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Unresponsiveness to Lymphoid-Mediated Signals at the Neonatal Follicular Dendritic Cell Precursor Level Contributes to Delayed Germinal Center Induction and Limitations of Neonatal Antibody Responses to T-Dependent Antigens¹

Maria Pihlgren,^{2*} Chantal Tougne,* Paola Bozzotti,* Alma Fulurija,* Michel A. Duchosal,[†] Paul-Henri Lambert,* and Claire-Anne Siegrist*

The factors limiting neonatal and infant IgG Ab responses to T-dependent Ags are only partly known. In this study, we assess how these B cell responses are influenced by the postnatal development of the spleen and lymph node microarchitecture. When BALB/c mice were immunized with alum-adsorbed tetanus toxoid at various stages of their immune development, a major functional maturation step for induction of serum IgG, Ab-secreting cells, and germinal center (GC) responses was identified between the second and the third week of life. This correlated with the development of the follicular dendritic cell (FDC) network, as mature FDC clusters only appeared at 2 wk of age. Adoptive transfer of neonatal splenocytes into adult SCID mice rapidly induced B cell follicles and FDC precursor differentiation into mature FDC, indicating effective recruitment and signaling capacity of neonatal B cells. In contrast, adoptive transfer of adult splenocytes into neonatal SCID mice induced primary B cell follicles without any differentiation of mature FDC and failed to correct limitations of tetanus toxoid-induced GC. Thus, unresponsiveness to lymphoid-mediated signals at the level of neonatal FDC precursors delays FDC maturation and GC induction, thus limiting primary Ab-secreting cell responses to T-dependent Ags in early postnatal life. *The Journal of Immunology*, 2003, 170: 2824–2832.

The level of IgG Ab responses to T-dependent Ags that can be generated in human neonates and infants under 2 mo of age is much lower than that achieved in older children and adults. For example, even the most immunogenic infant vaccines, such as *Haemophilus influenzae* type B or tetanus toxoid vaccines, fail to induce significant Ab responses in >90% of neonates (1, 2), whereas progressively higher serum Ab concentrations are elicited when immunization is given at 2–3, 4–6, or 8–10 mo of age (3). Such age-dependent limitations of primary Ab responses are observed with most vaccines (reviewed in Ref. 4). They represent a significant challenge for the development of novel vaccines against infant pathogens for which protection requires the induction of high titer Ab responses already in the first month of life. As an example, a novel live attenuated respiratory syncytial virus vaccine demonstrated to be strongly immunogenic at 3–5 mo of age failed to induce neutralizing serum IgG responses in 1- to 2-mo-old infants, the target group for prevention of severe respiratory syncytial virus disease (5).

Similar limitations of primary neonatal Ab responses to T-dependent Ags are observed when infant vaccines are used in murine models of neonatal and early life immunization, suggesting the existence of common limiting factors (6–9). The mechanisms limiting early life primary IgG responses are yet only partly understood. Immaturity at the level of APC, T cells, and/or B cells are all likely to contribute to these limitations (reviewed in Ref. 10). However, early life B cell responses are also likely to be influenced by the ongoing maturation of the spleen and lymph node microarchitecture. Recent studies have demonstrated that this development is a process that requires complex interactions between B cells and stromal cells, with the involvement of a large number of cytokines and chemokines including CXCL13 (B lymphocyte chemokine), TNF- α , and lymphotoxin (LT)³ $\alpha\beta$ (reviewed in Ref. 11). An essential step for primary Ag-specific B cell differentiation into IgG Ab-secreting cells (ASC) is the germinal center (GC) reaction. This step is critically influenced by the establishment of a functional follicular dendritic cell (FDC) network capable of retaining Ag-Ab complexes through their complement and Fc receptors and promoting the survival of GC B cells (reviewed in Ref. 12). In rodents, this FDC development extends throughout the postnatal period, through as yet poorly defined stages of maturation (13–16).

The objective of this study was to define the functional influence of distinct postnatal microarchitectural differentiation stages within secondary lymphoid organs on the development of Ag-specific primary IgG responses. Cellular and molecular determinants of ASC and GC responses were characterized at various stages of the murine early postnatal period. Specific questions were raised regarding 1) the capacity of neonatal stromal cells to recruit neonatal B

*World Health Organization Collaborating Center for Vaccinology and Neonatal Immunology, University of Geneva, Geneva, Switzerland; and [†]Department of Medicine, Division of Hematology, Center Hospitalier Universitaire Vaudois, Lausanne, Switzerland

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² Address correspondence and reprint requests to Dr. Maria Pihlgren, Department of Pathology, World Health Organization Collaborating Center for Vaccinology and Neonatal Immunology, University of Geneva, Centre Médicale Universitaire, Rue Michel Servet 1, 1211 Geneva 4, Switzerland. E-mail address: Maria.Pihlgren@medecine.unige.ch

³ Abbreviations used in this paper: LT, lymphotoxin; ASC, Ab-secreting cell; GC, germinal center; FDC, follicular dendritic cell; TT, tetanus toxoid; TNP, trinitrophenyl; PNA, peanut agglutinin.

cells into the splenic B cell zone; 2) the influence of transferred naive or activated adult B cells on the postnatal development of FDC in both normal and SCID recipients; and 3) the capacity of neonatal FDC precursors to undergo differentiation into mature FDC in response to *in vivo* activation signals.

Materials and Methods

Mice

Specific pathogen-free adult BALB/c mice (BALB/cByJ, H-2^d) and homozygous SCID mice (C.B.-17/Icr-*scid/scid*, H-2^d) were purchased from IFFA Credo (L'Arbresle, France) and kept and bred under specific pathogen-free conditions. Breeding cages were checked daily for births. Pups were kept with mothers until weaning at the age of 4 wk. SCID mice were tested for presence of mouse IgG at 6 wk of age. At this time point, leaky mice were eliminated, and nonleaky mice were not tested thereafter.

Ags, adjuvants, and immunization procedures

Tetanus Toxoid (TT; gift from the Vaccine and Serum Institute Berna, Bern, Switzerland) was used at a dose of 2 μ g (1 limit of flocculation)/mouse. Trinitrophenyl (TNP) hapten conjugated to OVA (TNP⁸-OVA) were obtained from Biosearch Technologies (Novato, CA) and used at a dose of 50 μ g/mouse. TT and TNP-OVA were absorbed to Al(OH)₃ (gift from Chiron, Siena, Italy) before immunization. The adult dose of Al(OH)₃ (1 mg) was weight-adjusted to 0.5 or 0.25 mg for immunization of 2- and 1-wk-old mice respectively, as previously experimentally defined. Oligonucleotides containing immunostimulating CpG sequences, CpG-ODN (1826, TCCATGACGTTCTGACGTT (7), kindly provided by A. M. Krieg, University of Iowa College of Medicine, Iowa City, IA), were used at a dose of 3 μ g in neonatal mice. Mice were immunized *i.p.* in groups of four to eight and bled at regular intervals for determination of TT- or TNP-specific serum Abs.

Quantification of Ag-specific Abs in serum

TT- and TNP-specific IgG Abs were determined by ELISA. Plates were coated with TT (1 μ g/ml) or TNP-BSA (10 μ g/ml; Biosearch Technologies) overnight at 4°C. After washing with PBS-0.05% Tween 20 and blocking with 1% BSA, serial dilutions of sera were added to the plates and incubated for 1 h (TT) or 2 h (TNP) at room temperature. After washing, the plates were incubated with peroxidase-conjugated goat anti-mouse IgG Abs (Zymed Laboratories, San Francisco, CA) for 30 min (TT) or 2 h (TNP) at 37°C, washed, and incubated with substrate. Results were expressed by reference to serial dilutions of a titrated pool of serum from immunized adult mice. In all tests, Ab titers below the cutoff of the assay were given an arbitrary titer of 1/2, the cutoff value to allow calculation of geometric mean Ab titers.

Enumeration of ASC by ELISPOT

The TT- and TNP-specific ASC number was assessed by ELISPOT as previously described for lymphocytic choriomeningitis virus-specific ASC (17) with minor modifications. Single-cell suspensions were obtained from the spleen and bone marrow at different times after immunization and resuspended in complete RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% FCS. The bone marrow and spleen cell suspensions were then treated with ACK lysing buffer (0.15 M NH₄CL, 10 mM KHCO₃ and 0.1 mM Na₂EDTA) to eliminate RBC. Multiscreen HA nitrocellulose-bottom plates (Millipore, Bedford, MA) were coated with 10 μ g/ml TT or TNP-BSA overnight at 37°C. After washing with PBS-0.1% Tween 20 and blocking with RPMI 1640 containing 10% FCS, serial dilutions of the single-cell suspension were added to the plates and incubated for 5 h at 37°C. After washing, plates were incubated with peroxidase-conjugated goat anti-mouse IgG (Zymed Laboratories) overnight at 4°C, washed, and incubated with substrate. Wells containing between 10 and 70 spots (linear zone) were counted by eye and with an ELISPOT reader (KS ELISPOT, Zeiss, Germany).

Immunohistochemistry

Spleens and lymph nodes (cervical, submedullary, axillary, brachillary, inguinal, and mesenteric) from immunized or naive mice were frozen in Tissue-Tek OCT compound (Sakura, Zouterwoude, The Netherlands) and cut into 10- μ m cryosections at three to four different levels separated by at least 200 μ m. Sections were fixed in acetone for 10 min and stored at -20°C until staining. For enumeration of GC, three to four sections from every spleen were stained with peanut agglutinin (PNA)-FITC (Vector Laboratories, Burlingame, CA) to detect GC and with IgM-Texas Red

(Southern Biotechnology, Birmingham, AL) to identify B cell follicles. The numbers of GC and follicles were counted under the microscope by two independent investigators, and the average of GC per follicle was calculated for every spleen so as to correct for difference in spleen size between mice of different ages. Mature FDC were detected with the FDC-M2 (209; kind gift from M. Kosco-Vilbois, Nouimmune, Geneva, Switzerland) or CR-1 (8C12, PharMingen, San Diego, CA) Abs followed by FITC-conjugated F(ab')₂ mouse anti-rat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and enumerated as described above for GC. For the measurement of the size of FDC and B cell follicles, a minimum of 20 FDC networks and follicles from every mouse were photographed with a digital camera (AXIOCAM; Zeiss, Oberkochen, Germany) and quantified using specifically designed macro functions of Zeiss KS400 3.0 software. Staining for CXCL13 was performed with goat anti-mouse BLC (R&D Systems, Minneapolis, MN) as previously described (18, 19). For detection of IgG-secreting B cells (defined as positive for cytoplasmic IgG), spleen sections were stained with IgG-Texas Red (Southern Biotechnology Associates, Birmingham, AL).

Transfer of splenocytes to SCID mice

Single spleen cell suspension from adult, 1-wk-old, or 2-day-old BALB/c mice were applied to Ficoll-Hypaque (Lympholyte-M; Cederlane Laboratories, Hornby, Ontario, Canada) gradient centrifugation. Cells were then washed twice with medium (DMEM; Life Technologies) and resuspended in PBS. Spleen cells were injected *i.v.* (1–3 \times 10⁷ spleen cells) into adult SCID mice or *i.p.* (2.5–10 \times 10⁷ spleen cells) into 2-day-old SCID mice. Seven days after transfer, mice were sacrificed and the spleens were frozen for immunohistochemistry. In some experiments, as indicated, the recipients were immunized with TT/Al(OH)₃ 5 days after transfer and sacrificed 7 or 10 days after immunization.

Transfer of TT-primed splenocytes to neonatal BALB/c mice

Splenocytes were harvested from adult BALB/c mice primed with TT/Al(OH)₃ and applied to Ficoll-Hypaque gradient centrifugation. Briefly, 10 \times 10⁷ cells were injected *i.p.* into 2-day-old BALB/c mice. Neonatal recipient mice were immunized 1 day after transfer with TT/Al(OH)₃ administered with CpG-ODN. Seven days after transfer, mice were sacrificed and bled and the spleens were frozen for immunohistochemistry.

Statistical analysis

Statistical analysis between results obtained from various groups of mice was performed using the Mann-Whitney *U* test. Differences with *p* > 0.05 were considered to be insignificant.

Results

Age-dependent limitations of GC induction in early life

To characterize *in vivo* primary B cell responses under optimal conditions, BALB/c mice were immunized *i.p.* at 1 wk of age or as adults with 2 μ g TT adsorbed to Al(OH)₃. This strong immunogenic signal was selected since it was shown to allow, even in early life, the quantification of various cellular parameters of the primary B cell response (20). In adults, a rapidly increasing TT-specific spleen ASC response peaked at day 10 (90 ASC/10⁶ spleen cells; Fig. 1A). In contrast, the TT-ASC response was significantly delayed and lower following immunization of 1-wk-old mice, whose stage of immune maturation best approximates that of human neonates (*i.e.*, <28 days of age; Ref. 4). TT-specific ASC were barely detectable 10 days after priming and reached a maximum of only 38 ASC/10⁶ spleen cells at day 14. From day 14 onward, TT-specific ASC declined at a similar rate in adult and infant mice, returning to baseline ~6 wk after priming (Fig. 1A). These ASC response patterns directly reflected serum anti-TT IgG responses which were significantly lower in mice immunized at 1 wk of age as compared with adults (day 10, 2.5 \pm 0.3 vs 5.0 \pm 0.2 log₁₀; day 24, 4.4 \pm 0.4 vs 5.4 \pm 0.2 log₁₀, respectively).

The intensity of the TT-induced GC reaction was assessed in the spleen at various time points after TT immunization. In preliminary experiments it was shown that, in our population of BALB/c mice kept under specific pathogen-free conditions, <0.1 GC was detectable per follicle in naive adults. In these adult mice, a

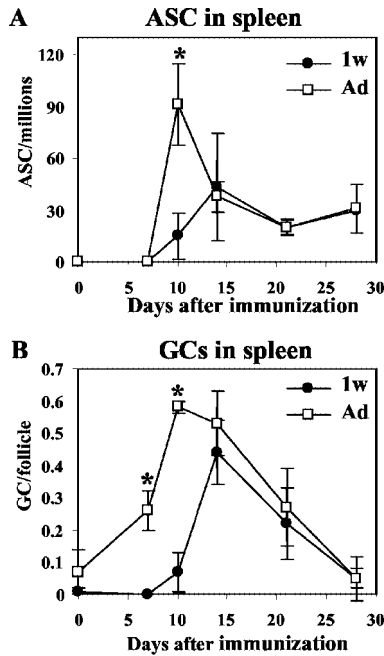


FIGURE 1. Delayed ASC and GC responses in early life. BALB/c mice were injected i.p. at 1 wk of age (1w) or as adults (Ad) with alum-adsorbed TT. At 7, 10, 14, 21, or 28 days after immunization, mice were sacrificed and TT-specific IgG ASC in spleen were measured by ELISPOT (A). Alternatively, the spleens were frozen and GC and B cell follicles were enumerated by staining spleen sections with PNA-FITC and IgM-Texas Red (B). Results are expressed as mean \pm SD obtained in groups of four to eight immunized mice sacrificed at each time point. TT-specific IgG ASC were not detected in nonimmunized control mice of any age and at any time point (data not shown). *, $p < 0.05$ comparing mice immunized as adults vs at 1 wk of age.

marked increase of the number of GC per follicle was identified 7 days after TT immunization, i.e., before detection of TT-specific ASC, reflecting active B cell proliferation (Fig. 1B). The number of GC per follicle peaked between days 10 and 14, waned thereafter, and returned to baseline levels at day 28. Thus, under our experimental conditions, the number of GC per follicle following TT immunization appeared to be a direct marker of the TT-specific GC response, as has been previously demonstrated for other Ags (21, 22). Following TT immunization of 1-wk-old mice, the GC reaction was undetectable at day 7 after immunization, remained weak at day 10, and only after day 14 were significant numbers of GC per follicle observed, i.e., in 3-wk-old mice (Fig. 1B). The subsequent decline of GC per follicle was similar and parallel to that seen in adults during the second phase of the GC reaction. Thus, the early life TT-induced GC response was both significantly delayed and less robust compared with adult GC responses, and this essentially reflected delayed GC initiation.

This age-dependent limitation of the early life ASC-GC reaction pattern was confirmed in another immunization model using AL(OH)₃-adsorbed TNP-OVA (50 μ g) as Ag. Seven days after immunization, the numbers of GC per follicle in spleen were significantly lower in BALB/c mice immunized at 1 wk of age than in adults (0.01 in 1-wk-old mice vs 0.24 in adults), which was mirrored by significantly lower TNP-specific spleen IgG ASC (35/million cells in 1-wk-old mice vs 413/million cells in adults). Thus, limitations of the initial IgG/ASC response following early life immunization are directly paralleled by limitations in the induction of the GC reaction.

To assess the influence of distinct postnatal developmental stages on induction of IgG and GC responses, BALB/c mice were

immunized with TT at various stages of their immune maturation, i.e., at 1, 2, or 3 wk of age or as adults. Mice were bled on day 9 after priming and sacrificed at day 10, allowing assessment of GC responses and serum TT-specific IgG in the same mice. With increasing age at immunization, there was an increase of the number of GC per follicle elicited: adult-like GC per follicle numbers were indeed observed 10 days after immunization of 2- or 3-wk-old mice, in contrast to the weak GC reaction in mice immunized at 1 wk of age (Fig. 2A). This developmental pattern was similar to that observed for day 9 TT-specific IgG titers (Fig. 2B). Thus, a marked increase in TT-IgG titers was obtained by postponing TT immunization from 1 to 2 wk of age, which was associated with a marked increase in the age-dependent GC induction, such that Ab responses elicited ≥ 2 wk of age were closer to that seen in adults (Fig. 2B).

Limitations in postnatal FDC network development

Within the context of a normal lymphoid organ microarchitecture, the induction of the GC reaction is known to require, among numerous factors, a mature FDC network capable of nucleating the GC reaction through Ag trapping, enhancing B cell activation, and protecting B cells from apoptosis (12, 23, 24). The question was thus raised whether the delayed and limited GC reaction seen after immunization of 1-wk-old mice, contrasting with the induction of an adult-like GC reaction in 2-wk-old mice, might reflect a delayed maturation of the FDC network. Two markers of mature FDC, FDC-M2 and CR-1, were selected to characterize the development of mature FDC networks in spleens and lymph nodes of naive, nonimmunized BALB/c mice at various stages of their immune maturation, i.e., between birth and 8 wk of age. Spleen cryosections were stained with either FDC-M2 or CR-1 Abs, both of which bind to mature FDC in primary and secondary follicles in adult mice. In mice aged 7 days, there were no FDC-M2-positive nor CR-1-positive FDC clusters and no apparent redistribution of FDC within the lymphoid microarchitecture (Fig. 3). In contrast, distinct although relatively small mature FDC clusters were readily

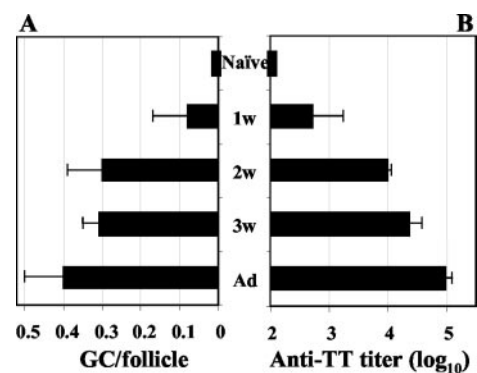


FIGURE 2. Parallel development of early life GC and Ab responses. BALB/c mice were injected i.p. at 1 wk (1w), 2 wk (2w), and 3 wk (3w) of age or as adults (Ad) with alum-adsorbed TT. Ten days after immunization, mice were sacrificed, their spleens were frozen, and GC and B cell follicles were enumerated by staining with PNA-FITC and anti-IgM-Texas Red (A). A group of naive 1-wk-old mice (Naive 1w) is shown for comparison. The number of GC per follicle was significantly lower in mice immunized at 1 wk compared with 2 wk of age ($p < 0.05$), whereas similar numbers of GC were elicited in 2 wk, 3 wk, or adult mice. Mice were bled on the day before sacrifice for determination of TT-specific IgG titers (B). Ab titers in all groups were statistically different from each other ($p < 0.05$). Results are expressed as mean \pm SD obtained in groups of three to eight immunized mice.

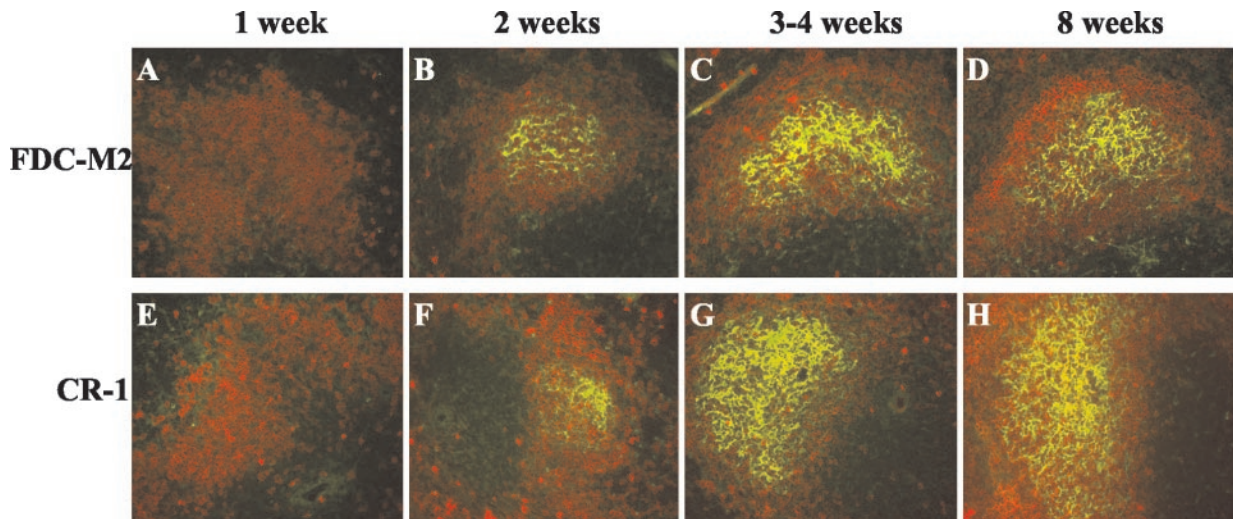


FIGURE 3. Delayed differentiation of mature FDC networks in early life. Cryostat sections were prepared from the spleens of naive mice of different ages, as indicated, and stained for mature FDC with FDC-M2 (A–D) or CR-1 (E–H) followed by FITC-conjugated F(ab')₂ mouse anti-rat IgG (green). B cells were stained with anti-IgM-Texas-Red (red). One representative section from each group of three mice is shown. Magnification, $\times 200$.

identified in spleens from 14-day-old mice at the expected localization within the B cell zone. FDC clusters only reached adult-like appearance after the age of 3 wk (Fig. 3). This postnatal development was not enhanced by active immunization of 1-wk-old mice with TT adsorbed to AL(OH)₃: clusters of mature FDC were seen at 2 wk of age, but their pattern remained as modest as that observed in 2-wk-old nonimmunized control mice (Fig. 3 and data not shown).

Differences are known to exist in the ontogenic development of spleen and lymph nodes (25, 26) and it has been suggested that the kinetics of immune maturation may be more rapid in murine lymph nodes than in spleen (27). Therefore, the appearance of mature FDC clusters was also studied in cervical, submedullary, axillary, brachillary, inguinal, and mesenteric lymph nodes. There was a complete absence of FDC-M2- or CR-1-positive FDC in nodes of mice younger than 14 days, whereas only few and small FDC clusters were detectable at 2 wk of age (data not shown). Thus, the delayed postnatal differentiation of FDC affects both spleen and lymph nodes.

Efficacy of CXCR5/CXCL13-mediated B cell recruitment in early life

The induction of FDC networks requires multiple complex interactions in the B cell zone (reviewed in Ref. 28). The functional efficacy of 1) CXCR5/CXCL13-mediated B cell recruitment, 2) TNF- α /LT α β -mediated signaling to FDC precursors by neonatal B cells, and 3) the differentiation and migration of mature FDC into B cell follicles was thus studied in relation to the identified time frame of the postnatal development of the FDC network, i.e., during the first 2 wk of life.

We first assessed the ability of neonatal stromal cells to attract B cells into the B cell zone. Immunohistochemical staining for CXCL13 was readily observed in the splenic B cell zone of 2-wk-old and adult mice, but not at 1 wk of age (Fig. 4 and data not shown), suggesting a potential insufficiency of CXCL13 production. Immunohistochemical detection of CXCL13 identified mature FDC-M2- and CR-1-positive FDC in B cell follicles. However, no CXCL13 staining was observed outside of FDC-containing B cell follicles or in spleens from adult SCID mice (data not shown), which contain FDC precursors able to rapidly differentiate into mature FDC (see below).

Thus, the production of CXCL13 by FDC precursors remains below detection level by immunohistochemistry.

Since a relative limitation of CXCL13 production by neonatal FDC precursors could not be excluded, we asked whether this was associated with a functional limitation of B cell recruitment by neonatal stromal cells. Adoptive transfer experiments were set up in SCID recipient mice to avoid competition between donor and recipient cells in the B cell zone. Adult BALB/c naive splenocytes ($3\text{--}10 \times 10^7$ cells) were adoptively transferred i.p. into 2-day-old SCID mice ($n = 6$). Seven days after transfer, mice were sacrificed and splenic cryosections were stained for B cells with an anti-IgM Ab. Well-demarcated splenic primary B cell follicles were visible in 9-day-old recipient SCID mice, contrasting with the total absence of follicles in age-matched noninjected control SCID mice (Fig. 5, C and G, and Table I). This demonstrated the capacity of the neonatal recipient splenic stromal cells to recruit transferred adult B cells. Furthermore, early postnatal B cells were as capable as adult B cells of migrating into the B cell zone after adoptive transfer (i.v.) into adult SCID mice (Fig. 5, B and F, and Table I). Thus, despite an apparently lower production of CXCL13, neonatal and early life B cells are able to functionally respond to chemoattractive signals and to be recruited by neonatal stromal cells of the B cell zone. This early homing capacity into the B cell zone is consistent with the presence of already demarcated T and B cell

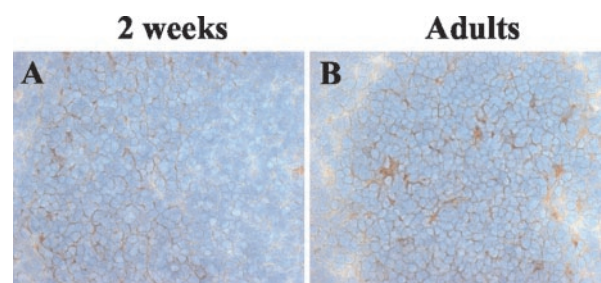


FIGURE 4. Expression of CXCL13 in early life. Cryostat sections were prepared from the spleens of naive 2-wk-old (A) or adult (B) mice and stained for CXCL13 (red). Sections were counterstained with hematoxylin (blue). One representative section from each group of three mice is shown. Magnification, $\times 400$.

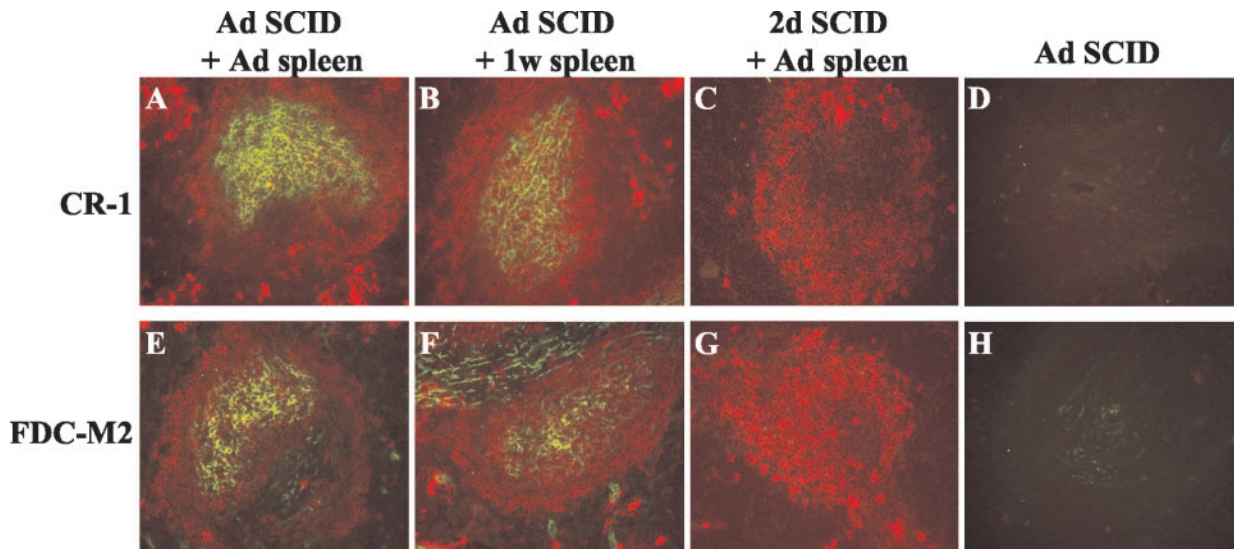


FIGURE 5. Induction of FDC networks in adult (Ad) SCID mice by neonatal lymphocytes. Splenocytes from adult (A and E) or from 1-wk-old (1w, B and F) BALB/c mice were transferred i.v. into adult SCID mice ($n = 3$ for transfer of adult splenocytes and $n = 5$ for transfer of 1-wk-old splenocytes). Alternatively, splenocytes from adult BALB/c mice were transferred i.p. into 2-day-old (2d) SCID mice (C and G, $n = 6$). Seven days after transfer, mice were sacrificed, their spleens were frozen, and cryosections were stained for mature FDC with FDC-M2 or CR-1 followed by FITC-conjugated F(ab')₂ mouse anti-rat IgG (green) and for B cells with anti-IgM-Texas-Red (red). One representative section from each group is shown. For comparison, a spleen section from one (of three) noninjected control SCID mice is shown (D and H).

zones in the spleen of 1 wk-old naive BALB/c mice (data not shown).

Neonatal B cell-mediated signaling to FDC precursors

Second, we explored the possibility that the lack of early postnatal FDC networks might reflect insufficient signaling (i.e., through LT α , LT β , and/or TNF- α) by B cells in early life. In SCID mice, which lack FDC as a result of the absence of mature B cells (29), the differentiation of FDC precursors into mature FDC can be induced within a few days after adoptive B cell transfer (30–32). This induction is dependent on LT α /LT β R signaling by B cells, since it is not elicited by transfer of LT α ^{-/-} B cells (32). Thus, B cells from 1-wk-old naive BALB/c mice were tested for their capacity to generate FDC differentiation following their i.v. adoptive transfer in an adult SCID environment. One-week-old or adult BALB/c splenocytes ($1\text{--}3 \times 10^7$ cells) were transferred i.v. into adult SCID mice ($n = 5$ for recipients of cells from 1-wk-old mice and $n = 3$ for recipients of adult cells). Clusters of mature FDC appeared within 7 days after adoptive transfer of 1-wk-old splenocytes into adult SCID mice (Fig. 5. B and F), with a pattern similar to that seen after transfer of naive adult splenocytes into adult SCID recipients (Fig. 5. A and E). Mature FDC clusters were sim-

ilar following transfer of 1-wk-old or adult splenocytes into adult SCID recipients, as assessed by their number and their size (Table I). Similar results were obtained when 2-day-old splenocytes were transferred i.v. into adult SCID mice. Therefore, neonatal lymphocytes are capable of providing sufficient *in vivo* signals for the rapid differentiation of adult FDC precursors into a mature FDC clusters, even in the context of the SCID defect.

Differentiation capacity of neonatal FDC precursors

These two series of experiments indicated that early life B cells are appropriately recruited into the B cell zone and are capable of providing the signals required for the differentiation of adult FDC precursors into mature FDC. This suggested that the lack of mature FDC in early life may reflect unresponsiveness of FDC precursors during the postnatal period. This hypothesis was first tested by a study of the development of FDC following transfer of naive adult splenocytes ($3\text{--}10 \times 10^7$ cells, i.p.) into 2-day-old SCID mice ($n = 6$). As previously mentioned, adoptively transferred adult B lymphocytes readily migrate into the neonatal B cell zone and, within a week, form primary follicle-like structures (Fig. 5, C and G, and Table I). However, these transferred adult B cells failed to induce

Table I. Induction of FDC networks in SCID mice^a

Age of		No. of		Size ($\times 10^{-3}$ squared/pixel) of	
Donor	Recipient	FDCs/section	Follicles/section	FDCs	Follicles
Adult	Adult	17 \pm 11	17 \pm 10	76 \pm 10	134 \pm 22
1 wk	Adult	21 \pm 6	22 \pm 6	71 \pm 11	130 \pm 13
2 days	Adult	22 \pm 4	22 \pm 3	147 \pm 13	229 \pm 5
Adult	2 days	0 \pm 0	13 \pm 3	ND	ND

^a Splen cells from BALB/c donors of different ages were injected into adult or 2-day-old recipient SCID mice. Seven days later, cryostat sections were prepared from the spleens of recipient SCID mice and stained for mature FDCs with CR-1 followed by FITC-conjugated F(ab')₂ mouse anti-rat IgG and for B cells with anti-IgM-Texas Red. The number and size of FDC networks and B cell follicles were evaluated as described in *Materials and Methods*. Spleen sections represent half a spleen in adult recipients and the whole spleen in neonatal recipients. Results are expressed as mean \pm SD obtained in groups of three to four mice.

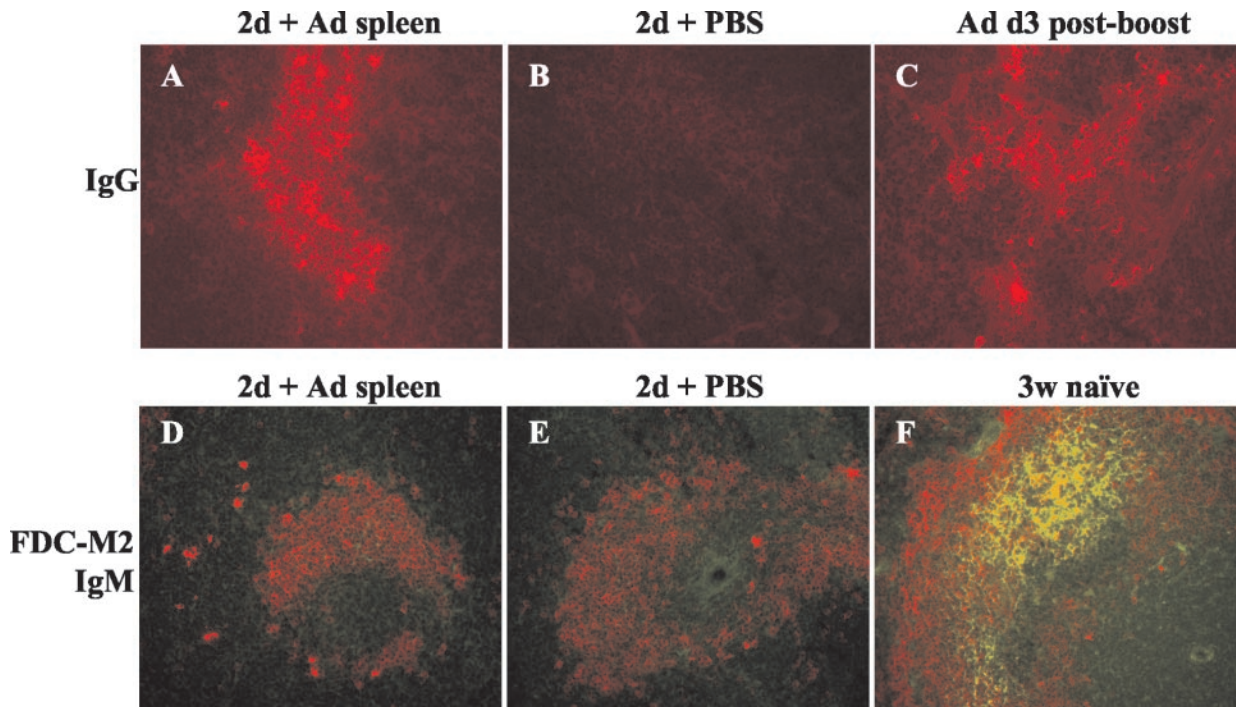


FIGURE 6. Activated adult (Ad) splenocytes fail to induce FDC maturation in neonatal BALB/c mice. Splenocytes from TT-primed adult BALB/c mice (A and D) or PBS (B and E) were injected i.p. into 2-day-old (2d) BALB/c mice ($n = 6$ for splenocyte-injected mice and $n = 2$ for PBS-injected mice). The next day, all mice were immunized with TT/Al(OH)₃ and CpG-ODN. Seven days after transfer, mice were sacrificed, their spleens were frozen, and cryosections were stained with IgG-Texas Red (A and B) to detect IgG-ASC (A–C) or double stained for mature FDC with FDC-M2 followed by FITC-conjugated F(ab')₂ mouse anti-rat IgG (green) and for B cells with anti-IgM-Texas Red (red, D–F). One representative section from each group is shown. As positive controls for IgG-ASC and FDC-M2 staining, spleen sections from an adult BALB/c mouse sacrificed 3 days (d3) after priming and boosting with TT/Al(OH)₃ (C) and from a naïve 3-wk-old mouse (F) were included.

the differentiation of mature FDC clusters in infant SCID recipients, as demonstrated by using both FDC-M2 and CR-1 Abs (Fig. 5, C and G), while readily eliciting FDC maturation in adult SCID mice (Fig. 5, A and E).

To demonstrate the importance of the FDC network in the early phase of the GC response to neonatal immunization, 2-day-old and adult SCID mice were transferred with 2.5×10^7 adult BALB/c splenocytes before immunization with TT(Al(OH)₃) 5 days later. Mice were sacrificed 7 days after immunization for evaluation of TT-specific responses. This schedule, in which neonatal mice are analyzed when 14 days old, was selected to allow 5 days for maturation of FDC progenitors into mature FDC before immunization, as well as sufficient time for detection of TT-induced GC responses. TT-induced GC were indeed readily elicited (mean GC per follicle in four recipient mice, 0.67 ± 0.11) in adult SCID recipients and reached numbers at least as high as those observed on day 10 after immunization of intact adult BALB/c mice (Fig. 1B). In contrast, the number of GC per follicle remained significantly lower (mean GC per follicle in eight recipient mice, 0.30 ± 0.18) following transfer of adult splenocytes into neonatal SCID mice. In this adoptive transfer model, TT-IgG and TT-ASC remained below detection level in both age groups at this time point. In another series of experiments where mice were sacrificed 10 days after immunization, the numbers of GC per follicle in adult SCID recipients were already significantly lower (0.37 ± 0.08) than at day 7. At this later time point, GC per follicle induced by TT immunization in 17-day-old neonatally transferred SCID recipients were also significantly lower than in adults (0.18 ± 0.18), confirming day 7 results. Thus, unresponsiveness at the FDC precursor level results in failure of adult B cells to induce FDC maturation in neonatal SCID recipients (Fig. 5, C and G), which is

associated with limited induction of GC responses to TT immunization.

Because these observations indicated a relative unresponsiveness of neonatal FDC precursors compared with those in adults, we assessed whether enhancing B cell-mediated signaling could trigger the maturation of neonatal FDC precursors. To maximally enhance neonatal APC, T cell, and B cell activation, TT Ag was formulated with a combination of two potent and synergistic adjuvants: Al(OH)₃ and oligonucleotides containing immunostimulating CpG sequences (CpG-ODN) (7, 33–35). Adult BALB/c mice were primed with TT(Al(OH)₃). Their splenocytes (10×10^7) were harvested 3 wk later and injected i.p. into 2-day-old BALB/c mice ($n = 6$). Neonatal recipient mice were immunized 1 day after transfer with TT/Al(OH)₃ administered with CpG-ODN (3 μ g). Mice were sacrificed at 9 days of age for determination of their TT-specific IgG response and assessment of their FDC networks. This adoptive transfer-boosting protocol led to a rapid induction of very high, adult-like TT-specific IgG serum Ab titers ($6.6 \log_{10}$) and to a massive accumulation of IgG-secreting B cells in the spleen (Fig. 6, A and B). However, even this massive transfer of activated adult B cells failed to induce the maturation of FDC clusters, which remained similar to that of 10-day-old naïve mice, as assessed by CR-1 and FDC-M2 staining (Fig. 6, D and E). Thus, even under conditions of enhanced in vivo activation, the signals generated by adult activated B cells remained unable to induce the differentiation of neonatal FDC precursors into mature FDC.

Discussion

The present study establishes that unresponsiveness at the FDC precursor level during the postnatal period is responsible for the delayed differentiation of a mature FDC network in early life and

demonstrates a direct correlation between this specific feature of the lymphoid microarchitectural structure and the limitations of early life GC, ASC, and Ab responses to T-dependent Ags. In contrast, it demonstrates the functional capacity of neonatal B cells to migrate into the B cell zone and to provide appropriate differentiation signals to adult FDC precursors.

Several factors might contribute to the absence of mature FDC networks in early life, since its induction requires multiple interactions and a complex interplay between lymphocytes, in particular B lymphocytes, and stromal cells. Studies in KO mice have indicated that this includes 1) the recruitment of B cells into the B cell zone by stromal cells, essentially mediated by CXCR5/CXCL13; 2) the expression of LT α , LT β , and TNF- α by B cells; and 3) the differentiation of putative stromal FDC precursors as a result of B cell-mediated TNFR1/LT β R-mediated signals (36–42). The lack of immunohistochemical detection of CXCL13 in spleens of 1-wk-old BALB/c mice may have suggested insufficient neonatal CXCL13 production for B cell recruitment into the B cell zone. However, CXCL13 immunostaining was unable to identify FDC precursors which produce weaker CXCL13 levels than mature FDC (19), as highlighted in adult SCID mice which lacked CXCL13 staining, despite the existence of FDC precursors able to rapidly differentiate into mature FDC after adoptive B cell transfer. Yet, the baseline chemokine production by putative neonatal FDC precursors is functionally sufficient to attract B cells. This is demonstrated by already demarcated B and T cell zones in 1-wk-old mice and more directly by the capacity of neonatal B cells to home into the B cell zone and form primary B cell follicles when transferred into 2-day-old SCID mice.

Insufficient TNF- α /LT $\alpha\beta$ signaling by neonatal splenocytes, demonstrated to be essential for FDC migration and differentiation (36, 37, 40), could also account for the absence of mature FDC during early postnatal life. However, splenocytes from either 2-day-old or 1-wk-old mice were capable of rapidly inducing the differentiation of adult FDC precursors into clusters of mature FDC, as described following adoptive transfer in adult SCID recipients. Because our adoptive transfer experiments were performed with total splenocytes and not with purified mature B cells, this indicates efficient signaling by neonatal B cells despite a subset repartition of immature, mature, B1, and marginal zone B cells distinct in neonatal mice from that of adult mice. Our observation is in agreement with the recent demonstration of surface LT α 1 β 2 on immature splenic B cells from 5-day-old mice (43) and suggests that early life B cells are both appropriately recruited into the B cell zone and capable of providing sufficient *in vivo* signaling for FDC maturation to occur.

In contrast, we identified an unresponsiveness at the level of FDC precursors during the first weeks of postnatal life, as the transfer of naive, or even of *in vivo*-activated, adult splenocytes into 2-day-old BALB/c or SCID mice was unable to induce FDC differentiation in 9-day-old mice. Furthermore, this adoptive transfer of adult splenocytes was unable to correct the limited induction of TT-specific GC observed in neonatal compared with adult recipients. Whether this reflects the physical absence of neonatal FDC precursors in the B cell zone of the spleen and nodes or functional unresponsiveness to differentiation signals can at present not be formally distinguished. The origin of FDC is yet unclear and their precursors remain relatively undefined. It is currently considered that FDC most probably do not differentiate from recirculating cells of hemopoietic origin but from locally present stromal cells (Ref. 44 and reviewed in Ref. 28). Our observations indicate that neonatal FDC precursors may be unresponsive to the TNF- α /TNFR1 signaling required for their migration from the periphery of the white pulp into splenic follicles (42, 45). Alternatively, neonatal FDC precursors may have migrated into the neo-

natal B cell zone, but remain unresponsive to subsequent differentiation signals until later in ontogeny. Although these two hypotheses would suggest distinct requirements of neonatal FDC precursors for additional differentiation stimuli, they have similar functional consequence: a delayed and limited induction of mature FDC network and thus of GC.

The fact that the absence of a mature FDC network in 1-wk-old mice results in delayed GC induction and limited ASC differentiation is indeed consistent with a large number of observations demonstrating the role of FDC for the nucleation of the GC reaction. In most adult mice deficient for either TNF- α , TNFR1, LT α , LT β , or LT β R, all of which lack FDC, no GC response is observed following immunization (26, 46–53). Although GC have been induced in the absence of FDC in the mesenteric lymph nodes of LT β -deficient mice (54), these GC disappear much more rapidly than in normal mice and do not lead to normal B cell affinity maturation. It is of interest to note that although early life GC responses to immunization are delayed and limited, they are not totally abrogated. The initiation of the GC reaction is postponed until 10–14 days after priming, *i.e.*, shortly after mature FDC clusters have been generated. This may reflect Ag persistence, favored by the use of the Al(OH)₃ depot adjuvant, giving time for FDC to eventually appear and nucleate a GC reaction as late as 2 wk after immunization. Similarly, increased Ag dose and time of Ag availability have been shown to substitute for FDC-stored Ab-complexed Ags in the induction of IgG responses in TNFR1^{-/-} mice devoid of classical GC (55). It is also of interest to note that although the delayed and limited early life GC reaction significantly limits ASC differentiation and primary IgG responses, it is nevertheless sufficient to allow the generation of memory B cells (20) and the Ab avidity maturation of certain T-dependent protein Ags (56). This is in accordance with the fact that memory B cells are generated relatively late in the GC reaction (57) while the avidity maturation process may extend beyond the GC compartment.

We report here a striking chronological and functional correlation between the delayed development of a mature FDC network, in the context of a maturing microarchitectural structure of spleen and nodes, and the timing and strength of GC, ASC, and IgG responses to T-dependent Ags during the first postnatal weeks. This demonstrates that the delayed FDC differentiation process is an essential factor limiting early life *in vivo* B cell responses, in addition to factors which may affect neonatal APC, T, and/or B cells and thus limit neonatal and early life Ab responses to T-dependent Ags (reviewed in Ref. 10). It had been previously observed that even the most potent immunostimulants, such as CpG-immunostimulating motifs which strongly activate neonatal as well as adult APC, T, and B cells (34), only significantly enhance primary neonatal IgG responses after a delay of 2–3 wk (7, 33). We show here that despite their multiple activation influences, these immunostimulating sequences do not accelerate the postnatal development of FDC, providing an explanation for their delayed influence on neonatal Ab responses and highlighting again the relative importance of FDC maturation for the development of neonatal responses to T-dependent Ags.

How do these observations in mice relate to the limitations of neonatal Ab responses observed in other species, particularly in human infants? The time required for completion of immune maturation differs widely among mammalian species. In mice, where postnatal immune maturation only requires ~6–8 wk, FDC first appear as immune complex-retaining cells at 2 wk (16, 58) and GC are absent from spleen and nodes until 3–4 wk of age (Ref. 59 and this report). This development is slightly slower in rats, where immune complex-retaining cells are reported only at 3 wk and GC

at 4–5 wk of age (13–15). Data related to FDC and GC development in humans are scarce but suggest yet much slower kinetics: although primary B cell follicles are already present during fetal life, existing studies report the appearance of mature FDC clusters around 2 mo of age and GC at 4 mo of age (Refs. 60 and 61) and W. Timens and A. Zandvoort, personal communication). Unresponsiveness at the neonatal FDC precursor level, and thus delayed differentiation into mature FDC and limited GC/ASC/Ab responses, could explain the lack of primary neonatal responses to even the most immunogenic T-dependent Ags (1, 2). Should this be the case, the identification of critical molecular interactions that may enhance the differentiation of neonatal FDC precursors into mature FDC may prove critical to the many early life immunization strategies requiring rapid induction of strong Ab responses.

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