without important participation of macrophages and NK cells. In our experiments we demonstrated directly in vivo that T cells were the main IFN-γ producers in the immune response to heat-killed BCG.

In conclusion, this study demonstrates the potentials of the immunohistochemical approach in the in vivo analysis of a cytokine-mediated immune response. We demonstrated its value for the investigation of the in vivo activity of IFN-γ in a thymus-dependent immune response. Moreover, this technique enables the study of the in vivo localization and cellular origin of IFN-γ. Provided that specific antisera are available, this method is also suitable to detect and characterize other cytokine-producing cells in vivo, thereby opening the way for validation of in vitro findings. Furthermore, this technique will facilitate, in combination with other techniques, the analysis of the local events and interactions of B-, T-, and antigen-presenting-cells during an in vivo immune response.

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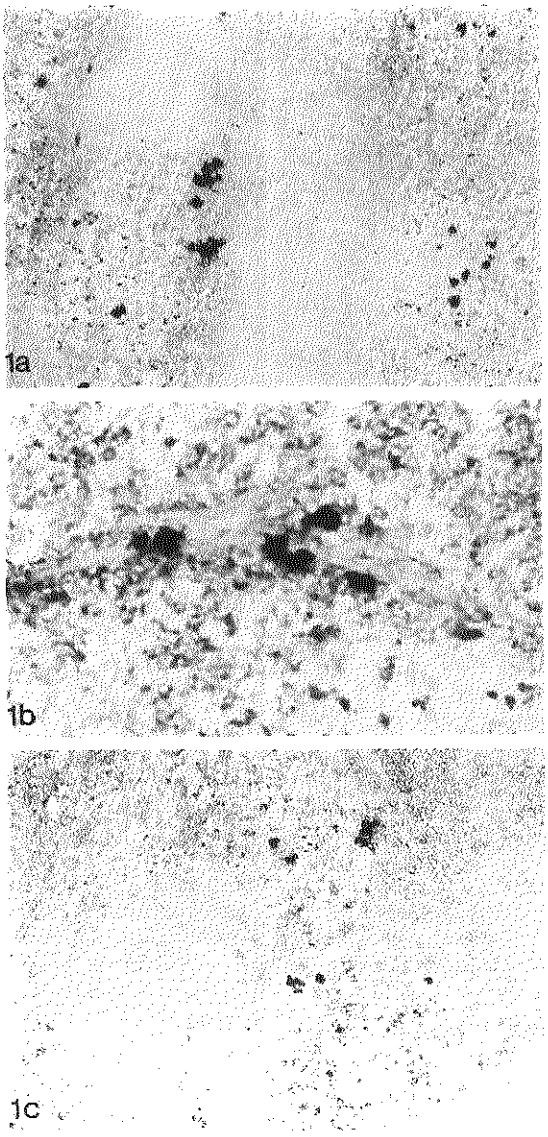

Figure 1 (a) IFN-γ–producing cells in a cryostat section of a spleen of a BCG-primed and Con A–stimulated mouse. Section was incubated with DB-1-AP (MAb specific for IFN-γ) and after AP cytochemistry the IFN-γ–producing cells with a blue cytoplasm were detected. Sections were counterstained with hematoxylin. For double-staining experiments, the sections were simultaneously incubated with DB-1-AP and rat MAb directed to different cell markers. followed by rabbit antirat-HRP. (b) After AP and HRP cytochemistry, the IFN-γ–producing cells and macrophages (MOMA2) were detected. Note the IFN-γ–producing cells in the coaxial sheath of lymphoid tissue surrounding the terminal arterioles in the spleen. (c) After AP and HRP cytochemistry, the IFN-γ–producing cells (cytoplasmic staining) and CD4 (membrane staining) were detected.

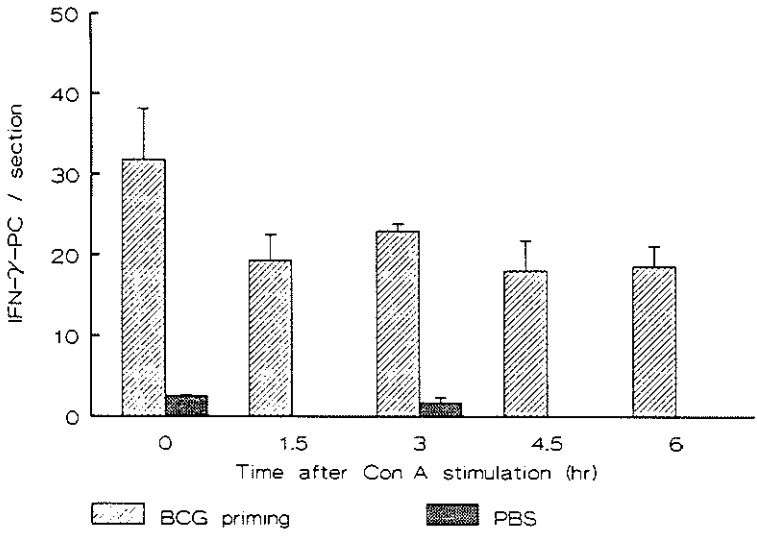

Figure 2 Kinetics of the IFN-γ-producing cells in spleens of mice after BCG priming and Con A stimulation. BALB/c mice were primed i.p. with heat-killed BCG (3.3×10^7) and stimulated i.v. with 200 μg Con A 3 weeks later. Subsequently, mice were sacrificed at the indicated intervals. Control mice were injected i.p. with 400 μl PBS. The INF-γ-producing cells in cryostat sections of spleens were stained and counted. Data presented are expressed as the mean ± SD of three different mice from each group.

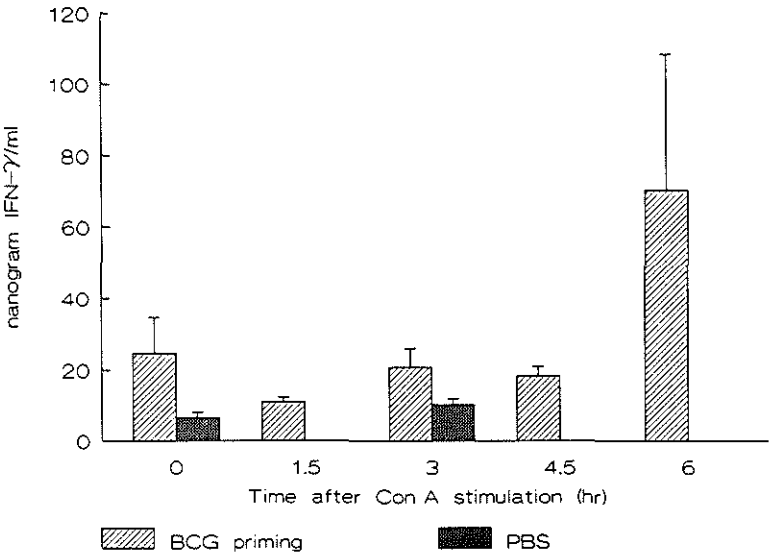

Figure 3 Kinetics of the IFN-γ serum levels of mice after BCG priming and Con A stimulation. BALB/c mice were primed i.p. with heat-killed BCG (3.3×10^7) and stimulated i.v. with 200 μg Con A 3 weeks later. Control mice were injected i.p. with 400 μl PBS. Subsequently, mice were bled at the indicated intervals. Sera were assayed for IFN-γ by a two-site sandwich ELISA. Data presented are expressed as the mean ± SD of three different mice from each group.

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CHAPTER 5

In vivo kinetics and characterization of IFN-γ-producing cells during a thymus-independent type 2 immune response

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IN VIVO KINETICS AND CHARACTERIZATION OF IFN-γ-PRODUCING CELLS DURING A THYMUS-INDEPENDENT IMMUNE RESPONSE

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Using immunohistochemical techniques, we studied IFN-γ-producing cells (IFN-γ-PC) in vivo during immune responses to thymus-independent type-2 (TI-2) Ag. Detection of IFN-γ-PC in cryostat sections of spleen-tissue was performed with an enzyme labeled mAb directed against IFN-γ. After TNP-Ficoll immunization, IFN-γ-PC and TNP-specific antibody-forming cells (TNP-AFC) displayed similar kinetics reaching a maximum number at day 5 to 7. The IFN-γ-PC were localized in the same compartment as TNP-AFC and a part of them in juxtaposition to TNP-AFC. Immunization with other TI-2 Ag resulted also in a significant increase of the number of IFN-γ-PC. In a parallel experiment we found both in vivo and in an ELISA-spot assay a significant increase of the number of IFN-γ-PC and IFN-γ-spot-forming-cells, respectively, in spleens of mice 6 to 7 days after TNP-Ficoll immunization. Double staining of spleen sections for IFN-γ and surface Ag revealed that 5 to 7 days after TNP-Ficoll immunization, ± 40% of the IFN-γ-PC expressed the MT4 Ag (CD4), ± 50% the Lyt-2⁺ Ag (CD8) and ± 10% the asialo-GM1 Ag (NK cell). This study represents the first description of the in vivo activity and characterization of IFN-γ-PC during a TI-2 immune response. Moreover, the presented data confirm suggestions from in vitro investigations that IFN-γ and T cells may play a direct role in the in vivo regulation of a primary immune response against a TI-2 Ag.

The spleen plays an important role in the protection against *Streptococcus pneumoniae, Neisseria meningitides,* and *Haemophilus influenzae,* for example (1). After splenectomy patients are at high risk for fulminant infections due to these bacteria. The protective immune response against these microorganisms is directed mainly against the polysaccharide component of the bacterial capsule, classified as a TI-2[2] Ag (2). The original definition of TI Ag reflects its ability to stimulate humoral immune responses in nude mice in vivo or in T cell-depleted cultures in vitro. These criteria are relatively nonstringent insofar as nude mice and the T cell-depleted cultures have been shown to possess both small numbers of T cells as well as large numbers of NK cells, which are able to secrete cytokines (3, 4). Rigorous elimination of T cells consistently and significantly reduces the in vitro immune response to TI Ag, indicating that these Ag are T cell dependent to a significant degree (5–7). The TI Ag are divided into type 1 and type 2 (TI-2) Ag, based on their ability to stimulate an immunodeficient strain of mice (CBA/N) to produce antibodies (8–10). The most common and extensively studied TI-2 Ag is TNP-Ficoll. As purified cytokines and antibodies directed to cytokines became available, their role in the immune response to TNP-Ficoll has been evaluated. Addition of IL-1 to cultures of lymph node cells plus TNP-Ficoll appeared to induce an increase in the number of TNP plaque-forming cells (11, 12). Several reports have shown that responses of purified populations of B cells to TNP-Ficoll were reduced markedly, compared with unfractionated spleen cells, and that their responsiveness could be restored by addition of IL-2 or T cells (3, 13). In contrast, IFN-γ reduced significantly the number of TNP plaque-forming cells and inhibited thymidin incorporation in cultures of TNP-specific B cells, suggesting that the suppressive effect on the TNP-Ficoll response is mediated, at least in part, by inhibition of the clonal expansion of TNP-Ficoll responsive cells (5). These in vitro experiments demonstrated that IFN-γ could play a downregulating role in TI-2 immune responses. However, these studies did not investigate the active participation of IFN-γ in in vivo TI-2 immune responses. In in vivo experiments, in contrast to in vitro experiments, there is no need to use manipulations like cell separation and culture, which may introduce artefacts and change essentially the context of immune cells. Furthermore, in vivo experiments, by means of immunohistochemical techniques, enable the study of immune cells in their natural context that is of great importance because regulation of immune responses by cytokines is predominantly a local and compartmentalized process (14). Therefore we conducted an in vivo study to the origin and activity of IFN-γ in the immune response to TI-2 Ag by means of immunohistochemical techniques.

Several reports have described the detection of cytokine producing cells at single cell level (15), in human tissues (16–18) and in murine tissues (19, 20) with cytokine specific mAb. The present study details the in vivo detection, localization, kinetics and characterization of IFN-γ-PC during a primary TI-2 immune response. Evidence

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[2] Abbreviations used in this paper: TI-2, thymus-independent type 2; AP, alkaline-phosphatase; HRP, horseradish-peroxidase; IFN-γ-PC, IFN-γ producing cells; PALS, periarteriolar lymfocyte sheath; TNP-AFC, TNP-specific antibody-forming cells; TNP-HES, TNP-hydroxyethyl starch.

that IFN-γ and T cells are active in the primary in vivo immune response to TNP-Ficoll is presented.

MATERIALS AND METHODS

Mice. Female BALB/c mice were bred at TNO, Rijswijk, The Netherlands and were used at 12 to 16 wk of age.

Chemicals. AP (P-6774, type VII-T, 1020 U/mg protein), 3-amino-9-ethylcarbazole (A-5754), 5-BCIP (5-bromo-4-chloro-3-indolyl-phosphate; B-8503), Fast blue BB Base (F-125), levamisole, naphthol AS MX phosphate (3-hydroxy-2-naphtoic acid 2,4-dimethyl-anilide), phosphatase substrate (Sigma 104, Sigma Chemical Co., St. Louis, MO). TNP sulfonic acid (TNBS, grade I) were obtained from Sigma. Con A was purchased from Pharmacia Fine Chemicals Inc., Uppsala, Sweden. All other reagents were analytical grade or better.

Ag. TNP-Ficoll was prepared as previously described (21). TNP-HES was a kind gift from the late Prof. J. Humphrey, Royal Postgraduate Medical School, London (22). TI-2 Ag characteristics based on the specific localization pattern in marginal zone macrophages were checked, as we described before (23). Dextran B1355 was a kind gift of Dr. J. Blomberg, University of Uppsala, Department of Immunology, Uppsala, Sweden (24). All the polysaccharides were extensively dialysed against PBS before administration.

Antibodies. The murine mAb DB-1 and DB-2, specific for rat IFN-γ and cross-reacting with murine IFN-γ, and affinity chromatography-purified rat rIFN-γ were a kind gift of Dr. P. H. van der Meide (25, 26), TNO Institute for applied Radiobiology and Immunology, Rijswijk, The Netherlands. Controls consisted of isotype and concentration-matched mAb directed to human IgG1 (27) and IgG3 (28).

The mAb XMG1.2 and XMG6.1, directed to murine IFN-γ, were a kind gift of Dr. R. L. Coffman (29), DNAX Research Institute, Palo Alto, CA. The rat mAb MOMA-2, which recognizes monocytes and macrophages (30), and cells of the rat hybridomas Lyt-2$^+$ (53.6.7), which marks the CD8 subset of T lymphocytes (31) and MT4 (H129.9), which recognizes the CD4 subset of T lymfocytes (32) were a kind gift of Dr. G. Kraal, Free University, Amsterdam, The Netherlands. Hybridomas were grown in RPMI 1640 with 10% FCS and obtained antibodies were purified from culture supernatants using affinity chromatography with a goat anti-rat Ig column. Rabbit anti-A.GM1 serum was obtained from WAKO Chemicals GmbH, FRG (33). Rabbit-HRP anti-rat Ig, Rabbit-HRP anti-mouse Ig, rabbit-peroxidase anti-peroxidase complex, Dakopatts, Copenhagen, Denmark and goat-HRP anti-rabbit-Ig, Miles-Yeda Ltd., Rehovot, Israel were used as secondary antibodies. AP-conjugated goat anti-mouse IgM (or IgM + IgG) was obtained from Tago, Burlingame, CA 94010. Streptavidin-AP conjugate was obtained from Jackson Immuno Research Labs, West Grove, PA.

Preparation HRP/AP conjugates. DB-1 and control mAb were conjugated to AP and HRP as previously described (34). TNP-AP was prepared as described before (35).

Experimental design. BALB/c mice were injected i.v. either with 16 μg TNP-Ficoll, 20 μg TNP-HES, 20 μg Dextran B1355, or with 20 μg (unhaptenated) Ficoll in 200 μl PBS. The TNP-Ficoll immunized mice (three mice/day) were anaesthetized, bled, and killed immediately thereafter and 1, 3, 5, 7, 10, 14, 21 days after immunization. Mice (five mice/day) injected with TNP-HES, Ficoll, and PBS were killed after 5 and 7 days. Dextran B1355 injected mice (five mice/day) were killed after 4, 5, 6, and 7 days immunization. Spleens were removed and immediately frozen in liquid nitrogen and stored at −70°C if not used immediately. Sera were stored at −20°C.

Immunostaining. Cryostat sections of spleens, one of every mouse, (−20°C, 8 μm) were picked up on the same glass slide and kept overnight under high humidity at room temperature. Slides were air dried and stored in air-tight boxes at 4°C until use. Cryostat sections were fixed for 10 min in acetone (p.a.) containing 0.02% H_2O_2 (freshly prepared). Subsequently slides were rinsed with PBS and incubated horizontally overnight at 4°C with reagents (DB1-AP, and/or MT4 or Lyt-2$^+$ or MOMA-2 or anti-asialo-GM1). All reagents were diluted in PBS containing 0.1% BSA and titrated to obtain optimal results. Subsequently, slides were washed with PBS (three times for 5 min) and incubated for 30 min at room temperature with secondary conjugates diluted optimally in PBS containing 0.1% BSA and 1% normal mouse serum. Histochemical revelation of the AP activity was done according to Claassen and Adler (34). Thereafter slides were washed with PBS, and HRP activity was demonstrated as previously described (34). Detection of TNP-AFC was performed as described before (21). Briefly, spleen sections of TNP-Ficoll immunized mice were incubated, horizontally overnight at 4°C, with TNP-AP (10 μg·ml^{-1}) in PBS + 0.1% BSA and treated for AP histochemistry.

Inhibition assay of IFN-γ specific staining. A total of 25 μg·ml^{-1} of the conjugate DB-1-AP was preincubated with dilutions (12.5–200 μg·ml^{-1}) of affinity purified IFN-γ for 2 h in the presence of 0.1% BSA. Subsequently, this solution was centrifuged (12,000 × *g*) for 2 min and acetone-fixed cryostat sections of spleens of three different mice, 5 days after TNP-Ficoll immunization, were incubated with supernatants overnight at 4°C. After histochemical revelation, the stained cells were quantitated by laser-scan microscopy.

Quantitation of IFN-γ-PC and TNP-AFC. The image of each spleen-section was acquired with an HTHMX-I camera (High Technology Holland bv, Eindhoven, The Netherlands). By means of image analysis (MCID software; Imaging Research Inc., Brock University-St. Catharines, Ontario, Canada) the total size of every spleen section in mm^2 was determined (36). The number of IFN-γ-PC or TNP-AFC was determined by laser scan microscopy (LSM-41; Carl Zeiss, Oberkochen, FRG) interfaced to a Micro Dutch-100 computer for data acquisition and processing (37). A He-Ne laser scanned the microscopic fields of spleen sections (sections of all mice within one experiment were picked up on the same glass slide). Transmitted light was received by a photo multiplier and digitized (eight bit) in a format of 512 × 512 pixels, each with a range of 255 possible gray values representing the amount of absorbed light. The images were displayed on a high resolution black and white monitor, to make real time/on line control of data acquisition possible at all times. Image analysis was performed by the Technical Command Language-image processor (developed by TNO-TPD, Delft, The Netherlands, in collaboration with the Technical University of Delft). The total stained surface of every spleen section was measured. The mean staining per cell was determined by analyzing 500 TNP-AFC or 500 IFN-γ-PC, respectively. The number of IFN-γ-PC or TNP-AFC was calculated by the division of the total spleen staining by the mean cell staining and was expressed as the number of IFN-γ-PC or TNP-AFC per mm^2 spleen section. Five spleen sections of each mouse were analyzed.

Detection of serum TNP-specific antibody levels. Serum antibodies to TNP were determined by means of a direct ELISA, as described previously (38).

Preparation of cell suspensions. Spleens were aseptically removed and single cell suspensions were prepared by gently teasing them through nylon gauze filters into balanced salt solution (39). Concentrations of spleen cells were determined with a Coulter Counter (Coulter Electronics, Luton, U.K.).

Enumeration IFN-γ-spot-forming cells. To quantitate the frequency of IFN-γ-spot-forming cells, an ELISA-spot assay was used according to the procedure described by Taguchi et al. (40) with minor modifications. Briefly, microtiter plates were coated overnight at 4°C with 100 μl of 1 μg XMG1.2·ml^{-1} in PBS, followed by blocking with 150 μl PBS containing 1% BSA for 30 min at 37°C. Spleen cells (1×10^2 to 1×10^5/well) suspended in RPMI plus 1% BSA were added to individual wells and stimulated with Con A (5 μg·ml^{-1}). Cells were incubated for 3 h at 37°C with 5% CO_2 in air. The plates were washed once with H_2O plus 0.05% Tween-20 and three times with PBS containing 0.1% BSA plus 0.05% Tween-20 and incubated overnight at 4°C with 100 μl of 0.2 μg XMG6.1-biotin·ml^{-1} in PBS containing 0.1% BSA and 0.05% Tween-20. Subsequently plates were washed three times with PBS containing 0.1% BSA plus 0.05% Tween-20 and incubated with 100 μl of a 1/1800 dilution of a streptavidin-AP conjugate in PBS containing 0.05% Tween-20 plus 0.1% BSA for 120 min. Trays were washed five times with PBS containing 0.1% BSA plus 0.05% Tween-20. Spots representing single IFN-γ-secreting cells were developed with 100 μl 5-BCIP (10 mg·ml^{-1}) in 2-amino-2-methyl-1 propanol buffer, pH 10.25. The number of spots was enumerated with the aid of a dissecting microscope.

ELISA for serum IFN-γ. Serum samples were assayed in duplo according to the procedure as described for the ELISA-spot assay. Instead of spleen suspensions serum samples and mouse rIFN-γ standards diluted in RPMI plus 1% BSA were added to each well. Absorbance was measured as described for the anti-TNP ELISA.

Statistical evaluation. Results were analyzed by the two-sample Student's *t*-test for comparison of two empirical means in a normally distributed population.

RESULTS

Detection of IFN-γ-PC. We investigated cryostat sections of spleens of BALB/c mice 0 to 21 days after TNP-Ficoll immunization. After incubation with the conjugate DB-1-AP and histochemical revelation, the IFN-γ-PC characterized by a blue cytoplasm were detected (Fig. 1*a*). No staining was seen in control slides from any specimen treated by omission or isotype matched substitution of primary antibody. To verify the specificity of the staining,

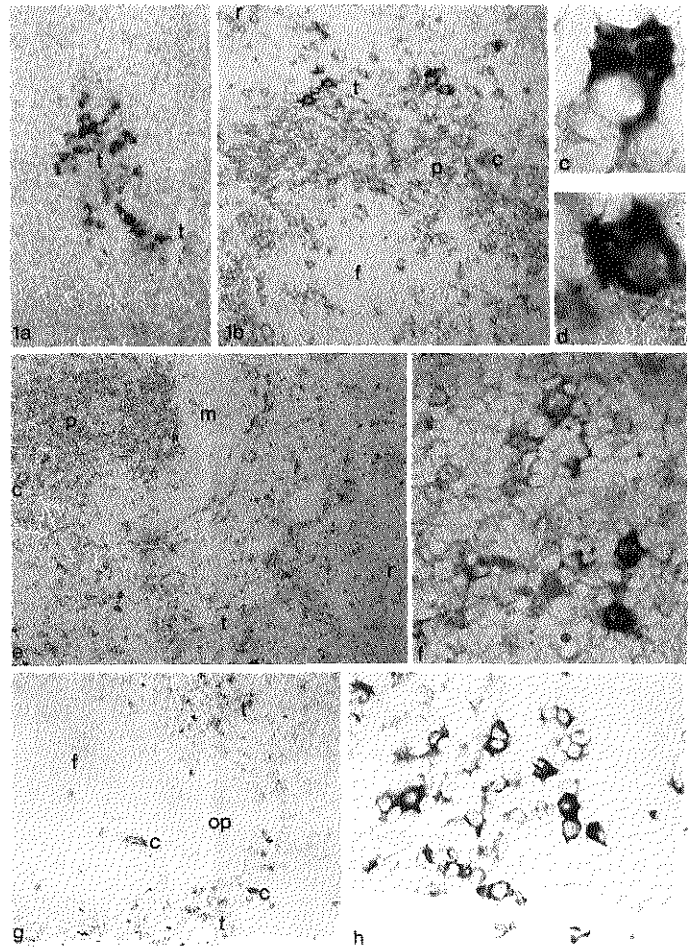

Figure 1. Immunohistochemical visualization and characterization of IFN-γ-PC and/or TNP-AFC in spleens of TNP-Ficoll immunized mice. *a*, Cryostat section was incubated with DB-1-AP and after AP cytochemistry the IFN-γ-PC with a blue cytoplasm could be seen around the terminal arteriole. Section was counterstained with hematoxylin. For double staining experiments the sections were incubated simultaneously with DB-1-AP, and antibodies directed to different cell markers, followed by secondary HRP-conjugates. AP and HRP cytochemistry revealed; *b* to *d*, IFN-γ-PC (blue cytoplasm), CD4 cells (red membrane staining) in the PALS and CD4 cells producing IFN-γ (double-stained cells) around the terminal arterioles. c to *d*, The difference between a single stained cell (*d*) and a double stained cell (c) was appreciated best under high power magnification (×2000). *e* to *f*, Overview and detail of IFN-γ-PC (blue cytoplasm) in red pulp. CD8 (red membrane staining) predominantly in PALS and CD8 cells producing IFN-γ (double-stained cells) in PALS, red pulp, and terminal arterioles. *g*, Cryostat section was incubated with rabbit-HRP anti-IgG±M and HRP cytochemistry was performed. Subsequently this section was incubated with DB-1-AP and AP cytochemistry was performed. IFN-γ-PC (blue cytoplasm) were localized around terminal arterioles, PALS, and red pulp. Note the absence of IFN-γ-PC in the follicle (within white rim). *h*, Cryostat section was incubated with DB-1-HRP and TNP-AP followed by PAP. After cytochemistry IFN-γ-PC (red cytoplasm) were found juxtaposed to TNP-AFC (blue cytoplasm). c, central arteriole; *f*, follicle; *m*, marginal zone; *op*, outer PALS; *p*, PALS; *r*, red pulp; *t*, terminal arteriole.

we performed a competition and an inhibition assay on spleen sections of mice 5 days after TNP-Ficoll immunization. Simultaneous incubation of DB-1-AP (20 $\mu g \cdot ml^{-1}$) with DB-2 (2000 $\mu g \cdot ml^{-1}$), a mAb directed to the same epitope as DB-1, eliminated completely the staining of the IFN-γ-PC. The ratios of the concentrations of the competing mAb in this assay, were similar to the ratios used in a competition ELISA, previously described by Van der Meide et al. (26). Preincubation of DB-1 (25 $\mu g \cdot ml^{-1}$) with IFN-γ (200 $\mu g \cdot ml^{-1}$) completely abolished the cytoplasmic staining of the IFN-γ-PC in these spleen sections. Preincubation of DB-1 with lower concentrations IFN-γ resulted in the detection of only a part of the IFN-γ-PC that were observed after preincubation with PBS (Fig. 2).

Localization of IFN-γ-PC and TNP-AFC. Morphologically, the spleen can be divided in red and white pulp. The red pulp consists of E and all types of nucleated cells such as lymphocytes, megakaryocytes, and macrophages. The splenic white pulp consists of three major compartments (38). The PALS, the major T cell area, surround the central arteries and branch off terminal arterioles, that traverses through the different compartments of the white pulp toward the red pulp. The follicles are globular structures attached to the PALS and contain mainly B cells. The marginal zone surrounds the PALS and follicles and contains B cells, macrophages, and relatively few T cells. Neutral polysaccharides, TI-2 Ag, are almost exclusively retained in the marginal zone macrophages, suggesting that this compartment may play an important role in the processing and presentation of TI-2 Ag (23). As described in previous studies (21), we found TNP-AFC predominantly in the lymphocyte sheaths around the terminal arterioles, in the outer parts of the PALS, whereas relatively few were located in the red pulp.

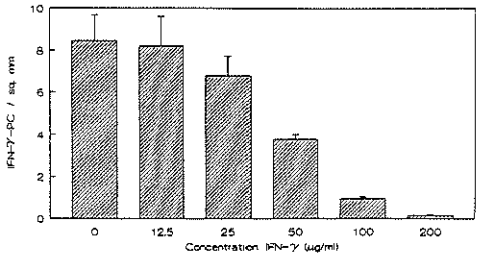

Figure 2. Inhibition assay of IFN-γ-specific staining. A total of 25 µg·ml^{-1} of the mAb DB-1 was pre-incubated with the indicated dilutions (0–200 µg·ml^{-1}) of IFN-γ for 2 h in the presence of 0.1% BSA. Subsequently the solutions were centrifuged at 12,000 × *g* for 2 min and cryostat sections of spleens of mice 5 days after TNP-Ficoll immunization were incubated with supernatants. After immunohistochemical revelation the number of IFN-γ-PC were quantitated by laser-scan microscopy as described in *Materials and Methods*. Data presented are expressed as the mean ± SD of three different mice.

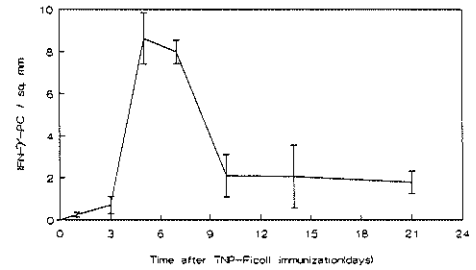

Figure 3. Kinetics of the IFN-γ-PC in spleens of mice after TNP-Ficoll immunization. BALB/c mice were immunized i.v. with 16 µg TNP-Ficoll and killed after 0 to 21 days. Subsequently the INF-γ-PC in cryostat sections (five of each mouse) were stained and quantitated by laser-scan microscopy as described in *Materials and Methods*. Data presented are expressed as the mean ± SD of three different mice from each group.

In our experiments the IFN-γ-PC were usually found in clusters of two to eight cells in the same compartment as the TNP-AFC. Double staining demonstrated that a part of the IFN-γ-PC were in direct cell contact with the TNP-AFC (Fig. 1*h*). No IFN-γ-PC were observed in the follicles and marginal zone. The IFN-γ-PC were found in roughly the same numbers in each section throughout the entire spleen. Double staining for mouse IgG + IgM and IFN-γ showed that IFN-γ-PC were juxtaposed to antibody-forming cells and were not found in B cell areas like follicles (Fig. 1*g*).

Kinetics of IFN-γ-PC. IFN-γ-PC were quantitated by laserscan microscopy. As shown in Figure 3., hardly any IFN-γ-PC were detectable 0 to 3 days after immunization with TNP-Ficoll. Thereafter, the number of IFN-γ-PC cells increased very rapidly and reached its maximum at day 5 to 7. Ten days after TNP-Ficoll immunization, the number of IFN-γ-PC was markedly decreased and remained at this level for the next 11 days. The effect of various polysaccharides on the number of IFN-γ-PC in spleens of mice is shown in Table I. The number of IFN-γ-PC in spleens of mice injected with the unhaptenated Ficoll was not different from the PBS-injected mice. Injection of TNP-HES resulted in a significant increase of the number of IFN-γ-PC in spleen sections of mice 5 and 7 days after immunization. Dextran B1355 immunized

TABLE I

IFN-γ-PC in spleen sections after administration of various polysaccharides[a]

Ag	Day 5	Day 7
TNP-Ficoll	8.6[b] ± 1.2[c]	8.0 ± 0.6*
TNP-HES	7.6 ± 1.0*	4.6 ± 0.3*
Dextran B1355	3.7 ± 0.7*	1.9 ± 1.8 (NS)
Ficoll	1.1 ± 0.3 (NS)	0.7 ± 0.3 (NS)
PBS (control)	1.2 ± 0.4	0.8 ± 0.3

[a] BALB/c mice were injected i.v. with (non-)haptenated polysaccharides. Mice were killed and spleens were removed at the indicated intervals. Cryostat sections were stained and IFN-γ-PC were quantitated according to procedure described in *Materials and Methods*.

[b] Number of IFN-γ-PC/mm^2 of spleen section ± SD.

[c] Statistical evaluation was performed by the two sample Student's *t*-test. *p* value against control.

* $p < 0.001$.

mice showed a significant increase of the number of IFN-γ-PC after 4 (2.9 ± 0.5), 5 (3.7 ± 0.7), and 6 (4.4 ± 0.3) days.

Phenotypic characterization of IFN-γ-PC. To determine the origin of IFN-γ, spleen sections were simultaneously incubated with DB-1-AP, and antibodies to NK-cells or CD4 cells or CD8 cells or macrophages, followed by secondary antibodies conjugated to HRP. Whereas cells with a blue cytoplasm were IFN-γ-PC, cells with a red membrane staining expressed the CD4 or CD8 or NK cell Ag (Fig. 1, *b*, *e*, and *f*). Thus, double stained cells, characterized by a violet cytoplasm were either NK cells (not shown) or CD4 (Fig. 1, *b* and *d*) or CD8 cells (*e* and *f*) producing IFN-γ. The double stained cells were appreciated best under high power magnification (Fig. 1, *c* and *d*). Light microscopic quantitation revealed that during the peak of the response (day 5–7) 38.6(± 21.6)% of the IFN-γ-PC were CD4, 49.4(± 19.2)% were CD8 and 9.6(± 4.8)% were NK cells. We investigated the kinetics of separate celltypes producing IFN-γ at different stages during the immune response to TNP-Ficoll (Fig. 4). As presented in Table II, we found that during the entire immune response to TNP-Ficoll the majority of the IFN-γ-PC were CD4 or CD8 cells. The total number of IFN-γ-PC per section was in the same range as the total double stained IFN-γ-PC, indicating that the majority of the IFN-γ-PC were expressing the above mentioned surface markers. No cells were double staining for MOMA-2 and IFN-γ.

Kinetics of TNP-AFC. As previously described (21), the number of TNP-AFC began to rise 3 days after TNP-Ficoll immunization and attained maxima 5 to 7 days after immunization. Thereafter the number of TNP-AFC decreased gradually.

Anti-TNP serum levels. The anti-TNP IgM response to TNP-Ficoll strongly increased after day 3 and reached a peak at days 7 to 10. The anti-TNP IgG response increased gradually after day 5 but remained low as compared to the IgM response.

Number of IFN-γ-SFC and IFN-γ-PC 6 and 7 days after TNP-Ficoll immunization. We assessed both in vivo and in vitro the number of cells producing IFN-γ in spleens of mice after TNP-Ficoll immunization. Therefore we quantitated the number of IFN-γ-PC, detected by immunohistochemistry, in one half of each spleen, and the number of IFN-γ spot-forming cells, by means of an ELISA-spot assay, in the other half of each spleen (eight mice/day). The control mice were injected with PBS. As demonstrated in Figure 5, the in vitro Con A stimulated

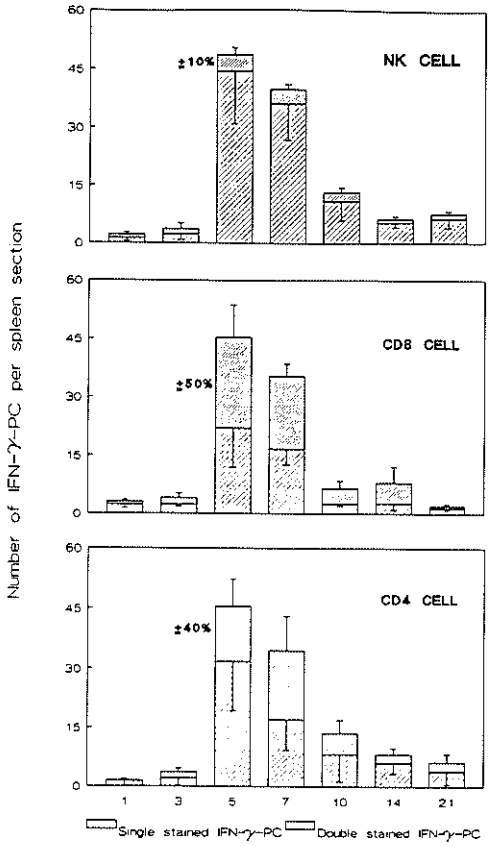

Figure 4. Kinetics of separate cell types producing INF-γ in spleens of mice after TNP-Ficoll immunization. BALB/c mice were immunized i.v. with 16 μg TNP-Ficoll and killed after 0 to 21 days. Subsequently cryostat sections were simultaneously incubated with antibodies specific for IFN-γ and the indicated cell markers. After immunohistochemical revelation the single and double stained cells were counted. Data presented are expressed as the mean ± SD of three different mice from each group.

splenocytes of the control mice were already producing IFN-γ in the ELISA-spot assay, whereas hardly any IFN-γ-PC in spleens of these mice were found. After TNP-Ficoll immunization the number of IFN-γ-SFC in vitro as well as the number of IFN-γ-PC in vivo increased significantly on days 6 and 7 (Fig. 5).

IFN-γ serum levels. To investigate whether IFN-γ production was also detectable at systemic level, we measured IFN-γ serum levels of TNP-Ficoll immunized mice and controls. Only in two mice, at 7 days after TNP-Ficoll immunization, an increase of the IFN-γ serum level was found (310 and 455 $ng \cdot ml^{-1}$). All other TNP-Ficoll immunized mice had IFN-γ serum levels similar to controls (5–15 $ng \cdot ml^{-1}$) (data not shown).

DISCUSSION

In this study we demonstrated in vivo that IFN-γ is active during immune responses to various TI-2 Ag. Moreover, we showed that IFN-γ-PC and TNP-AFC display similar kinetics and are localized in the same compartment. A part of the IFN-γ-PC were juxtaposed to TNP-AFC. T cells were the main source of IFN-γ during the immune response to TNP-Ficoll.

The original classification of TI Ag was based on their common ability to induce immune responses in nu/nu mice. Antibody responses to TI-2 Ag were later shown to be T cell regulated. IL-1 and IL-2 appeared to promote the TI-2 immune response, whereas IFN-γ suppressed the in vitro immune response to TI-2 Ag (5–7, 11–13). However, these studies did not show that IFN-γ participated actively in in vivo TI-2 immune responses. Therefore we investigated the in vivo IFN-γ production of spleen cells during TI-2 immune responses. Cleveland et al. (19) found, using indirect immunofluorescence techniques, an increased in vivo IFN-γ production in spleens of mice undergoing graft vs host disease. However, in their in vitro experiments they observed no increased IFN-γ production. They demonstrated that this discrepancy was due to the mixing of different cell populations, which destroyed the relative separation of these populations and changed their microenvironment. These data suggested that in vitro studies may not always truly represent the in vivo immune status of an animal. These studies and the fact that immunohistochemical techniques enable the investigation and characterization of immune cells in their original histologic context are the reasons for using these techniques.

After immunization of BALB/c mice with various TI-2 Ag, IFN-γ-PC were identified in spleen sections with an AP-labeled mAb (DB-1) specific for IFN-γ (Fig. 1*a*). The specificity and affinity of DB-1 has been extensively tested by Van der Meide et al. (25). Previously Ljungdahl et al. (42) used DB-1 for the in vivo detection of IFN-γ in the central nervous system of the rat. To validate the specificity of DB-1 for the detection of IFN-γ in murine tissue sections, a number of controls and verifying experiments were included in this study. In our experiments, the substitution or omission of DB-1-AP resulted in a lack of staining. Additional evidence for the specificity of this staining was provided by preincubation of DB-1-AP with IFN-γ, that resulted in a complete inhibition of the staining of IFN-γ-PC (Fig. 2). Simultaneous incubation with DB-2 completely abolished the staining of IFN-γ-PC. In our studies the cytoplasm of IFN-γ-PC stained intensively. The possibility that cytoplasmic staining represents uptake of IFN-γ rather than production, has already been ruled out previously by Andersson et al. (43). They observed that incubation of mononuclear cells with high concentrations of IFN-γ did not lead to any detectable IFN-γ staining. Moreover, the kinetics and localization of IFN-γ-PC we found after TNP-Ficoll immunization is also more suggestive for IFN-γ production than uptake. Administration of the same amount of unhaptenated Ficoll did not result in an increase of the number of IFN-γ-PC, indicating that haptenization of an intrinsically poor immunogen such as Ficoll (44) is responsible for the increased IFN-γ production, as expected. The number of IFN-γ-PC induced by dextran B1355, an unhaptenated TI-2 Ag, was lower than found in mice after immunization with the same amount of TNP-haptenated TI-2 Ag (Table I). This is possibly due to the less potent immunogenicity of this polysaccharide as

TABLE II

Actual number of IFN-γ-PC per spleen section displaying the surface Ag 1 to 21 days after TNP-Ficoll immunization[a]

Phenotype	Day	1	3	5	7	10	14	21
Asialo-GM1		1 ± 1[b]	1 ± 1	4 ± 2	4 ± 1	2 ± 1	1 ± 1	1 ± 1
MT4		0 ± 0	2 ± 1	14 ± 7	18 ± 9	6 ± 3	2 ± 1	2 ± 2
LYT2+		1 ± 0	2 ± 1	23 ± 8	19 ± 3	4 ± 2	5 ± 4	1 ± 1
MOMA-2		0	0	0	0	0	0	0
Total double stained cells[c]		2	5	41	41	12	8	4
Total number of IFN-γ-PC[d]		2 ± 1	4 ± 2	46 ± 12	37 ± 8	11 ± 5	7 ± 3	5 ± 2

[a] Three groups of BALB/c mice were immunized i.v. with 16 μg TNP-ficoll. Mice were killed and spleens were removed at the indicated intervals. Cryostat sections were simultaneously incubated with antibodies for IFN-γ and the different cell surface antigens. After histochemical revelation the double stained cells were counted.

[b] Number of double staining IFN-γ-PC per spleen section. Number of cells is shown as mean ± SD deviation from three mice.

[c] The total number of double staining IFN-γ-PC from three spleens, each stained for a different cell marker.

[d] The total number of IFN-γ-PC (double stained cells included) per spleen section. Number of cells is shown as mean ± SD from three mice.

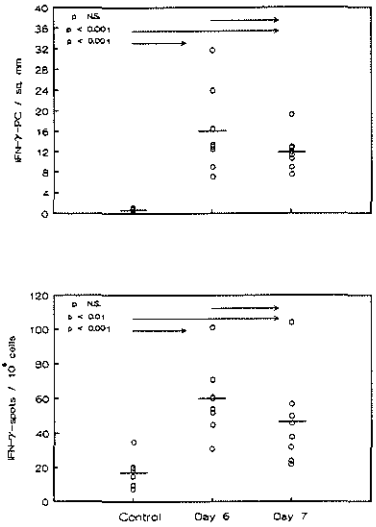

Figure 5. Effect of TNP-Ficoll immunization on the numbers of IFN-γ-PC and IFN-γ-SFC in spleens of mice. BALB/c mice were immunized i.v. with 16 μg TNP-Ficoll and killed after 6 and 7 days. Subsequently the IFN-γ-PC in cryostat sections and the IFN-γ-SFC in cell suspensions of spleens of the same mice were quantitated, according to the procedures described in *Materials and Methods*. Every *point* represents one mouse. Control mice were injected with PBS and were killed after 6 days. Mean values of eight mice from each group are shown. Statistical evaluation was performed by the two-sample Student's *t*-test.

compared to haptenated polysaccharides, as previously was demonstrated by Koch et al. (45). The kinetics of IFN-γ-PC we observed in this TI-2 immune response, starting at day 3 and attaining maxima on days 5 and 7, were remarkably consistent with the results of Gessner et al. (20) in lymphocytic choriomeningitis virus infected mice, considered as a thymus-dependent immune response.

To investigate whether spleen cells of mice, 6 and 7 days after TNP-Ficoll immunization, were also producing/secreting IFN-γ in vitro we performed an ELISA-spot assay. We found a two- to three-fold increase of the number of IFN-γ-SFC 6 and 7 days after TNP-Ficoll immunization. These results were again in agreement with the experiments of Gessner et al. (20), who found a 2.4-fold increase of the number of IFN-γ-SFC in spleens of mice infected with lymphocytic choriomeningitis virus. The number of IFN-γ-PC, determined with immunohistochemical techniques, in the same spleens showed a 40-fold increase after TNP-Ficoll immunization. This consistent gradual difference is probably due to the Con A addition to the spleen cell suspension during the ELISA spot assay. This mitogen may stimulate cells that produce lower quantities of IFN-γ to assemble and secrete higher amounts of IFN-γ, facilitating their detection. However, for the in vivo approach there was no amplification step applied, and probably therefore a low number of IFN-γ-PC in the nonimmunized mice and a marked increase in number of these cells was detected after TNP-Ficoll immunization.

We investigated whether the rise in local IFN-γ production also resulted in an increase of the IFN-γ serum levels. Increased IFN-γ serum was found in only two mice (day-7 TNP-Ficoll immunization). Possibly the amounts of IFN-γ produced locally in the spleen are not sufficient to cause an increase in the IFN-γ serum levels, e.g., Buchmeier and Schreiber (46) were unable to detect raised IFN-γ serum levels in primary *Listeria*-infected mice, although in vitro splenic cells were found to produce increased amounts of IFN-γ. Another possibility is complement-mediated inactivation of IFN-γ in sera (47).

In our studies, using TI-2 Ag, the IFN-γ-PC cells were often found in clusters of two to eight cells, located in the PALS, around the terminal arterioles and in the red pulp. The localization pattern of IFN-γ-PC in our experiments was comparable with results of other groups. Gessner et al. (20) have examined the IFN-γ-PC in spleens of mice infected with lymphocytic choriomeningitis virus. In this thymus-dependent immune response IFN-γ-PC were located in small clusters predominantly in the white pulp and less frequently in the red pulp. In graft vs host disease studies IFN-γ was mainly localized in the periarteriolar lymphocyte sheaths regions, and no IFN-γ immunofluorescence was found in the red pulp (19). In this study the IFN-γ production was proven to be T cell dependent and derived from the donor T cells.

We demonstrated that IFN-γ-PC were localized in the same compartment as TNP-AFC. Furthermore a part of the IFN-γ-PC were found in close conjunction to the TNP-AFC, suggesting a direct cell-cell interaction. Such an interaction was recently demonstrated by Kupfer et al. (48) in vitro who showed that after in vitro mixing of Ag-specific Th and B cells Th-B couples were formed. This

interaction resulted in cytokine production by these T cells near the Ag-specific B cells. Kupfer et al. (48) proposed that T cells were in this way able to induce selective and specific B cell responses. Dekruyff et al. (49) showed in vitro that a direct interaction between Ag-specific T cells with Ag-activated B cells was a prerequisite for T cell activation and cytokine production by T cells in TI-2 immune responses. They demonstrated that 72-h preculturing of B cells with TI-2 Ag resulted in maximal capacity of these B cells to stimulate Ag-specific T cells. This indirect activation pathway could be the explanation of the relatively late appearance of IFN-γ-PC in TI-2 immune responses. Mohler et al. (50) concluded from their experiments, using reverse transcription-polymerase chain reaction, that the generation of immune responses in vivo might develop according a specific sequential production of cytokines. They showed that the majority of IL-2 mRNA expression occurs from 1 to 3 days after antigenic exposure, whereas IL-4 mRNA expression occurred from day 3 through day 5. They suggested IL-2 was an up-regulating cytokine and IL-4 a down-regulating cytokine in this immune response. The late appearance of IFN-γ-PC in TI-2 immune responses and the juxtaposition of these cells to TNP-AFC observed in our experiments is also more suggestive for a maturing/regulating than for an inducing role of IFN-γ in TI-2 immune responses. This has also been suggested by Mond and Brunswick (51), who demonstrated in vitro that IFN-γ suppressed the number of plaque forming cells to TNP-Ficoll, whereas addition of IFN-γ neutralizing mAb enhanced the response to TNP-Ficoll. The in vivo localization and kinetics of IFN-γ-PC and TNP-AFC together with the in vitro data (51) discussed above suggest a (down)-regulating role of IFN-γ in the immune response to TNP-Ficoll. Further in vivo studies, by treating mice with IFN-γ or their neutralizing antibodies, are required to exactly elucidate the regulating role of IFN-γ in TI-2 immune responses.

The double staining experiments we performed allowed the phenotypical characterization of IFN-γ-PC during a TI-2 immune response. Analysis of the IFN-γ-PC at the peak of this response (days 5–7) revealed that ± 40% of the IFN-γ-PC were CD4 cells, ± 50% CD8 cells, and only ± 10% NK cells (Fig. 4). No cells double staining for IFN-γ and MOMA-2 were detected, indicating that IFN-γ is not produced by macrophages. We found that the total number of IFN-γ-PC per section was in the same range as the total double-stained IFN-γ-PC, indicating that the majority of the IFN-γ-PC were expressing the above mentioned surface markers (Table II). These results are in agreement with studies of Gessner et al. (52) in lymphocytic choriomeningitis virus-infected mice, who showed in an ELISA-spot assay, that after negative selection ± 50% of the IFN-γ-PC were either CD4 or CD8. We have demonstrated that both the major T cell subsets (CD4/CD8) were the main IFN-γ producers, whereas only a small proportion of the IFN-γ producers were NK cells, suggesting an important role of T cells in the regulation of the immune response to TNP-Ficoll. However, Mond and Brunswick (51) concluded from their experiments that NK cells may have a significant role in the responses to TI-2 Ag. They showed that responses of T cell-depleted, NK-depleted B cells to TNP-Ficoll were enhanced in the presence of IL-2. Responses from T cell-depleted B cell cultures from which NK cells were not eliminated or to which IFN-γ was added were significantly lower, suggesting a suppressive role for NK cells or IFN-γ in the immune response to TNP-Ficoll. The apparent discrepancy between these in vitro experiments and our in vivo results may be due to the localizing effect of the anatomical barriers in in vivo experiments, as earlier demonstrated by Cleveland et al. (19). These in vivo barriers may limit the contact between the TNP-AFC and NK cells. This in contrast to in vitro experiments, where the IL-2-activated NK cells may be able to produce IFN-γ close to the TNP-AFC, allowing them to regulate the TNP-specific plaque-forming cells.

In conclusion, this study describes for the first time the in vivo kinetics and characterization of IFN-γ-PC during primary TI-2 immune responses. The potentials of immunohistochemical techniques in the analysis of in vivo cytokine-mediated immunes response are demonstrated: we showed that IFN-γ is produced by CD4, CD8, and NK cells and that the IFN-γ-PC are localized in juxtaposition to and in the same compartment as TNP-AFC. Evidence that IFN-γ and T cells are active in the in vivo TI-2 immune response is presented. Further investigations into the details of the regulating role of IFN-γ in the immune response to TNP-Ficoll are underway.

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CHAPTER 6

Differential *in vivo* appearance of activated T cells, cytokine-producing cells and antibody-forming cells in peripheral lymph nodes and the spleen after immunization with thymus-independent type 2 antigens

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ABSTRACT

The putative defect of peripheral lymph nodes (PLN)[2] to mount an antibody response against thymus independent type 2 (TI-2) antigens was investigated. Mice were immunized with TNP-Ficoll and draining PLN were examined immunohistochemically, using a TNP-alkaline phosphatase conjugate, for the presence of TNP-specific antibody forming cells (TNP-AFC). Unexpectedly, high frequencies of TNP-AFC were detected in PLN of TNP-Ficoll immunized mice. Splenectomy experiments indicated that these TNP-AFC did not originate from the spleen. ELISPOT assays did not reveal the presence of TNP-specific secreting cells (TNP-ASC) in PLN of TNP-Ficoll immunized mice, whereas in the spleen of these mice high frequencies of TNP-ASC were observed, suggesting that TNP-Ficoll activated B cells in PLN do produce antibodies, but are not able to secrete them. Only after simultaneous injection of *Brucella abortus* (BA), a particulate TI-1 antigen, together with TNP-Ficoll, relatively high frequencies of TNP-ASC were detected in PLN. Four and five days after immunization with TNP-Ficoll relatively high frequencies of cytokine-PC (IL-2, IL-4, IL-5 and IFN-γ) were observed in the outer-periarteriolar sheath (PALS) and around the terminal arterioles of the spleen. TNP-AFC were localized in the same compartment as cytokine-PC indicating that these activated B cells were differentiating in a cytokine-rich microenvironment. In contrast, low frequencies of activated T cells and cytokine-PC were observed in PLN after TNP-Ficoll immunization, suggesting that the lack of cytokines in PLN was responsible for the maturation defect of TNP-AFC. Indeed, simultaneous immunization with TNP-Ficoll + BA induced high frequencies of activated T cells and cytokine-PC in draining PLN and also resulted in increased frequencies of TNP-ASC in PLN. However, *in vivo* administration of IL-4 and/or IL-5 could not overcome the putative maturation defect of TNP-Ficoll activated B cells in PLN.

In conclusion, this study shows that TNP-Ficoll is able to prime TNP-specific B cells in PLN. Data presented suggest that the differential capacity of PLN and spleens to generate fully differentiated TNP-ASC after TNP-Ficoll immunization is due to differences in cytokine production in these lymphoid organs. Furthermore, results suggest that combinations of cytokines or other cytokines than IL-4 and IL-5 are required for the *in vivo* maturation of TI-2 antigen activated PLN B cells into TNP-specific antibody-secreting cells.

INTRODUCTION

The original definition of thymus independent (TI) antigens reflects their ability to stimulate humoral immune responses in nude mice *in vivo* or in T cell depleted cultures *in vitro*. These criteria are relatively non-stringent insofar as both nude mice and T cell depleted cultures have been shown to possess small numbers of T cells. Rigorous elimination of T cells consistently and significantly reduces the *in vitro* immune response to TI antigens (1,2,3,4), indicating that these antigens are T cell dependent to a significant degree. The TI Ag are divided into type 1 and type 2 (TI-2) Ag, based on their ability to stimulate an immunodeficient strain of mice (CBA/N) to produce antibodies (5). The polysaccharide component of the bacterial capsule of bacteriae is classified as a TI-2 antigen (6). The spleen plays a critical role in the protection against capsular bacteria, e.g. *Streptococcus pneumoniae, Neisseria meningitides* and *Haemophilus influenzae* (7). Therefore, research has been focused on the delineation of the unique role of the spleen in TI-2 antibody responses. The most common and extensively studied TI-2 Ag is TNP-Ficoll. *In vitro* experiments indicated that cytokines produced by T cells or accessory cells are required for the development of an effective antibody response against TI-2 antigens in the spleen (2,3,4). In addition, *in vivo* experiments demonstrated that activated cytokine-producing T helper cells were active in the spleen during TI-2 immune responses (9). All these studies were performed in spleens *in vivo* or with splenocytes *in vitro*. *Ex vivo* analysis of TI-2 antibody responses in lymph nodes indicated that PLN B cells did not differentiate into TNP-specific antibody-secreting cells (TNP-ASC) after immunization with TNP-Ficoll (10). Only the addition of splenic accessory cells or IL-1 to *in vitro* PLN cultures enabled lymph node cells to mount an antibody response against TNP-Ficoll. Since PLN were able to mount an antibody response against TNP-BA, a TI-1 antigen, mice were immunized with TNP-Ficoll + BA, which resulted in high frequencies of TNP-ASC in draining PLN, comparable to the frequencies observed in the spleen of TNP-Ficoll immunized mice (10). These results suggest that the differential ability of lymph nodes and spleens to mount an antibody response against TI-2 antigens originates in the differences of the microenvironment in these secondary lymphoid organs during TI-2 immune responses. The delineation of the differences in the organ-specific microenvironment is important, because it may give us new information about the unique role of the spleen in the development of TI-2 antibody responses.

We have previously shown that injected TNP-Ficoll localized in the marginal zone and follicles of the spleen (11). Evidence was provided which showed that the typical follicular localization

of TNP-Ficoll is important for the development of TI-2 antibody responses. Furthermore, it was demonstrated that IFN-γ producing T cells colocalize with antigen-specific-AFC after immunization with TI-2 antigens (12). These data suggested that the localization of antigens and activity of cytokines are important factors in the microenvironment of lymphoid tissues during TI-2 antibody responses.

Therefore, in this study we explored the possible role of the lymphoid microenvironment in the development of TI-2 antibody responses. The *in vivo* localization of TNP-Ficoll in draining lymph nodes was compared to that of the spleen. The activity of activated T cells, cytokine-producing cells and TNP-specific antibody-forming cells in the spleen and lymph nodes of mice was investigated after immunization with TNP-Ficoll. Finally, by treating mice with IL-4 and/or IL-5, we investigated the capacity of these cytokines to restore the *in vivo* antibody response of PLN B cells against TI-2 antigens.

MATERIALS AND METHODS

Animals. BCBA.F1 (C57BL x CBA) mice were bred at the TNO breeding facilities, Rijswijk, The Netherlands. Animals were used at 11-19 weeks of age and were kept under standard protocol with free access to pelleted food and acidified water (pH 3). Experiments were performed under auspices of the Dutch Veterinary Inspection, as described in the Law on Animal Experiments.

Chemicals. Alkaline phosphatase (AP; P-6774, type VII-T, 1020 U/mg protein), 3-amino-9-ethylcarbazole (AEC; A-5754), 5-BCIP (5-bromo-4-chloro-3-indolyl-phosphate; B-8503), Fast blue BB Base (F-0125), Fast red, horse radish peroxidase (HRP), levamisole, naphthol AS-MX phosphate (3-hydroxy-2-naphtoic acid 2,4-dimethyl-anilide), TNP sulfonic acid (TNBS, grade I) were obtained from Sigma, St. Louis, MO, USA; N-hydroxysuccinimidyl-(biotinamido)-hexanoate and MHS (maleimidohexanoyl-n-hydroxysuccinimide ester) were obtained from Pierce, Rockford, IL, USA; β-galactosidase (β-gal; E. coli-derived β-D-galactoside galactohydrolase, MW 540 KD), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were obtained from Boehringer, Mannheim, FRG.

Antigens. Brucella abortus (BA) was a kind gift of Dr. S.H.M. Jeurissen, Central Veterinary Institute, Lelystad, The Netherlands. TNP-Ficoll and TNP-OVA were prepared according to previously described methods (13).

Reagents. AP-conjugated goat Ig anti-mouse IgM or IgG were obtained from Tago, Burlingame, CA 94010. Avidin-HRP and avidin-AP were obtained from Sigma, St. Louis, MO,

USA. The control hamster antibodies, the hamster mAb directed to gp39 (MR1; 14), and the murine mAb specific for rat/hamster Igκ (RG-7; 15) chain were a kind gift of Dr. R.J. Noelle (Department of Microbiology, Dartmouth School, Lebanon, NH 03757, USA) and purified by means of protein-A affinity chromatography. The murine mAb DB-1 directed to mouse IFN-γ, was a kind gift of Dr. P. H. van der Meide (16) from our institute. S4B6, directed to mouse IL-2, was a kind gift of Dr. T. Mosmann (17). The cells of the rat mAb 11B11, directed to mouse IL-4, and IL-4 were a kind gift of Dr. W.E. Paul, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, N.I.H., Bethesda, Maryland (18). The mouse-IL-5-specific rat mAb, TRFK5, was obtained from Dr. R.L. Coffman, DNAX Research Institute, Palo Alto, CA, USA (19). Control rat mAb (2A4, 1G11) were a kind gift of Dr. A. Zantema, Sylvius Laboratory, Leiden, The Netherlands (20). Recombinant mouse IL-2 was obtained from Genzym, Cambridge, MA, USA. The X6310 cell lines producing mouse IL-4 or IL-2 were a kind gift of Dr. F. Melchers, Basel Institute of Immunology, Basel, Switzerland (21). All rat antibodies were purified from culture supernatants using a goat anti-rat Ig column, obtained from Dr. J. Radl, IVVO, TNO, Leiden. Cytokine-specific and control antibodies were conjugated to AP and HRP as previously described (22). Biotinylation was performed as previously described (23). Conjugation to β-gal was performed according to the procedure described by Deelder and De Water (24) with minor modifications (11). TNP-AP was prepared according to previously described methods (13).

Experimental design. To investigate the development of TNP-specific antibody forming cells (TNP-AFC) mice were immunized s.c. in the footpad with 50 μg TNP-Ficoll in PBS and were killed at day 1, 4, 5, 6, 7, and 10. Serum was prepared and spleens and PLN were removed. To investigate the TNP-specific antibody response after immunization with TNP-Ficoll supplemented with BA, mice were immunized s.c. in both footpads with 50 μg TNP-Ficoll, 50 μg TNP-Ficoll and 1 mg BA, BA, or PBS and killed after five days. One PLN from each mouse was used for immunohistochemical analysis, while the other PLN was used for the ELISPOT assay. To investigate the role of the spleen in the antibody response against TNP-Ficoll in PLN, splenectomized mice and sham-splenectomized mice were immunized s.c. in both footpads with 50 μg TNP-Ficoll, 50 μg TNP-Ficoll and 1 mg BA, or PBS and killed after five days. One PLN from each mouse was used for immunohistochemical analysis, while the other PLN was used for the ELISPOT assay.

To investigate the effect of the *in vivo* administration of IL-4 and/or IL-5 on the TNP-specific antibody response, mice were implanted at day -2 with 2 x 10^6 CV/IL-4, 2 x 10^6 CV/IL-5, 2 x 10^6 CV/ IL-4 + 2 x 10^6 CV/IL-5, or empty Cytodex-3 beads encapsulated in alginate and immunized s.c. with 50 μg TNP-Ficoll in both footpads at day 0. Mice were sacrificed at day

5 and 7 and one PLN was prepared for immunohistochemistry, while the contralateral PLN was tested in the ELISPOT assay. For immunohistochemical analysis, lymphoid organs were frozen in liquid nitrogen and stored at -70°C.

Splenectomy. Mice were anaesthetized with nembutal. An incision was made in the peritoneum and the spleen was excised. The incision was closed with three sutures. The sham-splenectomized mice underwent the same operation procedures, but were not splenectomized (26).

Alginate encapsulation. Cells from the IL-4 or IL-5 producing cell line were encapsulated in alginate as described earlier (27). Monkey CV-1 cells were stably transfected with the murine IL-4 or IL-5 gene under control of the SV40 promotor. Cells were grown on Cytodex 3 beads (Pharmacia, Uppsala, Sweden). Fully covered beads were harvested after 2 days of culture and washed 3 times with sterile saline. The volume of the pellet was determined and 1 volume of saline followed by 2 volumes of a 1.2% sterile solution of alginate (FMC Bioproducts, Rockland, PA, USA) were combined in a syringe. This suspension was squirted through a 25 gauge needle into a fresh 80 mM $CaCl_2$ solution. The encapsulated cells were washed 3 times with saline and 2 x 10^6 encapsulated cells in 1 ml were injected i.p. with a 19 gauge needle.

Immunohistochemistry. Splenic cryostat sections (-20 °C, 8 μm), one of every mouse, were picked up on the same glass slide and kept overnight under high humidity at RT. Slides were air-dried and stored in air-tight boxes until use. Slides were fixed for 10 minutes in acetone containing 0.02% H_2O_2. Detection of cytokine-PC was performed as described previously (9,12,28). Slides were incubated horizontally overnight at 4 °C with cytokine-specific antibody-conjugates diluted in PBS containing 0.1% BSA, which were titrated to obtain optimal results. Immunohistochemical demonstration of gp39 was performed with MR1, followed by a hamster-Ig-specific mAb, RG-7, conjugated to HRP (9). Detection of TNP-specific antibody forming cells (TNP-AFC) was performed according to previously described methods (13). Detection of TNP-Ficoll was performed with a TNP-specific mAb (SH4.1C9) conjugated to β-gal as previously described (11). Subsequently, slides were washed with PBS (three times 5 min) and immuno-histochemical revelation was performed, as described previously; AP (22), HRP (22) and β-gal (24). To ensure that no over- or understaining occurred, slides with adherent substrate solution were monitored by light microscopy during histochemical reactions. Sections were counterstained with haematoxylin and mounted in glycerin-gelatin. Antibody-forming cells, $gp39^+$ cells and cytokine-PC were counted, and image-analysis of spleen sections was performed as previously described (12). A minimum of three sections of each mouse were examined. For the analysis of the *in situ* antibody response in PLN cryostat

sections were made of the entire lymph node. At least 20 sections of each PLN were examined for the presence of TNP-AFC. From the total number of sections of one PLN and the number of sections examined for each assay, the total frequencies of TNP-AFC per PLN were calculated. The frequency of cytokine-PC in PLN was expressed in number of cells per mm^2 and was based on at least 10 cryostat sections.

TNP-specific ELISA. Mouse serum TNP-specific IgM and IgG were determined by means of a direct enzyme-linked immunosorbent assay as described earlier (25). The TNP-specific titer was expressed as the dilution at which 50% of the maximal absorbance was found.

ELISPOT assay. To quantitate the frequency of TNP-ASC, an ELISPOT assay was used according to the procedure described by Taguchi et al. with minor modifications (29). Spleens were aseptically removed and single cell suspensions were prepared by gently teasing them through nylon gauze filters into RPMI plus 0.5% FCS. Cell suspensions were washed three times with RPMI plus 0.5% FCS. Microtitre plates were coated overnight at 4 °C with 50 μl of 5 μg OVA-TNP ml^{-1} in PBS, followed by blocking with 100 μl PBS containing 0.5% gelatine in PBS at 37 °C. Spleen cells (3 x 10^2 to 1 x 10^5/well) suspended in RPMI plus 0.5% FCS were added to individual wells and incubated for 3 h at 37 °C with 5% CO_2 in air. To remove the cells, plates were washed once with H_2O plus 0.05% Tween-20 and three times with PBS containing 0.1% gelatine plus 0.05% Tween-20 and incubated 1 hr at 37 °C with 50 μl of goat anti-mouse-IgG/IgM conjugated to AP in PBS containing 0.1% gelatine. Subsequently plates were washed three times with PBS containing 0.05% Tween-20. Spots representing single antibody-secreting cells were developed with 100 μl 5-BCIP (10 $mg.ml^{-1}$) in 2-amino-2-methyl-1-1 propanol buffer (pH=10,25). The number of spots was enumerated with the aid of a dissecting microscope.

RESULTS

TNP-Ficoll localizes in macrophages and follicles of the lymph node.

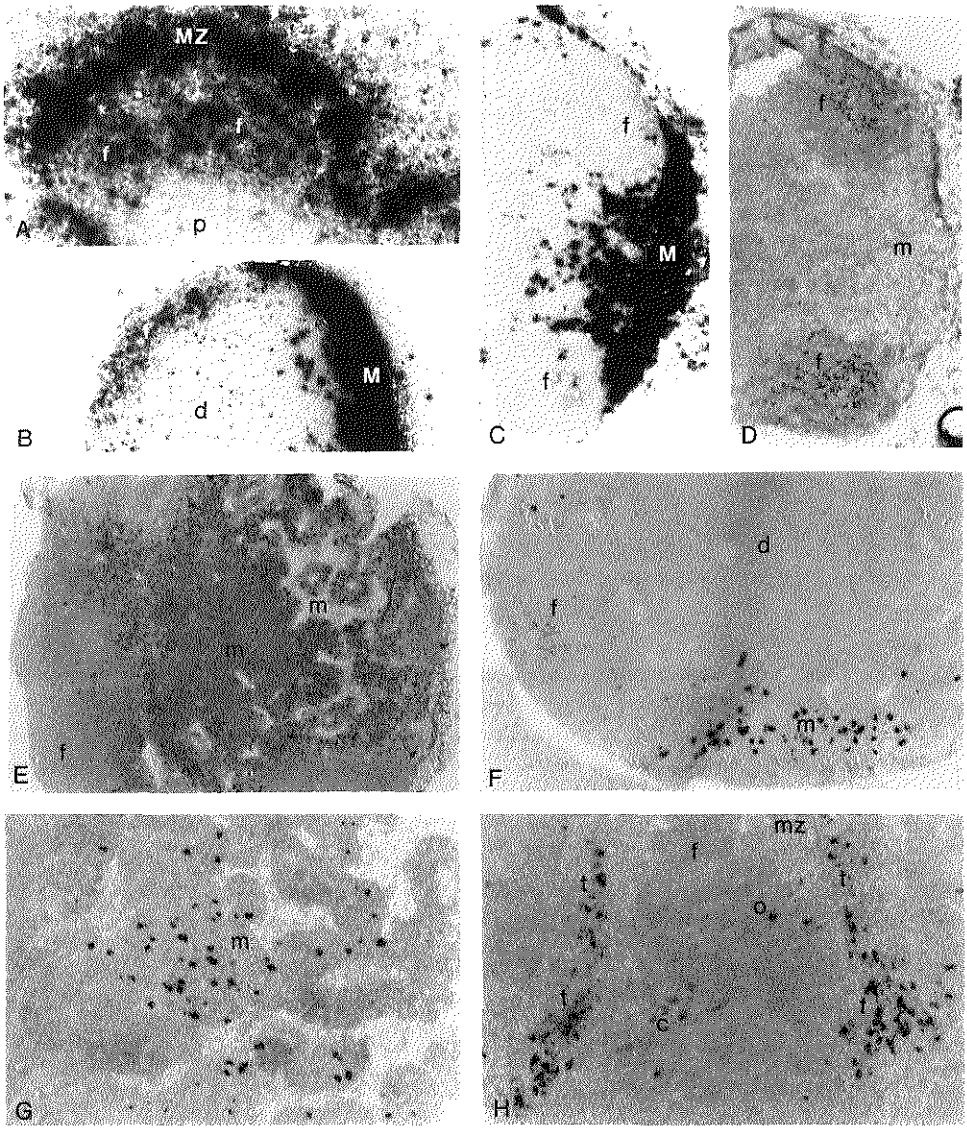
TNP-Ficoll localizes in the marginal zone macrophages and follicles of the spleen, irrespective of route of administration (11). Evidence was provided for an important role of follicular TNP-Ficoll in the induction of TI-2 antibody responses in the spleen (11). Therefore, the localization of TNP-Ficoll in the draining PLN node was investigated after s.c. immunization. Using a TNP-specific mAb conjugated to β-galactosidase, TNP-Ficoll was detected in the follicles of PLN at 7 h after injection (Fig. 1bc), a similar localization pattern as was observed in the spleen (Fig. 1a; 11). TNP-Ficoll predominantly localized in the medullary macrophages and to a

lesser degree in the subcapsular macrophages of the PLN. After s.c. immunization, TNP-Ficoll was also detected in the marginal zones and follicles of the spleen (Fig. 1a). Ten days after injection of TNP-Ficoll, the TI-2 antigen was still detectable in the follicles and macrophages of lymph nodes and the spleen.

PLN can mount an in situ antibody response against TNP-Ficoll.

To investigate whether murine PLN develop an *in situ* antibody response against TI-2 antigens, we examined PLN at different time-points after s.c. immunization with TNP-Ficoll. As shown in fig. 2, about 250 TNP-AFC were present in PLN at 4 days after TNP-Ficoll immunization. Maximum frequencies (± 2000/PLN) of TNP-AFC were observed 7 days after TNP-Ficoll immunization. TNP-AFC were predominantly found along the medullary cords of PLN (Fig. 1d, f). Besides the development of TNP-AFC, relatively high amounts of TNP-specific antibodies were observed in the follicles of lymph nodes 4 days after immunization (Fig. 1d, f). In serial sections we could demonstrate that TNP-Ficoll and TNP-specific antibodies had a similar localization pattern in the follicle, indicating that the observed staining represented trapped TNP-Ficoll-anti-TNP-antibodies immune complexes (Fig. 1c, d).

Figure 1) Localization of injected TNP-Ficoll, TNP-AFC and IL-5-PC in lymphoid organs after immunization. Cryostat sections of murine lymphoid tissue were incubated with specific immuno-conjugates, followed by immunohistochemical revelation. a) spleen section 7 h after injection of TNP-Ficoll: TNP-Ficoll, detected with a TNP-specific mAb conjugated to β-galactosidase, is localized in the marginal zone macrophages and follicles of the spleen; b) PLN 7 h after injection of TNP-Ficoll; TNP-Ficoll is predominantly localized in the medullary macrophages and in the follicles of PLN; c) PLN 5 days after injection of TNP-Ficoll; TNP-Ficoll is localized in the medullary macrophages and in the follicles of PLN (see serial section figure 1d); d) PLN 5 days after injection of TNP-Ficoll; TNP-specific antibodies, detected with TNP-alkaline phosphatase conjugate, are trapped in the follicle as immune complexes (see serial section figure 1c); e) PLN 5 days after injection of TNP-Ficoll + BA; TNP-AFC are localized along medullary cords and in deep cortex of PLN; f) PLN 5 days after injection of TNP-Ficoll; TNP-AFC are localized in the medulla and immune complexes in the follicles of PLN; g) PLN 5 days after injection of TNP-Ficoll + BA; IL-5-PC, detected with IL-5-specific mAb conjugated to alkaline phosphatase conjugate; h) IL-5-PC in spleen section, 4 days after immunization with TNP-Ficoll. c, central arteriole; d, deep cortex; f, follicle; m, medulla, mz, marginal zone; o, outer-PALS; p, PALS.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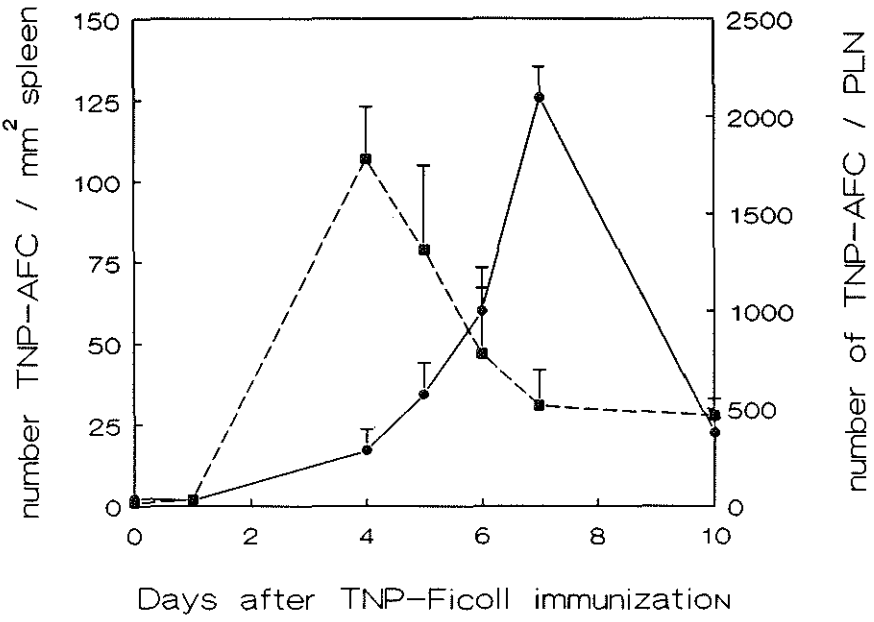

Figure 2) TNP-AFC appear earlier in the spleen than in the PLN after s.c. immunization with TNP-Ficoll. BCBA.F1 mice were injected s.c. with 50 μg TNP-Ficoll and sacrificed at the indicated time-points. Spleens were removed and immunohistochemistry and image analysis was performed as described in the materials and methods section. In the spleen values represent the mean ± SD of number of positive cells per mm^2; in PLN values represent the mean ± SD of the total frequencies of TNP-AFC per PLN from three mice. Broken line represents the development of TNP-AFC in the spleen, while the solid line represents the development of TNP-AFC in PLN.

The *in situ* TNP-specific antibody response in the spleen revealed maximum frequencies of TNP-AFC 4-5 days after s.c. immunization with TNP-Ficoll, 2 days earlier as compared to PLN. Thereafter, the frequency of TNP-AFC decreased gradually and remained at a constant level after day 7 of immunization. The TNP-AFC were predominantly found in the outer-PALS and around the terminal arterioles of the spleen, as described earlier (13).

TNP-AFC in PLN are not derived from the spleen.

The frequency of TNP-AFC in PLN reached maximum levels two days later than in the spleen, suggesting that TNP-AFC in the PLN originated from the spleen. To investigate this possibility, splenectomized mice were immunized s.c. with TNP-Ficoll. As shown in table I, no differences were found in the frequencies of TNP-AFC in PLN between splenectomized and control mice.

Table I

Effect of splenectomy on TNP-AFC and TNP-ASC in PLN after TNP-Ficoll immunization

Immunization	splenectomy	TNP-AFC/PLN	TNP-ASC/PLN
TNP-Ficoll	-	1295 ± 755	< 10
TNP-Ficoll	+	1287 ± 759	< 10
TNP-Ficoll + BA	-	90632 ± 21150	5170 ± 403
TNP-Ficoll + BA	+	87639 ± 24615	1266 ± 341
BA	-	not tested	170 ± 7

[a] Splenectomized mice and sham-splenectomized mice were immunized s.c. in both footpads with 50 μg TNP-Ficoll and/or 1 mg BA or PBS. [b] Mice were killed at day 5 after immunization and draining PLN were removed. In one PLN the frequency of TNP-AFC was determined immunohistochemically, while TNP-ASC were determined in the contralateral PLN by using the ELISA-spot assay.[c, d] Data represent the mean ± SD number of TNP-AFC or TNP-ASC per PLN from four mice.

TNP-specific antibody-secreting cells are not detectable in PLN after TNP-Ficoll immunization.

The TNP-AFC observed in PLN after TNP-Ficoll immunization represent the potential TNP-specific antibody-secreting cells. However, of all TNP-AFC only a part will be secreting antibodies at any given moment. Therefore, the frequency of TNP-ASC in PLN was determined after TNP-Ficoll immunization, by using an ELISPOT assay. In agreement with the experiments of Goud et al. (10) no TNP-ASC were observed in lymph nodes of TNP-Ficoll immunized mice (table I). Only s.c. injection of TNP-Ficoll along with BA induced high frequencies of TNP-ASC in draining PLN (table I), as was described earlier (10). Immunization of splenectomized mice with TNP-Ficoll + BA resulted in significantly lower frequencies of TNP-ASC in PLN than in sham-splenectomized mice, whereas the frequency of TNP-AFC was similar in draining PLN of these mice (Fig. 1d, table I). In contrast to the PLN, high frequencies of TNP-ASC were observed in the spleen after s.c. immunization with TNP-Ficoll (table III).

Low production of cytokines in lymph nodes relative to the spleen after immunization with TNP-Ficoll.

The previous experiments showed that TNP-Ficoll immunization induces the development of TNP-AFC in the PLN. However, the TNP-AFC failed to secrete TNP-specific antibodies, as was demonstrated in the ELISPOT assay, suggesting that PLN were lacking the factors which are essential for the final differentiation into TNP-ASC. Since high frequencies of TNP-ASC were detected in spleens of TNP-Ficoll immunized mice, these factors are most likely present in this secondary lymphoid organ. Therefore, we compared the expression of $gp39^+$ and cytokines

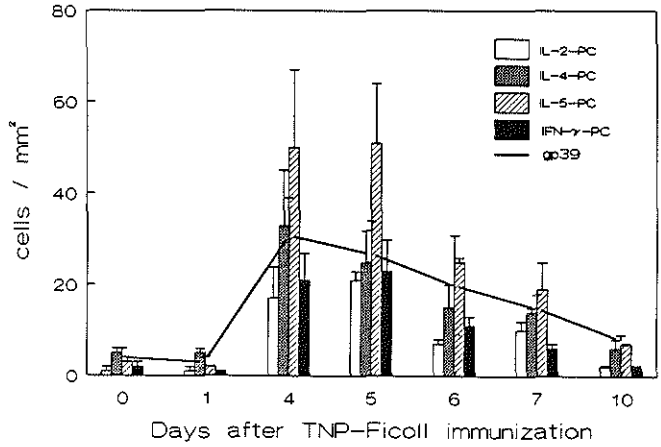

Figure 3) *Development of activated T cells and cytokine-producing cells in the spleen after immunization with TNP-Ficoll.* BCBA.F1 mice were injected s.c. with 50 μg TNP-Ficoll and sacrificed at the indicated time-points. Spleens were removed and immunohistochemistry and image analysis was performed as described in the materials and methods section. Values represent mean ± SD of number of positive cells per mm^2 from three mice.

in spleens and PLN after immunization with TNP-Ficoll. Gp39 is a ligand for the CD40-molecule on B cells and is only expressed on activated T helper cells *in vitro* (14) and *in vivo* (9). Analysis of gp39 expression in spleens of TNP-Ficoll immunized mice revealed high frequencies of gp39$^+$ cells in the outer-PALS and around the terminal arterioles. Maximum frequencies were observed 4 and 5 days after immunization with TNP-Ficoll (Fig. 3). In contrast, hardly any gp39$^+$ cells were found in PLN of TNP-Ficoll immunized mice, indicating that low frequencies of activated T helper cells were present in these PLN (Fig. 4).

To investigate whether cytokine-production was locally deficient in PLN of TNP-Ficoll immunized mice, the expression of cytokines in PLN and spleens was compared after immunization with TNP-Ficoll. Cytokines were detected with direct enzyme immuno-conjugates as described previously (9,12,28). High frequencies of IL-5-PC were observed in spleens of TNP-Ficoll immunized mice, reaching maximum levels at day 4 and 5 after immunization (Fig. 3). IL-2-, IL-4- and IFN-γ-PC developed according to similar kinetics as observed for IL-5-PC, though their frequencies were lower. Cytokine-PC were localized in the outer-PALS and around the terminal arterioles, as was described for IFN-γ-PC after immunization with TNP-Ficoll (12). The analysis of the *in situ* cytokine-production in PLN from TNP-Ficoll immunized mice revealed markedly lower frequencies of cytokine-PC than in the spleen (Fig. 4). In summary, these results show a differential cytokine-production in PLN and spleens after TNP-Ficoll immunization, which may be the reason for the putative maturation defect of TNP-Ficoll activated B cells in the PLN.

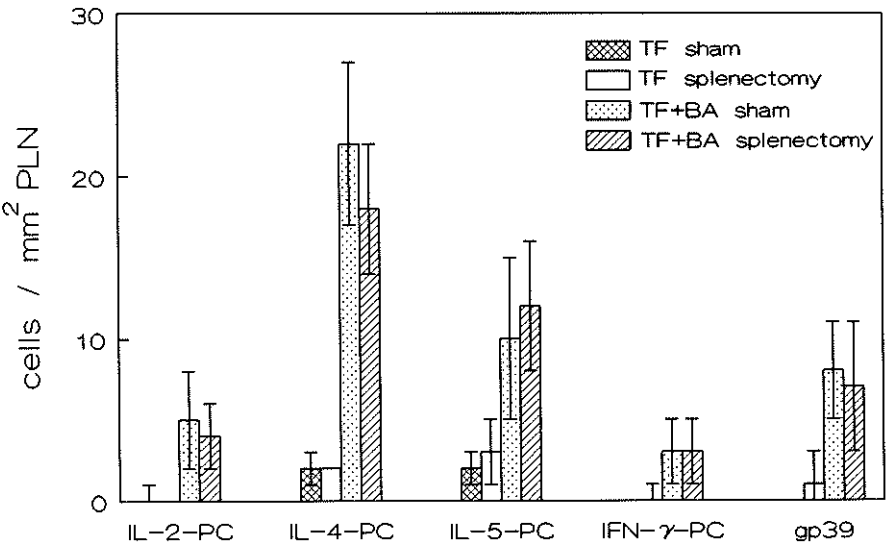

Figure 4) *Effect of splenectomy on the frequency of activated T cells and cytokine-producing cells in PLN after immunization with TI antigens.* BCBA.F1 mice were injected s.c. with 50 μg TNP-Ficoll or with 50 μg TNP-Ficoll supplemented with 1 mg BA and sacrificed 5 days after immunization. Draining PLN were removed and immunohistochemistry and image analysis was performed as described in the materials and methods section. Values represent mean ± SD of number of positive cells per mm^2 from three mice.

Co-immunization of BA with TNP-Ficoll increases the frequencies of activated T cells and cytokine-PC in PLN.

Goud et al. (10) showed that simultaneous immunization of TNP-Ficoll + BA induced high frequencies of TNP-ASC in draining PLN. To investigate whether these increased frequencies of TNP-ASC were related to the increased cytokine-production in PLN, we assessed the frequencies of $gp39^+$ cells and cytokine-PC in draining PLN after immunization with TNP-Ficoll + BA. As shown in fig. 4, relatively high frequencies of $gp39^+$ cells and cytokine-PC were found after this immunization protocol. In PLN of mice immunized with TNP-Ficoll + BA, higher frequencies of IL-4-PC and IL-5-PC relative to IL-2-PC and IFN-γ-PC were observed at day five of immunization. The cytokine-PC were predominantly localized in the medullary cords of the PLN, the same compartment where the majority of TNP-AFC were observed.

Splenectomy experiments suggested that cytokines or cells from the spleen stimulated the differentiation of TNP-ASC in PLN after immunization with TNP-Ficoll + BA (table I). To investigate whether the cytokine-PC investigated in this study were derived from the spleen, the cytokine-production in PLN of splenectomized mice was assessed after immunization with TNP-Ficoll + BA. As shown in fig. 4 the production of IL-2, IL-4, IL-5 and IFN-γ was not significantly changed after splenectomy.

Table II

Effect of *in vivo* IL-4 and/or IL-5 treatment on the frequency of TNP-AFC and TNP-ASC in PLN after TNP-Ficoll immunization

Immunization[a]	cytokine[b]	TNP-AFC / PLN day 5	TNP-AFC / PLN day 7	TNP-ASC / PLN day 5	TNP-ASC / PLN day 7
TNP-Ficoll	-	485 ± 169[c]	1683 ± 399	-[d]	-
TNP-Ficoll	IL-4	1487 ± 803	2416 ± 393	-	-
TNP-Ficoll	IL-5	1663 ± 951	1408 ± 312	-	-
TNP-Ficoll	IL-4 + IL-5	693 ± 249	2530 ± 296	-	-
PBS	IL-4 + IL-5	66 ± 13	47 ± 39	-	-

[a]Mice were immunized in both footpads with 50 μg TNP-Ficoll or PBS. [b]Two days earlier mice were infused i.p. with 2 x 10^6 cytokine-producing CV1 cells grown on cytodex beads and encapsulated in alginate. Mice were killed at day 5 or 7 and PLN were removed. In one PLN the frequency of TNP-AFC was determined immunohistochemically, while TNP-ASC were determined in the contralateral PLN by using the ELISA-spot assay.[c, d]Data presented are the mean ± SD number of TNP-AFC or TNP-ASC per PLN from four mice.

In vivo administration of IL-4 and/or IL-5 cannot restore the maturation defect of TNP-Ficoll activated B cells in PLN.

To test whether the absence of IL-4 and/or IL-5 in PLN of TNP-Ficoll immunized mice could account for the absence of TNP-ASC in PLN, these cytokines were administered continuously to mice immunized with TNP-Ficoll. This was performed by injecting mice i.p. with alginate-encapsulated CV-1 cells that were stably transformed with the murine IL-4 or IL-5 gene. Both

Table III

Effect of *in vivo* IL-4 and/or IL-5 treatment on the serum IgM-antibody titers and TNP-ASC in spleens after TNP-Ficoll immunization

Immunization[a]	cytokine[b]	TNP-specific IgM-Ab titer day 5	TNP-specific IgM-Ab titer day 7	TNP-ASC / 10^6 splenocytes day 5	TNP-ASC / 10^6 splenocytes day 7
TNP-Ficoll	-	1300 ± 200[c]	700 ± 200	2700 ± 112[d]	801 ± 132
TNP-Ficoll	IL-4	1300 ± 200	700 ± 383	2390 ± 959	857 ± 393
TNP-Ficoll	IL-5	1400 ± 239	800 ± 0	3500 ± 1060	1460 ± 830
TNP-Ficoll	IL-4 + IL-5	1600 ± 542	525 ± 150	3700 ± 1700	809 ± 251
PBS	IL-4 + IL-5	138 ± 189	75 ± 87	259 ± 407	117 ± 132

[a]Mice were immunized in both footpads with 50 μg TNP-Ficoll or PBS. [b]Two days earlier mice were infused i.p. with 2 x 10^6 cytokine-producing CV1 cells grown on cytodex beads and encapsulated in alginate. Mice were bled at day 5 or 7 and spleens were removed. TNP-specific IgM-antibody titers were determined in ELISA, while TNP-ASC were determined in the spleen by using the ELISA-spot assay. [c]Titers are expressed as the mean ± SD of the dilution at which 50% of the maximum absorbance was found. [d]Data represent the mean ± SD number of TNP-ASC / 10^6 splenocytes from four mice.

PLN of mice were removed at day 5 and 7 after TNP-Ficoll immunization and tested for the presence of either TNP-ASC or TNP-AFC. As shown in table II, administration of IL-4 and/or IL-5 did not increase significantly the frequency of TNP-AFC nor that of TNP-ASC in PLN of TNP-Ficoll immunized mice. In agreement with these results, no significant increases in the frequency of TNP-ASC in spleens and in the titers of TNP-specific antibodies in sera of TNP-Ficoll immunized mice were observed after *in vivo* treatment with IL-4 and/or IL-5 (table III).

DISCUSSION

In this report we investigated the inability of PLN to generate TNP-specific antibody-secreting cells after immunization with TNP-Ficoll. We demonstrated that TNP-Ficoll is able to induce the activation and proliferation of TNP-specific B cells in PLN. However, the microenvironment of PLN after TNP-Ficoll immunization was not capable to provide the signals required for the differentiation of TNP-specific antibody forming cells into TNP-specific antibody-secreting cells. Presented data suggest that the inability of TNP-Ficoll activated lymph node B cells to differentiate into TNP-specific antibody-secreting cells is due to the low frequencies of activated cytokine-producing T cells in PLN.

The fact that the spleen and PLN show a differential capacity to generate TNP-ASC upon TNP-Ficoll immunization suggests that the microenvironment of the spleen contains essential cells or cytokines which are lacking in PLN. In a previous report we presented data which suggested that the follicular localization of TNP-Ficoll in the spleen plays an important role in the generation of a TI-2 antibody response (11). Analysis of the localization of TNP-Ficoll in draining PLN revealed that TNP-Ficoll was taken up by the PLN macrophages and trapped in the follicles, in a similar way as observed in the spleen, suggesting that the way of antigen handling in PLN is not responsible for the putative defect.

The *in situ* examination of the TI-2 antibody response revealed, to our surprise, a significant number of TNP-AFC in draining PLN. Splenectomy experiments indicated that TNP-AFC were not derived from the spleen (table I). The usage of hapten-enzyme conjugates for the analysis of the *in situ* antibody production reveals all hapten-specific antibody-containing cells, and cannot determine if such a cell is actually secreting antibodies. The ELISPOT assay is an *ex vivo* method, which only detects antibody-secreting cells. By using this method for the analysis of the antibody-production in PLN, we demonstrated that no TNP-ASC were present in draining PLN of TNP-Ficoll immunized mice. These results suggest that TNP-Ficoll can

indeed activate TNP-specific lymph node B cells to produce antibodies, but that the lymph node microenvironment is not able to provide the stimuli required for the final differentiation of TNP-AFC into TNP-ASC. Only after immunization of TNP-Ficoll supplemented with BA, these TNP-AFC were able to differentiate into fully maturated antibody-secreting cells (table I). Similar conclusions were drawn from *in vitro* experiments by Goud et al. (30). They demonstrated, by using limiting dilution experiments, that TNP-Ficoll immunization led to an increased frequency of TNP-specific precursor B cells in lymph nodes. Only the addition of cytokines *in vitro* resulted in the final differentiation of TNP-Ficoll primed B cells into TNP-ASC, as was demonstrated in a plaque-forming assay (30). These results together suggest that the maturation defect of TNP-AFC might be due to a decreased cytokine production in PLN relative to the spleen after immunization with TNP-Ficoll. Indeed, after immunization of TNP-Ficoll no IL-2-PC and IFN-γ-PC were observed in draining PLN, whereas in spleens relatively high frequencies of these cytokine-PC were observed (Figs 3 and 4). In the spleen we detected about 30 times more IL-4-PC and IL-5-PC as compared to the PLN. These cytokine-PC were localized in the same splenic compartment as TNP-AFC, like was described for IFN-γ-PC (12), suggesting a regulating role of these cytokines in TI-2 antibody responses. In a previous report we demonstrated that high frequencies of activated T helper cells were present in spleens of TNP-Ficoll immunized mice (9). These activated T helper cells were detected with a mAb specific for gp39, the CD40 ligand. Although gp39 was not found to be critical for the induction of TI-2 antibody responses (31), these results indicated that T cells are activated *in vivo* after immunization with TI-2 antigens. In this study, in contrast to the spleen, only very few activated T helper cells were found in PLN after TNP-Ficoll immunization. This observation is in agreement with the low frequencies of cytokine-PC found in PLN (Fig. 4). Since BA co-administration results in increased frequencies of TNP-ASC, the effect of such an immunization protocol on the cytokine production in PLN was examined. As expected, supplementation of BA to TNP-Ficoll markedly increased the frequency of activated T helper cells and cytokine-PC in the draining PLN. Like was observed in the spleen, cytokine-PC and TNP-AFC were also colocalizing in PLN after immunization with TNP-Ficoll + BA, which suggests that these cytokines may have a regulatory role in this antibody response. All these data point to the relatively low cytokine-production in PLN after TNP-Ficoll immunization as the cause of the maturation defect of TNP-AFC.

The role of cytokines in TI-2 antibody responses was investigated by several groups. IL-1 produced by accessory cells was found to increase the *in vitro* antibody response against TNP-Ficoll (10,32,33). In some studies the addition of IL-2 was found to augment the *in vitro* TI-2 antibody response (2,33,34), whereas other studies did not report such an effect (10, 30).

Several studies demonstrated that the *in vitro* antibody response against TI-2 antigens was unchanged after the addition of IL-4 (30,34). In contrast, the addition of IL-5 to cultures did increase markedly the TNP-specific antibody response against TNP-Ficoll (30,34).

To investigate whether *in vivo* cytokine-treatment could overcome the cytokine-defect in PLN of TNP-Ficoll immunized mice, alginate encapsulated IL-4 and/or IL-5-producing cell lines were i.p. implanted. In a previous report is was demonstrated that the i.p. injection of these IL-4-producing cell lines resulted in a consistent IL-4 production for periods up to two weeks, which was proven to be sufficient to transform *in vivo* IgE non-responder mice into IgE high - responder mice (27). *In vivo* treatment with IL-5 producing cell lines resulted in increased frequencies of eosinophils in the circulation and bronchoalveolar lavage fluid of guinea pigs (35). However, the injection of these IL-4 and/or IL-5 producing cells in TNP-Ficoll immunized mice did not result in increased frequencies of TNP-AFC and TNP-ASC in draining PLN after TNP-Ficoll immunization (table II). Analogous to PLN, no significant increases of TNP-AFC (data not shown) or TNP-ASC were found in spleens of cytokine treated mice, which was also reflected by the TNP-specific IgM serum titers at day 5 and 7 after TNP-Ficoll immunization (table III). The observation that TNP-Ficoll immunization alone induced a significant IL-4 and IL-5 production in the spleen may explain the observation that the cytokine treatment did not augment the splenic TI-2 antibody response. The absence of a significant effect on *in vivo* TI-2 antibody responses in PLN might be due to the fact that these treatments do not give rise to the cytokine-levels in PLN, which are required for the maturation into TNP-ASC. However, since these cytokine-treatments have proven to be effective in other *in vivo* models (35,27), this possibility is not very likely. The absence of a significant effect may indicate that other cytokines than IL-4 and IL-5 are required for the maturation of TNP-AFC. *In vitro* experiments showed that the frequency of TNP-ASC after the addition of IL-5 to TNP-Ficoll primed PLN cells was much lower as compared to the cultures stimulated with BA, suggesting that more and other cytokines were involved in the maturation of TNP-AFC after TNP-Ficoll + BA administration.

In figure 4 it is shown that splenectomy did not affect the production of cytokines (IL-2, IL-4, IL-5 and IFN-γ) in PLN after TNP-Ficoll + BA immunization. However, splenectomy did markedly reduce the TNP-ASC after TNP-Ficoll + BA (table I), although it did not alter the frequency of TNP-primed B cells (TNP-AFC), indicating that the activity of investigated cytokines was not sufficient to restore the TI-2 antibody response in the PLN to the level observed in non-splenectomized mice. Goud et al. (10) demonstrated that the addition of adherent splenic cells or IL-1 to TNP-Ficoll primed lymph node cultures did increase the

frequency of TNP-ASC, suggesting that BA immunization induces the migration of adherent splenic cells, producing IL-1, to the PLN. The function of BA in this scenario would be the induction of chemotactic cytokines, e.g. IFN-γ, in PLN which would result in the migration of splenic macrophages to the PLN. The possibility that high local cytokine-production in the spleen might influence the microenvironment of PLN is not likely, since TNP-Ficoll immunization in draining PLN was not affected by BA immunization in the contralateral foot pad (10, and data not shown).

The observation that hardly any activated T cells or cytokine-PC were observed in PLN after TNP-Ficoll immunization suggests a defect in the activation of PLN T cells. The process of T cell activation has been described by two groups. DeKruyff et al. (36) showed that TI-2 antigen activated B cells were able to activate T cells in *vitro*. Similar results were described by Zisman et al. (37), who demonstrated that TI-2 antigens bind to class II molecules of adherent splenic antigen-presenting cells, enabling them to activate T cells *in vitro*. Accordingly, a deficiency in TI-2 antigen-presenting cells might explain the low frequencies of activated cytokine producing T cells in PLN as compared to the spleen. In that respect, the findings of Janeway et al. (38) are of interest. This group showed that in contrast to B cell depleted spleens, B cell depleted resting lymph nodes were not able to activate T cells *in vitro,* suggesting that resting lymph nodes are deficient in non-B cell antigen-presenting cells. Thus, it appears that the absence of a particular TI-2 antigen presenting cell in PLN is the cause of the low frequency of activated cytokine-PC in PLN. Because of the low cytokine-production in draining PLN of TNP-Ficoll immunized mice, splenic adherent cells are not stimulated to migrate to the PLN and consequently cannot regulate the differentiation of TNP-ASC. This all together makes the microenvironment of the PLN not suitable for the maturation of TNP-AFC after TNP-Ficoll immunization. Probably, BA, as a particulate antigen, uses different antigen-presenting cells for the activation of T cells in PLN than TI-2 antigens do.

The analysis of TI-2 antibody responses in PLN appears to be a valuable approach for the *in vivo* identification of the particular TI-2 antigen-presenting cells and cytokines, which are essential for the activation and maturation of B cells during TI-2 antibody responses. Such an approach may give us more insight in the unique microenvironment of the spleen which is critical in the protection against infections with *Streptococcus pneumoniae, Neisseria meningitides* and *Haemophilus influenzae*.

In conclusion, the present study suggest that a lack of cytokines in PLN after TNP-Ficoll immunization is responsible for the maturation defect of TNP-Ficoll activated PLN B cells. Furthermore, results indicate that combinations of cytokines or other cytokines than IL-4 and

IL-5 are required for the *in vivo* maturation of TNP-Ficoll activated PLN B cells into TNP-ASC. Finally, this *in vivo* study shows that activated T cells and cytokines are actively participating in TI-2 antibody responses.

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CHAPTER 7

In vivo gp39-CD40 interactions and cytokine-production in the spleen during thymus dependent antibody responses

7.1 *In vivo* CD40-gp39 interactions are essential for thymus dependent humoral immunity. I. *In vivo* expression of CD40 ligand, cytokines and antibody production delineates sites of cognate T-B cell interactions

Alfons J.M. Van den Eertwegh, Randolph J. Noelle, Meenakshi Roy, David M. Shepherd, Alejandro Aruffo, Jeffrey A. Ledbetter, Wim J. A. Boersma and Eric Claassen
J. Exp. Med. (in press)

7.2 *In vivo* gp39-CD40 interactions occur in non-follicular compartments of the spleen and are essential for thymus dependent antibody responses and germinal center formation

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In Vivo CD40-gp39 Interactions Are Essential for Thymus-dependent Humoral Immunity. I. *In Vivo* Expression of CD40 Ligand, Cytokines and Antibody Production Delineates Sites of Cognate T-B Cell Interactions

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ABSTRACT

T-B cell interactions have a central role in the development of antibody responses. Upon activation, Th cells express the ligand for CD40, gp39, which is essential for Th cell dependent B cell activation. The cytokines produced by activated Th cells have a regulatory role in B cell differentiation. In this study we investigated, using immunohistochemical techniques, the *in vivo* time course and localization of gp39-expression and cytokine-production in relation to the specific-antibody production. Both the immunization with KLH, a thymus dependent (TD) antigen, and TNP-Ficoll, a thymus independent type 2 (TI-2) antigen, induced Th cells to express gp39. The expression of gp39 was restricted to Th cells in the outer-periarteriolar lymphocyte sheaths (outer-PALS) and around the terminal arterioles (TA). Incidently, $gp39^+$ Th cells were found in the corona of follicles, whereas $gp39^+$ cells were never found in the germinal centers (GC) or marginal zones of the spleen. Maximum frequencies of $gp39^+$ cells were observed 3 and 4 days after primary and secondary immunization with KLH. After injection of TNP-Ficoll, a marked increase in $gp39^+$ cells was observed confirming previous observations that activated T cells are involved in TI-2 antibody responses. Analysis of the *in vivo* cytokine production revealed that IL-2-, IL-4- and IFN-γ-producing cells (IFN-γ-PC) developed according to similar kinetics as observed for $gp39^+$ cells. IL-2-PC and IL-4-PC were present in higher frequencies as were IFN-γ-PC in the immune response against TNP-KLH. Double staining experiments revealed $gp39^+$ Th cells producing IL-2, IL-4 or IFN-γ, suggesting that these cells were involved in both the initial activation as well as the differentiation process of B cells into antibody-forming cells. Dual immunohistochemical analysis revealed

gp39$^+$ T cells and cytokine-PC in close proximity to antigen-specific antibody-forming B cells. In conclusion, this study shows that *in vivo* gp39 is expressed on activated Th cells after immunization with TD and TI-2 antigens. Furthermore, the time course and compartmentalization of gp39$^+$ expression, cytokine production and antibody-formation after immunization suggest that cognate T-B cell interactions and T cell-regulated B cell differentiation occur in the outer-PALS and around the terminal arterioles of the spleen.

INTRODUCTION

The initiation of thymus-dependent (TD) antibody responses requires cognate interactions of class II-restricted antigen-specific Th cells with B cells. None of the molecularly cloned cytokines, alone or in combination, can replace the contact-dependent requirement for B cell activation (1-3). The molecule which mediates the contact-dependent signal was identified as a membrane protein that is expressed on the surface of activated Th cells (4, 5). This membrane protein, gp39, was identified as the ligand for the B cell membrane protein CD40. *In vitro* studies using gp39-bearing plasma membranes from activated Th cells showed that these plasma membranes induce the activation of resting B cells (6, 7). A soluble, CD40-Ig fusion protein and a gp39-specific mAb were able to block the activation of B cells by these plasma membranes (5). Recently, several groups showed that mutations in gp39 are responsible for the defective antibody production in patients with X-linked hyper-IgM syndrome, indicating that this molecule expressed by activated Th cells is essential for humoral immunity (8-11). Isolation of cDNA clones encoding murine (4) and human gp39 (12) showed that this molecule encodes a type II membrane protein. Comparison of the predicted gp39 amino acid sequence with those of other published protein sequences shows that gp39 is homologous to TNF-α and -β (12, 13).

After the initial B cell activation by gp39, Th cell derived cytokines regulate the differentiation of B cells into antibody-forming B cells (6, 7). The pattern of cytokine secretion by Th cells has been suggested to be decisive in the selection of isotype produced by antibody-forming B cells (15-17). It has been shown *in vitro* that Th1 cells (14), which secrete IL-2 and IFN-γ, promote IgM and IgG2a secretion by B cells, whereas Th2 cells (IL-4 and IL-5) stimulate B cells to produce antibodies of IgG1 and IgE isotypes (15-17). The putative central role of gp39 and cytokines in the initiation and development of humoral immunity is mainly based on *in vitro* studies. To date, no data has been presented about the simultaneous expression of gp39 and cytokines during *in vivo* antibody responses. Furthermore, the role of gp39 in *in*

vivo antibody responses has not been addressed. A companion paper demonstrates that gp39 is essential for the induction of *in vivo* antibody responses against various TD antigens (18). This study describes the *in vivo* development and localization of $gp39^+$ cells and cytokine-producing cells in relation to antigen-specific antibody-formation.

MATERIALS AND METHODS

Animals. BCBA.F_1 (C57BL x CBA) mice were bred at the TNO breeding facilities, Rijswijk, The Netherlands. Animals were used at 16-24 weeks of age and were kept under a standard protocol with free access to pelleted food and acidified water (pH 3). Experiments were performed under auspices of the Dutch Veterinary Inspection, as described in the law on Animal experiments.

Chemicals. Alkaline phosphatase (AP; P-6774, type VII-T, 1020 U/mg protein), 3-amino-9-ethylcarbazole (AEC; A-5754), Complete Freunds Adjuvant (CFA), 3,3-diaminobenzidine-tetrahydrochloride (DAB), Fast blue BB Base (F-0125), Fast red, horse radish peroxidase (HRP), Incomplete Freunds Adjuvant (IFA), levamisole, naphthol AS-MX phosphate (3-hydroxy-2-naphtoic acid 2,4-dimethyl-anilide), were obtained from Sigma, St. Louis, MO, USA. N-hydroxysuccinimidyl-(biotinamido)-hexanoate and MHS (maleimidohexanoyl-n-hydroxysuccinimide ester) were obtained from Pierce, Rockford, IL, USA. β-galactosidase (β-gal; E. coli-derived β-D-galactoside galactohydrolase, MW 540 KD), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were obtained from Boehringer, Mannheim, FRG.

Reagents. TNP-Ficoll and TNP-KLH were prepared as previously described (19, 20). The rat mAb Lyt2+ (CD8)(clone 53.6.7.2)(21) and L3T4 (CD4)(clone GK-1.5)(22) were used as cell markers. The control hamster antibodies and ascites from the cell lines MR1 (5), a mAb directed to gp39 and RG7, a mAb specific for rat/hamster Igκ (RG-7; 23) chain were purified by means of a protein-A column. Anti-human IgG1 (3.1.1; 24) and Human IgG1 were obtained from Nordic Immunol. Labs, Tilburg, The Netherlands. The murine mAb DB-1 directed to IFN-γ, was a kind gift of Dr. P. H. van der Meide (25) from our Institute. The cells of the rat mAb 11B11, directed to IL-4, and IL-4 were a kind gift of Dr. W.E. Paul, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, N.I.H., Bethesda, Maryland (26). The IL-2 specific mAb, S4B6, was a kind gift of Dr. T. Mosmann (14). Control rat mAb (2A4, 1G11) were a kind gift of Dr. A Zantema, Sylvius Laboratory, Leiden, The Netherlands (27). Recombinant mouse IL-2 was obtained from Genzyme, Cambridge, MA, USA. The X6310 cell lines producing IL-4 or IL-2 were a kind gift of Dr. F. Melchers, Basel Institute of Immunology,

Basel, Switzerland (28). Antibodies were purified from culture supernatants using an affinity chromatography goat anti-rat Ig column. Cytokine-specific, gp39-specific and control antibodies were conjugated to AP and HRP, as previously described (29). Conjugation to β-gal was performed according to the procedure described by Deelder and De Water (30) with minor modifications (31). TNP-AP and KLH-HRP was prepared according to the previously described methods (19, 20).

Experimental design. BCBA.F_1 mice were injected i.v. with 100 μg of KLH or TNP-KLH or 20 μg of TNP-Ficoll and killed after 0, 1, 2, 3, 4, 5, 6, and 7 days. Another group of mice was injected with 100 μg of KLH, boosted 16 weeks later with 100 μg TNP-KLH and killed after 0, 1, 2, 3, 4, 5 and 7 days. In a parallel experiment, mice were immunized s.c. with TNP-KLH in CFA, boosted 4 weeks later s.c. with 20 μg TNP-KLH in IFA and killed 6 days after injection. Spleens and draining popliteal lymph nodes were removed and immediately frozen in liquid nitrogen and stored at -70°C.

Immunohistochemistry. Splenic cryostat sections (-20 °C, 8 μm), one of every mouse, were picked up on the same glass slide and kept overnight under high humidity at RT. Slides were air-dried and stored in air-tight boxes until use. Slides were fixed for 10 minutes in acetone containing 0.02% H_2O_2. Slides were incubated horizontally overnight at 4 °C with primary cytokine-specific antibody-conjugates diluted in PBS containing 0.1% BSA and titrated to obtain optimal results. Immunohistochemical demonstration of gp39 was performed in two ways: first, with a gp39-specific hamster mAb, MR-1, followed by a hamster-Ig-specific mAb, RG-7, conjugated to peroxidase; second, with a fusion-protein of the gp39 receptor, CD40-IgG1 (5), followed by a human-IgG1-specific mAb conjugated to peroxidase. For double staining of gp39 and CD4 or CD8, spleen sections were incubated simultaneously with MR-1-AP and L3T4-HRP or $Lyt2^+$-HRP. Detection of KLH-specific antibody forming cells (KLH-AFC) and TNP-AFC were detected according to previously described methods (19, 20). Slides were washed with PBS (three times 5 minutes) and immuno-histochemical revelation was performed as described previously; AP (29), HRP (29) and β-gal (30). For double staining the immunohistochemical revelation of AP was performed prior to HRP, and the β-gal staining prior to AP or HRP, because both the peroxidase substrate and the AP substrate were found to inhibit the β-gal activity. To ensure that no over- or understaining occurred, slides with adherent substrate solution were monitored by light microscopy during histochemical reactions. Sections were counterstained with hematoxylin and mounted in glycerin-gelatin. AFC, $gp39^+$ cells and cytokine-PC were counted, and image-analysis was performed as previously described (32). A minimum of three sections of each mouse were examined.

RESULTS

Gp39 expression in immune spleen.

Spleen sections from mice immunized with KLH were stained for the expression of gp39. Two gp39-specific reagents were used for detection: first, MR1, a gp39-specific mAb, and second, CD40-IgG1, a soluble, recombinant fusion protein of the gp39 receptor and IgG1. In serial spleen sections CD40-IgG1 and anti-gp39 identified the same number of cells, which were localized in identical anatomical locations (Fig. 1a,b, table I). Moreover, MR1 blocked the staining with CD40-IgG1 in a dose-dependent manner, confirming the fact that MR1 and CD40-IgG1 recognize the same molecule, gp39 (5). Immunohistochemical double staining, using anti-gp39 and anti-CD4 mAb, showed that gp39 expression was restricted to the CD4 lineage cells (Fig. 1c).

Table 1. Localization of gp39$^+$ cells in different compartments of the spleen after immunization with thymus (in)dependent antigens

Immunization[a]	Day[b]	Reagent[c]	White pulp					Red pulp
			Fc[d]	GC	iP	oP	MZ	TA
Saline	4	MR-1	0[e]	0	0	2±2	0	7±5 0
TNP-KLH: primary	4	CD40-Ig	0	0	0	16±7	0	115±16 0
	4	MR-1	0	0	0	20±5	0	106±30 0
TNP-KLH: secondary	4	MR-1	1±1	0	0	36±12	0	165±19 0
TNP-Ficoll: primary	5	MR-1	0	0	0	60±18	0	222±30 0

[a]Mice were immunized and sacrificed at the indicated day[b]. [c]Cryostat sections were prepared from spleens and immunohistochemical demonstration of gp39 was performed with a anti-gp39 mAb, or with CD40-Ig followed by secondary peroxidase-conjugates. Cells were counted in each anatomical compartment[d] of the spleen: Follicular corona, Fc; germinal center, GC;inner-PALS, iP; outer-PALS, oP; marginal zone, MZ; terminal arterioles, TA. [e]Values represent mean ± SD number of positive cells in each compartment of spleen sections from three mice.

Localization and kinetics of gp39$^+$ cells in lymphoid tissue.

Gp39$^+$ Th cells were found predominantly in the outer-periarteriolar lymphocyte sheaths (PALS) and around the terminal arterioles (TA) of the spleen (Table I, Fig. 1a, 5b). Double staining for CD4$^+$ and gp39 clearly revealed that CD4$^+$ cells in primary follicles were not gp39 positive (Fig. 1c). After secondary immunization, only a few gp39$^+$ cells were observed in the

follicular corona, but not in the GC of secondary follicles (Fig. 1a). Examination of lymph node sections of TNP-KLH immunized mice revealed that gp39$^+$ cells were localized in the deep cortex and along the medullary cords (Fig. 1b). Incidently, gp39$^+$ cells were observed in the corona, but not in the GC, of follicles in lymph nodes (data not shown).

At day 3 and 4 after injection of KLH we observed the maximum number of gp39$^+$ cells (Fig. 2). Thereafter, the number decreased and remained stable during the next three days. Another group of mice was boosted 16 weeks later with KLH. Already during the first two days after secondary immunization we observed a striking increase of the number of gp39$^+$ cells, which was markedly higher as compared to the the primary response. After four days, the gp39$^+$ cells reached maximum numbers, which were about two times higher as we observed during the primary immune response against KLH (Fig. 2). Immunization with TNP-Ficoll, a TI type II antigen, resulted in an increase in the frequency of gp39$^+$ Th cells, attaining maximum frequencies 5 days after injection (Fig. 3). As was observed for TD antigens, in the antibody response against TNP-Ficoll, gp39$^+$ cells were localized in the outer-PALS and around the TA of the spleen (Fig. 5b). No gp39 expression was observed in the splenic follicles of mice immunized with TNP-Ficoll.

Localization and kinetics of cytokine-producing cells in the spleen.

Th cell-derived cytokines have a decisive role in isotype-selection (15-17). As antibody responses against TNP-KLH are dominated by IgG1 antibodies, we investigated whether a Th subset (Th1/Th2) was preferentially activated and where these cytokine-PC were localized in the spleen. Detection of cytokine-PC was performed with cytokine-specific mAb conjugated to different enzymes on splenic cryostat sections of TNP-KLH immunized mice. The IL-4-specific mAb (11B11) conjugated to β-gal was used for the *in situ* demonstration of IL-4-PC. After immunohistochemical revelation, IL-4-PC characterized by a turquoise cytoplasm were detected (Fig. 1d). No staining was observed in control slides from any specimen treated by omission or substitution of the primary antibody. Preincubation of 11B11 conjugated to β-gal with recombinant IL-4 inhibited the staining of IL-4-PC, in a dose-dependent manner. Moreover, the specificity of the staining was confirmed on cytospin prepararations of cells from an IL-4-producing cell line (X6310-IL-4). IL-2-PC were demonstrated with an IL-2-specific mAb (S4B6) conjugated to HRP and were characterized by a red stained cytoplasm, respectively (data not shown). Control immuno-conjugates showed no staining. Furthermore, the specific staining was inhibited by recombinant IL-2 in a dose dependent manner. In addition, specificity was confirmed on cytospin preparations of cells from an IL-2-producing

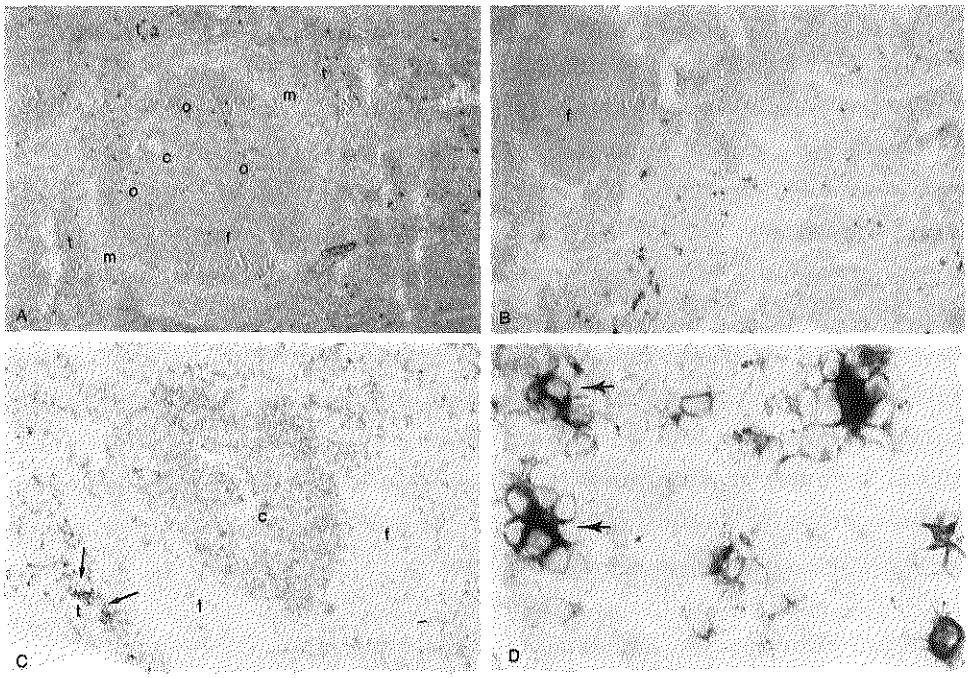

Figure 1) Immunohistochemical localization and characterization of gp39$^+$ cells *in lymphoid organs.* Cryostat sections of murine lymphoid tissue were incubated with specific immuno-conjugates, followed by immunohistochemical revelation. Conjugates and substrates used are indicated between the square brackets. a) 4 days after secondary i.v. immunization with KLH; red stained cells are gp39$^+$ cells localized in outer-PALS (o), around the terminal arteriole (t) and in the follicle (f) of the spleen [MR1 + RG7-HRP; AEC]. b) cryostat section of lymph node 6 days after secondary s.c. immunization with TNP-KLH; red stained cells are gp39$^+$ cells localized in deep cortex and along the medullary cords. In this section no gp39$^+$ cells are localized in the follicle (f) [MR1 + RG7-HRP; AEC]. c) 3 days after KLH immunization; red membrane positive cells are CD4$^+$ cells, while violet double staining cells are CD4$^+$ cells expressing gp39 (arrow). (note that CD4$^+$ cells are present in the follicle (f), while CD4$^+$ cells expressing gp39 are found around terminal arterioles (t) and not in the primary follicle (f) [L3T4-HRP, AEC; MR1-AP, Fast blue] d) 4 days after KLH immunization, red stained cells are gp39$^+$ cells, turquoise stained cell are IL-4-PC, whereas violet stained cells are gp39$^+$ cells producing IL-4 (arrows). [MR1-AP, Fast red; 11B11-β-Gal, X-Gal].

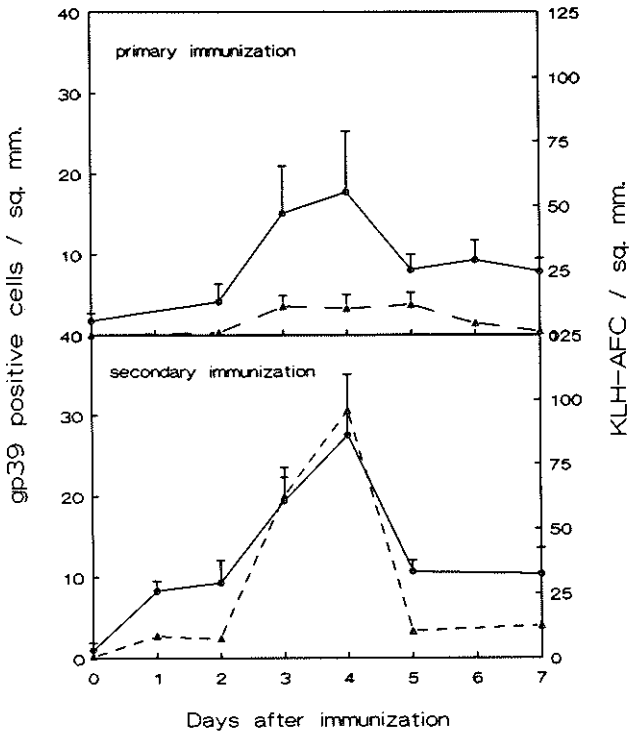

Figure 2) Kinetics of gp39$^+$ T cells and KLH-AFC after primary and secondary immunization with KLH. BCBA.F1 mice were injected i.v. with 100 μg KLH (18) and sacrificed at the indicated time-points. Another group of BCBA.F1 mice was injected with 100 μg of KLH, boosted 16 weeks later with 100 μg KLH i.v. and sacrificed at the indicated time-points. Spleens were removed and immunohistochemistry and image analysis was performed as described in materials and methods section. Values represent mean ± SD of number of positive cells per mm^2 from three mice. Closed triangles, KLH-AFC; closed circles, gp39-bearing cells.

cell line (X6310-IL-2). The specific demonstration of IFN-γ-PC, with an IFN-γ specific mAb (DB-1) conjugated to alkaline phosphatase, was performed as described previously (32, 33). In all spleen sections examined, cytokine-PC were observed in the outer-PALS and around the terminal arterioles (TA) of the spleen. Cytokine-PC were never observed in the follicles or marginal zone of the spleen. Analysis of the kinetics of cytokine-PC revealed higher frequencies of IL-2-PC and IL-4-PC, relative to IFN-γ-PC, in the antibody response against TNP-KLH, reaching maximum frequencies at 3-4 days after immunization (Fig. 4).

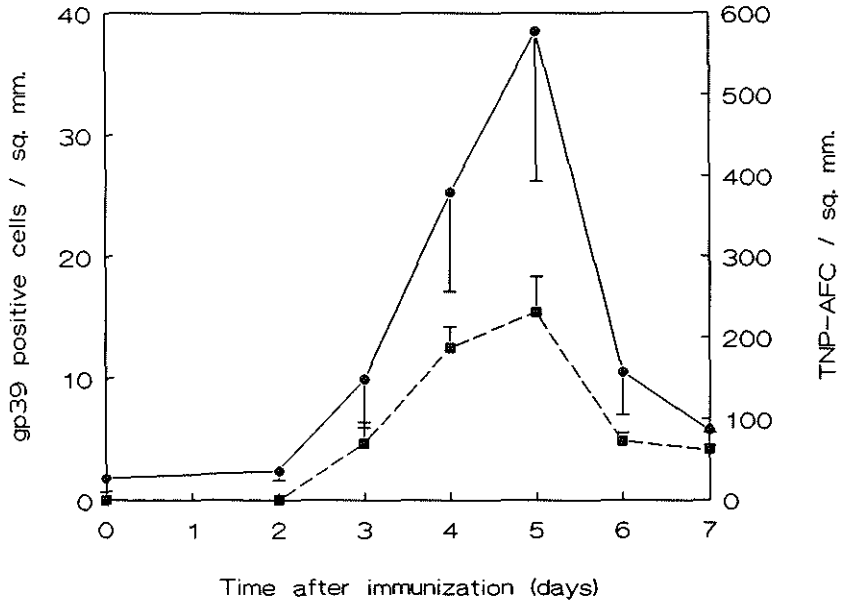

Figure 3) *Kinetics of gp39$^+$ cells and TNP-AFC may be superimposed after immunization with TNP-Ficoll.* BCBA.F1 mice were injected i.v. with 20 μg TNP-Ficoll and sacrificed at the indicated time-points. Spleens were removed and immunohistochemistry and image analysis was performed as described in the materials and methods section. Values represent mean ± SD of number of positive cells per mm^2 from three mice. Closed squares, TNP-AFC; closed circles, gp39-bearing cells.

Cytokine-production by gp39 positive cells.

Gp39$^+$ cells are a prerequisite for B cell activation *in vitro* (5) and TD antibody responses *in vivo* (18). Thereafter, cytokine-producing Th cells are thought to be active as regulators of antibody responses. In order to investigate whether gp39$^+$ cells have the capacity to produce cytokines, as was suggested by *in vitro* experiments (34), we performed double-staining experiments. MR-1 (gp39) was conjugated to AP, whereas S4B6 (IL-2) and 11B11 (IL-4) were conjugated to HRP and β-galactosidase, respectively. Double staining for IFN-γ and gp39 was performed with a direct IFN-γ-specific conjugate and MR-1 followed by a secundary HRP-conjugated mAb directed to hamster Ig. Double staining for IL-2 and gp39 revealed red stained cells producing IL-2, blue stained gp39$^+$ cells and violet double staining cells, representing gp39$^+$ cells producing IL-2 (data not shown). In case of IL-4 and gp39, the

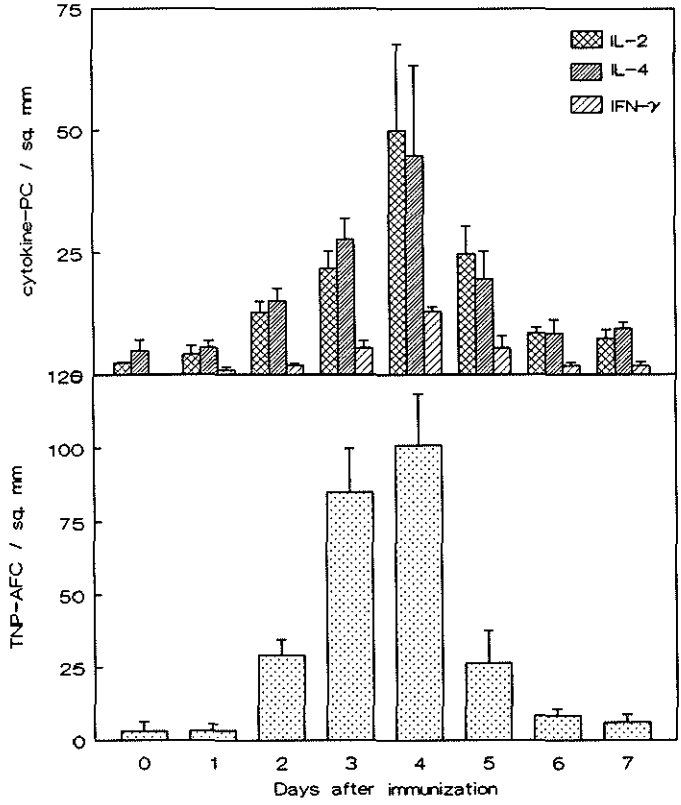

Figure 4) *Cytokine-PC and TNP-AFC develop according to similar kinetics after immunization with TNP-KLH.* BCBA.F1 mice were injected i.v. with 100 μg TNP-KLH (18) and sacrificed at the indicated time-points. Spleens were removed and immunohistochemical demonstration of IL-2-, IL-4-, IFN-γ-PC and TNP-AFC was performed as described in materials and methods section. Values represent mean ± SD of number of positive cells per mm^2 from three mice.

turquoise stained cells were IL-4-producing cells, the red stained cells were gp39 positive, whereas double staining violet cells were gp39$^+$ cells producing IL-4 (Fig. 1d). After double staining for IFN-γ and gp39, we observed blue stained IFN-γ-PC, red stained gp39$^+$ cells and double staining violet cells, representing gp39$^+$ cells producing IFN-γ (data not show). In the spleen, gp39$^+$ cells producing IL-2, IL-4 or IFN-γ e found both during TD and TI-2 antibody responses.

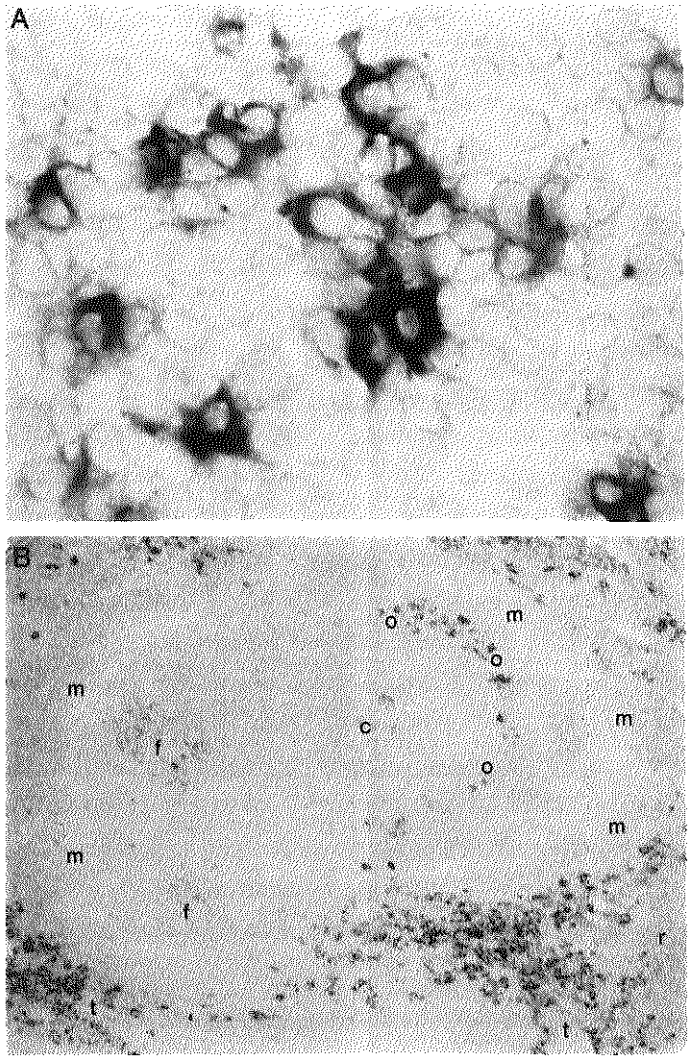

Figure 5. Gp39$^+$ cells and antigen-specific B cells are colocalizing in the immune spleen a) Four days after secondary KLH immunization; KLH-AFC, stained red, were found in close proximity to gp39$^+$ cells, stained blue [KLH-HRP, AEC; MR1-AP, Fast blue] b) Five days after TNP-Ficoll immunization; Gp39$^+$ cells, stained red, and TNP-AFC, stained blue, are found in the same compartments. (Note the gp39$^+$ cells in close proximity to TNP-AFC in outer-PALS and around TA, but not in follicles and marginal zone. Immune-complexes are found in the follicles) [TNP-AP, Fast blue; MR1+RG7-HRP, AEC]. c= central arteriole, f= follicle, m= marginal zone, o= outer-PALS, r= red pulp, t= lymphocyte sheath around terminal arteriole.

Kinetics and localization of antigen-specific AFC.

Using KLH-HRP and TNP-AP conjugates, we were able to study the development of KLH-AFC and TNP-AFC in the spleen after immunization with KLH or TNP-KLH (35). As demonstrated in figure 2 and 4, in both experiments the kinetics of KLH-AFC or TNP-AFC developed according to similar patterns attaining maximum frequencies at 4 days after immunization. The frequency of TNP-(hapten)-specific AFC was about 10 times higher than the detected frequency of AFC specific for the carrier (KLH). After boosting with KLH we found about 8 to 10 times more KLH-AFC as compared to the primary immunization at the peak of the response (Fig. 2). Immunization with TNP-Ficoll resulted in a gradual increase of the number of TNP-AFC attaining the maximum number at day 5 after immunization (Fig. 3).

Gp39 positive cells co-localize with antigen-specific B cells.

As $gp39^+$ Th cells have been found to be essential for the activation of B cells *in vitro* and *in vivo*, their anatomical localization in relation to resting and antibody-producing B cells was examined. Double immunohistochemical staining for resting B cells (membrane IgM-bearing) or B plasma blasts (cytoplasmic Ig) and gp39, revealed that the majority of $gp39^+$ Th cells were co-distributed amongst both B cell types in the outer-PALS and TA (data not shown). In addition, when antibody-forming B cells, specific for the immunizing antigen, were revealed, the KLH-AFC/TNP-AFC were found in close proximity to the $gp39^+$ Th cells (Fig. 5a). Also in the immune response against TNP-Ficoll we observed antigen-specific B cells (TNP-AFC) co-localizing with $gp39^+$ cells in the outer-PALS and around the TA (Fig. 5b).

DISCUSSION

The present study demonstrates that during *in vivo* antibody responses gp39 expression and cytokine-production develop simultaneously and are predominantly localized in the outer-PALS and around the TA of the spleen. The $gp39^+$ cells and cytokine-PC are found in close proximity to antigen-specific B cells. These results suggest that the initial Th cell induced B cell activation and the subsequent Th cell regulated B cell differentiation occur in restricted compartments of the spleen. Furthermore, high frequencies of IL-4-PC, relative to IFN-γ-PC, are found in the immune response against TNP-KLH.

In the primary antibody response against KLH, maximum frequencies of $gp39^+$ Th cells were observed 3-4 days after i.v. immunization (Fig. 2). Later during the course of the immune response, reduced numbers of $gp39^+$ Th cells were present. Parallel studies using

KLH-primed mice showed that upon secondary immunization with KLH, a significant increase in gp39$^+$ Th cells was observed within 24 hrs (Fig. 2). Maximum frequencies in the secondary response were reached at day 4 and were about twice as high as those observed during primary responses. This observation is in agreement with the results described in the accompanying paper (18), which shows a twofold increase in helper activity after adoptive transfer of SRBC immunized spleen cells. Furthermore, the kinetics of appearance of gp39$^+$ Th cells and KLH-AFC were superimposable (Fig. 2). *In vitro* studies using Th cell clones have demonstrated that gp39 is rapidly expressed upon triggering with anti-CD3 mAb (5). Data presented herein document that *in vivo* administration of antigen induces a rapid expression of gp39 on CD4$^+$ Th cells. The simultaneous development of gp39$^+$ cells and KLH-AFC, together with the demonstration of gp39$^+$ Th cells in close proximity to KLH-AFC, suggest that gp39 expression plays a role in the specific antibody production. This is substantiated by experiments described in the accompanying paper which show that anti-gp39 mAb were able to significantly reduce the antibody responses to KLH *in vivo* (18).

It is well known that rigorous T cell depletion completely abrogates the anti-TI-2 antibody response, indicating that T cells are necessary for a bona fide TI-2 response (36-38). The observed high frequency of gp39$^+$ Th cells after immunization with TNP-Ficoll was surprising, given the fact that the antibody responses to TNP-Ficoll were unaffected *in vivo* following administration of anti-gp39 mAb (18). This finding shows that gp39 expression on T cells in the spleen by itself is not enough to activate B cells or could indicate that gp39 detected in the spleen sections is not the same gp39 that seems to be necessary for TD antibody responses *in vivo.* The process of T cell activation, revealed *in vivo* as an increase in the number of gp39$^+$ Th cells, could be explained as suggested by DeKruyff et al. (39), who showed *in vitro* that in TI-2 immune responses activated B cells were responsible for the activation of T cells. Similar results were described by Zisman et al. (40), who demonstrated that TI-2 antigens, composed of D amino acids which bind to class II molecules of antigen-presenting cells, were able to activate T cells. These activated T cells can produce cytokines, as was demonstrated *in vitro* (40) and *in vivo* (32), and may regulate the proliferation and differentiation of B-cells activated by TI-2 antigens (41).

Analysis of the kinetics of cytokine-PC revealed that IL-2-PC and IL-4-PC were predominant in the antibody response against KLH with maximum frequencies at 3-4 days after immunization. The IFN-γ-PC were also found to be active in this immune response, reaching maximum levels at day 3, but the number of IFN-γ-PC was low as compared to the number of IL-2- and IL-4-PC. Bradley et al. (42) observed that the kinetics of appearance of effector

$CD4^+$ T cells that produce cytokines upon restimulation with KLH *in vitro* were similar for each of the cytokines investigated. These results confirm our *in vivo* findings and suggest that after immunization and subsequent antigen presentation, T cells with potency to produce IL-2, and/or IL-4, IL-5 and/or IFN-γ are activated at about the same time and differentiate into cytokine-PC, following a similar time course. At the peak of the immune response, we observed relatively high frequencies of IL-2-PC and IL-4-PC in the outer-PALS and around the TA. As these cytokine-PC were observed in close conjunction, it is likely that these cells create a microenvironment which is rich in IL-2 and IL-4. Such a microenvironment has been suggested to be essential for the development IL-4-PC, as was demonstrated *in vitro* (43-45). These experiments suggested that IL-2 was required for the optimum proliferation of cytokine-producing T cells (43-46), while IL-4 would propagate the preferential development of IL-4-PC (43, 44). After double staining we were able to detect TNP-AFC in close proximity to cytokine-PC (data not shown), suggesting that these cytokines have a role in B cell differentiation. Nossal and Riedel (47), demonstrated a rapid rise in precursors of KLH-binding IgG1-secreting B cells in the spleen 5 to 7 days after KLH immunization. IL-4 has been suggested to play a role in B cell switching to IgG1 and in the propagation of $IgG1^+$ AFC (17). Consequently, the close proximity of IL-4-PC to KLH-specific B cells may stimulate the preferential development of $IgG1^+$ B cells.

In this study we observed incidently (<1%) $gp39^+$ cells in the corona of the follicles, but no $gp39^+$ cells or cytokine-producing T cells were found in the GC. These observation are compatible with studies in humans (48, 49, 50) and mice (32,52), which demonstrated that none of the B cell differentiation factors, such as IL-2, IL-4 and IFN-γ, were localized in the follicles of lymphoid tissue. Butch et al (51) showed *in vitro* that IL-4 mRNA was the only cytokine expressed by germinal center T cells, whereas these T cells expressed no mRNA of the other 9 tested cytokines. As this group examined cytokine mRNA expression *in vitro*, which is not always correlated with protein synthesis *in vivo* (53), these data are not incompatible with our *in vivo* results.

Recently, Lederman et al. (54) demonstrated in human lymphoid tissue that the T-cell-B-cell activating molecule (T-BAM), the human equivalent of gp39, was expressed on $CD4^+$ cells in the PALS of the spleen and in lymphoid follicles of tonsils, lymph nodes and spleens. Essentially the human and murine studies give identical localization patterns for gp39 expressing T-cells. The observed difference in the number of positive cells in the follicles is most likely due to the fact that the murine studies are performed with a non-replicating antigen whereas the human material is most probably chronical inflamed (more activated T-

cells).

During primary immune responses, antibody production in PALS and around TA precedes the germinal center formation, indicating that the initial B cell activation occurs outside the follicles (55,56,57). This is in agreement with the results presented in this report, showing no $gp39^+$ cells and cytokine-PC in the follicles, but in the outer-PALS and around the terminal arterioles. Only after secondary immunization, we observed a low frequency of $gp39^+$ cells in the corona of lymphoid follicles. The similar localization of AFC (35), $gp39^+$ cells and cytokine-PC as observed in the immune response against KLH or TNP-Ficoll, suggests that T-B cell interactions during primary and secondary antibody response against TD or TI-2 antigens occur in the same splenic compartments, e.g. the outer-PALS and terminal arterioles. Furthermore, it validates *in vitro* experiments demonstrating that the kinetics of cytokine production and CD40-ligand expression are superimposable (7). The observation that during the entire experimental period antigen-specific AFC and $gp39^+$ cells were found in close proximity, in addition to *in vitro* experiments which showed that extended contact (more than 48 hrs) is required for maximal proliferative responses (58) or cytokine production (59), suggests that T-B conjugates may persist for several days *in vivo*.

Figure 6 shows a model for the development of TD antibody responses in the spleen based on presented data and on the localization and migration of immune cells (52). We suggest that during the primary antibody response, TD antigens are presented by interdigitating cells in the PALS, leading to increasing numbers of antigen-specific T cells which subsequently encounter antigen-specific B cells in the PALS, forming T-B cell conjugates (60, 61). During this cognate T-B cell interaction, $CD4^+$ Th cells will be activated by antigen-presenting B cells and express the ligand for CD40. Gp39 will trigger B cell growth and differentiation. Part of the activated B cells migrates to the follicles to undergo follicular processes, such as B cell selection, somatic mutation, affinity maturation and memory formation (55, 56). Another part of the activated B cells migrates to the TA and differentiates into antigen-specific AFC regulated by activated cytokine-producing T cells. After secondary immunization, antigen-specific memory B cells acquire the antigen in the follicle, where it is presented by follicular dendritic cells in the form of immune complexes (55, 56). These B cells will migrate to the PALS and meet antigen-specific T cells and will subsequently follow the pathway as described for primary immune responses. In case of secondary TD antibody responses, the follicles already contain relatively high frequencies of antigen-specific B cells, which increases the likelihood that B cells encounter antigen-specific T cells in the PALS. This may explain the relatively high frequency of activated $gp39^+$ T cells and antigen-specific AFC found during

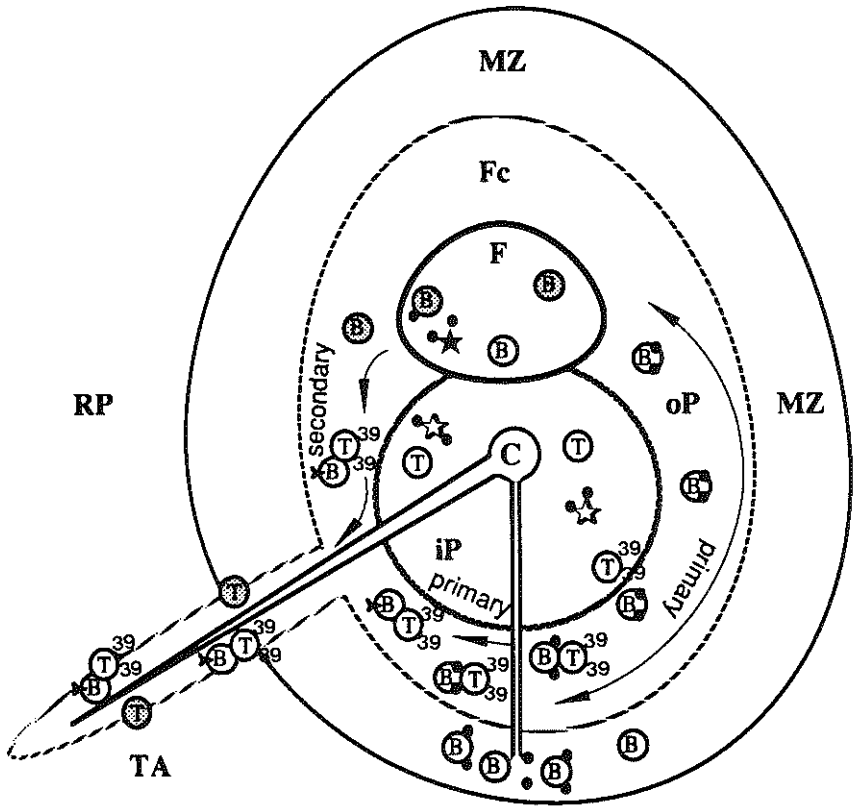

Figure 6) *Schematic representation of the activation and migration of T and B cells in the spleen during the TD immune response.* Arrows indicate migration of B and T cells in the spleen. C= central arteriole, ip= inner-PALS, F= follicle, Fc= follicle corona, MZ= marginal zone, iP= inner-PALS, oP= outer-PALS, R= red pulp, S= sinus, ta= lymphocyte sheath around terminal arteriole. • = antigen, Ⓑ = resting B cell, ● = memory B cell, Ⓑ* = differentiating antigen-specific B cell Ⓑ = antibody-forming B cell, Ⓣ = resting T cell, Ⓣ^{39}_{39} = activated antigen-specific T cell (gp39 positive), ⓒ = cytokine-producing T cell, ✶ = FDC, ☆ = IDC.

secondary immune responses. The observation of higher frequencies of antigen-specific AFC relative to gp39$^+$ T cells (Fig. 2), suggests that one T cell may be able to activate more than one B cell. Alternatively, the relatively low frequencies of gp39$^+$ T cells could be due to the shorter lasting expression of gp39 by T cells (7, 62) as compared to the expression of antigen-specific antibodies by B cells.

In conclusion, this study demonstrates that gp39$^+$ T cells and cytokine-PC are simultaneously upregulated *in vivo* after immunization. These gp39$^+$ cells and cytokine-PC are observed in close proximity to antigen-specific B cells. The data presented suggest that the initial cognate B cell activation and the subsequent regulation of B cell differentiation by T cells occur in the non-follicular areas of the spleen, namely the outer-periarteriolar sheaths and around the terminal arterioles.

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IN VIVO gp39-CD40 INTERACTIONS OCCUR IN THE NON-FOLLICULAR COMPARTMENTS OF THE SPLEEN AND ARE ESSENTIAL FOR THYMUS DEPENDENT ANTIBODY RESPONSES AND GERMINAL CENTER FORMATION

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INTRODUCTION

The initiation of thymus-dependent (TD) antibody responses requires class II-restricted antigen-specific helper T cell cognate interactions with B cells. None of the molecularly cloned cytokines, alone or in combination, can replace the contact-dependent requirement for B cell activation[1]. The molecule which mediates the contact-dependent signal is a 39 kD membrane protein which is expressed on the surface of activated T-helper (Th) cells[2,3]. This membrane protein, gp39, was identified as the ligand for the B cell membrane protein CD40. CD40, a mitogenic receptor expressed on all mature B lymphocytes[4], is a type I membrane protein and member of the TNF receptor family[5]. Evidence that CD40 is an important receptor on B cells is derived from studies that anti-CD40 mAb and cofactors such as anti-immunoglobulin (Ig) and cytokines initiate both B cell growth and differentiation[6,7].
The ligand for CD40, gp39 is a type II membrane protein which is homologous to TNF-α and -β[8,9]. It is transiently expressed on activated $CD4^+$ cells *in vitro*[3]. It was demonstrated that gp39-bearing plasma membranes (PM) from activated Th cells can activate resting B cells[10]. A soluble, CD40-Ig fusion protein and a gp39-specific monoclonal antibody (mAb) were able to block the activation of B cells by these PM[3]. After activation, the Ig-secretion of activated B cells is regulated by Th cell derived cytokines[10,11,12]. Recently, we demonstrated that $gp39^+$ cells are upregulated after immunization with TI-2 and TD antigens and are localized in juxtaposition to antigen-specific antibody-forming cells in restricted compartments of the spleen[13]. It was demonstrated that these putative gp39-CD40 interactions are critical for the development of secondary antibody responses against soluble TD antigens[14].
Germinal centers (GC) are clusters of B lymphoblastoid cells which develop after antigenic stimulation in follicles of peripheral lymphoid organs and are thought to play a major role in the generation of B cell memory[15]. GC formation is known to be TD[16], although it is not known how T cells are involved in the induction of the GC reaction. *In vitro* studies suggested that the CD40-molecule is important for GC formation[17]. Therefore, we investigated in this study the localization of CD40-ligand bearing cells in the spleen during the formation of GC. Furthermore, the *in vivo* localization of gp39-CD40 interactions in the spleen, and their role in primary antibody responses and GC formation was assessed.

MATERIALS AND METHODS

Animals BCBA.F_1 (C57BL x CBA) and BALB/c mice were bred at the TNO breeding facilities, Rijswijk, The Netherlands. Animals were used at 16-24 weeks of age and were kept under a standard protocol with free access to pelleted food and acidified water (pH 3).

Chemicals Alkaline phosphatase (AP; P-6774, type VII-T, 1020 U/mg protein) 3-amino-9-ethylcarbazole (A-5754), Fast blue BB Base (F-0125), horse radish peroxidase (HRP), levamisole, naphthol AS-MX phosphate (3-hydroxy-2-naphtoic acid 2,4-dimethyl-anilide), TNP sulfonic acid (TNBS, grade I) were obtained from Sigma, St. Louis, MO, USA.

Reagents TNP-Ficoll and TNP-KLH were prepared as previously described[16]. The control hamster antibodies and ascites from the cell lines MR1 (3), a mAb directed to gp39 and RG7, a mAb specific for rat/hamster Igκ (RG-7[19]) chain were purified by means of a protein-A column. TNP-AP was prepared according to the previously described methods[18].

Experimental design BCBA.F_1 mice were injected i.v. with 100 μg of TNP-KLH or 20 μg of TNP-Ficoll and killed after 0, 1, 2, 3, 4, 5, 6, and 7 days. To determine the functional role of gp39$^+$ cells in TD and TI-2 antibody responses, BALB/c mice were injected i.v. with either 100 μg of TNP-KLH or 20 μg TNP-Ficoll on day 0. Subsequently, at day 0, 2, 4 mice were given 250 μg of purified anti-gp39 mAb (MR1) or 250 μg purified hamster Ig or PBS, i.p., as previously described[14]. Mice were bled at day 7 and 14, sacrificed at day 14 and serum was prepared. In all experiments spleens were removed and immediately frozen in liquid nitrogen and stored at -70°C.

Immunohistochemistry Immunohistochemistry was done as earlier described[21,22].

TNP-specific ELISA TNP-specific IgM and IgG were determined in mouse serum by means of a direct ELISA as described earlier[23]. The isotype-specific capture ELISA was performed as described by Vos et al.[24]. The TNP-specific titer was the dilution at which 50% of the maximum absorbance was found. Titers of each isotype were related to that of a control (PBS) group, which was set at 100%.

RESULTS AND DISCUSSION

Localization and kinetics of gp39$^+$ cells in lymphoid tissue. Spleen sections from immunized mice were stained for the expression of gp39[13]. Gp39$^+$ Th cells were found predominantly in the outer-periarteriolar lymphocyte sheaths (PALS) and around the terminal arterioles (TA) of the spleen (Fig. 1), but not in the follicles or marginal zone of the spleen. At day 3 and 4 after injection of TNP-KLH we observed the maximum number of gp39$^+$ cells[13]. Thereafter, the number decreased and remained stable during the next 10 days. Immunization with TNP-Ficoll, a TI type 2 antigen, resulted in an increase in the frequency of gp39$^+$ Th cells, reaching maximum frequencies 5 days after injection[13]. Similar as observed for TD antigens, in the antibody response against TNP-Ficoll gp39$^+$ cells were localized in the outer-PALS and around the TA of the spleen.

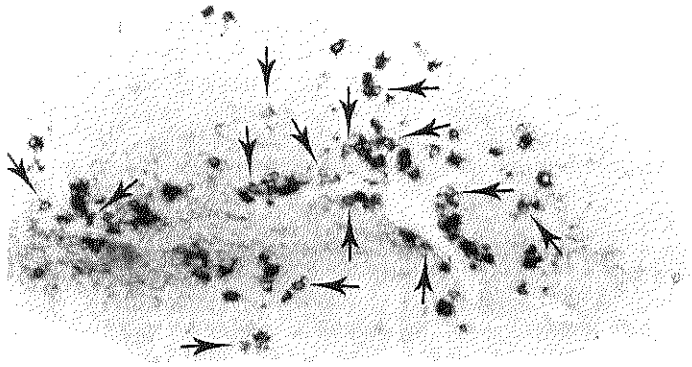

Fig. 1. Gp39+ cells (arrow) are found in close conjunction to TNP-AFC around the terminal arterioles of the spleen 4 days after immunization with TNP-KLH

Gp39+ cells co-localize with antigen-specific B cells.
As gp39$^+$ Th cells are essential for the activation of B cells *in vitro* and *in vivo*, their anatomical localization in relation to resting and antibody-producing B cells was examined. Double immunohistochemical staining for resting B cells (membrane IgM-bearing) or B plasma blasts (cytoplasmic Ig) and gp39, revealed that the majority of gp39$^+$ Th cells were co-distributed amongst both B cell types in the outer-PALS and TA (data not shown). In addition, when antibody-forming B cells, specific for the immunizing antigen, were revealed, the TNP-AFC were found in close conjunction to the gp39$^+$ Th cells (Fig. 1). Also in the antibody response against TNP-Ficoll we observed antigen-specific B cells (TNP-AFC) co-localizing with gp39$^+$ T cells in the outer-PALS and around the TA. These results suggest that during TI-2 as well as TD antibody responses T-B cell interactions occur in the non-follicular compartments of the spleen[13].

Anti-gp39 administration inhibits the primary IgG antibody responses to TD antigens.
In vitro studies indicated that anti-gp39 mAb blocked the Th-dependent activation of B cells[3]. We observed high frequencies of gp39$^+$ cells in juxtaposition to TNP-AFC at 2-4 days after TNP-KLH immunization. In order to investigate whether these putative gp39-CD40 interactions were essential for the primary antibody response against TNP-KLH *in vivo*, mice were treated with anti-gp39 mAb after immunization. The primary anti-TNP antibody response was completely inhibited when we examined the IgG subclasses (Fig. 2). The antigen-specific IgM response was not completely inhibited after treatment with anti-gp39 mAb, confirming that this part of the response was not fully T cell (gp39)-dependent[25].

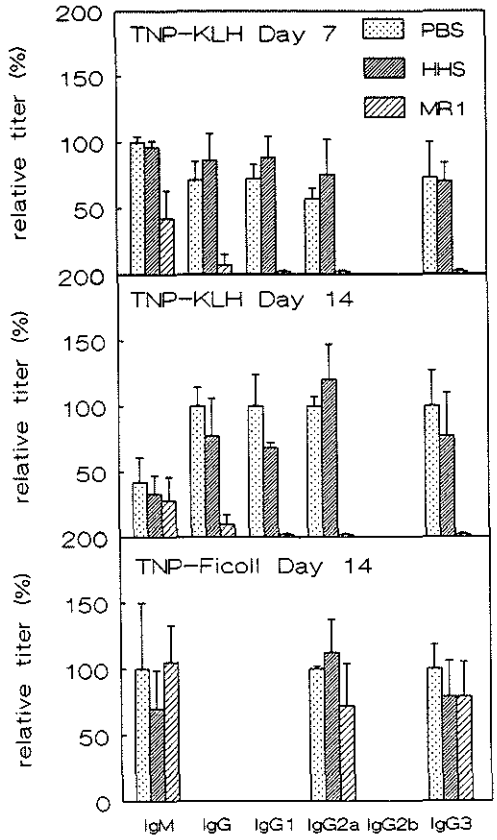

Fig. 2. Anti-gp39 antibodies (MR1) inhibit the primary antibody response against TNP-KLH

Anti-gp39 administration does not inhibit the antibody response to TI-2 antigens

We observed that gp39$^+$ cells were upregulated after administration of TNP-Ficoll, and localized in close conjunction to TNP-AFC, suggesting that gp39-CD40 interactions play a role in the initiation of TI-2 antibody responses. In order to address this possibility we treated TNP-Ficoll immunized mice with anti-gp39 mAb. Figure 2 demonstrates that the *in vivo* IgM, IgG2a and IgG3 antibody responses against TNP-Ficoll were not blocked by gp39-specific antibodies. These results indicate that the gp39 is not functional in the induction of TI-2 antibody responses. The gp39$^+$ cells localized in juxtaposition to TNP-AFC may represent interactions between activated cytokine-producing Th cells and antibody-forming cells[22]. The demonstration of activated T cells in the spleen after TNP-Ficoll immunization substantiates previous reports, which showed that T cells are important for the development of antibody responses against TI-2 antigens[26,27].

Anti-gp39 administration inhibits GC formation

GC formation is a T cell dependent process. Nude mice do not develop GC upon antigenic challenge, but are able to produce GC after reconstitution with T cells[16]. To investigate whether gp39$^+$ (activated) T cells play a role in GC formation, we studied the influence of the anti-gp39 treatment on the formation of GC at day 14 after TNP-KLH immunization. Interestingly, anti-gp39 treatment completely inhibited GC formation. Since we demonstrated in previous reports[14] that gp39$^+$ cells are not depleted after injection of anti-gp39, these results suggest that gp39-CD40 interactions play a role in GC formation. Gp39$^+$ cells were never observed in the follicles of spleens after primary immunization, indicating that the initial B cell activation occurs outside the follicles, as was earlier suggested[28,29,30]. Apparently, after activation by gp39$^+$ cells, B cells get the competence to proliferate in the microenvironment of follicles, which will lead to the development of germinal centers.

Conclusions

Data presented and previous studies on the localization and migration of immune cells[30] suggest that during primary antibody responses, TD antigens are presented by interdigitating cells in the PALS, leading to increasing numbers of antigen-specific T cells which subsequently encounter antigen-specific B cells in the PALS, forming T-B cell conjugates. During this cognate T-B cell interaction, Th cells will be activated by antigen-presenting B cells and express the ligand for CD40. Gp39 will trigger B cell growth and differentiation. Part of the activated B cells migrates to the follicles to undergo follicular processes, such as somatic mutation and memory formation[15]. Another part of the activated B cells migrates to the TA and differentiates into antigen-specific AFC regulated by activated cytokine-producing T cells[13,30]. In conclusion, presented data suggest that the gp39-CD40 interactions which occur in the outer-PALS and around the terminal arterioles of the spleen are essential for the induction of TD antibody responses as well as germinal center formation.

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CHAPTER 8

In vivo antigen localization, gp39 expression, cytokine production and antibody-formation are sequential events after injection of anti-mouse-IgD antibodies

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ABSTRACT

In thymus dependent (TD) antibody responses a physical interaction between T and B cells is required for the activation of Th cells. After activation, Th cells will express a 39 Kd protein (gp39) on their membranes and produce cytokines. Binding of gp39 to the CD40 molecule on B cells provides the initial B cell activating signal, while the Th cell derived cytokines regulate the B cell differentiation into antibody-forming cells. In order to determine the timing and localization of these phenomena during *in vivo* TD antibody responses, we investigated *in vivo* the successive events of antigen localization, gp39 expression, cytokine production and antibody production after injection of rabbit anti-mouse IgD (RαIgD) antibodies. Shortly after immunization, injected RαIgD was observed in all the follicles of the spleen. At 36 h after RαIgD injection, the frequency of gp39$^+$ cells started to increase and attained maximum levels at day 4. Double staining revealed gp39$^+$ cells producing IL-2, IL-4, or IFN-γ, and explains the observation that the kinetics of cytokine-producing cells (cytokine-PC) were superimposable to that of activated gp39$^+$ Th cells. IL-2-PC and IL-4-PC were observed in markedly higher frequencies as were IFN-γ-PC. Analysis of the *in vivo* cytokine profile revealed low frequencies of cells producing IL-2 and IFN-γ, or IL-4 and IFN-γ, or IL-2 and IL-4 consistent with the Th2 character of this response. All gp39$^+$ cells and cytokine-PC were predominantly localized in the peri-arteriolar lymphocyte sheaths (PALS) and around the terminal arterioles (TA) of the spleen, whereas the injected RαIgD was preferentially localized in the follicles, implicating that antigen-acquisition and presentation occur in different compartments. Three and four days after RαIgD injection, high frequencies of gp39$^+$ cells and cytokine-PC were observed in juxtaposition to IgM$^+$ cells, which preceded the high IgE and IgG1 production observed after day 7. Treatment of RαIgD immunized mice with anti-gp39-specific mAb, resulted in an almost complete suppression of the polyclonal antibody response after RαIgD injection. The possibility that the anti-gp39 treatment depleted gp39$^+$ cells and cytokine-PC *in vivo*, was ruled out by showing that injection of anti-gp39 mAb did not affect the expression of gp39 and cytokines *in vivo*.

In conclusion, presented data suggests that cognate T-B cell interactions, leading to isotype-switching and antibody-production, occur predominantly in the PALS and around the terminal arterioles of the spleen. Furthermore, it was demonstrated that IL-2 and IL-4 are two of the principal active cytokines in the *in vivo* immune response to RαIgD. Moreover, the *in vivo* localization, kinetics and function of gp39$^+$ cells, cytokine-PC, in relation to IgM$^+$ cells and IgG and IgE antibody production, indicate a major regulating role of gp39$^+$ Th cells and their products in the antibody response after RαIgD injection.

INTRODUCTION

T-B cell interactions have a central role in the development of humoral immunity. B cells capture antigen via their antigen-specific receptor and present the processed antigen to specific Th cells. The recognition of complexes of processed antigens in context of MHC on B cells, together with the interactions of the costimulatory molecule B7 on B cells with CD28 on Th cells (1), leads to the initial class II restricted, antigen-specific activation of Th cells. These activated Th cells express novel surface proteins on their membranes and produce cytokines, which enable them to activate the B cells. The activation of B cells requires a physical contact with activated Th cells which cannot be replaced by presently known cytokines (2, 3). Recently, it was demonstrated *in vitro* that a 39 kD surface protein (gp39), selectively expressed on activated Th cells, and its receptor CD40 on B cells were critical for the initial Th dependent B cell activation (4, 5). After the initial B cell activation, Th cell derived cytokines are thought to regulate the differentiation of B cells into antibody-forming cells (2,6,7). The pattern of cytokine secretion of Th cells appears to be crucial in the selection of isotype produced by antibody-forming B cells (8,9,10). It has been shown *in vitro* that Th1 cells (11), which secrete interleukin-2 (IL-2) and interferon-γ (IFN-γ), promote IgM and IgG2a secretion by B cells, whereas Th2 cells (IL-4 and IL-5) stimulate B cells to produce antibodies of IgG1 and IgE isotypes (8,9,10).

Most of the above discussed data are derived from *in vitro* experiments. However, whereas cells can interact relatively randomly in cell suspension, peripheral lymphoid tissues like lymph nodes and spleen have distinct compartments, each consisting of particular cell types, which dictate the cell-cell interactions that are allowed or impossible. *In vitro* experiments necessarily disregard this complex organization of lymphoid organs, and may therefore not be applicable to *in vivo* immune responses. In order to get insight in the microenvironment of lymphoid tissues, studies were initiated, which addressed the *in vivo* activity and anatomical compartmentalization of immune cells during antibody responses (12). *In vivo* analysis of TD antibody responses have demonstrated that the frequency of $gp39^+$ cells and cytokine-producing cells (PC) were upregulated after administration of KLH, a TD antigen (13). These effector Th cells were found in juxtaposition to activated KLH-specific B cells. In a separate report evidence was provided that gp39 was essential for antibody responses against TD antigens (14). For the investigation of the role of cytokines in isotype switching several *in vivo* models are used (15). Injection of mice with goat antibodies directed to mouse IgD (GαIgD) leads to a strong polyclonal activation of the murine immune system (16,17). These antibodies

bind to and cross link mouse B cell membrane IgD, resulting in the activation of B cells. GαIgD is internalized, processed, and presented to goat-Ig-specific T cells, leading to massive T cell activation and cytokine production (18,19). Recognition of the injected anti-IgD antibody as foreign is required for the induction of IgG1 and IgE production (17, 20), isotypes which are suggested to be regulated by a.o. IL-4 (8,9,10). The high degree of T cell activation and cytokine-production, which is critical for the large antibody production, make this model ideal for analysis of the sequence of immunological events during *in vivo* TD immune responses. To get insight in the splenic microenvironment during antibody responses, we analyzed the localization and development of antigen-handling, gp39 expression, cytokine-production and antibody-formation in spleens of mice after injection of RαIgD. Furthermore, we investigated the *in vivo* role of $gp39^+$ cells in this polyclonal antibody response.

MATERIALS AND METHODS

Animals. BALB/c and BCBA.F_1 (C57BL x CBA) mice were bred at the TNO breeding facilities, Rijswijk, The Netherlands. Animals were used at 16-24 weeks of age and were kept under standard protocol with free access to pelleted food and acidified water (pH 3). Experiments were performed under auspices of the Dutch Veterinary Inspection, as described in the law on Animal experiments.

Chemicals. Alkaline phosphatase (AP; P-6774, type VII-T, 1020 U/mg protein), 3-amino-9-ethylcarbazole (AEC; A-5754), 3,3-diaminobenzidine-tetrahydrochloride (DAB), 5-BCIP (5-bromo-4-chloro-3-indolyl-phosphate; B-8503), Fast blue BB Base (F-0125), Fast red, horse radish peroxidase (HRP), levamisole, naphthol AS-MX phosphate (3-hydroxy-2-naphtoic acid 2,4-dimethyl-anilide), were obtained from Sigma, St. Louis, MO, USA; N-hydroxysuccinimidyl-(biotinamido)-hexanoate and MHS (maleimidohexanoyl-n-hydroxysuccinimide ester) were obtained from Pierce, Rockford, IL, USA; β-galactosidase (β-gal; E. coli-derived β-D-galactoside galactohydrolase, MW 540 KD), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were obtained from Boehringer, Mannheim, FRG.

Reagents. RαIgD, goat-Ig (GIg) specific for mouse IgE conjugated to HRP, anti-human IgG1 (3.1.1; 21) and human IgG1 were obtained from Nordic, Tilburg, The Netherlands. AP-conjugated GIg to mouse IgM or IgG were obtained from Tago, Burlingame, CA 94010. GIg anti-mouse IgG2a was obtained from Southern Biotechnology, USA. Biotinylated RIg anti-mouse IgG2a was obtained from Pharmagen, San Diego, USA. Rabbit antibodies against peroxidase complexed with peroxidase (RPAP), and swine anti-RIg conjugated to HRP were

obtained from Dakopatts, Copenhagen, Denmark. Avidin-HRP and avidin-AP were obtained from Sigma, St. Louis, MO, USA. The control hamster antibodies and ascites from the cell line MR1 (5), a mAb directed to gp39 and RG7, a mAb specific for rat/hamster Igκ (RG-7; 22) chain were purified by means of a protein-A column. The murine mAb DB-1 directed to IFN-γ, was a kind gift of Dr. P. H. van der Meide (23) from our Institute. The cells of the rat mAb 11B11, directed to IL-4, and IL-4 were a kind gift of Dr. W.E. Paul, Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, N.I.H., Bethesda, Maryland (24). S4B6, directed to IL-2, was a kind gift of Dr. T. Mosmann (11). Control rat mAb (2A4, 1G11) were a kind gift of Dr. A Zantema, Sylvius Laboratory, Leiden, The Netherlands (25). Recombinant mouse IL-2 was obtained from Genzyme, Cambridge, MA, USA. The X6310 cell lines producing IL-4 or IL-2 were a kind gift of Dr. F. Melchers, Basel Institute for Immunology, Basel, Switzerland (26). Antibodies were purified from culture supernatants using an affinity chromatography goat anti-rat Ig column. Cytokine-specific and control antibodies were conjugated to AP and HRP as previously described (27). Biotinylation was performed as previously described (28). Conjugation to β-gal was performed according to the procedure described by Deelder and De Water (29) with minor modifications (30). TNP-AP and KLH-HRP was prepared according to the previously described methods (31).

Experimental design. BCBA.F_1 mice were injected i.v. with 800 μg of RαIgD and killed after 1.5, 3, 4, 5, 6, 7, 9, and 11 days. Mice were bled and serum was prepared. Spleens were weighed, removed and immediately frozen in liquid nitrogen and stored at -70°C.

To determine the functional role of $gp39^+$ cells in this antibody response, high responder BALB/c mice were injected i.v. with 800 μg of RαIgD on day 0. Subsequently, on day 0, 2, 4 mice were given 250 μg of purified anti-gp39 mAb (MR1) or 250 μg purified hamster Ig or PBS, i.p.. Mice were bled and sacrificed at day 7, and serum was prepared. Spleens were removed, weighed and a part was immediately frozen in liquid nitrogen and stored at -70°C. The other part of the spleen was used for ELISPOT assay. To investigate the effect of anti-gp39 treatment on the splenic expression of gp39 and cytokines, BCBA.F1 were injected with 800 μg RαIgD on day 0. Subsequently, on day 0, 2, 4 mice were given 250 μg of purified anti-gp39 mAb (MR1) or 250 μg purified hamster Ig or PBS, i.p.. At day 4, 2 h after the last injection of anti-gp39 mAb, mice were killed and spleens were removed.

Immunohistochemistry. Splenic cryostat sections (-20 °C, 8 μm), one of every mouse, were picked up on the same glass slide and kept overnight under high humidity at RT. Slides were air-dried and stored in air-tight boxes until use. Slides were fixed for 10 minutes in acetone containing 0.02% H_2O_2. Detection of cytokine-PC was performed as described

previously (13,32). Slides were incubated horizontally overnight at 4 °C with cytokine-specific antibody-conjugates diluted in PBS containing 0.1% BSA and titrated to obtain optimal results. Specificity was confirmed on cytospin preparations of cells from an IL-2- or IL-4-producing cell line (X6310-IL-2). Immunohistochemical demonstration of gp39 was performed in two ways (13): first, with a gp39-specific hamster mAb, MR-1, followed by a hamster-Ig-specific mAb, RG-7, conjugated to HRP; second, with a fusion-protein of the gp39 receptor, CD40-IgG1 (5), followed by a human-IgG1-specific mAb conjugated to HRP. For double staining of gp39 and IL-2 or IL-4, spleen sections were incubated simultaneously with MR-1-AP and cytokine-specific immuno-conjugates. Detection of KLH-specific antibody forming cells (KLH-AFC) and TNP-AFC was performed according to previously described methods (31). IgM and IgG positive cells were detected with specific antibodies conjugated to AP. Subsequently, slides were washed with PBS (three times 5 minutes) and immuno-histochemical revelation was performed, as described previously; AP (27), HRP (27) and β-gal (29). For the double staining experiments, the immunohistochemical revelation of AP was performed prior to HRP, and the β-gal staining prior to AP or HRP, because both the peroxidase substrate as well as the AP substrate were found to inhibit the β-gal activity. To ensure that no over- or understaining occurred, slides with adherent substrate solution were monitored by light microscopy during histochemical reactions. Sections were counterstained with haematoxylin and mounted in glycerin-gelatin. Antibody-forming cells, $gp39^+$ cells and cytokine-PC were counted, and image-analysis was performed as previously described (32). For quantitation of follicular RαIgD, all positive follicle were counted and expressed as the percentage of all follicles in each cryostat section. A minimum of three sections of each mouse were examined.

ELISA Microtitre plates (Flow labs, PVC flat-bottomed, 96 wells, # 77-172-05 highly activated) were coated overnight at 4°C with 100 μl of 5 μg RαIgD ml^{-1}, or 5 μg OVA-TNP ml^{-1}, or 5 μg KLH ml^{-1} in PBS, followed by blocking with 150 μl 0.5% Gelatine in 0.1 M sodium phosphate buffer + 0.9% NaCl. The plates were washed with PBS plus 0.05% Tween-20. Subsequently, plates were incubated with sera from immunized mice for 1 hr at 37 °C, washed and incubated with appropriately diluted goat-anti mouse IgM or IgG antibodies conjugated to alkaline phosphatase for 1 hr at 37 °C. Plates were again washed five times with PBS + 0.1% Tween-20 and 100 μl of substrate solution (1 g p-nitrophenylphosphate.l^{-1} in 10 mM diethylenel glycol + 10 mM $MgCl_2$, pH 9.6) was added to each well. The absorbance at 405 nm was measured after 30 min, on an automated micro-ELISA reader (Titertek Multiskan, Flow Lab., Irvine, Scotland). The antigen-specific titer was expressed as dilution at which 50% of the maximal absorbance was found. Total serum IgM, IgE, IgG1, and IgG2a were measured by isotype-specific ELISA (33).

ELISPOT assay. To quantitate the frequency of antibody-secreting cells, an ELISPOT assay was used according to the procedure described by Taguchi et al. with minor modifications (34). Spleens were aseptically removed and single cell suspensions were prepared by gently teasing them through nylon gauze filters into RPMI plus 0.5% FCS. Cell suspensions were washed three times with RPMI plus 0.5% FCS. Microtitre plates were coated overnight at 4 °C with 50 μl of 5 μg RaIgD ml^{-1} in PBS, followed by blocking with 100 μl PBS containing 0.5% Gelatine in PBS at 37 °C. Spleen cells (3 x 10^2 to 1 x 10^5/well) suspended in RPMI plus 0.5% FCS were added to individual wells and incubated for 3 h at 37 °C with 5% CO_2 in air. The plates were washed once with H_2O plus 0.05% Tween-20 and three times with PBS containing 0.1% gelatine plus 0.05% Tween-20 and incubated 1 hr at 37 °C with 50 μl of Goat anti-mouse-IgG/IgM conjugated to AP in PBS containing 0.1% gelatine. Subsequently plates were washed three times with PBS containing 0.05% Tween-20 and spots representing single antibody-secreting cells were developed with 100 μl 5-BCIP (10 mg.ml^{-1}) in 2-amino-2-methyl-1-1 propanol buffer (pH=10,25). The number of spots was enumerated with the aid of a dissecting microscope.

Statistical evaluation. Results were analyzed by the two-sample Student's t-test for comparison of two empirical means in a normally distributed population.

RESULTS

Injected RαIgD localizes in the follicles of the spleen.

Injected RαIgD was detected with Rig-specific antibodies conjugated to HRP and was found in all splenic follicles at 36 h after injection (Fig. 1a, 2). In an experiment separately performed, already six h after injection we observed RαIgD in all follicles of the spleen (data not shown). Staining for mouse IgD revealed an identical follicular localization pattern, indicating that immune-complexes consisted of RαIgD and mouse-IgD. The localization strongly resembled the staining pattern as is observed after injection of preformed immune-complexes (30, 35, 36), suggesting that circulating *in vivo* formed mouse-IgD-RαIgD-immunecomplexes were trapped by follicular dendritic cells. After 36 h, the frequency of RαIgD positive follicles decreased, but at 7 days after RαIgD injection the staining of follicular RαIgD intensified simultaneously with the development of Rig specific antibody-forming cells, albeit the number of RαIgD positive follicles did not increase significantly (Fig. 2). Staining with RPAP, showed that beyond day 6 after immunization the follicular immune complexes consisted of Rig-specific mouse-Ig.

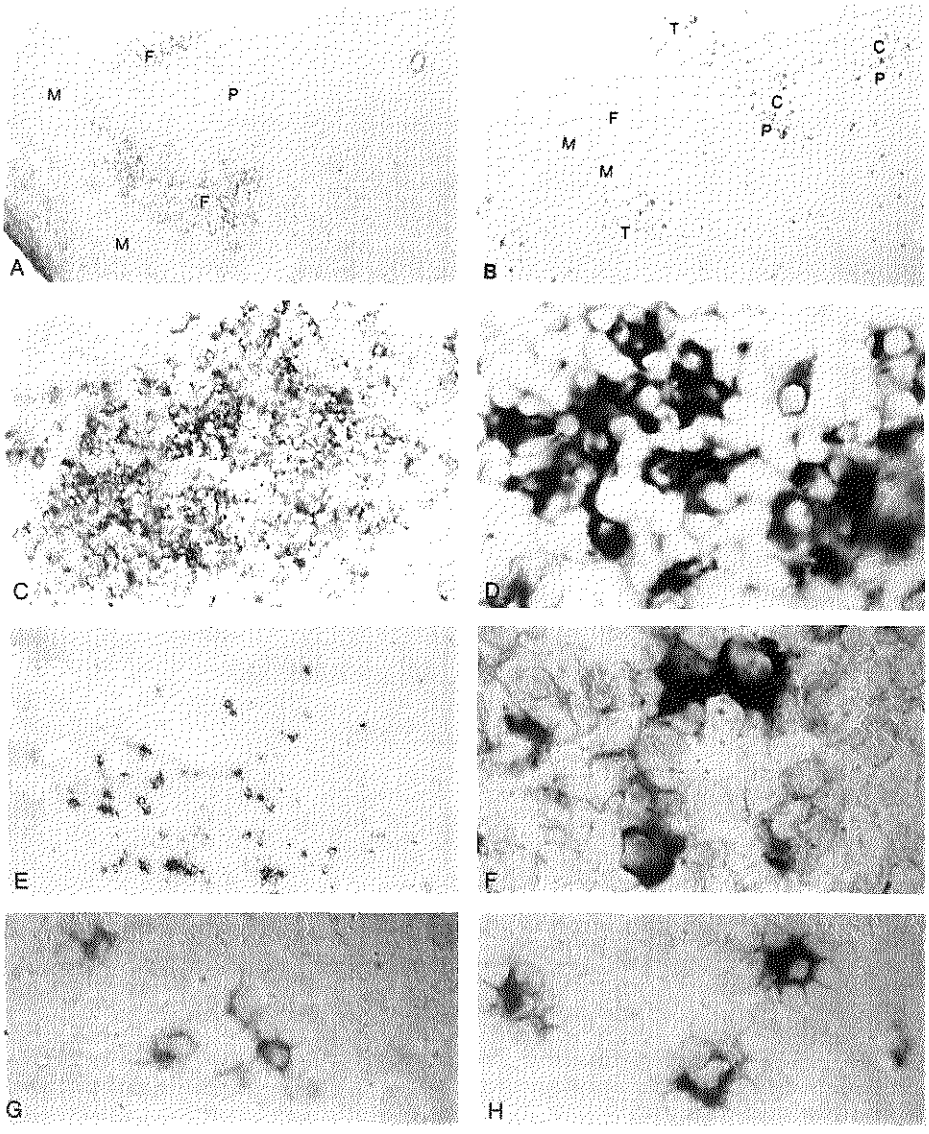
The frequency of gp39$^+$ cells is upregulated after injection of RαIgD.

The *in situ* demonstration of gp39$^+$ cells in splenic cryostat sections was performed as previously described (13). Incubation with the gp39 specific mAb (MR1) was followed by anti-hamster-HRP and after immunohistochemical revelation the gp39$^+$ cells stained red (Fig. 1b,c). Analysis of gp39 expression, revealed already at 36 h after injection RαIgD an increase in the frequency of gp39$^+$ cells which reached maximum levels at day 4 (Fig. 2b). Thereafter, the frequency of gp39$^+$ cells decreased gradually. The gp39$^+$ cells were localized in the PALS and around the TA, like was described in the antibody response against KLH (13). Relatively few gp39$^+$ cells were found in the splenic follicles (Fig. 1b, table I).

Cytokine production in the spleen.

Detection of cytokine-PC was performed with cytokine-specific mAb conjugated to different enzymes (13,32). The IL-4-specific mAb (11B11) conjugated to β-gal was used for the *in situ* demonstration of IL-4-PC. After immunohistochemical revelation, IL-4-PC, characterized by a turquoise stained cytoplasm, were detected (Fig. 1d, e, g). IL-2-PC were demonstrated with an IL-2-specific mAb (S4B6) conjugated to HRP and were characterized by a red stained cytoplasm (Fig. 1f, h). The specific demonstration of IFN-γ-PC (Fig. 1f), with an IFN-γ specific mAb (DB-1) conjugated to AP, was performed as described previously (32,37). All cytokine-PC investigated, were observed in the PALS and around the TA of the spleen. No cytokine-PC were observed in the follicles or marginal zone of the spleen (table I). Double staining for RαIgD and individual cytokines showed clearly, that in all cases the injected antigen localized

Figure 1. Immunohistochemical visualization of antigen, cytokine-PC , gp39$^+$ cells and antibody-forming cells in the spleen after injection of RαIgD. Cryostat section of spleens of RαIgD treated mice were incubated with specific-conjugates, followed by immunohistochemical revelation. Conjugates and substrates used are indicated between the square brackets a) 1 day after RαIgD injection: red staining represents RαIgD which is localized in the splenic follicles (F) and not in the PALS (P) [Swine anti-RIg-PO; AEC]; b) 4 days after RαIgD injection: gp39$^+$ cells [MR1 + RG7-HRP; AEC] are localized in PALS (P) around central arteriole (C), around TA (T) and incidently in the follicles (F); c) 4 days after RαIgD injection: red stained gp39$^+$ cells [MR1 + RG7-HRP; AEC] are localized in close conjunction to blue stained IgM$^+$ B cells [GIgM-AP; Fast blue]; d) 4 days after RαIgD injection: red stained IL-4-PC [11B11-biotine + avidin-HRP; AEC] are localized in close conjunction to activated cytoplasmic-IgM$^+$ B cells [GαIgM-AP; Fast blue]; e) 4 days after RαIgD injection: red stained cells are gp39$^+$ cells [MR-1-AP; Fast red], the turquoise stained cells are IL-4-PC [11B11-β-gal; X-Gal], while the double staining violet cells are gp39$^+$ cells producing IL-4; f) 4 days after RαIgD injection: red stained cells are IL-2-PC [S4B6-PO; AEC], blue stained cells are IFN-γ-PC [DB-1-AP; Fast blue], while double staining violet cells are producing IL-2 and IFN-γ; g) 4 days after RαIgD injection: turquoise stained cells are IL-4-PC [11B11-β-gal; X-Gal], brown stained cells are IL-2-PC [S4B6-PO; DAB], while the double staining green cells produce both IL-2 and IL-4; h) 4 days after RαIgD injection: red stained cells are IFN-γ-PC [DB-1-AP; Fast red], the turquoise stained cells are IL-4-PC [11B11-β-gal; X-Gal], while the double staining violet cells produce both IFN-γ and IL-4.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in the follicles, a compartment different from that where cytokine-PC were found. After injection of RαIgD, high frequencies of IL-2-PC and IL-4-PC, relative to IFN-γ-PC, were observed attaining maximum levels at day 3 and 4 (Fig. 2b). Thereafter, the frequencies of cytokine-PC decreased and were still detectable at day 11 after injection.

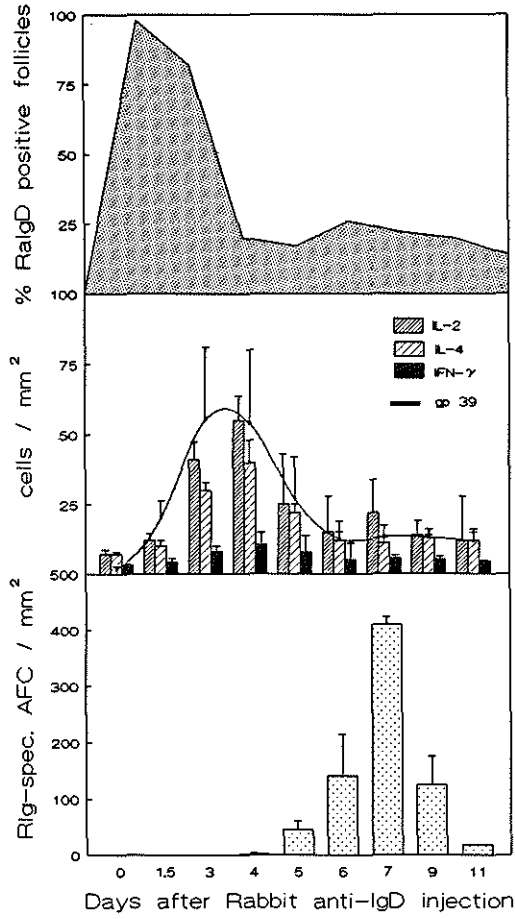

Figure 2. Follicular localization of antigen and the development of gp39⁺ cells, cytokine-PC and Rlg-AFC are sequential events in the immune response against RαIgD. BCBA.F1 mice were injected with 800 μg RαIgD and sacrificed at the indicated time-points. In order to detect injected RαIgD, gp39, IL-2, IL-4 and Rlg-AFC, splenic cryostat sections were incubated with specific immuno-conjugates, followed by the appropriate substrates, as described in the materials and methods section. Follicular localized RαIgD is expressed as the percentage positive follicles in each cryostat section. Cytokine-PC, gp39⁺ cells and antibody-forming cells were counted and expressed as the number of positive cells per mm² spleen section. Data presented are the mean ± SD of three mice at each time-point.

Table 1

Localization of gp39$^+$ cells and cytokine-PC in the different compartments of the spleen after RαIgD immunization.

Cell type[c]	White pulp					Red pulp
	Fc[a]	GC	PALS	MZ	TA	
GP39$^+$ cell	1.4 ±1.1[b]	0	43.8 ±9.4	0	54.8 ±9.5	0
IL-2-PC	0	0	30.4 ±5.4	0	69.6 ±5.4	0
IL-4-PC	0	0	39.3 ±8.8	0	60.7 ±8.8	0
IFN-γ-PC	0	0	31.7 ±6.8	0	68.3 ±6.8	0

BCBA.F1 mice were injected with RαIgD and sacrificed at day 4. Cryostat sections were prepared from spleens and immunohistochemical demonstration of gp39 and cytokine-PC was performed as described in the materials and methods section. Cells were counted in each anatomical compartment[a] of the spleen: follicular corona, Fc; germinal center, GC; periarteriolar lymphocyte sheaths, PALS; marginal zone, MZ; terminal arterioles, TA. [b]Values represent the specific cell types[c] localized in each compartment of the spleens and are expressed as the mean ± SD percentage of the total number of positive cells found in examined spleen section. Results are based on spleen sections from three mice.

Cytokine-PC can produce all combinations of cytokines.

To determine whether cytokine-PC were able to produce simultaneously more then one cytokine and whether the cells doing so would belong to either the Th0, Th1 or Th2 subset, we performed double staining experiments for all combinations of the three cytokines investigated. Figure 1f, shows red stained IL-2-PC and blue stained IFN-γ-PC, while the double staining violet cells are cells that produce both cytokines. About 3-11% of the IFN-γ-PC were found to produce IL-2 in the immune response against RαIgD. In figure 1g, the brown stained IL-2-PC and the turquoise stained IL-4-PC turquoise are demonstrated. The double staining green cells are cells that produce both IL-2 and IL-4. After injection of RαIgD, about 1-4% of the IL-2-PC were found to produce IL-4. In figure 1h, the red stained cell is an IFN-γ-PC and the turquoise stained cell an IL-4-PC, while the double stained violet cell produces IL-4 as well as IFN-γ. During the whole experimental period we found that 1-11% of the IFN-γ-PC were producing IL-4.

In vitro experiments suggested that after activation, Th cells express the ligand for CD40 simultaneously with the production of cytokines (7). In accordance with these *in vitro* observations, we detected *in vivo* gp39$^+$ cells producing IL-2, IL-4 or IFN-γ in spleens of mice after RαIgD injection (Fig 1e).

IgM expression increases and IgD expression decreases on follicular B cells after injection of RαIgD.

In non-immunized mice, B cells in the marginal zone of the spleen are characterized by a relatively high expression of membrane IgM and low expression of membrane IgD, whereas the follicular B cells have a relatively low expression of membrane IgM and high expression of membrane IgD (12). Thirty-six h after injection of RαIgD, a striking increase of IgM expression on especially the follicular B cells, was observed. After day 3, a marked increase in the frequency of cytoplasmic-IgM$^+$ cells (cIgM$^+$)was observed, localized in the same compartment as where gp39$^+$ cells and cytokine-PC were observed: in the PALS and around the TA. These cytoplasmic IgM$^+$ cells, were large cells with a central nucleus and a high nuclear to cytoplasmic ratio, and appeared therefore rather activated B cell blasts than mature plasma cells (Fig 1c). In contrast, 36 h after RαIgD injection, IgD$^+$ B cells were almost completely absent in the spleen. Only the follicles, which trapped RαIgD-IgD immune-complexes, were found to be positive for mouse-IgD.

Cytokine-PC and gp39$^+$ cells are localized in close conjunction to IgM$^+$ B cells

Since gp39$^+$ cells and cytokines have a role in B cell activation and B cell differentiation, respectively, the localization of gp39$^+$ cells and cytokine-PC in relation to IgM$^+$ B cells was studied. After double staining, gp39$^+$ cells were found in close conjunction to activated B cells (cIgM$^+$) in the PALS and around TA. Since gp39$^+$ cells produce cytokines it was not surprising to find cytokine-PC, most likely expressing gp39, juxtaposed to IgM$^+$ in the same compartment as where gp39$^+$ cells were observed. Analogous to the observation of higher frequencies of IL-4-PC and IL-2-PC, relative to IFN-γ-PC, we found also more IL-2-PC and IL-4-PC in juxtaposition to cIgM$^+$ B cells after injection of RαIgD (Fig. 1d).

Majority of the antibody-forming cells in the spleen are IgG$^+$ and in part RIg-specific

The predominance of IgG1 in the immune response to GαIgD was already demonstrated in several reports (15,16,17). To investigate the relation between cytokine-production and antibody production, we analyzed the development of IgG expression in the spleen after RαIgD injection. In non-immunized mice we observed only a few IgG$^+$ AFC. Five days after injection of RαIgD a gradual increase in the number of IgG$^+$ cells was observed, leading to maximum frequencies of IgG$^+$ AFC at day 7. Thereafter, the number of IgG$^+$ cells decreased gradually. To investigate the antigen-specificity of the antibody response, we incubated cryostat sections with RPAP, which revealed that the RIg-specific AFC started to develop at

day 5 and reached peak levels at day 7 after injection of RαIgD (Fig. 2c). Double staining for IgG and Rlg at day 7, revealed that about 42±6% of the IgG^+ cells were specific for Rlg. Incubation with swine-Ig peroxidase conjugate, Glg alkaline-phosphatase conjugate, KLH-HRP, or TNP-AP revealed only a few AFC specific for these antigens.

Serum IgG1 increases substantially and is in part specific for Rabbit-IgD.

Several experiments have suggested that the isotypes IgG1 and IgE are regulated by IL-4, whereas IFN-γ was suggested to have a regulating role in the IgG2a isotype selection (15, 38). Therefore, we assessed the serum levels of IgG1,2a and IgE after injection of RαIgD. As expected, both the total-IgG1 as well as the total-IgE serum levels increased about 7 times, whereas no increase of the serum IgG2a was observed (Fig. 3). IgM levels reached at day 5 maximum levels but were not specific for RαIgD (Fig. 3). In BCBA.F1 mice, the increase of serum IgE and IgG1 did not reach such high levels, as found in the high responding BALB/c mice after RαIgD injection (compare Fig. 3 and 6, (16)) but the levels were still remarkably high for a primary antibody response. To determine the specificity of the IgG production we tested immune sera on different antigens. We found that the total IgG antibody response after RαIgD injection reached a titer of 10^4 against RαIgD, whereas no specific response was found against OVA-TNP, KLH, CGG or rat Ig, which is in agreement with the results found in splenic cryosections of RαIgD immunized mice.

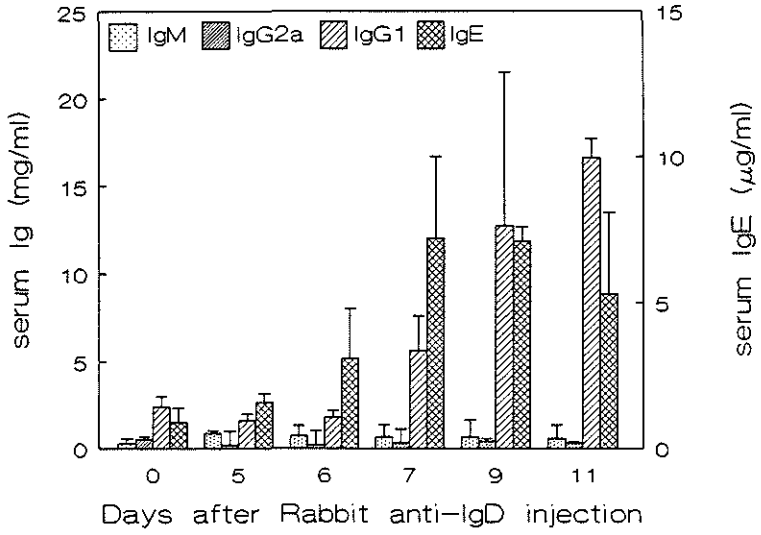

Figure 3. IgG1 and IgE serum levels increase markedly after injection of RαIgD. BCBA.F1 mice were injected with 800 μg RαIgD and bled at the indicated time-points. IgM, IgG1, IgG2a and IgE serum levels were determined by ELISA. Data presented are the mean ± SD of three mice at each time-point.

Treatment of mice with anti-gp39 mAb suppresses the antibody response after RαIgD injection. *In vitro* and *in vivo* studies indicated that gp39-CD40 interactions are essential for TD humoral immunity (4, 5, 14). To address whether cognate T-B interactions were essential in this model polyclonal immune response, we assessed the effect of anti-gp39 treatment on the antibody response after RαIgD injection. Seven days after immunization with RαIgD a fourfold increase in splenic weight was observed in BALB/c mice. As shown in Fig. 4, anti-gp39 treatment completely prevented the increase in splenic weight after immunization with RαIgD.
In contrast, the increase of IgM expression on follicular B cells after RαIgD injection, was not affected by the anti-gp39 treatment, confirming the T cell independency of the RαIgD induced IgM expression. Furthermore, the administration of anti-gp39 mAb did not significantly influence the splenic cytokine production and gp39 expression after RαIgD injection, indicating that $gp39^+$ cells and cytokine-PC were not depleted by this treatment (table 2).

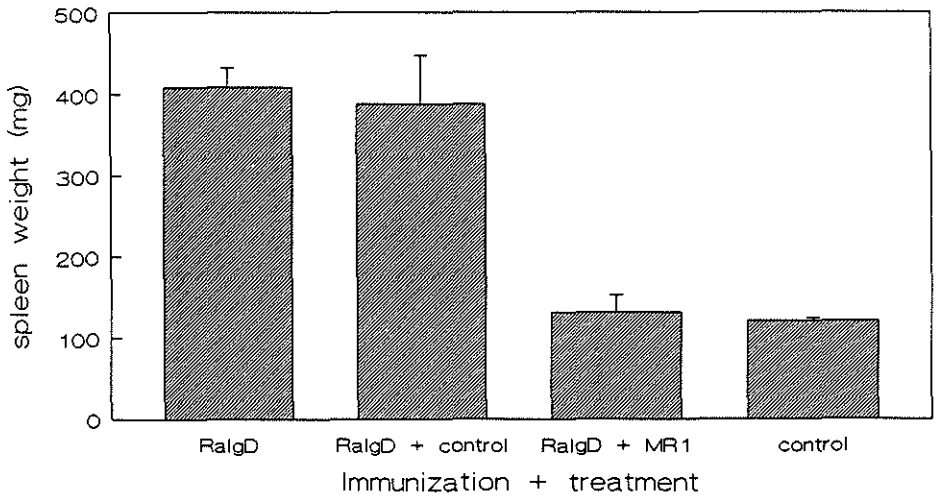

Figure 4. *Increase in spleen weight after RαIgD injection is completely prevented by the treatment with anti-gp39 mAb.* BALB/c mice were immunized with 800 μg RαIgD and simultaneously injected with 250 μg anti-gp39 mAb (MR1), control hamster antibodies or PBS on days 0, 2 and 4. At 7 days after injection mice were killed and spleens were removed and weighed. Data presented are the mean ± SD weights of spleens from four mice.

Table 2

Effect of anti-gp39 treatment on the expression of gp39 and cytokines in spleens of mice after RαIgD immunization.

Treatment[a]	Cell type[b]			
	GP39$^+$ cell	IL-2-PC	IL-4-PC	IFN-γ-PC
RαIgD + MR1	37.7 ±5.3[c]	30.5 ±4.5	25.8 ±3.6	7.8 ±0.5
RαIgD + control	31.2 ±7	28.3 ±5.2	25.3 ±2.7	7.7 ±1.4
RαIgD + PBS	35.2 ±3.5	25.5 ±3.6	27.1 ±4.1	6.6 ±1.4

[a]BCBA.F1 mice were injected with 800 μg of RαIgD. Mice were treated at day 0, 2 and 4 with anti-gp39 mAb, control antibodies or PBS. At day 4, two hours after the last treatment, mice were sacrificed. Cryostat sections were prepared from spleens and immunohistochemical demonstration of gp39 and cytokine-PC was performed as described in the materials and methods section. [c]Values represent mean ± SD of number of positive cells per square mm of three spleen sections from three mice.

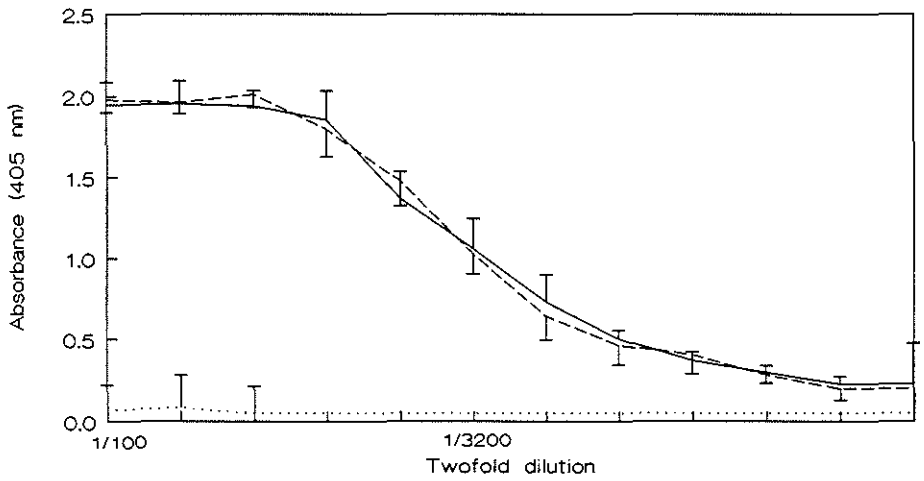

Figure 5. RαIgD-specific antibody response is completely suppressed after treatment with anti-gp39 mAb. BALB/c mice were immunized with 800 μg RαIgD and simultaneously injected with 250 μg anti-gp39 mAb (MR1), control hamster antibodies or PBS on days 0, 2 and 4. Mice were bled and killed 7 days after immunization. RαIgD-specific IgG was determined by ELISA. Data presented are the mean ± SD absorbance of sera from four mice after twofold dilution starting at 1/100. Solid, broken and dotted line represent, respectively, the sera of PBS, hamster-antibody and anti-gp39 treated RαIgD immunized mice.

By using the hamster-specific HRP-conjugate, we tried to detect at day 4, 2 h after the last injection, the i.p. administered anti-gp39 mAb (MR1). Only a faint staining, identical to that observed after injection of control hamster antibodies, was observed in the marginal zone of the spleen (data not shown). No individual cells binding anti-gp39 were detected *in situ*, indicating that not all gp39 molecules on activated Th cells were covered with anti-gp39 mAb.

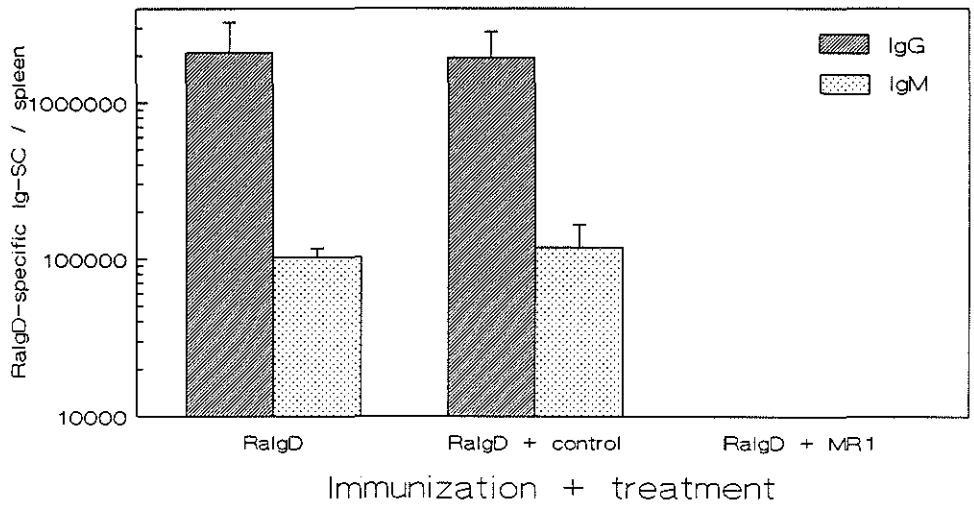

Figure 6. RαIgD-specific antibody response in the spleen is completely suppressed after treatment with anti-gp39 mAb. BALB/c mice were immunized with 800 μg RαIgD and simultaneously injected with 250 μg anti-gp39 mAb (MR1), control hamster antibodies or PBS on days 0, 2 and 4. Mice were killed 7 days after immunization and RαIgD-specific ELISPOT assay was performed. Data presented are the mean ± SD number of RαIgD-specific antibody-secreting cells in spleens of four mice.

Analysis of the RαIgD-specific antibody response, showed a complete disappearance of RαIgD-specific antibodies in sera of RαIgD immunized mice after treatment with anti-gp39 mAb (Fig. 5). In agreement with serum data, hardly any RαIgD-specific antibody secreting cells *in vitro* (Fig. 6) and RIg-AFC *in situ* (data not shown) were found in spleens of anti-gp39 treated mice at 7 days after RαIgD injection. Since modulation experiments were performed in the high responding BALB/c mice the increases of serum IgG1 and IgE were about 3-5 time higher as found in BCBA.F1 mice (compare fig. 3 and 7). Even this pronounced increase of serum IgG1 was completely blocked by anti-gp39 treatment (Fig. 7). Interestingly, only the increase of IgE serum levels after RαIgD injection, was not completely suppressed by the anti-gp39 treatment. In conclusion, this data indicates that $gp39^+$ cells are essential for the antigen-specific and polyclonal antibody response after RαIgD injection.

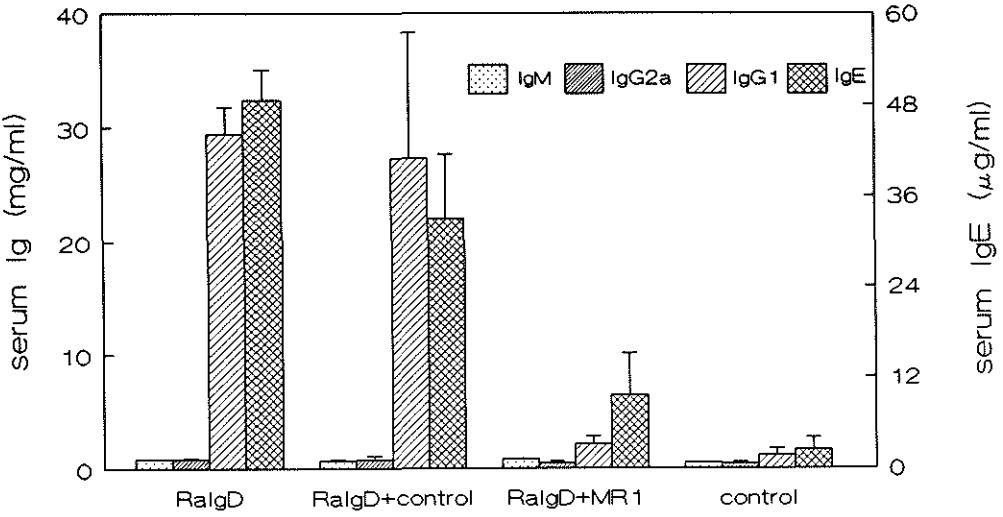

Figure 7. Effects of anti-gp39 treatment on the generation of polyclonal Ig responses in RαIgD antibody-injected mice. BALB/c mice were immunized with 800 μg RαIgD and simultaneously injected with 250 μg anti-gp39 mAb (MR1), control hamster antibodies or PBS on days 0, 2 and 4. Mice were killed 7 days after immunization and total IgM, IgG1, IgG2a, and IgE was determined by ELISA. Data presented are the mean ± SD of four mice.

DISCUSSION

In this *in vivo* study we demonstrated that RαIgD is localized, as RαIgD-IgD immune complexes, in the follicles of the spleen shortly after i.v. injection. Thereafter, a simultaneous increase of $gp39^+$ cells and cytokine-PC was observed which were predominantly localized in the PALS and around the TA of the spleen. At day 3-5 after RαIgD injection, $gp39^+$ cells and cytokine-PC were found in close conjunction to activated IgM^+ B cells. *In vivo* blocking experiments suggested that these T-B cell interactions were essential for the high IgG1 and IgE serum levels observed at later time-points. Furthermore, cytokine-PC with an unrestricted cytokine profile were demonstrated, though Th2-like cells were found to dominate in the antibody response after RαIgD injection.

The localization pattern of the injected RαIgD in the splenic follicles was similar as observed for immune-complexes (30,35,36). It is likely that the injected RαIgD binds to free IgD in the circulation and to mouse-IgD shedded from the by RαIgD activated IgD^+ B cells. During the whole experimental period we could detect RαIgD-IgD immune complexes in the follicles of the spleen, indicating that RIg specific epitopes were continuously presented to the circulating B cells. Finkelman et al. (42) showed that injection of FITC-GIg after GαIgD treatment increased the specific FITC response, indicating that a sustained availability of antigen was responsible for the increased specific antibody response. Consequently, the observed persistence of antigen in the follicles may explain the relatively high RIg-specific antibody response observed after RαIgD injection. The trapping of RαIgD-IgD immune-complexes in the follicles of the spleen may also contribute to the high cytokine-production and antibody production observed after RαIgD injection, as has been demonstrated after injection of preformed immune-complexes (39,40,41).

None of the injected RαIgD was detected in the inner-PALS of the spleen, the compartment where dendritic cells are localized. The impossibility to detect RαIgD in this compartment could be due to the very low amount of antigen in antigen-presenting cells, or due to processing by antigen presenting cells which may change their antigenic structure. Kyewski et al. (43), showed *in vitro* that after i.v. injection of myoglobin, splenic DC were the major cell types that could stimulate myoglobin-specific T cell clones, in spite of the fact that no myoglobin was detected in the dendritic cells of the spleen. Our study suggests that B cells are the important antigen-presenting cells in the antibody response after RαIgD injection. However, it does not exclude the absolute requirement of dendritic cells for the activation of naive Th cells (44,45).

Analysis of gp39 expression revealed already 36 h after injection of RαIgD an increase in the frequency of $gp39^+$ cells in the spleen, reaching maximum levels at days 3 and 4. At day 2-5 after injection we observed $gp39^+$ cells in juxtaposition to activated B cells, predominantly localized in the PALS and around the TA of the spleen. The observation that treatment of mice with anti-gp39 mAb resulted in a complete suppression of the polyclonal antibody response, suggests that the *in situ* revealed interactions between $gp39^+$ cells and IgM^+ cells represent functional T-B cell interactions. As gp39 is exclusively expressed on $CD4^+$ cells (5, 13), these results are in agreement with experiments showing that $CD4^+$ cells are essential for the large increases of IgE and IgG1 serum levels at day 7 after injection of GαIgD (20). We demonstrated that anti-gp39 treatment did not affect the *in situ* cytokine production and gp39 expression after RαIgD injection (table 2), indicating that activated $gp39^+$ cells were not

depleted by this treatment. This supports previous experiments (14), which demonstrated that unaltered Th function can be transferred from mice treated with anti-gp39, suggesting that blockage of gp39 was responsible for the *in vivo* suppression of TD antibody responses. The observation that we were not able to detect *in situ* the injected anti-gp39 mAb on $gp39^+$ cells, suggests that blockage of only a part of the gp39-molecules on activated Th cells is sufficient to prevent B cell activation by $gp39^+$ Th cells. These results are in agreement with experiments of Morris et al. (49), which showed that for the generation of an IgG response after GαIgD injection, intense physical interactions are required between antigen-presenting B cells and antigen-specific Th cells. This group (49) demonstrated that even bystander B cells , in spite of their close localization to IL-4-PC and $gp39^+$ cells, were not stimulated to produce IgG1. The study of Morris et al. together with the herein presented data, suggest that injected αIgD antibodies are presented to antigen-specific Th cells, which are subsequently activated and express gp39. Most likely antigen-presentation results in a close physical interaction between Th cells and B cells, which may be essential for the binding of gp39 to the CD40 molecule on the antigen-presenting B cell. In that way, antigen-specific interactions may be preserved and will lead to the initial activation of B cells required for the differentiation into AFC. The localization and function of gp39-CD40 interactions, as demonstrated in this study, resembles a previous *in vivo* study in KLH immunized mice (13), and indicate that the immune response after RαIgD injection, albeit polyclonal, behaves like other TD antibody responses.

The kinetics of IL-2 protein expression in this study are in agreement with experiments of Svetic et al. (46), who demonstrated that maximum IL-2-gene activity is found 3 days after injection of GαIgD. The observation that peak IL-2 protein secretion coincides with peak IL-2-gene expression, supports *in vitro* experiments of Kelso et al. (50), who showed that IL-2-gene expression and protein production by T cell clones were correlated. In contrast, *in vitro* studies by this group did not show a correlation between cytokine-gene expression and protein production for IL-4 and IFN-γ. The experiments of Svetic et al. demonstrated maximum expression of IL-4-genes between day 3 and day 6 after GαIgD injection (46), whereas in our study maximum IL-4 protein expression was found 3 and 4 days after RαIgD injection. A clear discrepancy was only found for IFN-γ gene expression and protein production, as in our study peak production was observed at day 3 and 4, while in the study of Svetic et al. IFN-γ gene expression started to raise at day 2 reaching maximum levels at day 7. The lack of correlation could be explained by the cellular heterogeneity in mRNA expression, and/or by differences in translational or secretorial regulation (50,51). The *in vivo* kinetics of IL-4-PC as described

in this study, is consistent with experiments of Finkelmann et al. who showed that anti-IL-4 treatment of GαIgD immunized mice was only effective in blocking IgE-production when it was administrated from 0-4 days after immunization with GαIgD (47,48).

At the peak of the immune response we observed relatively high frequencies of IL-2-PC and IL-4-PC in the outer-PALS and around the TA. As these cytokine-PC were observed in close conjunction, it is likely that these cells create a microenvironment which is rich in IL-2 and IL-4. Such a microenvironment was suggested to be essential for the development IL-4-PC, as was demonstrated *in vitro* (52,53,54). These experiments suggested that IL-2 was required for the optimum proliferation of cytokine-producing T cells (52,53,55), while IL-4 would propagate the preferential development of IL-4-PC (52,53). The suggested role of IL-2 in T cell proliferation is supported by FACS analysis, showing an increased IL-2R expression on T cells at day 3-5 after GαIgD injection (19). The presented data, showing higher frequencies of IL-4-PC relative to IFN-γ-PC is consistent with the marked increase of IgE and IgG1 serum levels found after RαIgD injection, as these isotypes are suggested to be regulated by IL-4 (9, 10). Peak IL-4-production was observed at day 3 and 4 after RαIgD injection, 3 days before the maximum frequency of antigen-specific AFC was observed. Interestingly, at these time-points high frequencies of IL-4-PC were found juxtaposed to activated IgM^+ B cells. This observation substantiates experiments of Finkelman et al. (42), which provided evidence that T-B cell interactions, leading to high IgG1 serum levels, occur at day 2-4 after GαIgD treatment. Moreover, the evidence that produced IL-4 is functional at day 3 and 4, was provided by serum-IgE modulation studies with anti-IL-4 or anti-IL-4R antibodies (47,48).

In this study, analysis of cytokine-profiles of cytokine-PC failed to define a clearcut Th1/Th2 dichotomy. The majority of IL-4-PC were not producing IL-2 or IFN-γ and consequently compatible with Th2 cells. However, we found also Th0 cells, although in relatively low frequencies (1-5%), producing IL-2 and IL-4, or IL-4 and IFN-γ. In addition, we observed a relatively low percentage of cells producing simultaneously IL-2 and IFN-γ, compatible with Th1 cells. These results may be explained by the strong Th2 character of this antibody response (47,48,52), resulting in high frequencies of cells producing only IL-4. The observation that low percentages of cells were producing both IL-2 and IL-4 is supported by experiments of Powers et al. (55), who demonstrated, by limiting dilution of KLH stimulated lymph node cells, that *in vitro* produced IL-2 and IL-4 was not derived from the same cells.

This study clearly showed that injected RαIgD localized in the follicles (Fig. 1a), one of the major B cell compartments, whereas cytokine-PC and activated B cells were localized in the PALS and around the TA (table I). Consequently, antigen acquisition and antigen-presentation

may occur in different compartments, which would imply that in the spleen antigen presenting B cells migrate from follicle to PALS and TA. The localization pattern of RαIgD suggests that follicular B cells are the major antigen-presenting cells in this antibody response. The dominant presentation of RαIgD by B cells can lead to the preferential activation of Th2 cells (56,57) and may explain the observed high frequencies of IL-4-PC, relative to IFN-γ-PC, after RαIgD injection.

Figure 2 summarizes the successive immunological phenomena of antigen localization, $gp39^+$ cytokine production and specific-antibody production, and suggest the following sequence of events after RαIgD injection. IgD^+ B cells, which are predominantly found in the follicles, are activated by RαIgD, and subsequently migrate to the PALS and TA where they encounter Rlg-specific T cells. In these compartments B cells will present processed Rlg to specific T cells, leading to T cell activation, proliferation and cytokine production. As the microenvironment of cytokine-PC is rich of IL-2 and IL-4, the development of IL-4-PC will be preferentially propagated. Activated Th cells express gp39 and provide B cells the initial activating signal. This stimulus in conjunction with IL-4 rich microenvironment, created by IL-4-PC, propagates the preferential development of B cells, producing IgG1 and IgE. In case of an antigen-specific antibody response Rlg-specific IgM^+ cells acquire RαIgD in the follicle and follow subsequently the same pathway as described for IgD^+ B cells.

In conclusion, in this *in vivo* study we demonstrate for the first time *in situ*, that IL-2-PC and IL-4-PC are dominating over IFN-γ-PC in the antibody response after RαIgD injection. Moreover, 3-5 days after RαIgD injection high frequencies of $gp39^+$ cells and cytokine-PC were observed in close conjunction to activated IgM^+ B cells. Functional studies suggest that these T-B cell interactions, predominantly restricted to the PALS and around the TA, are essential for the high IgG1 and IgE serum levels found in the antibody response after RαIgD injection.

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CHAPTER 9

GENERAL DISCUSSION

A part of the discussion has been and will be published in:

Immunological functions and *in vivo* cell-cell interactions of T lymphocytes in the spleen

Alfons J.M. Van den Eertwegh, Wim. J.A. Boersma, and Eric Claassen
Crit. Rev. Immunol., 11: 337-380, 1992

In vivo T-B cell interactions and cytokine-production in the spleen

Alfons J.M. van den Eertwegh, Randolph J. Noelle, Wim J.A. Boersma and Eric Claassen
Seminars in Immunology (in press)

9.1 Cells involved in antibody formation

A variety of cells are involved in the development of antibody responses. Antigen-presenting cells (APC) like macrophages, dendritic cells (DC) and B cells take up antigen, process it and present fragments of the antigen (T cell epitopes) to T cells. The presentation of the T cell epitope in the context of MHC molecules, together with the costimulatory signals, provided by antigen-presenting cells, results in the activation of T cells. Under specific conditions B cells are not able to activate naive T cells (1,2). Therefore, it has been proposed that DC or macrophages are required for the primary activation of naive T cells. Thereafter, T-B cell interactions have a central role in the development of humoral immunity (Fig. 1). B cells capture antigen via their antigen-specific receptor (membrane immunoglobulin) and present the processed antigen to specific T-helper (Th) cells. The recognition of fragments of processed antigens bound in the groove of MHC class II molecules on B cells, together with the interactions of the costimulatory molecule B7 on B cells with CD28 on Th cells (3), leads to the initial class II restricted, antigen-specific activation of Th cells. These activated Th cells express gp39 and produce cytokines, which enable them to regulate B cell differentiation. Binding of gp39 to its receptor CD40 on B cells results in the initial activation of B cells (4,5), while Th cell derived cytokines are thought to regulate the differentiation of B cells into antibody-forming cells (6,7,8). The pattern of cytokine secretion of Th cells appears to be crucial in the selection of isotype produced by antibody-forming B cells (9,10,11). The cell-cell interactions and regulatory role of cytokines in antibody responses, as discussed above, is mainly based on a combination of results from *in vitro* experiments which may not be applicable to *in vivo* antibody responses.

In this thesis, the critical events in the development of antibody responses, as suggested by *in vitro* experiments, were investigated *in vivo*. Most of the presented studies were performed in the murine spleen, as this secondary lymphoid organ is the major site of antibody production after primary i.v. immunization. The localization of injected antigen and the subsequently induced expression of gp39 and cytokines were investigated in the spleen. Furthermore, the localization and kinetics of appearance of $gp39^+$ cells and cytokine-PC was related to that of antigen-specific antibody-forming cells (AFC). Functional studies were performed to analyze the role of cytokines and gp39 in experimental antibody responses. In this last chapter, we shall discuss the main points presented in this thesis. Finally, *in vivo* models are presented, which show the cell-cell interactions in the

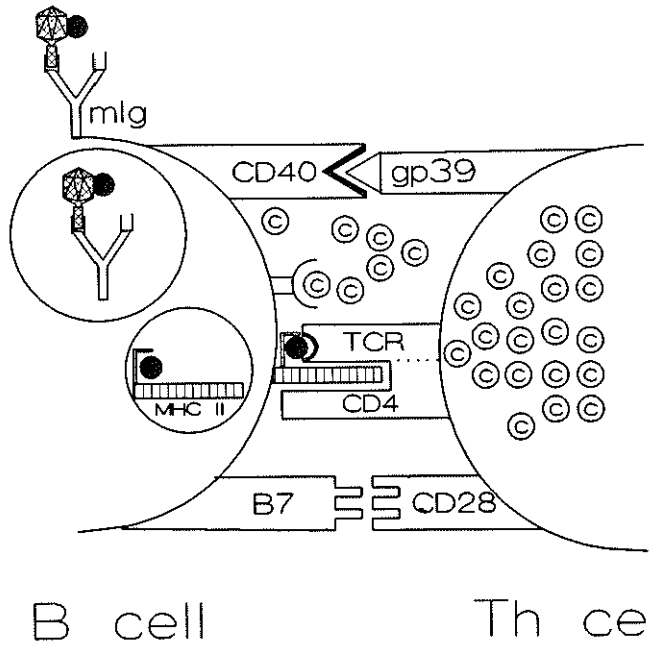

Figure 1 *An "immunological synapse" formed between B cells and Th cells assures the antigen-specific activation of B cells by activated Th cells.* B cells capture antigen via their antigen-specific receptor (mIg) and present fragments of the processed antigen (● ;T cell epitope) to specific T-helper (Th) cells. The recognition of the T cell epitope bound in the groove of MHC class II molecules on B cells, together with the interactions of the costimulatory molecule B7 on B cells with CD28 on Th cells, leads to the class II restricted, antigen-specific activation of Th cells. These activated Th cells express gp39 and produce cytokines, which enable them to regulate B cell differentiation. Binding of gp39 to its receptor CD40 on B cells results in the initial activation of B cells, while Th cell derived cytokines (©) regulate the proliferation and differentiation of B cells.

spleen during thymus dependent (TD) and thymus independent type 2 (TI-2) antibody responses.

9.2 Localization of antigens in the spleen

A. Localization of particulate antigens in the spleen

In chapter 3.2 we described the splenic localization of i.v. injected heat killed *Group B streptococci*, particulate TI-1 antigens. After injection, *Group B streptococci* were predominantly found in the marginal zone macrophages and to a lower degree in red pulp macrophages. These results are in agreement with studies which showed that i.v. injected particulate antigens, irrespective of their nature (TD: TNP-SRBC, BSA-liposomes; TI: TNP-liposomes, *Brucella abortus*), localize in the marginal zone, taken up by marginal zone macrophages and marginal metallophils (13). The similar localization observed for all types of particulate antigens suggests a common function of these macrophages. Since marginal zone macrophages do not express detectable MHC class II antigens (14), it is highly unlikely that these cells are active as antigen-presenting cells. *In vitro* (15,16) and *in vivo* (17,18) experiments suggested that macrophages can be active as pre-processors of particulate antigens. Specific elimination of marginal zone macrophages *in vivo* resulted in a decreased antibody response against liposome-associated albumin (18) and TNP-SRBC (17). These experiments indicated that marginal zone macrophages fragmentate (pre-process) particulate antigens, which subsequently are transferred to either B cells or DC (19). The latter cells enter the spleen via the marginal zone and migrate to the periarteriolar lymphocyte sheath (PALS) were they can present the processed antigen to specific T cells (chapter 2). These data suggest that marginal zone macrophages clear particulate antigens from the circulation, and may have a role as pre-processors in antibody responses against particulate antigens/bacteriae.

B. Localization of soluble TD antigens in the spleen

Using immunohistochemical techniques we tried to detect injected TD antigens in the spleen, e.g. TNP-KLH and TNP-BSA. In the first days after injection these haptenated TD antigens were exclusively localized in the red pulp macrophages (chapter 3.1). The localization in red pulp macrophages could represent the scavenging of bulk antigen,

whereas antigen which is involved in the induction of the humoral immune responses may be localized in other compartments. The impossibility to detect these immunological relevant antigens *in situ* could be due to the very low amount of antigen in APC, or due to the rapid processing of protein antigens by APC which may change their antigenic structure, as was suggested by experiments of Kyewski et al. (20). They demonstrated that after i.v. injection of myoglobin, splenic DC were the major cell types that could stimulate myoglobin-specific T cell clones *in vitro*, although the antigen could not be detected in the inner-PALS of the spleen.

Immune complexes are formed as soon as antigen-specific antibodies appear in the circulation and bind to injected antigen. Immune complexes are also formed if specific antibodies recognizing the antigen are already present in the circulation when antigen is administered, e.g. during secondary antibody responses. In these situations or after the injection of preformed immune complexes, we were able to detect immune-complexes (peroxidase-anti-peroxidase and TNP-Ficoll-anti-TNP immune complexes, chapter 3.1, 6; mouse-IgD-RαIgD immune complexes, chapter 8) in the follicles of the spleen. These antibody-complexed antigens are trapped by the processes of follicular dendritic cells (FDC) via Fc receptors or complement receptors and can be retained for long periods of time (21, 22). The trapped complexed antigen plays a crucial role in B cell memory and regulation of antibody responses (22, 23). The follicles have also been suggested to have a role in affinity maturation of B cells (24), which is the result of the competition between free antibody, antibody trapped in the form of immune-complexes by FDC and membrane Ig on B cells that are undergoing somatic mutation. Since T cell memory for soluble protein is also antigen-dependent (25) and FDC are the only cells known which can preserve antigens for months to years, it is likely that follicular trapped antigen also plays a role in the maintenance of T cell memory.

The data discussed suggest that the localization of injected TD antigens in the spleen is not identical for primary and secondary immune responses. In both responses macrophages take up the bulk of antigen. However, after the first antigenic challenge antigen localizes in the inner-PALS, containing DC and T cells, and plays a role in the induction of the primary immune response. Whereas after a secondary challenge, antigen localizes also in the follicle, containing FDC, B and T memory cells, which may be crucial for induction and maintenance of secondary immune responses.

C. Localization of TI-2 antigens in the spleen

In chapter 3, the immunohistochemical detection of injected haptenated TI-2 antigens in lymphoid tissue was described. Within minutes after i.v. injection, TI-2 antigens (TNP-Ficoll, TNP-HES) localized in the marginal zone, attached to marginal zone macrophages and B cells. Twenty minutes after injection the antigen was also detected in the follicles and gradually accumulated there up till 7 hours after injection. Thereafter, the amount of follicular antigen gradually decreased but was still detectable up to 14 days after immunization (chapter 2: fig. 6). It was demonstrated that the immediate follicular localization of TNP-Ficoll was complement dependent and not antibody mediated. Immunohistochemically, it is difficult to determine whether FDC or B cells are the principal TI-2 binding cells in the follicles. However, the staining pattern suggests that injected TI-2 antigens are predominantly bound by FDC.

Because of their specific uptake of fluoresceinated TI-2 antigens, it was thought that marginal zone macrophages play a role in the presentation of TI-2 antigens (26, 27). However, *in vivo* elimination/blocking of marginal zone macrophages did not affect the antibody response against TI-2 antigens (28,29,30), which suggested that other cells or mechanisms were involved in this antibody response. The data presented in this thesis show that injected TNP-Ficoll localizes in the major B cell compartments, namely the marginal zone and the follicle. In chapter 3.1 it was demonstrated that TNP-Ficoll can activate complement *in vivo*, as was described for other TI-2 antigens (31,32,33,34). The subsequently formed complexes of TNP-Ficoll and C3d (a complement degradation product) are able to activate B cells directly (34,35,36), without the requirement of antigen presenting macrophages. Direct complement dependent activation of B cells by TI-2 antigens is compatible with the *in vivo* localization of TI-2 antigens in the major B cell compartment of the spleen and with *in vivo* experiments, which indicated the trivial role of marginal zone macrophages in the induction of TI-2 antibody responses (28,29,30).

The localization pattern of TNP-Ficoll suggests that B cells in the marginal zone as well as B cells in the follicle can be activated by TI-2 antigens. This is supported by experiments showing that B cells from these two compartments do not differ in their responsiveness to TI-2 antigens (37).

In chapter 3 it was demonstrated that *in vivo* macrophage elimination drastically increased the amount of injected TNP-Ficoll in the follicles. In a previous study it was shown that the antibody response against low doses of TI-2 antigen increased significantly after *in vivo*

macrophage depletion (38). These data suggest that in intact animals, macrophages obscure the antigen from the immune system, by removing the injected TNP-Ficoll from the circulation, and prevent in that way the direct activation of B cells by TI-2 antigens. In contrast, complement depletion of mice abrogated the localization of TI-2 antigens in the follicles (chapter 3), and was found to lead to a decreased humoral TI-2 immune response (39, 40). These results together suggest that follicular-trapped TI-2 antigens, FDC and B cells have a role in the induction of antibody responses against TI-2 antigens. Furthermore, it is concluded that the role of marginal zone macrophages in TI antibody responses is not primarily antigen-presentation but rather removal from the circulation (TI-2 antigens), killing and pre-processing of particulate antigen/bacteriae.

9.3 The expression of gp39 in the spleen

In vitro studies revealed that upon activation (by ConA or anti-CD3 mAb) Th cells express gp39, a 39 kD surface protein. Further analysis indicated that gp39 is the ligand for the CD40 surface molecule on B cells. It was demonstrated that a mAb specific for gp39 and a CD40-IgG1 fusion protein block the activation of B cells by activated Th cells, suggesting that these molecules are critical for antibody responses against TD antigens (5).
The studies presented in this thesis investigated the expression of gp39 in lymphoid tissues after immunization with TD and TI-2 antigens. Furthermore, the function of gp39 in TD and TI-2 antibody responses was assessed. In chapter 7 it was shown that *in vivo* gp39 was exclusively expressed on $CD4^+$ cells. High frequencies of $gp39^+$ cells were observed in spleens of mice after immunization with TD (KLH, RαIgD) and TI-2 (TNP-Ficoll) antigens. During all antibody responses examined, $gp39^+$ cells were localized predominantly in the (outer)-PALS and around the terminal arterioles of the spleen. After immunization with RαIgD and during secondary antibody response against KLH, relatively few $gp39^+$ cells were also observed in the splenic follicles. Lederman et al. (41) demonstrated in human lymphoid tissue that the T-cell-B-cell activating molecule, the human equivalent of gp39, was expressed on $CD4^+$ cells in the PALS of the spleen, like was described in this thesis. In contrast to our studies, they found relatively high frequencies of $gp39^+$ cells in lymphoid follicles of tonsils, lymph nodes and spleens. This discrepancy could be explained by the relatively undefined character of the lymphoid tissue examined by Lederman et al. In non-immune murine lymphoid tissue hardly any $gp39^+$ cells were detected, whereas in the study of Lederman high frequencies of activated T cells were

observed. Since gp39 is only temporarily expressed on T cells (42), the results obtained by Lederman et al. are an indication that the lymphoid tissue examined was most likely chronically inflamed and consequently not comparable with lymphoid tissue of the laboratory mouse, mounting a well defined antibody response against a non-replicating antigen. In the murine spleen, $gp39^+$ T cells were localized in the outer-PALS and around the terminal arterioles, the same compartments where activated B cells are found. These results suggest that T-B cell interactions occur predominantly in these splenic compartments. Indeed, double staining experiments revealed multiple (antigen-specific) activated B cells in close conjunction to $gp39^+$ T cells both during TD (KLH, RαIgD) and TI-2 antibody responses. To determine whether $gp39^+$ cells had a functional role in these *in vivo* antibody responses, modulation studies were performed. Treatment of mice with gp39 specific mAb resulted in a complete inhibition of the primary antigen-specific antibody response against TD antigens (chapters 7 and 8; 43). Even the huge increase in serum IgG1 levels, observed after RαIgD injection, was completely suppressed by the anti-gp39 treatment. Only the secondary antibody response against KLH and the polyclonal increase of IgE after RαIgD injection were not completely suppressed. Examination of spleens of immunized mice revealed that cytokine-production and gp39 expression were not affected by the anti-gp39 treatment. Collectively, these observations suggest that the unchanged cytokine-production after anti-gp39 treatment provides a microenvironment which stimulates activated memory B cells and memory IgE^+ AFC to secrete antibodies. The observation that anti-gp39 treatment did not affect the frequency of $gp39^+$ cells substantiates previous experiments (43), which demonstrated that unaltered Th function can be transferred from mice treated with anti-gp39, and suggests that blockage of gp39 is responsible for the *in vivo* suppression of TD antibody responses. It was not possible to demonstrate *in situ* that injected anti-gp39 mAb were binding to $gp39^+$ cells, which suggests that blockage of only a part of the gp39-molecules on activated Th cells is sufficient to prevent B cell activation by $gp39^+$ Th cells. These results are in agreement with experiments of Morris et al. (44), which showed that for the generation of an IgG response after GαIgD injection, intense physical interactions are required between antigen-presenting B cells and antigen-specific Th cells. This group (44) demonstrated that even bystander B cells, in spite of their localization close to IL-4-PC and $gp39^+$ cells, were not stimulated to produce IgG1. Most likely antigen-presentation results in a intimate physical interaction between Th cells and B cells, which may be essential for the binding of gp39 to the CD40 molecule on the antigen-presenting B cell (Fig. 1). These interactions assure the

preferential activation of antigen-specific B cells, which is required for the development of an antigen-specific antibody response.

The experiments presented indicate that gp39 is essential for TD antibody responses *in vivo*. A similar molecule was identified on activated Th cells of humans (42). Its essential role in humoral immunity was clearly illustrated in patients with the X-linked hyper-IgM syndrome, whose defective IgG production was found to be caused by mutations in the gp39 molecule (45,46,47,48).

After immunization with TNP-Ficoll, a TI-2 antigen, high frequencies of $gp39^+$ cells were observed in the outer-PALS and around the terminal arterioles of the spleen (chapters 6 and 7), indicating that T cells were activated during TI-2 antibody responses. The observation that antibody responses against TNP-Ficoll were unaffected *in vivo* after administration of anti-gp39 mAb (43, chapter 7) confirms that the initiation of this response is not dependent on gp39 and therefore actually T cell independent. These data indicate that gp39 is a marker for activated T cells and consequently not always related to T cell dependent B cell activation. The process of T cell activation, revealed *in vivo* as an increase in the frequency of $gp39^+$ Th cells, can be explained as was suggested by DeKruyff et al. (49), who showed that B cells, activated by TI-2 antigens, were able to activate T cells *in vitro*. Similar results were described by Zisman et al. (50), who demonstrated that TI-2 antigens composed of D-amino acids and binding directly to class II molecules of antigen-presenting cells, were able to activate T cells. The observation of high frequencies of $gp39^+$ cells during TI-2 antibody responses is also in agreement with experiments showing that rigorous depletion of presumedly activated Th cells reduces the antibody response against TI-2 antigens (51,52,53). As will be discussed in the next section, the cytokines produced by activated Th cells have an important regulating role in the proliferation and differentiation of B cells activated by TI-2 antigens.

Independent of the route of administration and the nature of the antigen, $gp39^+$ cells and activated antigen-specific B cells were found in the PALS and around the terminal arterioles of the spleen. These observations suggest that T-B cell interactions occur in restricted compartments of the spleen. This was already suggested by van Rooijen et al. (54), who found that specific-AFC were localized in the same compartments during TD, TI-1 and TI-2 antibody responses. They hypothesized that there was only one migration pathway in the spleen for antigen-reactive B cells which are differentiating into AFC and that this pathway is independent of the type of antigen. Our experiments, describing the localization of antigen-specific B cells and activated T cells in the spleen, support this

hypothesis and suggest that T-B cell interactions occur in the PALS and around the terminal arterioles of the spleen during all types of antibody responses.

9.4 Cytokine-production in the spleen

Besides a role in the initial B cell activation, Th cells have also a major function in B cell differentiation. This regulatory ability is exerted by the cytokines produced by Th cells (Fig. 1). Several different *in vitro* models have been used to study the effects of cytokines on proliferation and differentiation of B cells (55, chapter 2). These *in vitro* experiments indicated the potential interactions of immune cells and cytokines, but did not show whether these cytokines are active or have similar effects during *in vivo* antibody responses. Therefore, *in vivo* validation of *in vitro* observations is required to delineate the precise role of cytokines in immune responses.

The role of IL-4 and IFN-γ in *in vivo* antibody responses has been extensively studied (56, chapter 2). In this thesis the actual *in vivo* participation of cytokines in TD and TI-2 antibody responses is described. Furthermore, cytokine-production was related to antigen-localization, gp39 expression and antibody production in the spleen during TD and TI-2 antibody responses.

The historical definition of a TI antigen reflects its ability to stimulate antibody responses in nude mice *in vivo* and in T cell depleted cultures *in vitro* (57). However, more recent experiments showed that rigorous elimination of T cells consistently reduced the *in vitro* antibody response against TI antigens, indicating that these antigens are T cell dependent to a significant degree (51,52,53). Addition of cytokines to cultures of cells from secondary lymphoid organs, indicated a regulatory role of cytokines in *in vitro* antibody responses against TI-2 antigens (53,58,60,61).

Chapters 5 and 6 of this thesis describe the *in vivo* activity of cytokine-PC in TI-2 antibody responses. After immunization with (non)-haptenated TI-2 antigens relatively high frequencies of IFN-γ-PC were observed in spleens of mice. It was demonstrated that IFN-γ was mainly produced by CD4 and CD8 positive T cells and to a lesser degree by NK cells. Furthermore, it was demonstrated that these cells were actively secreting IFN-γ. The IFN-γ-PC were predominantly localized in the outer-PALS and around the terminal arterioles. In these compartments high frequencies of IFN-γ-PC were found in juxtaposition to TNP-AFC, suggesting a regulatory role of IFN-γ in TI-2 antibody responses. A regulatory role of IFN-γ in TI-2 antibody responses was also suggested by *in vitro* experiments of Snapper et al.

(60), which demonstrated that IFN-γ stimulated the production of IgG2a and IgG3 of B cells activated by TI-2 antigens. Interestingly, these results were supported by *in vivo* data, showing increased antigen-specific IgG3 serum levels after immunization with TI-2 antigens (62). Altogether, these results suggest that the *in situ* visualized juxtaposition of IFN-γ-PC and TNP-AFC represent T-B cell interactions which stimulate the development of $IgG3^+$ antigen-specific AFC. However, *in vivo* treatment with anti-IFN-γ mAb inhibited only slightly the IgG3 TNP-specific antibody response after TNP-Ficoll immunization (unpublished observation, Van den Eertwegh et al.). This observation may indicate that the physical interaction between IFN-γ-PC and TNP-AFC does not allow the neutralization by IFN-γ specific antibodies, or that IFN-γ is one of the cytokines participating in the complex cytokine network that regulates TI-2 antibody responses.

Indeed, in chapter 6 it was shown that besides IFN-γ-PC also high frequencies of IL-2-PC, IL-4-PC and IL-5-PC were found in close conjunction to TNP-AFC in spleens of TNP-Ficoll immunized mice. In contrast, relatively low frequencies of $gp39^+$ cells and cytokine-PC were demonstrable in lymph nodes after s.c. immunization with TNP-Ficoll. Examination of the *in situ* antibody response, revealed significant frequencies of TNP-AFC in lymph nodes after immunization with TNP-Ficoll. Since no TNP-ASC were observed in TNP-Ficoll immunized lymph nodes, these data suggest that the lack of cytokines in the microenvironment of lymph nodes from TNP-Ficoll immunized mice is responsible for the absence of TNP-specific antibody secreting cells (TNP-ASC) in these draining lymph nodes. This was substantiated by the observation that simultaneous immunization with TNP-Ficoll and *Brucella abortus*, a particulate TI-1 antigen, resulted in a simultaneous increase in the frequencies of cytokine-PC (IL-2, IL-4, IL-5 and IFN-γ) and TNP-ASC in draining lymph nodes. Similar results were obtained by Goud et al. (61) *in vitro*. They demonstrated that in suspensions of peripheral lymph nodes, TNP-Ficoll could induce the priming and proliferation of hapten-specific B cells. However, the differentiation into TNP-ASC was not possible in the microenvironment created by lymph node cells. Only the addition of cytokines (IL-1, IL-5 and IL-6) could overcome this maturation defect. Therefore, we investigated the effect of *in vivo* IL-4 and/or IL-5 treatment on the antibody response against TNP-Ficoll in draining lymph nodes of immunized mice. Continuous *in vivo* administration of these cytokines did not significantly increase the frequencies of TNP-AFC or TNP-ASC in lymph nodes of TNP-Ficoll immunized mice. These results may indicate that administered cytokines do not increase the cytokine-concentrations in the microenvironment of lymph nodes to the level required for differentiation of TNP-ASC or that other cytokines are

involved as well in the differentiation of TNP-AFC into TNP-ASC. In conclusion, it appears that the lack of cytokines in the microenvironment of lymph nodes is responsible for the absence of TNP-ASC after TNP-Ficoll immunization. Furthermore, presented data suggest that during TI-2 antibody responses the initial activation of B cells is T cell (gp39) independent, whereas the further differentiation into antibody-secreting cells is dependent on cytokines produced by, e.g. T cells, NK cells, and macrophages.

In antibody responses against (TNP-)KLH, a TD antigen, IgG1 is the principal isotype expressed by the antigen-specific AFC (63,64). In addition, IgE is produced only after immunization with TD (protein) antigens (65). Since IL-4 is suggested to be involved in the isotype selection of IgG1 and IgE, a role for this cytokine might be predicted in the response to KLH. We examined the *in vivo* cytokine-production during the antibody response against (TNP-)KLH. In the spleen IL-2-PC and IL-4-PC were observed in relatively high frequencies as compared to IFN-γ-PC. Similar results were observed after immunization with RαIgD, a TD antibody response which is also dominated by IgG1 and IgE. The observation that all cytokine-PC reached maximum frequencies at day 3 and 4 after immunization with KLH and RαIgD are consistent with experiments of Bradley et al. (67), who demonstrated that the kinetics of appearance of effector $CD4^+$ T cells, that produce cytokines upon restimulation with KLH *in vitro*, were similar for each of the cytokines investigated. These results suggest that after immunization and subsequent antigen presentation, T cells with potency to produce IL-2, IL-4, IL-5 and/or IFN-γ are activated at about the same time and differentiate into cytokine-PC, following a similar time course. The dominance of a particular cytokine-PC (IL-4-PC or IFN-γ-PC) in an antibody response most likely regulates the isotypes produced by individual AFC. Analysis of the *in situ* TD antibody response revealed high frequencies of antigen-specific AFC at days 3 and 4 after TNP-KLH immunization. These IgM^+ antigen-specific-AFC were found in juxtaposition to predominantly IL-2-PC and IL-4-PC and preceded the antigen-specific IgG1 production, suggesting that these cytokines play a role in isotype-switching. Indeed, cultures of purified KLH-specific $CD4^+$ cells, predominantly secreting IL-2 and IL-4, and hapten-specific B cells resulted in a relatively high production of antigen-specific IgG1 (67), confirming the suggested role of IL-4 in IgG1 production (10). Furthermore, the observation that *in vivo* anti-IL-4 treatment can completely block the increase in serum IgE after immunization with TNP-KLH (68), indicates that IL-4 is involved in the regulation of IgE production.

Finkelmans group described extensively the model immune response observed after

injection of a high dosis of GαIgD (56). The high degree of T cell activation and cytokine-production, which is critical for the huge increase in serum IgG1 and IgE, makes this model suitable for the analysis of *in vivo* TD antibody responses. In chapter 8 we investigated the *in vivo* cytokine expression in the antibody response after RαIgD injection. High frequencies of IL-2-PC and IL-4-PC relative to IFN-γ-PC were observed in close conjunction to cytoplasmic-IgM$^+$ B cells 2-5 days after immunization. The high cytokine-production preceded the IgG1 and IgE production which started 5 days after injection. These observations substantiate *in vivo* experiments, which suggested that T-B cell interactions, required for high IgG1 serum levels, occur at day 2-5 after GαIgD treatment (65). This is consistent with the observation that IgE production is blocked by the injection of mice with anti-IL-4 mAb at day 3 and 4 after GαIgD immunization (69,70). These data together, suggest that the *in vivo* visualized interactions between IL-4-PC and IgM$^+$ cells, localized in the PALS and around the terminal arterioles of the spleen, are essential for the high IgE production observed after injection of RαIgD.

In vivo studies demonstrated that anti-IL-4 mAb treatment did not completely inhibit IgG1 responses (70). In addition, the substantial amount of IgG1 demonstrable in sera of IL-4 deficient mice (71), indicates that isotype switching to IgG1 is IL-4 independent. Recently, it was demonstrated that activated Th cell membranes, most likely expressing gp39, induced the germline transcription of Cγ1, suggesting that this mechanism was responsible for the switching to IgG1 (72). These *in vitro* data were substantiated by the studies described in this thesis, which showed *in vivo* that anti-gp39 treatment completely abrogated the IgG1 response after RαIgD injection.

In contrast to the large differences in IgG1 and IgE production after RαIgD and KLH immunization, the splenic cytokine-production revealed similar frequencies of cytokine-PC in these two TD antibody responses. These results suggest that the type of cytokine (IL-4) determines the quality (isotype) of the antibody response, whereas other factors may be responsible for the quantity of antibody production. Analysis of the gp39 expression in both types of antibody responses revealed 3-4 times more gp39$^+$ cells after RαIgD immunization. In addition, *in vivo* blocking of gp39 prevented the four-fold increase in splenic weight, as is observed after RαIgD injection. This suggests that differences observed in B cell proliferation/antibody production may be attributed to different frequencies of gp39$^+$ cells.

Another important difference between the antibody responses against KLH and RαIgD may be found in the APC. Because of the relatively low frequency of antigen-specific B

cells in non-immune mice, DC are most likely the important APC during the primary antibody response against KLH. In contrast, after injection of RαIgD, the antigen binds directly to IgD^+ B cells. Hence it is likely that B cells are the principal APC in the immune response to RαIgD. This implicates that in the antibody response against KLH, a large part of the $gp39^+$ cells is activated by DC and does not interact with antigen-specific B cells. This in contrast to the immune response after RαIgD injection, where most $gp39^+$ cells are activated by antigen presenting B cells, which increases the frequency of T-B cell conjugates. The formation of these T-B cell conjugates (Fig. 1) facilitates the activation of B cells by activated Th cells, and will lead to the activation of relatively high frequencies of B cells. In the secondary response against KLH, high frequencies of KLH-specific memory B cells are present in the spleen, which will preferentially bind KLH and increase the relative contribution of the antigen-presenting B cells in the activation of Th cells. This will increase the frequency of T-B cell conjugates, and may explain the significant increase in antigen-specific AFC after secondary immunization (chapter 7).

At the peak of cytokine production relatively high frequencies of IL-2-PC and IL-4-PC were observed in the PALS and around the terminal arterioles during the antibody responses against KLH and RαIgD. As these cytokine-PC were observed in close conjunction, it is likely that these cells create a microenvironment which is rich in IL-2 and IL-4. Such a microenvironment was suggested to be essential for the development IL-4-PC, as was demonstrated *in vitro* (73,74,75). It was suggested that IL-2 provides the optimum conditions for proliferation of cytokine-producing T cells (73,74,76), while IL-4 propagates the preferential development of IL-4-PC (73, 74). *Ex vivo* evidence that IL-2 was involved in T cell proliferation was provided by FACS analysis, showing an increased IL-2R expression on T cells at day 3-5 after GαIgD injection (77).

$Gp39^+$ cells, antigen-specific AFC, but also cytokine-PC were predominantly found in the PALS and around the terminal arterioles of the spleen, irrespective of type of antibody response (primary, secondary, TD, or TI). These results support the hypothesis of Van Rooijen et al (54), who suggested one single differentiation pathway for B cells in the spleen during all types of antibody responses, as was discussed in the previous section.

Cytokine-PC were never observed in the follicles of the spleen, similar to studies in humans (78,79,80), which demonstrated that none of the putative B cell differentiation factors, such as IL-2, IL-4 and IFN-γ, were localized in the follicles of lymphoid tissue. Butch et al (81) showed *in vitro* that IL-4 mRNA was the only cytokine expressed by human germinal center T cells, whereas these T cells expressed no mRNA of the other 9

tested cytokines. Levels of mRNA expression *in vitro*, however, not always correlate with protein synthesis *in vivo* (82). Therefore these data are not incompatible with our *in vivo* results. Since cytokines are thought to be the determining factors in the regulation of isotype-switching and hardly any evidence was found for the expression of cytokines in the follicles, it is likely that isotype-switching does not occur in the follicles, as has been suggested by Kraal et al. (83). They demonstrated that during a primary antibody response follicular B cells were mIgM-positive, whereas after secondary immunization germinal center B cells were mIgG positive, suggesting that isotype switching occurred in the follicles. However, they could not exclude that these mIgG$^+$ B cells were generated in another compartment, as is suggested in this thesis.

The studies described in this thesis show that gp39$^+$ cells and cytokine-PC were predominantly localized in the PALS and around the terminal arterioles of the spleen. *In vivo* experiments suggested that B cell migrate from the PALS to the follicle (84). Based on the localization and migration of the immune cells involved, we hypothesize that part of the differentiating B cells regulated by activated Th cells switch from IgM to IgG1 in the PALS, and migrate subsequently to the follicle. In this compartment, B cells undergo follicular processes, such as B cell selection, somatic mutation, affinity maturation and will stay there as memory IgG$^+$ B cells (85,86). In conclusion, *in situ* analysis of cytokine- and antibody-production suggests that T-B cell interactions occur predominantly in the PALS and around the terminal arterioles of the spleen.

9.5 *In vivo* cell-cell interactions in the spleen

In this thesis we described the localization and cytokine production of the cells which are assumed to be involved in antibody formation in the spleen. The characteristic localization of T cells, B cells, macrophages, DC, gp39$^+$ cells, cytokine producing cells, antibody-forming cells, and other immuno-competent cells within the splenic compartments was discussed in relation to antigen presentation. In addition we tried to evaluate the vast amount of *in vitro* data on the role of various cytokines and cell-cell interactions suggested to be involved in the generation of antibody responses. As a basis for understanding of *in vivo* processes, discrete phenomena of *in vivo* immune responses, like cell migration patterns of immunocompetent cells, antigen localization/trapping, T cell activation, cytokine production and antibody formation in the different compartments of the spleen, were related to *in vitro* observations for individual cells as well as bulk populations.

In this section we will try to bring order to the sequence of events that occur in the spleen after antigenic challenge. Both *in vitro* and *in vivo* data will be incorporated in the models presented. As antigen localization and T cell activation are completely different for TI and TD humoral immune responses, these models will be discussed separately (Figs. 2-4).

A. *In vivo* cell-cell interactions during TI-2 antibody responses

After i.v. injection, TI antigens arrive in the marginal zone of the spleen (Fig. 2). Particulate TI antigens (e.g. *Brucella abortus*) are taken up and processed by marginal zone macrophages. These processed antigens will subsequently be transferred to neighbouring B cells or act as soluble TI antigens. Soluble TI-2 antigens activate complement and bind, mediated by complement degradation products, to B cells in the marginal zone, and to B cells and FDC in the follicles. In these compartments, TI-2 antigen-complement complexes can activate B cells directly, without the requirement of antigen presentation by marginal zone macrophages. Apart from direct B cell activation in the marginal zone, FDC expose the antigen to B cells which migrate through the follicles, resulting in the activation of virgin B cells with appropriate specificity. TI-2 antigens drive resting TI-2 antigen binding B cells into the G_1 stage of the cell cycle, resulting in the acquisition of the competency to respond to T cell derived cytokines. Subsequently these primed TI-2 antigen binding B cells migrate through the outer-PALS of the spleen and have cell-cell contact with T cells migrating from the inner PALS. These T cells will be activated by the TI-2 antigen activated B cells and will then express gp39. Subsequently activated T cells will secrete cytokines which in turn regulate the proliferation and differentiation of G_1 B cells into antigen-specific antibody-forming and -secreting B cells. These differentiating antigen-specific B cells and a part of the antigen-binding G_1 B cells leave the white pulp along the sheaths of lymphoid tissue surrounding terminal arterioles. A part of these antigen-specific differentiating B cells will stay in the lymphoid tissue surrounding the terminal arterioles and develop into antigen-specific antibody-forming cells, whereas another part will leave the spleen through the red pulp into the circulation and migrate into extrasplenic sites, such as bone marrow and other lymphoid tissues.

The role of marginal zone macrophages is not primarily antigen-presentation but rather removal (TI-2 antigens), killing and pre-processing of particulate antigens/bacteriae, whereas FDC and B cells are most likely involved in the induction of antibody responses to TI-2 antigens. Cytokines secreted by T cells seem to be crucial for B cell differentiation

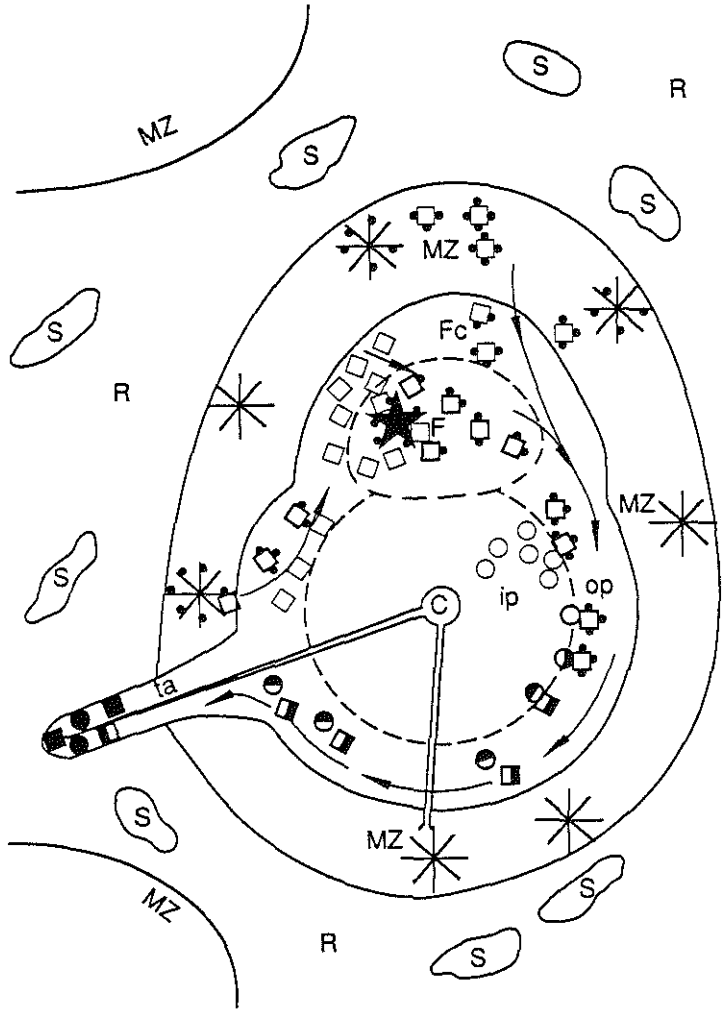

Figure 2. Schematic representation of activation and migration of immune cells in the spleen during TI-2 antibody responses Arrows migration of B and T cells in the spleen. C= central arteriole, ip= inner-PALS, F= follicle, Fc= follicle corona, MZ= marginal zone, op= outer-PALS, R= red pulp, S= sinus, ta= lymphocyte sheath around terminal arteriole, • =antigen, □= B cell, ◘ = activated antigen-specific B cells (G_1), ◧ = differentiating antigen-specific B cell, ■ = antibody-forming cell, ○ = T cell, ◯ = activated T cell, ◐ = differentiating T cell, ● = cytokine producing T cell, ★ = FDC, ✳ = marginal zone macrophage.

and isotype selection in the antibody response to TI-2 antigens (Fig. 2).

B. *In vivo* cell-cell interactions during TD antibody responses

Like TI antigens, TD antigens also arrive primarily in the marginal zone of the spleen after i.v. injection (Fig. 3; see also Van Rooijen [19]). Particulate antigens will be taken up and pre-processed by macrophages in the marginal zone. The macrophages in the marginal zone will transfer the processed antigen to B cells in the marginal zone, or migrate into the while pulp and there transfer the antigen to DC in the inner-PALS. Alternatively, the DC may pick up the antigen in the marginal zone and migrate subsequently into the PALS of the spleen. Upon contact with antigen, non-antigen specific T cells leave the clusters formed by IDC and T cells, while antigen-specific T cells are activated to proliferate. The induction of proliferation of antigen-specific T cells by IDC leads to an expansion of antigen-specific T cells. B cells which bind soluble or processed antigens migrate from the marginal zone into the adjacent outer parts of the PALS. On arrival in the PALS, B cells encounter numerous T cells, among which are T cells of the appropriate antigen-specificity activated by IDC, migrating in an opposite direction. The counter flow of antigen-specific B and T cells optimizes the chance that these two cell types meet and interact with each other. Antigen-specific B cells will process and present the antigen in context of MHC class II to antigen-specific T cells, by forming a T-B cell conjugate (Fig. 1). Antigen-presentation results in the activation of Th cells and the expression of gp39 by these cells. The T-B cell conjugate formed, facilitates the binding of gp39 to the CD40-molecule on the B cells, which will lead to the initial activation of B cells. Besides the expression of new membrane antigens, activated T cells will also produce cytokines which will regulate the proliferation and differentiation of B cells into antibody forming cells. All these cell-cell interactions occur most likely during their migration from the PALS along the sheaths of lymphoid tissue surrounding the terminal arterioles towards the red pulp (Fig. 3). Whether T cells or B cells will migrate as single cells, as T-B cell conjugates or encounter more than one antigen-specific B or T cell is not yet clear. After all these interactions B cells eventually differentiate into antibody-forming/secreting cells. The primary antibody response will be reflected in increasing serum concentrations of antibodies. These antibodies will form complexes with circulating antigen. A minor proportion of the antibody-antigen complexes will be trapped by FDC and can be retained there for long periods of time. These complexes provide the proper microenvironment for B cell affinity

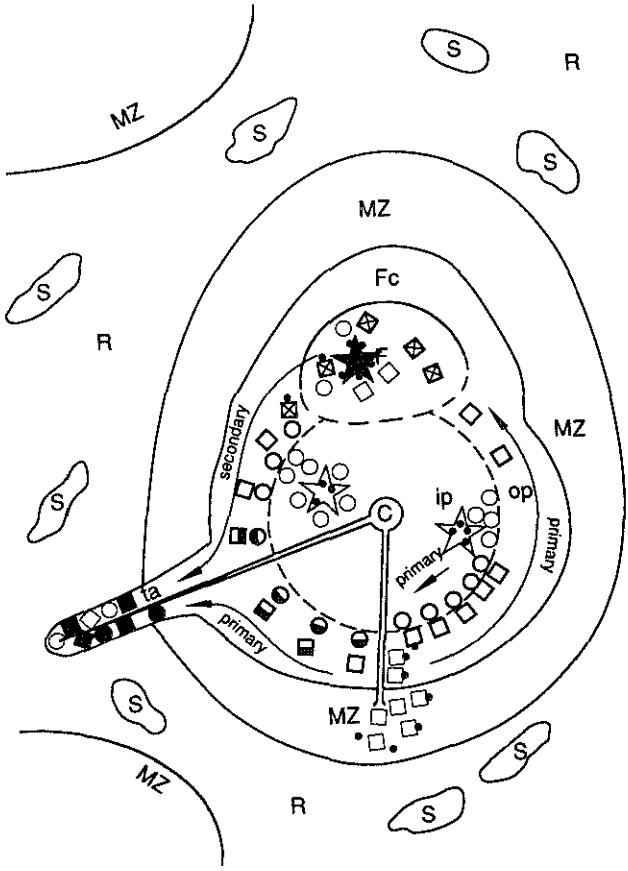

Figure 3) Schematic representation of the activation and migration of T and B cells in the spleen during TD antibody responses. Arrows indicate migration of B and T cells in the spleen. C= central arteriole, ip= inner-PALS, F= follicle, Fc= follicle corona, MZ= marginal zone, iP= inner-PALS, oP= outer-PALS, R= red pulp, S= sinus, ta= lymphocyte sheath around terminal arteriole. • = antigen, □ = resting B cell, ⊠ = memory B cell, ◧ = differentiating antigen-specific B cell, ■ = antibody-forming B cell, ○ = resting T cell, ○ = activated antigen-specific T cell (gp39 positive), ● = cytokine-producing T cell, ★ = FDC, ✡ = IDC.

maturation and are involved in the generation and maintenance of B cell and possibly T cell memory.

After secondary challenge with the same antigen, the majority of the formed antigen-antibody complexes are phagocytized by macrophages in the marginal zone and red pulp. A part of the immune complexes or free antigen is trapped in the follicles (Fig. 3). The FDC dendrites bead and form iccosomes, which are dispersed in the developing germinal centers where they are trapped and endocytosed by germinal center memory B cells. Germinal center B cells process the iccosome derived antigen, and present the antigen to T cells. The exact localization of the assumed T-B cell interaction is not known. On one hand the presence of many T cells, with unknown specificity, in the light zone of the follicle suggests that this interaction may occur in the follicle. On the other hand the high rate of T cell circulation in the PALS and the predominance of antibody-forming cells as well as $gp39^{+}$/cytokine-producing cells in this compartment after the initiation of the secondary immune response are more suggestive for the occurrence of T-B cell interactions in the PALS, as was discussed for primary antibody responses. The cytokine profile of T cells will determine the isotype of antibodies produced by B cells: e.g. Type 1 pathway regulated immune responses with a predominance of IFN-γ-PC may lead to a preferential production of IgG2a, whereas the type 2 pathway regulated immune responses with a predominance of IL-4 may favour the preferential production of IgG1 and IgE. These cytokine-producing T cells can only exert their regulatory role when they are localized in juxtaposition to antigen-specific B cells. During formation of T-B cell conjugates (Fig. 1), the expression of gp39, CD40, cytokines, cytokine-receptors are all critical for the further differentiation of the antigen-specific B cells. Part of these isotype switched high affinity B cells will localize in the follicle as memory B cells and will follow the above described route when FDC present a free epitope of the antigen. In the case that all epitopes of antigen are covered with antibody no B cell activation will occur. In this way follicular trapped antigen is able to fine-tune the antigen-specific antibody response. Furthermore, by presenting the antigen to antigen-specific T cells, B memory cells may preserve T cell memory.

9.6 CONCLUDING REMARKS

In this thesis we demonstrate that the spleen participates significantly in host defence mechanisms. All immune cells and pathogens present in the circulation will pass the

spleen, where their sequestration may lead to a variety of antigen-specific immune responses. The characteristics of an antigen, i.e. soluble vs. particulate, protein vs. polysaccharide, complement-activating capability, determine the fate of the antigen in the spleen. The splenic compartments where antigen localizes and the cell types which take up the antigen have a decisive role in the selection of immune cells that will be involved in the development of immune responses.

Macrophages, DC and B cells are the main APC, although their relative contribution to *in vivo* immune responses requires more study. *In vitro* experiments characterized the properties of isolated APC and suggested different roles of each of the APC in immune responses. However, *in vitro* experiments did not reveal the actual cells and compartments which are involved in antigen-presentation. *In vivo* studies on the localization of injected antigen and antigen presenting cells, combined with *in vivo* elimination studies of particular antigen presenting cells provide more direct clues to the contribution of each of the APC in the development of *in vivo* immune responses.

The possible role of T cells, B cells, macrophages, $gp39^+$ cells, cytokine producing cells and cytokines in *in vivo* immune responses, as suggested by *in vitro* experiments, was discussed. However, it was demonstrated that not all *in vitro* models correspond to the actual *in vivo* situation. We emphasized the advantages of the *in vivo* approach, which is powerful in the combined description of localization, characterization and activity of immune cells and the factors they produce in separate stages of immune responses. Furthermore, we demonstrated the valuable qualities of *in vivo* modulation studies for the understanding of antibody responses.

The *in vivo* studies presented indicate that immune responses are regulated by a complex cytokine network, and show that the treatment of mice with cytokines or their neutralizing antibodies do not reveal the same clear-cut effects, as *in vitro*. These observations indicate that the delineation of the regulating role of the cytokine-network in *in vivo* antibody responses first requires a detailed *in vivo* description of all the cells and factors involved. Only after this stage of research, when cytokines and cell types producing cytokines during particular immune responses are characterized, modulation studies with multiple antibodies and/or cytokines may give us insight in the role of cytokines in *in vivo* immune responses. In conclusion, this thesis clearly shows that a combined *in vitro/in vivo* approach increases our comprehension of the important events during *in vivo* antibody responses.

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SUMMARY

The aim of the studies presented in this thesis was to investigate *in vivo* the immunological events taking place during the development of various types of antibody responses. Special emphasis was put on time-course and localization of the different immunological events, e.g. antigen-presentation, T cell activation, cytokine-production and antibody-formation in the spleen, as these processes are thought to have a decisive role in the development of *in vivo* antibody responses. To this end we have investigated cryostat sections of lymphoid tissue from mice immunized with various types of model antigens at different time points. The results revealed by specific immunohistochemistry is taken to be representative for the *in vivo* situation at that moment of the immune response. Furthermore, *in vivo* modulation experiments were performed to determine the role of antigen, cytokines and immune cells during *in vivo* antibody responses.

Chapter 2 discusses the complex anatomical organization of the spleen, consisting of distinct compartments which are characterized by the localization of unique and specialized cell types. The migration patterns of B cells, T cells and dendritic cells are described. The model antigens used in immunological experiments were characterized and their localization in the spleen after injection is discussed. Furthermore, the vast amount of *in vitro* data dealing with antigen-presentation, cell-cell interactions, T and B cell activation, and the immunoregulatory role of cytokines is reviewed. Finally, the experimental questions addressed in this thesis are presented.

Chapter 3 describes a highly sensitive immunohistochemical technique for the detection of trinitrophenylated antigens in the spleen and lymph nodes. The localization pattern of TI-2 antigens is compared to the localization of particulate TI-1, soluble TI-1 and soluble TD antigens. It is demonstrated that TI-2 antigens and particulate antigen localize in the marginal zone of the spleen. The rapid uptake of these antigens by marginal zone macrophages emphasizes their important role in the primary defense against bloodborn pathogens. Furthermore, the putative role of marginal zone macrophages as important pre-processors of particulate antigens is discussed. It is demonstrated that TI-2 antigens localize in the follicles of lymphoid organs, a hitherto undescribed phenomenon. Evidence is provided that the early follicular localization of TNP-Ficoll is mediated by complement. *In vivo* macrophage elimination drastically increased the amount of TNP-Ficoll in the follicles, and enhanced the

humoral immune response to relatively low doses of antigen. In contrast, complement deprivation of mice abrogated the follicular localization of TI-2 antigen and led to a decreased antibody response, suggesting that the follicular localization of antigen plays a role in the induction of TI-2 antibody responses. The mechanisms and *in vivo* cell-cell interactions which occur during the development of TI-2 antibody responses are discussed. It is suggested that marginal zone macrophages are not primarily involved in antigen-presentation, but rather in removal (i.c. of TI-2 antigens), killing and pre-processing of particulate antigen/bacteriae, whereas follicular dendritic cells and B cells are most likely involved in the induction of antibody responses against TI-2 antigens.

In *chapter 4*, a new method is described for the immunohistochemical detection of cytokine-producing-cells (cytokine-PC) in murine spleens. To this end, the *in situ* detection of IFN-γ was used as example. Immunization with Bacille Calmette Guèrin (BCG) was used as model immune response, since experiments have indicated that such a protocol might result in a high splenic IFN-γ production. In order to detect IFN-γ-PC, a monoclonal antibody directed to IFN-γ was conjugated to alkaline-phosphatase. After incubation of cryostat sections with this conjugate and immunohistochemical revelation, IFN-γ-PC, characterized by a blue stained cytoplasm, were detected. Immunization with BCG resulted in increased frequencies of IFN-γ-PC in the spleen. However, no raised IFN-γ serum levels were detected in BCG immunized mice, indicating that IFN-γ is locally produced and consumed in the splenic microenvironment. Only i.v. administration of Concanavalin A, a T cell mitogen which activates probably all IFN-γ-PC, resulted in increased IFN-γ serum levels in BCG immunized mice. Simultaneous incubation of the IFN-γ-specific conjugate and an antibody specific for a membrane marker, followed by a secondary peroxidase conjugate, enabled the characterization of IFN-γ-PC. It is concluded that immunohistochemical techniques indeed are suitable for the detection and characterization of cytokine-PC, provided that specific antibodies are available.

Chapter 5 describes the development and localization of IFN-γ-PC during immune responses against various TI-2 antigens. It is demonstrated that the frequency of IFN-γ-PC is markedly upregulated during TI-2 antibody responses. The IFN-γ-specific ELISPOT assay confirmed the results obtained *in vivo* and suggest that IFN-γ is not only produced but also secreted in the spleen. Double staining experiments revealed that IFN-γ is predominantly produced by $CD4^+$ and $CD8^+$ cells, while relatively few IFN-γ-PC were found to be NK cells. These data substantiate previous reports which suggested that T cells are involved in TI-2 antibody

responses. IFN-γ-PC were predominantly localized in the outer-PALS and around the terminal arterioles of the spleen after immunization with TNP-Ficoll. No IFN-γ-PC were localized in the follicles and marginal zone of the spleen. Antigen-specific antibody-forming cells (AFC) were localized in the same compartments as IFN-γ-PC. Furthermore, double staining revealed that TNP-AFC were localized in juxtaposition to IFN-γ-PC, suggesting a regulatory role of T cells in the antibody response against TI-2 antigens.

In *chapter 6* the investigation of the putative defect of peripheral lymph node cells to mount an antibody response against TI-2 antigens is described. Unexpectedly, analysis of the *in situ* antibody production revealed the presence of TNP-AFC in draining lymph nodes after s.c. immunization with TNP-Ficoll. Splenectomy experiments indicated that these TNP-AFC did not originate from the spleen. However, ELISPOT assays did not show TNP-specific antibody-secreting cells (TNP-ASC) in draining lymph nodes, whereas in the spleen relatively high frequencies of TNP-ASC were observed after TNP-Ficoll immunization. These results suggest that TNP-Ficoll can prime TNP-specific lymph node B cells, but the microenvironment of lymph nodes appears not capable to provide the signals required for the final differentiation of TNP-AFC into TNP-ASC. Only after simultaneous injection of TNP-Ficoll and *Brucella abortus*, a particulate TI-1 antigen, relatively high frequencies of TNP-ASC were detected. To investigate the cause of the differential ability of the spleen and peripheral lymph nodes to mount an antibody response against TI-2 antigen, we compared the micro-environment of the (high responding) spleen and (low responding) lymph node after immunization with TNP-Ficoll. It was demonstrated that injected TNP-Ficoll localized in the follicles and macrophages of the lymph nodes, as was described for the spleen. Analysis of T cell activation and cytokine-production revealed relatively high frequencies of activated T cells ($gp39^+$ cells), IL-2-, IL-4-, IL-5- and IFN-γ-PC in spleens of mice immunized with TNP-Ficoll. Cytokine-PC were found in the same compartment as where TNP-AFC were localized, namely the outer-PALS and around the terminal arterioles, suggesting a regulatory role of these cytokines in TI-2 antibody responses. In contrast, hardly any expression of gp39 and cytokines was observed in lymph nodes after immunization with TI-2 antigens. These results suggest that the absence of activated cytokine-producing T cells is responsible for the lack of TNP-ASC cells in lymph nodes after TNP-Ficoll immunization. Consistent with this suggestion was the observation that the simultaneous injection of *Brucella abortus* and TNP-Ficoll resulted in the development of high frequencies of $gp39^+$ cells, cytokine-PC and TNP-ASC in draining lymph nodes. However, treatment of TNP-Ficoll immunized mice with alginate encapsulated IL-4

and/or IL-5-producing cell lines did not result in increased frequencies of TNP-ASC in draining lymph nodes. These data indicate that combinations of cytokines or other cytokines than IL-4 and IL-5 are required for the *in vivo* maturation of TI-2 antigen activated lymph node B cells into TNP-specific antibody-secreting cells. In conclusion, the *in vivo* data presented in chapter 6 substantiate previous reports which suggested that activated T cells and cytokines are actively participating in TI-2 antibody responses.

Chapter 7 describes the detection and activity of gp39$^+$ cells and cytokine-PC in the antibody response against a TD antigen (KLH) and a TI-2 antigen (TNP-Ficoll). Gp39$^+$ cells were detected using a specific mAb, or a fusion protein of the receptor of gp39, CD40-IgG1. Immunization with TNP-Ficoll and KLH resulted both in the upregulation of the frequency of gp39$^+$ cells. It was demonstrated *in vivo* that gp39 is only expressed by CD4$^+$ cells, as was suggested by *in vitro* experiments. Gp39$^+$ cells were predominantly localized in the outer-PALS and around the terminal arterioles of the spleen, but only incidently in the corona of the follicles. The cytokine-PC were only observed in the outer-PALS and around the terminal arterioles of the spleen. Gp39$^+$ cells produced IL-2, IL-4, and IFN-γ, and the kinetics of the development of gp39$^+$ cells and cytokine-PC were superimposable. In the antibody response against TI-2 and TD antigens, the gp39$^+$ cells and cytokine-PC were demonstrated in close conjunction to antigen-specific B cells, suggesting a role of these cells in B cell activation and differentiation. Indeed, treatment of TNP-KLH immunized mice with anti-gp39 antibodies completely inhibited the antigen-specific antibody response, indicating that gp39-CD40 interactions are essential for TD immune responses. As expected, the antibody response against TNP-Ficoll was unaffected after *in vivo* treatment of mice with anti-gp39 antibodies, confirming the T cell (gp39) independency of this antibody response. The high frequencies of IL-4-PC, relative to IFN-γ-PC, observed after injection of TNP-KLH were consistent with the relatively high amount of antigen-specific IgG1 and IgE demonstrable in sera of mice immunized with TD antigens. In conclusion, it is shown that during primary and secondary antibody responses against TD and TI-2 antigens T-B cell interactions and cytokine-production occur in the outer-PALS and around the terminal arterioles of the spleen.

Chapter 8 describes immunological events such as antigen-localization, gp39 expression, cytokine-production and antibody formation in spleens of mice after injection of rabbit antibodies directed to mouse-IgD (RαIgD). Immunization with RαIgD results in high frequencies of activated T cells and cytokine-PC, which are critical for the huge polyclonal

IgG1 and IgE production observed beyond day 7 after primary immunization. Therefore, this is a suitable model to investigate the localization and function of (activated) immune-cells and cytokines in *in vivo* TD antibody responses. Shortly after i.v. injection, RαIgD localized in all the follicles of the spleen as RαIgD-mouse IgD immune complexes. A relatively high frequency of $gp39^+$ cells was detectable 36 h after injection and reached maximum frequencies 3 and 4 days after RαIgD injection. The maximum frequencies of IL-2-PC and IL-4-PC observed at day 3 and 4 were about 5 times higher than the maximum frequencies of IFN-γ-PC, and were followed by a striking raise in the serum levels of IgG1 and IgE. Analysis of the *in vivo* cytokine profile revealed low frequencies of cells producing IL-2 and IFN-γ, or IL-4 and IFN-γ, or IL-2 and IL-4 consistent with the Th2 character of this response. $Gp39^+$ cells were predominantly found in the PALS, around terminal arterioles and incidently in the follicles. The high frequencies of activated T cells, induced after RαIgD injection, may explain the observation that in this polyclonal response $gp39^+$ cells were not only localized in the outer-PALS, but also in the inner-PALS. Analysis of the antibody response revealed a striking increase in serum IgG1 and IgE after RαIgD injection. It was demonstrated that a part of the polyclonal antibody response was directed to the injected RαIgD. The *in situ* antigen-specific-antibody response reached maximum levels at 7 days after immunization. Earlier during the immune response, at days 2-5 after RαIgD injection, $gp39^+$ cells and cytokine-PC were observed in close conjunction to $cIgM^+$ B cells, suggesting that activated T cells have a role in B cell activation and differentiation. Indeed, treatment of RαIgD immunized mice with gp39-specific antibodies resulted in an almost complete inhibition of the polyclonal antibody response. These data suggest that the *in situ* visualized T-B cell conjugates represent functional gp39-CD40 interactions, which are essential for the antibody response after RαIgD injection. This study substantiates the hypothesis presented in chapter 7, by showing that, even in a strong polyclonal antibody response, T-B cell interactions occur in the PALS and around the terminal arterioles of the spleen.

In *chapter 9* the main points emerging from the experimental studies are discussed. Results are related to *in vitro* and *in vivo* data dealing with antigen-localization/presentation, immune cell localization/migration, cell-cell interactions and the immunoregulatory role of cytokines. In spite of the fact that antigen-presentation, T cell and B cell activation are completely different for TI-2 and TD antibody responses, no differences were found between these two types of antibody responses with regard to the splenic compartmentalization of T-B cell interactions and cytokine production. These observations suggest a common differentiation

pathway for B cells into clones of AFC in the spleen. Finally, models are presented which show the cell-cell interactions in the spleen during TI-2 and TD antibody responses.

GENERAL CONCLUSIONS

TI-2 antigens also localize in the follicles of lymphoid tissues which may have an important role in the induction of TI-2 antibody responses.

T cells are activated and produce cytokines during *in vivo* TI-2 antibody responses.

Thymus independent antibody responses are dependent on the cytokines produced by T cells but not on the gp39 molecule expressed by activated T cells.

In vivo gp39-CD40 interactions are essential for TD humoral immunity.

T-B cell interactions and cytokine-production occur in the periarteriolar sheaths and around the terminal arterioles of the spleen during TI-2 and TD antibody responses.

T-B cell interactions leading to isotype switching of B cells do not seem to occur in the follicles of the spleen.

SAMENVATTING

De mens komt gedurende zijn leven in contact met een grote verscheidenheid aan infectieuze micro-organismen, zoals bacteriën, schimmels, virussen en parasieten. Het feit dat deze micro-organismen niet de kans krijgen ons lichaam te infecteren, is voornamelijk te danken aan het immuunsysteem.

Het immuusysteem heeft een specifiek en een niet-specifiek gedeelte. Het laatste, ook wel genoemd het aangeboren immuunsysteem, is de primaire defensie tegen micro-organismen en bestaat uit de huid, trilhaarepitheel, mucus, maagzuur, cel-dodende enzymen in lichaamsvloeistoffen, acute fase eiwitten, fagocyten etc.. De tweede lijn van defensie wordt het specifieke, ook wel verworven immuunsysteem genoemd, dat bestaat uit een cellulair en een humoraal deel. In de cellulaire immuniteit spelen cytotoxische en helper T cellen een centrale rol. Cytotoxische T cellen doden o.a. virus geïnfecteerde en tumor cellen, terwijl T helper cellen de functie van andere immuuncellen reguleren door de expressie en secretie van eiwitten (cytokinen).

In de humorale immuniteit spelen de antilichaam-vormende B cellen een centrale rol. Antilichamen zijn eiwitten die worden gesecreteerd in lichaamsvloeistoffen en die met hoge specificiteit aan een klein onderdeel van een ziekteverwekker (antigeen) binden en deze daardoor onschadelijk kunnen maken. Hierbij spelen neutralisatie, complement activatie, opsonisatie en antilichaam gemediëerde cytotoxiciteit een belangrijke rol. Er worden vijf klassen immunoglobulinen onderscheiden op basis van hun grootte, lading, aminozuur compositie en glycosylering: IgA, IgD, IgE, IgG en IgM. In de muis bestaan er nog 4 subklassen (isotypen) van IgG, namelijk IgG1, IgG2a, IgG2b en IgG3. De diversiteit in de structuur van deze subklassen bepaalt de functie van deze effector moleculen in de immuniteit.

B cellen rijpen in het beenmerg waar ze uitgerust worden met antigeen-specifieke receptoren, membraan immunoglobuline, en migreren vervolgens naar de perifere lymfoïde organen. Daar kunnen zij differentiëren tot antilichaamvormende cellen, mits zij het juiste antigeen tegen komen in het geschikte micromilieu. De interactie tussen antigeen en membraan immunoglobuline op de B cel is de eerste interactie in een antilichaamrespons. De meeste antigenen kunnen de B cel niet direct activeren en hebben de hulp nodig van dendritische cellen, macrofagen en T cellen voor de inductie van een antilichaamrespons. Macrofagen en dendritische cellen zijn potente antigeen-presenterende cellen en hebben een rol in de initiële activatie en expansie van virginale T cellen. Na activatie brengen T

cellen nieuwe membraan-antigenen (o.a. gp39) tot expressie en gaan cytokinen produceren, hetgeen hen in staat stelt de B cel activatie en differentiatie te reguleren. De interactie tussen het gp39-molekuul op geactiveerde T cellen en het CD40-molecuul op B cellen is essentieel voor de initiële activatie van B cellen gedurende thymus afhankelijke (TD) immuunresponsen. Dit in tegenstelling tot T cel onafhankelijke antigenen (TI) die in staat zijn B cellen direct te activeren. De door diverse cellen, waaronder T cellen, geproduceerde cytokinen reguleren vervolgens de verdere differentiatie van geactiveerde B cellen tot antilichaam-vormende B cellen. Cytokinen zijn kleine geglycosyleerde eiwitten, geproduceerd door zowel hematopoïetische alsmede non-hematopoïetische cellen. Oorspronkelijk werden ze ook wel lymfokinen, monokinen of interleukinen genoemd, doelend op hun bron of rol in de communicatie tussen leucocyten. Het bleek echter al snel dat deze polypeptiden door meer dan één cel kunnen worden geproduceerd en dat ze effect hebben op verschillende cellen. Daarom wordt de term cytokinen nu algemeen gebruikt binnen het onderzoek. Cytokine-producerende cellen zijn parakriene cellen, dat wil zeggen dat hun regulatoire functie gericht is op de doel-cellen welke in de buurt zijn gelegen. Er komen steeds meer aanwijzingen dat met name interleukine 2 (IL-2), IL-4, IL-5, IL-6 en interferon-γ (IFN-γ) betrokken zijn by de B cel proliferatie en differentiatie.

Om de interacties tussen immuuncellen te vergemakkelijken, zijn deze cellen georganiseerd in een aantal weefsels en organen, het lymfoïde systeem. De primaire lymfoïde organen zijn verantwoordelijk voor de vorming van de lymfoïde cellen. De thymus verzorgt de rijping van T cellen, terwijl in de foetale lever en het beenmerg de B cellen uitrijpen. De secundaire lymfoïde organen, zoals de lymfklieren, milt, en het darm-, huid- en longgeassocieerd lymfoïd weefsel, vormen een micromilieu voor de immuuncellen dat hen in staat stelt tot een immuunrespons tegen antigenen. De lymfklier en milt kunnen worden gezien als de immunologische filters van respectievelijk de lymfe en het bloed. Dat wil zeggen dat antigenen die in het bloed voorkomen een immuunrespons opwekken in de milt, terwijl de antigenen die voorkomen in de lymfe dat primair doen in de drainerende lymfklieren. Het lokale micromilieu van de lymfoïde organen speelt een belangrijke rol in de totstandkoming van een immuunrespons. Dit micromilieu bestaat uit de extracellulaire matrix, niet-lymfoïde cellen en lymfoïde cellen. De niet-lymfoïde cellen bestaan uit twee groepen. De eerste zijn de interdigiterende dendritische cellen en macrofagen en de tweede groep zijn de stromale cellen, die bestaan uit reticulaire cellen, folliculaire dendritische cellen en endotheelcellen. De lymfoïde cellen produceren cytokinen en antilichamen die op een antigeen-specifieke wijze bijdragen tot het micromilieu. Al deze lokaal actieve

cellen en factoren hebben een sturende invloed op de vorming van een antilichaamrespons. Daarom is voor een goed begrip van de mechanismen die leiden tot een antilichaamrespons inzicht vereist in het micromilieu van lymfoïde organen.
Het doel van de studies beschreven in dit proefschrift was om *in vivo* te onderzoeken waar en wanneer de verschillende immunologische gebeurtenissen plaatsvinden in lymfoïde organen gedurende een aantal verschillende typen antilichaamresponsen. Daarbij is speciaal gekeken naar de timing en lokalisatie van antigeenpresentatie, T cel activatie, cytokine-produktie en antilichaamvorming in de milt, omdat deze processen een beslissende rol spelen in de ontwikkeling van een antilichaamrespons. Hiervoor zijn vriescoupes van lymfoïd weefsel van muizen onderzocht op verschillende tijdstippen na immunisatie met antigenen. De resultaten verkregen na specifieke immunohistochemie worden als representatief beschouwd voor de lokale *in vivo* situatie in het onderzochte lymfoïde orgaan op dat tijdstip gedurende een antilichaamrespons. Daarnaast zijn *in vivo* modulatie experimenten gedaan om de rol van antigeen, cytokinen en immuuncellen in antilichaamresponsen te bepalen.

In *hoofdstuk 2* wordt de complexe anatomische organisatie van de milt besproken, welke bestaat uit duidelijk gescheiden compartimenten die gekarakteriseerd worden door de lokalisatie van unieke en gespecialiseerde celtypen. De migratiepatronen van B cellen, T cellen en dendritische cellen worden beschreven. De antigenen die gebruikt worden in immunologisch onderzoek worden gekarakteriseerd en hun lokalisatie in de milt na injectie wordt beschreven. Verder worden *in vitro* experimenten besproken betreffende antigeenpresentatie, cel-cel interacties, T en B cel activatie en de immuno-regulatoire rol van cytokinen. Als laatste worden de experimentele vragen geformuleerd die in dit proefschrift onderzocht worden.

In *hoofdstuk 3* wordt de lokalisatie van geïnjecteerde thymus onafhankelijke type 2 (TI-2) antigenen in de milt en lymfklieren beschreven. Deze lokalisatie wordt vergeleken met die van particulate (deeltjes) TI-1, oplosbare TI-1 en thymus afhankelijke (TD) antigenen. Het blijkt dat TI antigenen en particulate antigenen beide in de marginale zone lokaliseren. De snelle opname van deze antigenen door de marginale zone macrofagen benadrukt nog eens hun belangrijke rol in de primaire bescherming tegen infecties in de circulatie. Daarnaast wordt een geheel nieuw fenomeen beschreven, namelijk dat TI-2 antigenen ook in de follikels van lymfoïde organen lokaliseren. Het blijkt dat deze vroege folliculaire

lokalisatie van TI-2 antigenen complement gemedïeerd is. *In vivo* depletie van de marginale zone macrofagen resulteerde in een opmerkelijke toename van folliculair gelokaliseerd TI-2 antigeen, terwijl de antilichaamrespons tegen lage dosis antigeen ook bleek toe te nemen. Complement depletie resulteerde juist in een verminderde folliculaire lokalisatie van TI-2 antigenen, en gaf ook een verlaagde antilichaamrespons te zien. Dit tezamen suggereert dat de folliculaire lokalisatie van TI-2 antigenen een rol speelt in de inductie van TI-2 antilichaamresponsen. De mechanismen en de *in vivo* cel-cel interacties welke plaatsvinden in de milt gedurende TI-2 antilichaamresponsen worden besproken. Er wordt geconcludeerd dat marginale zone macrofagen niet primair bij antigeen-presentatie betrokken zijn, maar wel by de verwijdering (in het geval van TI-2 antigenen), zuivering van het bloed, doden van bacteriën, en "pre-processing" van particulate antigenen. Terwijl B cellen en folliculair dendritische cellen betrokken zijn bij de inductie van TI-2 antilichaamresponsen.

In *hoofdstuk 4* wordt een nieuwe methode beschreven voor de immunohistochemische detectie van cytokine-producerende cellen (cytokinen-PC) in lymfoïd weefsel van muizen. De *in situ* detectie van IFN-γ werd gebruikt als model voor de ontwikkeling van de techniek. Muizen werden geïmmuniseerd met *Bacille Calmette Guèrin* (BCG), aangezien dit protocol zou moeten resulteren in een hoge IFN-γ produktie in de milt. Om IFN-γ-PC te kunnen detecteren werd een IFN-γ-specifiek monoclonaal antilichaam gekoppeld aan alkalische fosfatase. Incubatie van een vriescoupe met dit conjugaat, gevolgd door het juiste substraat, stelden ons in staat de IFN-γ-PC, gekarakteriseerd door een blauw cytoplasma, te detecteren. Immunisatie met BCG resulteerde inderdaad in verhoogde frequenties van IFN-γ-PC in de milt. Echter de IFN-γ serum spiegels bleken niet verhoogd te zijn. Dat wijst erop dat IFN-γ alleen lokaal in de milt werd geproduceerd en gebonden. Alleen in geval van de toediening van Concanavaline A, een T cel mitogeen welke waarschijnlijk IFN-γ-PC activeert tot IFN-γ secretie, leidde tot een toename van de IFN-γ serumspiegels in met BCG geïmmuniseerde muizen. Incubatie van een vriescoupe met het IFN-γ-specifieke alkalische fosfatase conjugaat en een antilichaam dat specifiek is voor een membraan antigeen (CD4 én CD8), maakte de karakterisering van IFN-γ-PC mogelijk. Er wordt geconcludeerd dat immunohistochemische technieken geschikt zijn voor het detecteren en karakteriseren van cytokine-PC *in vivo*, mits de juiste reagentia beschikbaar zijn.

Hoofdstuk 5 beschrijft de ontwikkeling en lokalisatie van IFN-γ-PC gedurende antilichaamresponsen tegen verschillende TI-2 antigenen. De frequentie van IFN-γ-PC bleek aanmerkelijk te stijgen na immunisatie met TI-2 antigenen. ELISPOT assay bevestigde deze resultaten en suggereerde bovendien dat deze cellen ook IFN-γ secreteerden. Dubbelkleuringen lieten zien dat IFN-γ werd geproduceerd door $CD4^+$ and $CD8^+$ cellen, terwijl relatief weinig IFN-γ-PC tot de natural killer cellen behoorden. Deze resultaten bevestigen voorgaande studies die suggereerden dat cytokine-producerende T cellen betrokken zijn bij de regulatie van TI-2 antilichaamresponsen. De IFN-γ-PC waren voornamelijk gelokaliseerd in het buitenste deel van de periarteriolaire lymfocyten schede (outer-PALS) en rondom de terminale arteriolen. IFN-γ-PC werden niet gevonden in de marginale zone of follikels van de milt. Antigeen-specifieke antilichaam-vormende cellen bleken in hetzelfde compartiment te lokaliseren als waar IFN-γ-PC gevonden werden. Dubbelkleuringen lieten zien dat IFN-γ-PC dicht tegen de TNP-specifieke antilichaam-vormende cellen (TNP-AFC) lagen, hetgeen een regulatoire rol suggereert van IFN-γ-producerende T cellen in de antilichaamrespons tegen TI-2 antigenen.

In *hoofdstuk 6* wordt het vermeende defect van perifere lymfklieren, met betrekking tot het vermogen om een antilichaamrespons tegen TI-2 antigenen te ontwikkelen, onderzocht. Onverwacht gaf de immunohistochemische *in situ* analyse van de antilichaamrespons toch een relatief grote hoeveelheid TNP-AFC te zien in drainerende popliteale lymfklieren (PLN) van met TNP-Ficoll geïmmuniseerde muizen. De verwijdering van de milt had geen effect op de frequentie van TNP-AFC in PLN, hetgeen betekent dat deze TNP-AFC niet uit de milt komen. De ELISPOT assay kon echter geen TNP-specifieke antilichaam-secreterende cellen (TNP-ASC) aantonen in drainerende PLN, terwijl grote hoeveelheden TNP-ASC werden aangetoond in de milt. Deze resultaten suggereren dat TNP-Ficoll wel in staat is TNP-specifieke B cellen in PLN te activeren, maar dat het micromilieu in de PLN niet de juiste stimuli kan verschaffen voor de uiteindelijke differentiatie tot TNP-ASC. Co-immunisatie van TNP-Ficoll en *Brucella abortus* (BA), een particulaat TI-1 antigeen, resulteerde in relatief hoge frequenties van TNP-ASC in drainerende PLN. Om te onderzoeken waarop dit het verschil tussen milt en lymfklier berust, werd het micromilieu van deze lymfoïde organen vergeleken na immunisatie met TNP-Ficoll. De lokalisatie van het geïnjecteerde TNP-Ficoll in de macrofagen en follikels van de lymfklier was vergelijkbaar met wat in de milt gevonden werd. Echter, de relatieve frequenties van geactiveerde T cellen en cytokinen-PC (IL-2, IL-4, IL-5 en IFN-γ) in de milt bleken vele malen hoger te zijn dan in de PLN

na immunisatie met TNP-Ficoll. Cytokinen-PC werden gevonden in hetzelfde compartiment als waar TNP-AFC waren gelokaliseerd, namelijk in de outer-PALS en rondom de terminale arteriolen. Deze resultaten suggereren een regulatoire rol van deze cytokinen in TI-2 antilichaamresponsen en doen vermoeden dat de afwezigheid van cytokinen-PC in PLN na immunisatie met TNP-Ficoll verantwoordelijk is voor het maturatie defect van TNP-AFC in PLN. In overeenstemming met deze suggestie was de waarneming dat immunisatie met TNP-Ficoll + BA resulteerde in relatief hoge frequenties van geactiveerde T cellen, cytokinen-PC en ook TNP-ASC in drainerende PLN. Behandeling van TNP-Ficoll geïmmuniseerde muizen met in alginaat gekapselde IL-4 en/of IL-5 producerende cellijnen gaf echter geen toename van het aantal TNP-ASC in PLN te zien. Dit zou mogelijk kunnen betekenen dat combinaties van cytokinen of andere cytokinen dan IL-4 en IL-5 vereist zijn voor de *in vivo* maturatie van TNP-AFC in TNP-ASC. Concluderend kan men stellen dat geactiveerde T cellen en cytokine-producerende cellen een rol spelen in antilichaamresponsen tegen TI-2 antigenen.

In *hoofdstuk 7* wordt de detectie en de activiteit van $gp39^+$ cellen en cytokine-PC in de antilichaamrespons regen TD (KLH) en TI-2 (TNP-Ficoll) antigenen beschreven. $Gp39^+$ cellen werden aangetoond met een specifiek monoclonaal antilichaam, of met een fusie-eiwit van de receptor van gp39, het CD40-IgG1. Immunisatie met TNP-Ficoll of KLH resulteerde in een toename van de frequentie van $gp39^+$ cellen. In overeenstemming met *in vitro* waarnemingen lieten dubbelkleuringen zien dat gp39 alleen tot expressie komt op $CD4^+$ cellen. De $gp39^+$ cellen werden met name gevonden in de outer-PALS en rondom de terminale arteriolen van de milt, en slechts enkele $gp39^+$ cellen werden gevonden in de corona van de follikels. Opnieuw werden de cytokine-PC alleen in de outer-PALS en rondom de terminale arteriolen van de milt gevonden. Er werden in de milt $gp39^+$ cellen aangetoond die IL-2, IL-4 of IFN-γ produceerden. De kinetiek van de ontwikkeling van $gp39^+$ cellen en cytokine-producerende cellen verliep op een identieke wijze na immunisatie met TNP-Ficoll of TNP-KLH. In zowel de antilichaamresponsen tegen TI-2 antigenen alsmede tegen TD antigenen werden $gp39^+$ cellen aangetoond in dichte nabijheid van antigeen-specifieke B cellen. Deze resultaten suggereren een rol van deze cellen in de activatie en differentiatie van B cellen. De *in vivo* behandeling van met TNP-KLH geïmmuniseerde muizen met gp39 blokkerende antilichamen onderdrukte volledig de antigeen-specifieke antilichaamrespons, hetgeen betekent dat gp39-CD40 interacties essentieel zijn voor TD antilichaamresponsen. Zoals mocht worden verwacht, werd de antilichaamres-

pons tegen TNP-Ficoll niet beïnvloed door de behandeling met gp39-blokkerende antilichamen. Daarmee werd het T cel onafhankelijke karakter van de antilichaamrespons tegen TI-2 antigenen bevestigd. De hoge frequentie van IL-4-PC t.o.v. IFN-γ-PC, die werd gevonden na immunisatie met TNP-KLH, is in overeenstemming met de relatief grote hoeveelheid antigeen-specifiek IgG1 en IgE dat aangetoond kon worden in sera van muizen geïmmuniseerd met TD antigenen. Geconcludeerd kan worden dat de T-B cel interacties en cytokine-produktie gedurende zowel primaire als secundaire antilichaamresponsen tegen TD en TI-2 antigenen plaats vinden in de outer-PALS en rondom de terminale arteriolen van de milt.

In *hoofdstuk 8* worden de antigeen-lokalisatie, gp39-expressie, cytokine-produktie en antilichaam-vorming beschreven in de milt van muizen na injectie van konijne-antilichamen gericht tegen muis-IgD (RαIgD). Immunisatie met RαIgD resulteert in hoge frequenties van geactiveerde T cellen en cytokine-producerende cellen, die essentieel zijn voor de enorme polyclonale IgG1 and IgE produktie welke gezien wordt op dag 7 van de primaire immunisatie. Daarom is dit een zeer geschikt model om de lokalisatie en functie van (geactiveerde) immuuncellen en cytokinen gedurende *in vivo* antilichaamresponsen te bestuderen. Al kort na intraveneuze injectie kon het RαIgD worden aangetoond als RαIgD-muis-IgD immuuncomplexen in de follikels van de milt. Een relatief hoge frequentie van gp39$^+$ cellen werd aangetoond in de milt na 36 uur en bereikte maximale frequenties 3 en 4 dagen na immunisatie. Op deze tijdstippen werd ook de maximale frequentie van cytokine-PC in de milt aangetoond. De frequenties van IL-2-PC én IL-4-PC waren daarbij ongeveer 5 maal hoger als de frequentie van IFN-γ-PC, wat in overeenstemming is met de enorme toename van de concentraties van IgG1 en IgE in het serum van de met RαIgD geïmmuniseerde muizen. Analyse van het cytokine-profiel liet cellen zien die IL-2 én IL-4 (Th0), IL-2 én IFN-γ (Th1), IL-4 én IFN-γ (TH0) of alleen IL-4 (Th2) produceerden. Gp39$^+$ cellen werden met name gevonden in de PALS en rondom de terminale arteriolen van de milt. Slechts enkele gp39$^+$ cellen werden aangetoond in de follikels. Analyse van het serum liet een opmerkelijke toename van IgG1 en IgE zien na injectie van RαIgD. Zowel in het serum als in de milt werd aangetoond dat de polyclonale antilichaamrespons voor een deel specifiek was voor het geïnjecteerde antigeen. De *in situ* aangetoonde antigeen-specifieke AFC bereikten maximale frequenties op 7 dagen na immunisatie met RαIgD. Iets eerder in de antilichaamrespons, op dag 2-5 na injectie van RαIgD, werden relatief grote hoeveelheden gp39$^+$ cellen en cytokine-PC aangetoond dicht nabij cIgM$^+$ cellen,

suggererend dat geactiveerde T cellen de B cel activatie en differentiatie reguleren. Behandeling van muizen met blokkerende gp39-specifieke antilichamen resulteerde in een bijna volledige inhibitie van de polyclonale en antigeen-specifieke antilichaamrespons. Deze gegevens suggereren dat de *in situ* gevisualiseerde T-B cel conjugaten functionele gp39-CD40 interacties representeren die essentieel zijn voor de antilichaamrespons na RαIgD injectie. Bovendien bevestigt deze studie de voorgestelde hypothesen, door aan te tonen dat zelfs in een zeer sterke polyclonale antilichaamrespons, T-B cel interacties plaatsvinden in de PALS en rondom de terminale arteriolen van de milt.

In *hoofdstuk 9* worden de belangrijkste resultaten uit de voorgaande hoofdstukken besproken. Deze worden gerelateerd aan *in vitro/in vivo* experimenten betreffende antigeen-lokalisatie/presentatie, immuuncel lokalisatie/migratie, cel-cel interacties en de immunoregulatoire rol van cytokinen zoals die beschreven zijn in de literatuur. Ondanks het feit dat antigeen-presentatie, T cel en B cel activatie voor TI-2 en TD antilichaamresponsen volledig verschillen, werd geen verschillen gevonden tussen deze twee typen antilichaamresponsen wat betreft de compartimentalisatie van T-B cel interacties en cytokine-produktie in de milt. Deze waarneming suggereert dat er voor de differentiatie van B cellen één gemeenschappelijke weg gevolgd wordt in de milt gedurende zowel TI-2 als TD antilichaamresponsen. Tenslotte worden modellen gepresenteerd die de cel-cel interacties in de milt laten zien gedurende de ontwikkeling van TI-2 en TD antilichaamresponsen.

ALGEMENE CONCLUSIES

TI-2 antigenen lokaliseren ook in de follikels van lymfoid weefsel hetgeen mogelijk een rol speelt in de inductie van TI-2 antilichaamresponsen.

T cellen worden geactiveerd en produceren cytokinen *in vivo* na immunisatie met TI-2 antigenen.

Thymus-onafhankelijke antilichaamresponsen zijn afhankelijk van de cytokinen geproduceerd door T cellen, maar niet van het gp39-molekuul op geactiveerde T cellen.

In vivo gp39-CD40 interacties zijn essentieel voor thymus-afhankelijke humorale immuniteit.

T-B cel interacties en cytokine-produktie vinden plaats in de periarteriolaire lymfocyten schede en rondom de terminale arteriolen van de milt gedurende TI-2 en TD antilichaamresponsen.

T-B cel interacties die leiden tot isotype switching van B cellen lijken niet plaats te vinden in de follikels van de milt.

ABBREVIATIONS

AP	alkaline phosphatase
AFC	antibody-forming cell
APC	antigen-presenting cells
β-Gal	β-galactosidase
BCG	bacille Calmette Guèrin
BA	Brucella abortus
BSA	bovine serum albumin
BGG	bovine γ-globulin
clg	cytoplasmic Immunoglobulin
ConA	concanavalin A
CTL	cytolytic T cell
DC	dendritic cell
DNP	2,4 dinitrophenyl
DTH	delayed type hypersensitivity
FCS	fetal calf serum
FDC	follicular dendritic cells
FITC	fluorescein isothiocyanate
ELISA	enzyme-linked immuno sorbent assay
GαIgD	goat antibodies anti-IgD
H	hour
HRP	horseradish peroxidase
HSA	human serum albumin
Ia	major histocompatibility complex class II
IDC	interdigitating cells
Ig	immunoglobulin
IL	interleukin
IFN	interferon
i.p.	intraperitoneal
i.v.	intravenous
KLH	keyhole limpet hemocyanin
LCMV	lymphocytic choriomeningitis virus
LPS	lipopolysaccharide

MHC	major histocompatibility complex
Min	minutes
mIg	membrane immunoglobulin
NK	natural killer cell
NIP	4-hydroxy-5-iodo-3-nitrophenyl
OVA	ovalbumin
PC	producing cell
PALS	periarteriolar lymphocyte sheath
RαIgD	rabbit antibodies anti-IgD
SRBC	sheep red blood cell
TA	terminal arterioles
Th	T helper
TD	thymus dependent
TI-1(2)	thymus independent type 1(2)
TNP	2,4,6-trinitrobenzenesulfonic acid
TNP-HES	TNP-hydroxyethyl starch

NAWOORD

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CURRICULUM VITAE

Alfonsus Johannes Maria van den Eertwegh werd op 15 januari 1963 geboren te Nijmegen. In 1981 werd het VWO-diploma behaald op de Werkplaats Kindergemeenschap te Bilthoven. Datzelfde jaar startte hij met de studie geneeskunde aan de Vrije Universiteit te Amsterdam. Hij deed een wetenschappelijke stage bij medische informatica onder leiding van dr. J.L. Talmon met als onderwerp: "3de orde polynoomfitting; een methode ter detectie van late-potentials". Gedurende zijn studietijd was hij actief als assistent-docent bij de vakgroep fysiologie aan de medische faculteit. In 1988 liep hij klinische stages haematologie/oncologie in het Worcester Memorial Hospital en infectieziekten aan de University of Massachusetts Medical School te Worcester, Massachusetts, U.S.A. In 1988 werd het Arts-diploma behaald. Hierna was hij gedurende 9 maanden werkzaam op de afdeling bloedcelchemie (Hoofd: prof. dr. D. Roos) van het Centraal Laboratorium van de Bloedtransfusiedienst te Amsterdam aan het project "aggregatie-onderzoek van bewaarde thrombocyten". In de periode 1989-1993 werd op de afdeling Immunologie en Medische Microbiologie van het Medisch Biologisch Laboratorium (TNO-MBL) onder leiding van dr. E. Claassen het onderzoek verricht dat geleid heeft tot dit proefschrift. Gedurende deze tijd heeft hij de opleiding tot Immunoloog gevolgd op de afdeling Immunologie (Hoofd: prof. dr. R. Benner) van de Erasmus Universiteit te Rotterdam. Vanaf 1 juli 1993 is hij internist in opleiding in de regio Rotterdam.

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