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

By 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*

*From the *Department of Immunology and Medical Microbiology, TNO Medical Biological Laboratory, 2280 HV, Rijswijk, The Netherlands; the ‡Department of Microbiology, Dartmouth Medical School, Lebanon, New Hampshire 03757; and §Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle, Washington 98121*

Summary

T-B cell interactions have a central role in the development of antibody responses. Upon activation, T helper (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 keyhole limpet hemocyanin (KLH), a thymus-dependent (TD) antigen, and trinitrophenyl (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). Incidentally, gp39$^+$ Th cells were found in the corona of follicles, whereas gp39$^+$ cells were never found in the germinal centers or marginal zones of the spleen. Maximum frequencies of gp39$^+$ cells were observed 3 and 4 d 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 interleukin 2 (IL-2)–, IL-4– and interferon γ (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 TA of the spleen.

The initiation of thymus-dependent (TD)[1] 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 that 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

[1] *Abbreviations used in this paper:* AEC, ethylcarbazole; AFC, antibody-forming cell; AP, alkaline phosphatase; GC, germinal center; HRP, horseradish peroxidase; PALS, outer periarteriolar lymphocyte sheath; PC, producing cell; TA, terminal arteriole; TD, thymus dependent; TI-2, thymus independent.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/11/1555/11 $2.00
Volume 178 November 1993 1555–1565

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 (8–11) 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. 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 (14–16). It has been shown in vitro that Th1 cells (17), 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 (14–16). 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 by Foy et al. (18) demonstrates that gp39 is essential for the induction of in vivo antibody responses against various TD antigens. 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 × CBA) mice were bred at the TNO breeding facilities (Rijswijk, The Netherlands). Animals were used at 16–24 wk of age and were kept under a standard protocol with free access to pelleted food and acidified water (pH 3). Experiments were performed under the auspices of the Dutch Veterinary Inspection, as described in the law on animal experiments.

Chemicals. Alkaline (AP; P-6774, type VII-T, 1,020 U/mg protein), 3-amino-9-ethylcarbazole (AEC; A5754), CFA, 3,3-diaminobenzidine-tetrahydrochloride, Fast blue BB Base, Fast red, horseradish peroxidase (HRP), IFA levamisole, and naphthol AS-MX phosphate (3-hydroxy-2-naphtoic acid 2,4-dimethyl-anilide were obtained from Sigma Chemical Co. (St. Louis, MO) *N*-hydroxysuccinimidyl-(biotinamido)-hexanoate and maleimidohexanoyl-*n*-hydroxysuccinimide ester were obtained from Pierce (Rockford, IL). β-Gal (*Escherichia coli* derived β-D-Gal galactohydrolase, 540,000), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) were obtained from Boehringer Mannheim (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 Immunological 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, National Institutes of Health, Bethesda, MD [26]). The IL-2–specific mAb, S4B6, was a kind gift of Dr. T. Mosmann (17). Control rat mAbs (2A4, 1G11) were a kind gift of Dr. A. Zantema (Sylvius Laboratory, Leiden, The Netherlands [27]). Recombinant mouse IL-2 was obtained from Genzyme Corp. (Cambridge, MA). 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 were prepared according to the previously described methods (19, 20).

Experimental Design. BCBA.F_1 mice were injected intravenously 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 d. Another group of mice was injected with 100 μg of KLH, boosted 16 wk later with 100 μg TNP-KLH, and killed after 0, 1, 2, 3, 4, 5, and 7 d. In a parallel experiment, mice were immunized subcutaneously with TNP-KLH in CFA, boosted 4 w later subcutaneously with 20 μg TNP-KLH in IFA, and killed 6 d after injection. Spleens and draining popliteal lymph nodes were removed, 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 room temperature. Slides were air-dried and stored in airtight boxes until use. Slides were fixed for 10 min 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 for 5 min) and immunohistochemical revelation was performed as described previously; AP (29), HRP (29), and β-Gal (30). For double staining, the immunohistochemical revelation of AP was performed before HRP, and the β-gal staining before 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-producing cells (PC) were counted, and image analysis was performed as previously described (32). A minimum of three sections of each mouse were examined.

Results

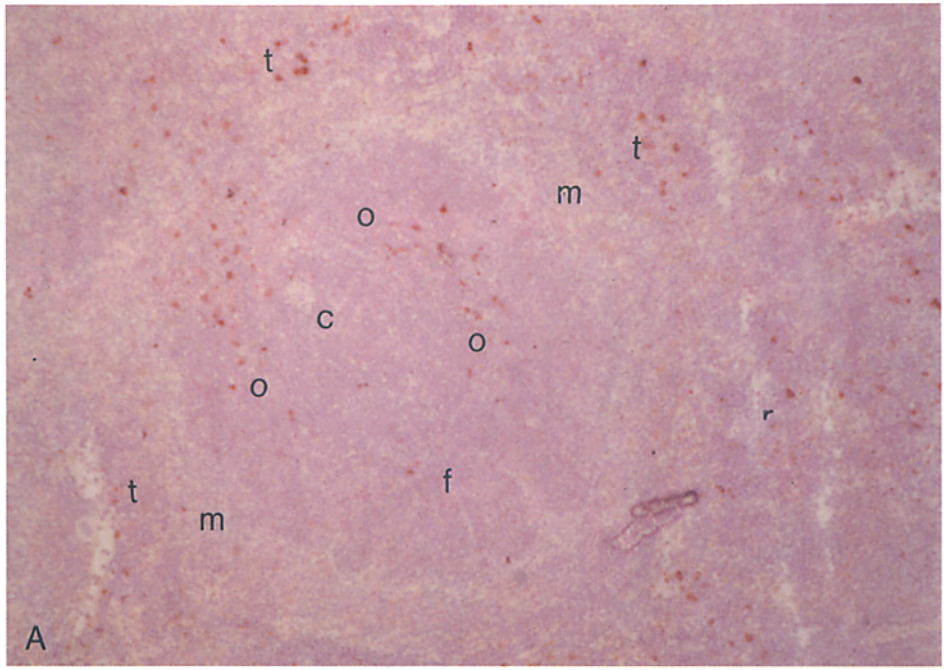
Gp39 Expression in Immune Spleen. Spleen sections from mice immunized with KLH were stained for the expression of gp39. Two gp39-specific reagents were used for detection: first, MR1, a gp39-specific mAb, and second, CD40-IgG1, a soluble, recombinant fusion protein of the gp39 receptor and IgG1. In serial spleen sections, CD40-IgG1 and anti-gp39 identified the same number of cells, which were localized in identical anatomical locations (Fig. 1, *A* and *B*; Table 1). 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. 1 *C*).

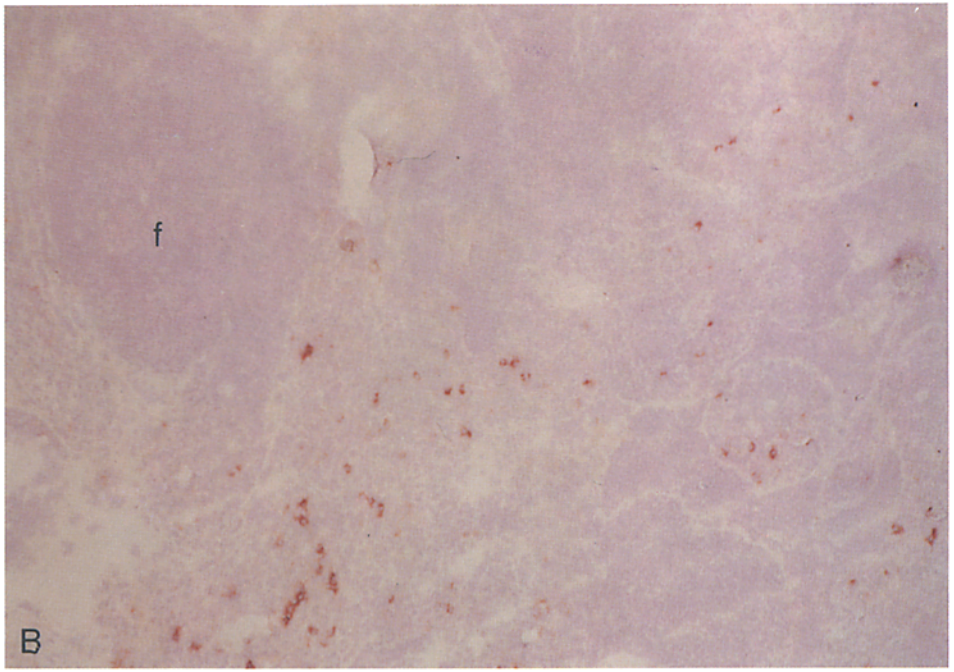
Localization and Kinetics of gp39$^+$ Cells in Lymphoid Tissue. Gp39$^+$ Th cells were found predominantly in the outer-periarteriolar lymphocyte sheaths (PALS) and around the terminal arterioles (TA) of the spleen (Table 1, Figs. 1*A* and 2*B*). Double staining for CD4$^+$ and gp39 clearly revealed that CD4$^+$ cells in primary follicles were not gp39 positive (Fig. 1 *C*). After secondary immunization, only a few gp39$^+$ cells were observed in the follicular corona, but not in the germinal centers (GC) of secondary follicles (Fig. 1 *A*). 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. 1 *B*). Incidentally, 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. 3). Thereafter, the number decreased and remained stable during the next 3 d. Another group of mice was boosted 16 wk later with KLH. Already during the first 2 d after secondary immunization we observed a striking increase in the number of gp39$^+$ cells, which was markedly higher as compared to the primary response. After 4 d, the gp39$^+$ cells reached maximum numbers, which were about two times higher as we observed during the primary immune response against KLH (Fig. 3). Immunization with TNP-Ficoll, a thymus-independent type 2 (TI-2) antigen, resulted in an increase in the frequency of gp39$^+$ Th cells, attaining maximum frequencies 5 d after injection (Fig. 4). 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. 2 *B*). No gp39 expression was observed in the splenic follicles of mice immunized with TNP-Ficoll.

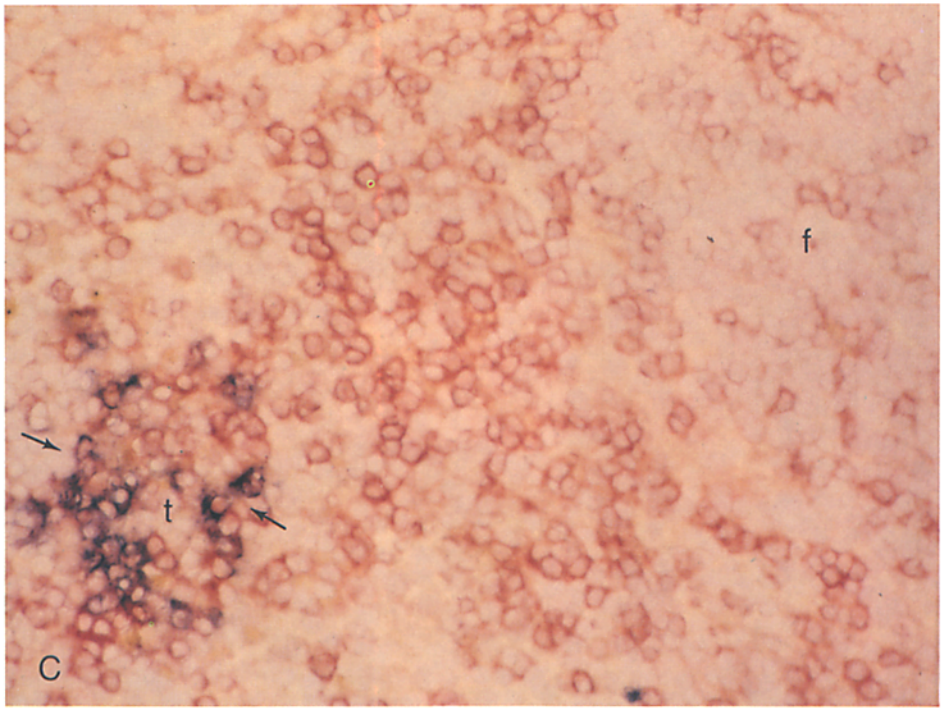
Localization and Kinetics of Cytokine-producing Cells in the Spleen. Th cell-derived cytokines have a decisive role in isotype selection (14–16). As antibody responses against TNP-KLH are dominated by IgG1 antibodies, we investigated whether a Th subset (Th1/Th2) was preferentially activated and where 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. 1 *D*). 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 preparations 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 immunoconjugates 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 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 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 d after immunization (Fig. 5).

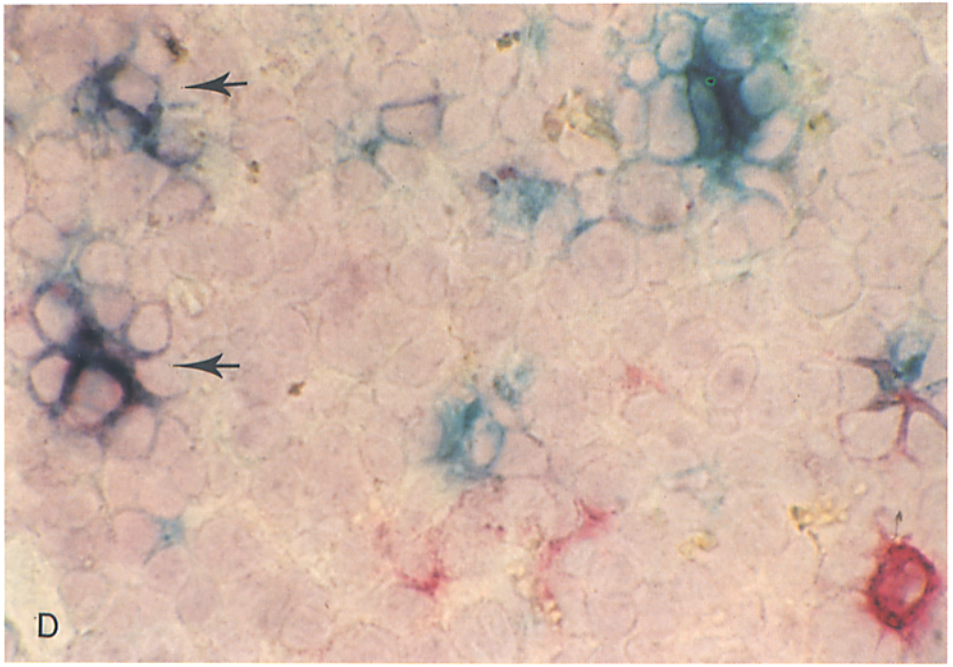
Cytokine Production by gp39$^+$ Cells. Gp39$^+$ cells are a prerequisite for B cell activation in vitro (5) and TD antibody responses in vivo (18). Thereafter, cytokine-producing Th cells are thought to be active as regulators of antibody responses. To investigate whether gp39$^+$ cells have the capacity 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-Gal, respectively. Double staining for IFN-γ and gp39 was performed with a direct IFN-γ–specific conjugate and MR-1 followed by a secondary 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 the case of IL-4 and gp39, the turquoise-stained cells were IL-4-PC and the red-stained cells were gp39$^+$, whereas double-staining violet cells were gp39$^+$ cells producing IL-4 (Fig. 1 *D*). 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 shown). In the spleen, gp39$^+$ cells producing IL-2, IL-4, or IFN-γ were found both during TD and as TI-2 antibody responses.

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 Figs. 3 and 4, in both experiments the kinetics of


t
t
m
o
c
o
o
r
t
m
f
A


f
B


f
t
C


D

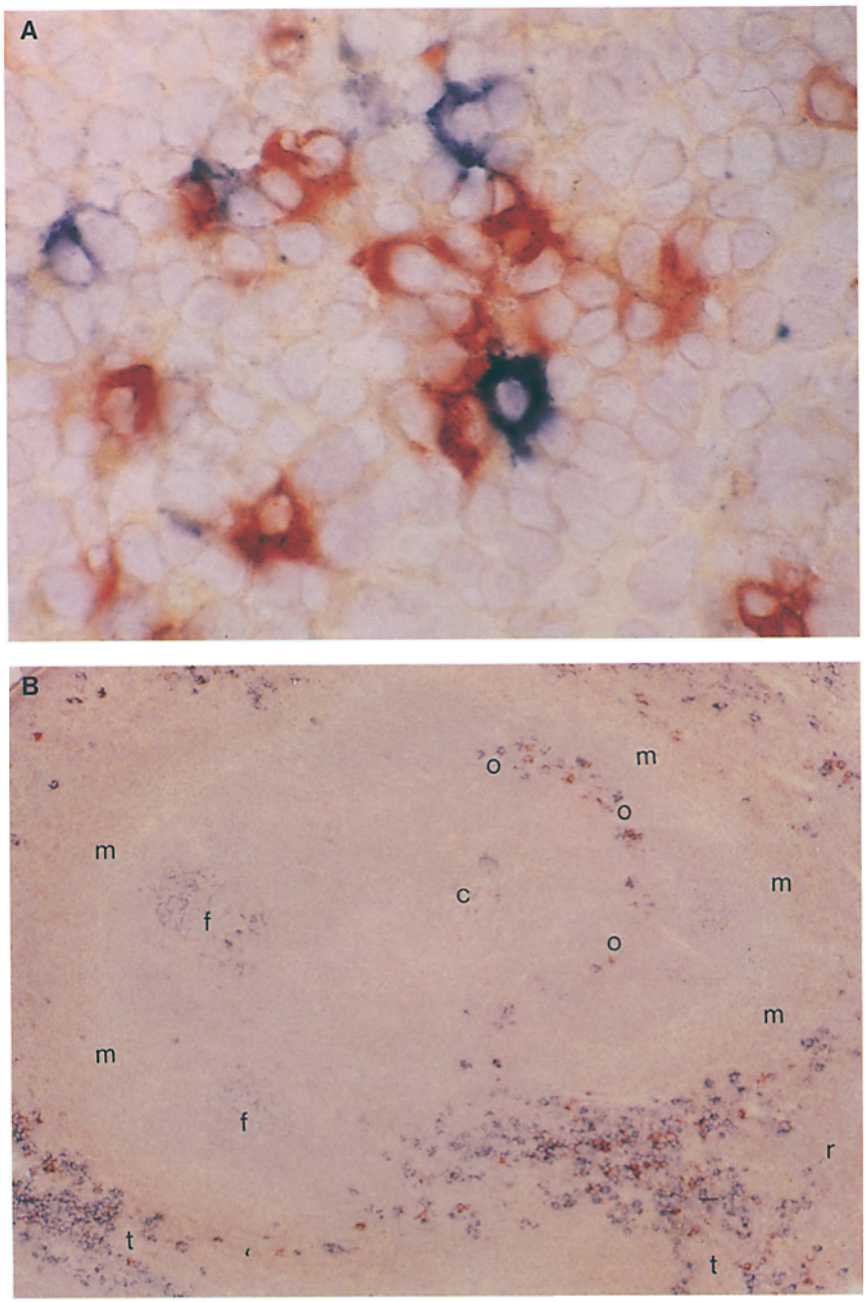


Figure 2. Gp39$^+$ cells and antigen-specific B cells are colocalizing in the immune spleen. (*A*) 4 d after secondary KLH immunization, KLH-AFC, stained red, were found in close proximity to gp39$^+$ cells, stained blue (KLH-HRP, AEC; MR1-AP, Fast blue). (*B*) 5 d after TNP-Ficoll immunization, Gp39$^+$ 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.

Figure 1. Immunohistochemical localization and characterization of gp39$^+$ cells in lymphoid organs. Cryostat sections of murine lymphoid tissue were incubated with specific immunoconjugates, followed by immunohistochemical revelation. Conjugates and substrates used are indicated between parentheses. (*A*) 4 d after secondary intravenous immunization with KLH; red stained cells are gp39$^+$ cells localized in outer-PALS (*o*), around the TA (*t*), and in the follicle (*f*) of the spleen (MR1 + RG7-HRP; AEC). (*B*) Cryostat section of lymph node 6 d after secondary subcutaneous immunization with TNP-KLH; red stained cells are gp39$^+$ cells localized in the deep cortex and alon the medullary cords. In this section, no gp39$^+$ cells are localized in the follicle (*f*) (MR1 + RG7-HRP; AEC). (*C*) 3 d after KLH immunization; red membrane positive cells are CD4$^+$ cells, whereas violet double-staining cells are CD4$^+$ cells expressing gp39 (*arrow*). Note that CD4$^+$ cells are present in the follicle (*f*), whereas CD4$^+$ cells expressing gp39 are found around TA (*t*) and not in the primary follicle (*f*) (L3T4-HRP, AEC; MR1-AP, Fast blue). (*D*) 4 d after KLH immunization, red stained cells are gp39$^+$ cells, turquoise stained cells are IL-4–PC, whereas violet stained cells are gp39$^+$ cells producing IL-4 (*arrows*). (MR1-AP, Fast red; 11B11-β-Gal, X-Gal).

Table 1. *Localization of gp39⁺ Cells in Different Compartments of the Spleen after Immunization with Thymus-(In)dependent Antigens*

Immunization	Day	Reagent	White pulp						Red pulp
			Fc	GC	iP	oP	MZ	TA	
Saline	4	MR-1*	0‡	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

Mice were immunized and killed on the indicated day.

* Cryostat sections were prepared from spleens and immunohistochemical demonstration of gp39 was performed with an anti-gp39 mAb, or with CD40-Ig followed by secondary peroxidase conjugates. Cells were counted in each anatomical compartment of the spleen: *Fc*, follicular corona; *iP*, inner-PALS; *oP*, outer-PALS; MZ, marginal zone.

‡ Values represent mean ± SD number of positive cells in each compartment of spleen sections from three mice.

KLH-AFC or TNP-AFC developed according to similar patterns attaining maximum frequencies at 4 d 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 ~8–10 times more KLH-AFC as compared with the primary immunization at the peak of the response (Fig. 3). 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. 4).

Gp39⁺ Cells Colocalize 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 codistributed among both B cell types in the outer-PALS and TA (data not shown). In addition,

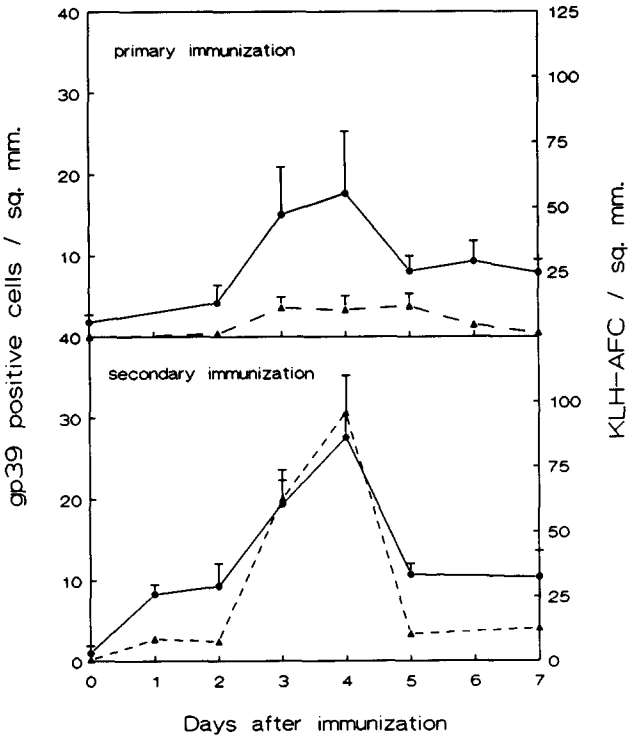


Figure 3. Kinetics of gp39⁺ T cells and KLH-AFC after primary and secondary immunization with KLH. BCBA.F_1 mice were injected intravenously with 100 μg KLH (19) and killed at the indicated time points. Another group of BCBA.F_1 mice was injected with 100 μg of KLH, boosted 16 wk later with 100 μg KLH i.v. and killed at the indicated time points. Spleens were removed and immunohistochemistry and image analysis were performed as described in Materials and Methods. Values represent mean ± SD of number of positive cells per mm² from three mice. (▲) KLH-AFC; (●) gp39-bearing cells.

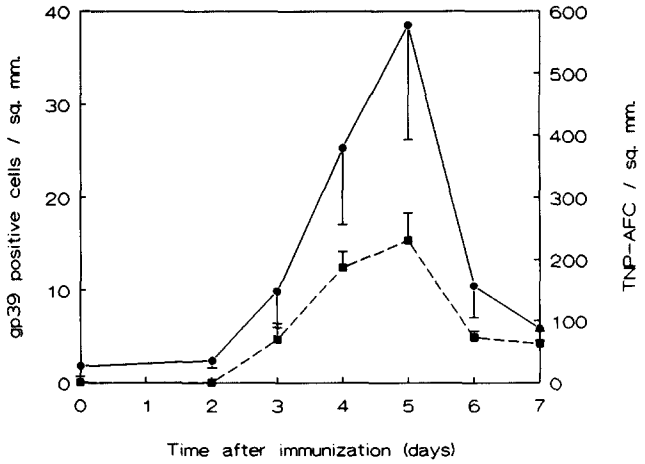


Figure 4. Kinetics of gp39⁺ cells and TNP-AFC may be superimposed after immunization with TNP-Ficoll. BCBA.F_1 mice were injected intravenously with 20 μg TNP-Ficoll and killed at the indicated time points. Spleens were removed and immunohistochemistry and image analysis were performed as described in Materials and Methods. Values represent mean ± SD of number of positive cells per mm² from three mice. (■) TNP-AFC; (●) gp39-bearing cells.

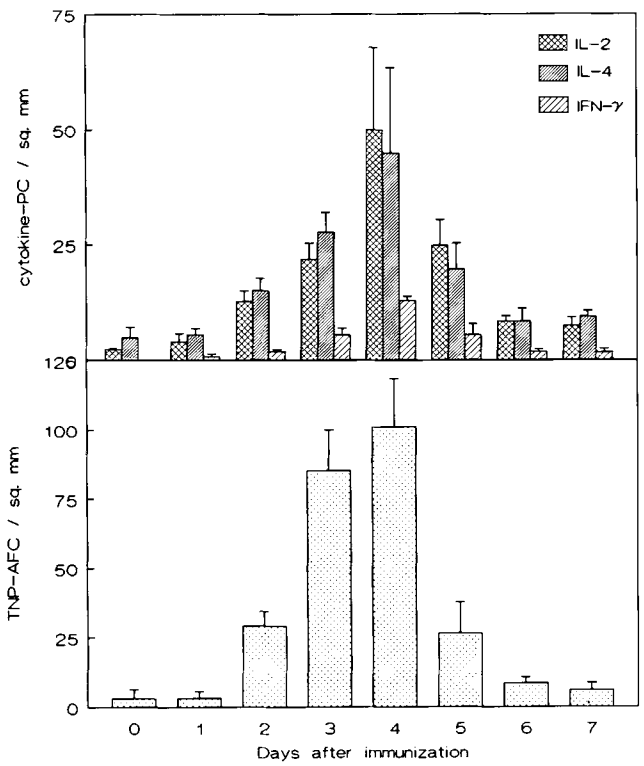


Figure 5. Cytokine PC and TNP-AFC develop according to similar kinetics after immunization with TNP-KLH. BCBA.F_1 mice were injected intravenously with 100 μg TNP-KLH (19) and killed at the indicated time points. Spleens were removed and immunohistochemical demonstration of IL-2-, IL-4-, IFN-γ-PC, and TNP-AFC were performed as described in Materials and Methods. Values represent mean ± SD of number of positive cells per mm^2 from three mice.

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. 2 *A*). Also in the immune response against TNP-Ficoll we observed antigen-specific B cells (TNP-AFC) colocalizing with gp39$^+$ cells in the outer-PALS and around the TA (Fig. 2 *B*).

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 d after intravenous immunization (Fig. 3). 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 h (Fig. 3). 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. 3). 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 (18) which show that anti-gp39 mAb were able to significantly reduce the antibody responses to KLH in vivo.

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 after 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 APCs, 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 d 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 with the number of IL-2-PC 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), whereas 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 5–7 d 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 (16). 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 observations are compatible with studies in humans (48–50) and mice (32, 51), 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. (52) showed in vitro that IL-4 mRNA was the only cytokine expressed by GC T cells, whereas these T cells expressed no mRNA of the other nine 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-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 nonreplicating 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 GC formation, indicating that the initial B cell activation occurs outside the follicles (55–57). This is in agreement with the results presented in this report, showing no $gp39^+$ cells or cytokine-PC in the follicles, but in the outer-PALS and around the TA. Only after secondary immunization, did we observe 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 TA. Furthermore, it validates in vitro experiments demonstrating that the kinetics of cytokine production and CD40-ligand expression can be superimposed (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 h) is required for maximal proliferative responses (58) or cytokine production (59), suggests that T-B conjugates may persist for several days in vivo.

Fig. 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 (51). 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). An-

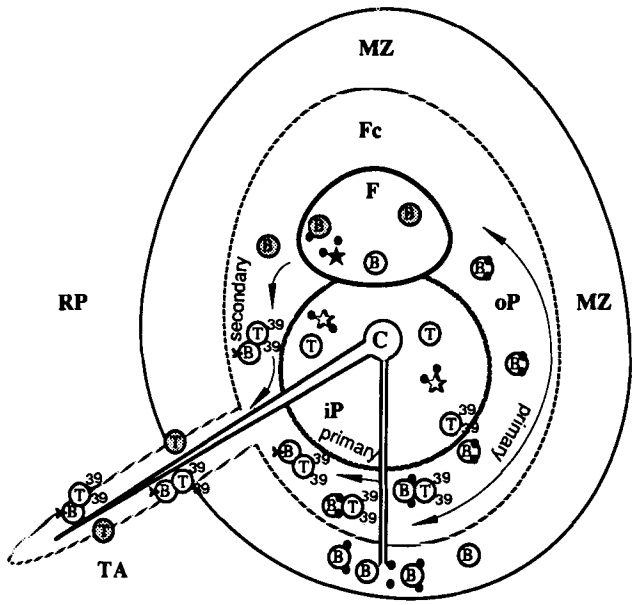


Figure 6. Schematic representation of the activation and migration of T and B cells in the spleen during the TD immune response. (*Arrows*) Migration of B and T cells in the spleen. (*C*) Central arteriole, (*F*) follicle, (*Fc*) follicle corona, (*MZ*) marginal zone, (*iP*) inner-PALS, (*oP*) outer-PALS, (*RP*) red pulp, (*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, (T_{39}) activated antigen-specific T cell ($gp39^+$), (Ⓣ) cytokine-producing T cell, (★) FDC, (☆) IDC.

other 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 secondary immune responses. The observation of high frequencies of antigen-specific AFC relative to gp39$^+$ T cells (Fig. 3), 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 with 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 IL-4-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 nonfollicular areas of the spleen, namely the outer-PALS and around the TA.

We thank Drs. A. Kruisbeek, J. D. Laman, W. E. Paul, B. O. Roep, and B. H. Waksman for their suggestions on the manuscript; and Mrs. N. Vermeulen for excellent technical assistance.

Address correspondence to Dr. E. Claassen, Department of Immunology and Medical Microbiology, TNO Medical Biological Laboratory, Lange Kleiweg 137-139, P.O. Box 5815, 2280 HV Rijswijk, The Netherlands.

Received for publication 30 March 1993 and in revised form 1 June 1993.

References

1. Snow, E.C., and R.J. Noelle. 1987. Thymus dependent antigenic stimulation of hapten-specific B cells. *Immunol. Rev.* 99:173.
2. Snow, E.C., R.J. Noelle, J. Uhr, and E.S. Vitetta. 1983. Activation of antigen-enriched B cells. II. Role of linked recognition in B cell proliferation to thymus dependent antigens. *J. Immunol.* 130:614.
3. Parker, D.C. 1990. How does the helper T cell activate the resting B cell when it recognizes antigen on the B cell surface, in "32nd Forum in Immunology", *Res. Immunol.* 141:405.
4. Armitage, R.J., W.C. Fanslow, L. Strockbine, T.A. Sato, K.N. Clifford, B.M. Macduff, D.M. Anderson, S.D. Gimpel, T. Davis-Smith, C.R. Maliszewski, et al. 1992. Molecular and biological characterization of a murine ligand for CD40. *Nature (Lond.).* 257:80.
5. Noelle, R.J., M. Roy, D.M. Shepherd, I. Stamenkovic, J.A. Ledbetter, and A.A. Aruffo. 1992. A novel ligand on activated helper T cells bind CD40 and transduces the cognate activation of B cell. *Proc. Natl. Acad. Sci. USA.* 89:6550.
6. Noelle, R.J., J. Daum, W.C. Bartlett, J. McCann, and D.M. Shepherd. 1991. Cognate interactions between helper T cells and B cells. V. reconstitution of T helper cell function using purified plasma membranes from activated Th1 and Th2 T helper cells and lymphokines. *J. Immunol.* 146:1118.
7. Hodgkin, P.D., L.C. Yamashita, R.L. Coffman, and M.R. Kehry. 1990. Separation of events mediating B cell proliferation and Ig production by using T cell membranes and lymphokines. *J. Immunol.* 145:2025.
8. Aruffo, A., M. Farrington, D. Hollenbaugh, X. Li, A. Milatovich, S. Nonoyama, J. Bajorath, L.S. Grosmaire, R. Stenkamp, M. Neubauer, et al. 1993. The CD40 ligand, gp39, is defective in activated T cells from patients with X-linked hyper-IgM syndrome. *Cell.* 72:291.
9. Allen, R.C., R.J. Armitage, M.E. Conley, H. Rosenblatt, N.A. Jenkins, N.G. Copeland, M.A. Bedell, S. Edelhoff, C.M. Disteche, D.K. Simoneaux, et al. 1993. CD40 ligand gene defects responsible for X-linked hyper-IgM syndrome. *Science (Wash. DC).* 259:990.
10. Korthauer, U., D. Graf, H.W. Mages, F. Brière, P. Munoreedevi, S. Malcolm, A.G. Ugazio, L.D. Notarangelo, R.J. Levinsky, and R.A. Kroczek. 1993. Defective expression of T cell CD40 ligand causes X-linked immunodeficiency with hyper-IgM. *Nature (Lond.).* 361:539.
11. Disanto, J.P., J.Y. Bonnefoy, J.F. Gauchat, and A. Fischer. 1993. CD40 ligand mutations in X-linked immunodeficiency with hyper-IgM. *Nature (Lond.).* 361:541.
12. Hollenbaugh, D., L.S. Grosmaire, C.D. Kullas, N.J. Chalupny, S. Braesh-Andersen, R.J. Noelle, I. Stamenkovic, J.A. Ledbetter, and A. Aruffo. 1992. The human T cell antigen gp39, a member of the TNF gene family, is a ligand for the CD40 receptor: expression of a soluble form of gp39 with B cell co-stimulatory activity. *EMBO (Eur. Mol. Biol. Organ.) J.* 11:4313.
13. Farah, T., and C.A. Smith. 1992. Emerging cytokine family. *Nature (Lond.).* 358:26.
14. Snapper, C.M., and W.E. Paul. 1987. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science (Wash. DC).* 236:944.
15. Snapper, C.M., F.D. Finkelman, and W.E. Paul. 1988. Regu-

lation of IgG1 and IgE by interleukin 4. *Immunol. Rev.* 102:51.
16. Stevens, T.L., A. Bossie, V.M. Sanders, R. Fernandez-Botran, R.L. Coffman, T.R. Mosmann, and E.A. Vitetta. 1988. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature (Lond.).* 334:255.
17. Mosmann, T.R., H. Cherwinski, M.W. Bond, M.A. Giedlin, and R.L. Coffman. 1986. Two types of murine helper T cell clone. I. definition according to profiles of lymphokine activities and secreted proteins. *J. Immunol.* 136:2348.
18. Foy, T.M., D.M. Shepherd, F.H. Durie, A. Aruffo, J.A. Ledbetter, and R.J. Noelle. 1993. *In vivo* CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39[1]. *J. Exp. Med.* 178:1567.
19. Claassen, E., N. Kors, and N. Van Rooyen. 1986. Influence of carriers on the development and localization of anti-trinitrophenyl antibody forming cells in the murine spleen. *Eur. J. Immunol.* 16:271.
20. Claassen, E., N. Kors, and N. Van Rooijen. 1986. Influence of carriers on the development and localization of anti-2,4,6-trinitrophenyl (TNP) antibody forming cells in the murine spleen. II. Suppressed antibody response to TNP-Ficoll after elimination of marginal zone cells. *Eur. J. Immunol.* 16:492.
21. Ledbetter, J.A., and L.A. Herzenberg. 1979. Xenogeneic monoclonal antibodies to mouse lymphoid differentiation antigens. *Immunol. Rev.* 47:63.
22. Dialynas, D.P., Z.S. Quan, K.A. Wall, A. Pierres, J. Quitans, M.R. Loken, M. Pierres, and F.W. Fitch. 1983. Characterization of the murine T cell surface molecule designated L3T4, identified by monoclonal antibody GK1.5: similarity of L3T4 to the Leu-3/T4 molecule. *J. Immunol.* 131:2445.
23. Springer, T.A., A. Bhattacharya, J.T. Cardoza, and F. Sanchez-Madrid. 1982. Monoclonal antibodies specific for rat IgG1, IgG2a, and IgG2b subclasses, and kappa chain monotypic and allotypic determinants: reagents for use with rat monoclonal antibodies. *Hybrid.* 1:257.
24. Boersma, W.J.A., J.E.M. van Leeuwen, C. Deen, J. Radl, and J.J. Haaijman. 1989. A monoclonal antihuman IgG1 subclass antibody produced with secondary in vitro immunization. *Proc. 36th Colloquium Protides of the Biological Fluids.* 36:157.
25. Van der Meide, P.H., A.H. Borman, H.G. Beljaars, M.A. Dubbeld, C.A.D. Botman, and H. Schellekens. 1989. Isolation and characterization of monoclonal antibodies directed to rat interferon-gamma. *Lymphokine Res.* 8:439.
26. Ohara, J., and W.E. Paul. 1985. Production of a monoclonal antibody to and molecular characterization of B-cell stimulatory factor-1. *Nature (Lond.).* 315:333.
27. Zantema, A., J.A.M. Fransen, A. Davis-Oliver, F.C.S. Ramaekers, G.P. Vooys, B. Deleys, and A.J. Van der Eb. 1985. Localization of the E1B proteins of adenovirus 5 in transformed cells, as revealed by interaction with monoclonal antibodies. *Virology.* 142:44.
28. Karasuyama, H., and F. Melchers. 1988. Establishment of mouse cell lines which constitutively secrete quantities of interleukin 2, 3, 4, or 5, using modified cDNA expression vectors. *Eur. J. Immunol.* 18:97.
29. Claassen, E., and L. Adler. 1988. Sequential double immunocytochemical staining for the in situ identification of an auto-anti-allotype immune response in allotype suppressed rabbits. *J. Histochem. Cytochem.* 36:1455.
30. Deelder, A.M., and R.J. De Water. 1981. A comparative study on the preparation of immunoglobulin-gal conjugates. *J. Histochem. Cytochem.* 29:1273.
31. Van den Eertwegh, A.J.M., J.D. Laman, M.M. Schellekens, W.J.A. Boersma, and E. Claassen. 1992. Complement mediated follicular localization of T-independent type 2 antigens: the role of marginal zone macrophages revisited. *Eur. J. Immunol.* 22:719.
32. Van den Eertwegh, A.J.M., M.J. Fasbender, M.M. Schellekens, A. Van Oudenaren, W.J.A. Boersma, and E. Claassen. 1991. *In vivo* kinetics and characterization of IFN-γ-producing cells during a thymus independent immune response. *J. Immunol.* 147:439.
33. Van den Eertwegh, A.J.M., M.J. Fasbender, W.J.A. Boersma, and E. Claassen. 1991. *In vivo* detection, kinetics and characterization of interferon-gamma producing cells during a thymus dependent immune response: an immunohistochemical study. *In* Lymphatic Tissues and *In Vivo* Immune Responses. B. Imhof, S. Berrih-Aknin, and S. Ezine, editors. Marcel Dekker, Inc., New York. 207–214.
34. Hodgkin, P.D., L.C. Yamashita, B. Seymour, R.L. Coffman, and M.R. Kehry. 1991. Membranes form both Th1 and Th2 T cell clones stimulate B cell proliferation and prepare B cells for lymphokine-induced differentiation to secrete Ig. *J. Immunol.* 147:3696.
35. Claassen, E., K. Gerritse, J.D. Laman, and W.J.A. Boersma. 1992. New immuno-enzyme-cytochemical stainings for the *in situ* detection of epitope specificity and isotype of antibody forming B-cells in experimental and natural (auto) immune responses in animals and man: a review. *J. Immunol. Methods.* 150:207.
36. Mond, J.J., P.K.A. Mongini, D. Sieckmann, and W.E. Paul. 1980. Role of T lymphocytes in the response to TNP-AECM-Ficoll. *J. Immunol.* 125:1066.
37. Mond, J.J., J. Farrar, W.E. Paul, J. Fuller-Farrar, M. Schaefer, and M. Howard. 1983. T cell dependence and factor reconstitution of in vitro antibody responses to TNP-B. abortus and TNP-Ficoll: restoration of depleted responses with chromatographed fractions of a T-cell derived factor. *J. Immunol.* 131:633.
38. Endres, R., E. Kushnir, J.W. Kappler, P. Marrack, and S.C. Kinsky. 1983. A requirement for T cell help factors in antibody responses to "T-independent" antigens. *J. Immunol.* 130:781.
39. Dekruyff, R.H., C. Clayberger, and H. Cantor. 1985. Monoclonal helper T cells induce B cell responses to T-independent antigens: antigen-specific T cells are directly stimulated by activated B cells in the absence of antigen. *J. Immunol.* 134:86.
40. Zisman, E., M. Dayan, M. Sela, and E. Mozes. 1993. Ia-antigen–T cell interactions of a thymus-independent antigen composed of D amino acids. *Proc. Natl. Acad. Sci. USA.* 90: 994–998.
41. Snapper, C.M., T.M. McIntyre, R. Mandler, L.M.T. Pecanha, F.D. Finkelman, A. Lees, and J.J. Mond. 1992. Induction of IgG3 secretion by interferon γ: a model for T cell–independent class switching in response to T cell–independent type 2 antigens. *J. Exp. Med.* 175:1367.
42. Bradley, L.M., D.D. Duncan, S. Tonkonogy, and S.L. Swain. 1991. Characterization of antigen-specific $CD4^+$ Effector T cells in vivo: immunization results in a transient population of MEL-14-CD45RB-helper cells that secretes interleukin 2 (IL-2), IL-3, IL-4, and interferon γ. *J. Exp. Med.* 174:547.
43. Le Gros, G., S.Z. Ben-Sasson, R. Seder, F.D. Finkelman, and W.E. Paul. 1990. Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4–PC producing cells. *J. Exp. Med.* 172:921.

44. Swain, S.L., A.D. Weinberg, M. English, and G. Huston. 1990. IL-4 directs the development of Th2-like helper effectors. *J. Immunol.* 145:3796.
45. Swain, S.L., L.M. Bradley, M. Croft, S. Tonkonogy, G. Atkins, A.D. Weinberg, D.D. Duncan, S.M. Hedrick, R.W. Dutton, and G. Huston. 1991. Helper T cell subsets: phenotype, function and the role of lymphokines in regulating their development. *Immunol. Rev.* 123:115.
46. Powers, G.D., A.K. Abbas, and R.A. Miller. 1988. Frequencies of IL-2 and IL-4–secreting T cells in naive and antigen-stimulated lymphocyte populations. *J. Immunol.* 140:3352.
47. Nossal, G.J.V., and C. Riedel. 1989. Sudden appearance of anti-protein IgG1-forming cell precursors early during primary immunization. *Proc. Natl. Acad. Sci. USA.* 86:4679.
48. Hoefakker, S., E.H.M. van 't Erve, C. Deen, A.J.M. Van den Eertwegh, W.J.A. Boersma, W.R.F. Notten, and E. Claassen. 1993. Immunohistochemical detection of co-localizing cytokine and antibody producing cells in the extrafollicular area of human palatine tonsils. *Clin. Exp. Immunol.* 93:223.
49. Bosseloir, A., E. Hooghe-Peters, E. Heinen, C. Marcoty, N. Cormann, C. Kinet-DeNoel, and L.J. Simar. 1991. *In* B. Imhof, S. Berrih-Aknin, and S. Ezine, editors. Lymphatic Tissues and In Vivo Immune Responses. Marcel Dekker, Inc., New York. 315–319.
50. Bowen, M.B., A.W. Butch, C.A. Parvin, A. Levin, and M.H. Nahm. 1991. Germinal center T cells are distinct helper-inducer T cells. *Hum. Immunol.* 31:67.
51. Van den Eertwegh, A.J.M., W.J.A. Boersma, and E. Claassen. 1992. Immunological functions and *in vivo* cell–cell interactions of T-lymphocytes in the spleen. *Crit. Rev. Immunol.* 11:337.
52. Butch, A.W., G.H. Chung, J.W. Hoffmann, and M.H. Nahm. 1993. Cytokine expression by germinal center cells. *J. Immunol.* 150:39.
53. Kelso, A., A.B. Troutt, E. Maraskovsky, N.M. Gough, M.H. Pech, and J.A. Thomson. 1991. Heterogeneity in lymphokine profiles of $CD4^+$ and $CD8^+$ T cells and clones activated *in vivo* and *in vitro*. *Immunol. Rev.* 123:85.
54. Lederman, S., M.J. Yellin, G. Inghirami, J.L. Lee, D.M. Knowles, and L. Chess. 1992. Molecular interactions mediating T-B lymphocyte collaboration in human lymphoid follicles. Roles of T cell–B-cell–activating molecule (5c8) and CD40 in contact-dependent help. *J. Immunol.* 149:3817.
55. Liu, Y., G.D. Johnson, J. Gordon, and C.M. Maclennan. 1992. Germinal centres in T-cell dependent antibody responses. *Immunol. Today.* 13:17.
56. Kosco, M.H., and D. Gray. 1992. Signals involved in germinal center formation. *Immunol. Rev.* 126:63.
57. Jacob, J., R. Kassir, and G. Kelsoe. 1991. In situ studies of the primary response to (4-hydroxy-3-nitrophenyl)acetyl. I. The architecture and dynamics of responding cell populations. *J. Exp. Med.* 173:1165.
58. Kehry, M.R., L.C. Yamashita, and P.D. Hodgkin. 1990. B cell proliferation and differentiation mediated by Th cell membranes and lymphokines, 32nd Forum in Immunology. *Res. Immunol.* 141:421.
59. Swoboda, R., U. Bommhardt, and A. Schimpl. 1991. Regulation of lymphokine expression in T cell activation. I. Rapid loss of interleukin-specific RNA after removal of the stimulating signal. *Eur. J. Immunol.* 21:1691.
60. Inaba, K., M.D. Witmer, and R.M. Steinman. 1984. Clustering of dendrotic cells, helper T lymphocytes, and histocompatible B cells during primary antibody responses *in vitro*. *J. Exp. Med.* 160:858.
61. Inaba, K., and R.M. Steinman. 1985. Protein-specific helper T-lymphocyte formation initiated by dendritic cells. *Science (Wash. DC).* 229:475.
62. Lederman, S., M.J. Yellin, A. Krichevsky, J. Belko, J.L. Lee, and L. Chess. 1992. Identification of a novel surface protein on activated $CD4^+$ T cells that induces contact-dependent B cell differentiation (help). *J. Exp. Med.* 175:1091.