

# Development of T–B cell collaboration in neonatal mice

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## Abstract

The neonatal immune response is impaired during the first weeks after birth. To obtain a better understanding of this immaturity, we investigated the development of T cell interactions with B cells in mice. For this purpose, we analyzed the immune response to three T-dependent antigens *in vivo*: (i) the polyclonal antibody response induced by vaccinia virus; (ii) the production of polyclonal and specific antibodies following immunization with hapten–carrier conjugates; (iii) the mouse mammary tumor virus superantigen (sAg) response involving an increase in sAg-reactive T cells and induction of polyclonal antibody production. After vaccinia virus injection into neonates, the polyclonal antibody response was similar to that observed in adult mice. The antibody response to hapten–carrier conjugates, however, was delayed and reduced. Injection with sAg-expressing B cells from neonatal or adult mice allowed us to determine whether B cells, T cells or both were implicated in the reduced immune response. In these sAg responses, neonatal T cells were stimulated by both neonatal and adult sAg-presenting B cells but only B cells from adult mice differentiated into IgM- and IgG-secreting plasma cells in the neonatal environment *in vivo*. Injecting neonatal B cells into adult mice did not induce antibody production. These results demonstrate that the environment of the neonatal lymph node is able to support a T and B cell response, and that immaturity of B cells plays a key role in the reduced immune response observed in the neonate.

## Introduction

Shortly after birth, antibody responses to type 1 T-independent antigens are high, whereas T-dependent antibody secretion is often delayed and reduced (for review see 1,2). For most T-dependent antigens, only weak antibody production has been found until 2–8 weeks after birth (3–5). Immaturity of B cells, T cells and macrophages as well as the presence of maternal antibodies and reduced antigen trapping by follicular dendritic cells have been implicated in this impaired T-dependent immune response (2,6–9). Although T cells can be primed shortly after birth (10–13), they have been shown to secrete a limited panel of cytokines when compared to adult mice (2,14). Priming of mice shortly after birth with T-dependent antigens can result in strong T<sub>h</sub>1 or T<sub>h</sub>2 responses when re-challenged *in vitro* or *in vivo* later in life (11,15).

To investigate the role of B and T cells in the reduced

neonatal immune response we compared the *in vivo* antibody production of mice at different ages within the first 10 days after immunization. Three T-dependent antigens with different immune-stimulation properties were used: (i) protein–hapten complexes [NP-CGG (4-hydroxy-3-nitrophenyl) acetyl conjugated to chicken gammaglobulin] (16), (ii) vaccinia virus (17) and (iii) mouse mammary tumor virus (MMTV) superantigens (sAg) (18).

It has been shown that injection of adult mice with NP-CGG in alum (with or without addition of pertussis toxin) results in a strong IgG response which becomes detectable 5–6 days after immunization and further increases by day 10 (16). Similarly, vaccinia virus induces a strong T–B cell collaboration, and polyclonal B cell differentiation to IgG secretion within 6 days of injection into adult mice. The specific anti-vaccinia IgG response becomes detectable later (17). MMTV

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exists either as an endogenous provirus (e.g. *Mtv-7*) or as infectious retrovirus particles (MMTV). Endogenous *Mtv-7* and infectious MMTV express sAg on the surface of B lymphocytes (18–20), which stimulate T cells expressing one or several TCR V $\beta$  elements (18,21,22). Injection of adult mice with *Mtv-7*-expressing B cells or infection with MMTV(SW) result in a strong and rapid activation of sAg-specific T cells (23,24). Thereafter, B cells receive sAg-mediated 'cognate' help from activated T cells and differentiate into IgG-secreting plasma cells within 5–6 days of virus injection (25,26). T cell priming as well as extrafollicular and follicular B cell differentiation are very similar to responses to classical antigens (16). For MMTV(SW) and *Mtv-7*, the sAg-presenting cells are B cells and the responding CD4<sup>+</sup> T cells express TCR V $\beta$ 6 (21,24,25).

Shortly after birth, the spleen contains only few mature B and T cells, whereas neonatal lymph nodes have comparable proportions of B and T cells to adult mice. Therefore, we decided to analyze the immune response in peripheral lymph nodes. Our study demonstrated that neonatal mice produced IgG antibodies after vaccinia immunization at levels similar to adult mice. Since responses to classical antigens were reduced and delayed in neonatal mice, we used MMTV sAg to show that (i) the lymph node architecture was not responsible for the neonatal unresponsiveness; (ii) T cell stimulation by either neonatal or adult B cells expressing MMTV sAg was similar; (iii) antibody secretion was only observed after injection of B cells derived from adult mice and (iv) neonatal B cells did not differentiate into antibody-secreting cells in an adult or neonatal environment. These data suggest that unless additional factors are present, neonatal B cells are unable to respond to or to induce T cell help.

## Methods

### *Mice*

BALB/c mice were obtained from Harlan Olac (London, UK). MMTV(SW)-infected BALB/c breeders were selected from mice originally purchased from IFFA Credo (L'Arles, France). *Mtv-7* congenic BALB/c (BALB.D2) (27) were bred at the Swiss Institute for Cancer Research (ISREC).

### *MMTV isolation*

Milk was aspirated from lactating MMTV(SW)-infected BALB/c mice after i.p. injection of 0.5 IU of oxytocin (Sandoz, Basel, Switzerland) (24). Before storing as aliquots at  $-70^{\circ}\text{C}$ , the milk was diluted 1:3 in PBS, pH 7.4, centrifuged at 600 *g* for 10 min to skim and remove cells. The virus was titrated as previously described and  $\sim 5 \times 10^7$ – $10^8$  virus particles were injected per mouse (24).

### *Antigens and immunizations*

NP (Cambridge Research Biochemicals, Northwich, UK) was conjugated to CGG (Sigma, St Louis, MO). Mice of different ages were injected either with 20  $\mu\text{g}$  alum-precipitated (Sigma) NP-CGG with or without  $5 \times 10^8$  *Bordetella pertussis* organisms (Lederle, Gosport, UK) (these reagents were kindly provided by I. C. M. MacLennan, Birmingham, UK),  $10^6$  p.f.u. of wild-type vaccinia virus (kindly provided by F. Luthi, Lausanne,

Switzerland),  $10^8$  MMTV(SW) particles or  $3 \times 10^5$  lymph node cells of *Mtv-7*-congenic BALB.D2 mice. Popliteal lymph nodes of BALB.D2 mice were removed and mechanically dissociated in sterile PBS. T cell-depleted lymph node cells were obtained after incubation of total lymph node cells with anti-Thy-1 (AT83) (28), anti-CD4 (3.168.8.1) (29) and anti-CD8 (RL172-4) (30) antibodies and rabbit complement (Saxon Europe, Suffolk, UK). After washing and counting, cells were injected into both hind footpads of neonatal mice. To ensure that the transferred cells reached the neonatal lymph nodes, control cells were labeled for 1 min with the aliphatic fluorescent chromophore PKH26 (Sigma). At different time points after injection into the neonates, transferred cells were visualized on frozen sections obtained from the draining popliteal lymph nodes using fluorescence microscopy.

### *Flow cytometry*

Lymphocytes were labeled with the following antibodies: FITC-conjugated anti-V $\beta$ 6 (44-22-1) (31) FITC-conjugated anti-B220, phycoerythrin (PE)-conjugated anti-CD4 (GK1.5; Becton Dickinson, Basel, Switzerland), PE-conjugated anti-CD8 (Boehringer, Mannheim, Germany). The cells were analyzed on a FACScan (Becton Dickinson) after exclusion of dead cells using forward and side scatter analysis.

### *ELISPOT assay*

The numbers of Ig-secreting cells as well as Ig isotypes were determined as previously described (32). Briefly, microtiter plates (Maxisorp; Nunc, Oslo, Norway) were coated with goat anti-mouse IgG and IgM (Tago, Burlingame, CA), and cells isolated from lymph nodes were serially diluted from  $10^5$  cells/well and incubated for 4 h at  $37^{\circ}\text{C}$ . The Ig-secreting cells were then determined with biotinylated goat anti-mouse IgM, IgG1, IgG2a, IgG2b and IgG3 (Caltag, San Francisco, CA). After incubation with a streptavidin-conjugated alkaline phosphatase (Boehringer), the plates were developed with a solution of 5-bromo-4-chloro-3-indolyl phosphate (Sigma). The spots were counted under a dissection microscope.

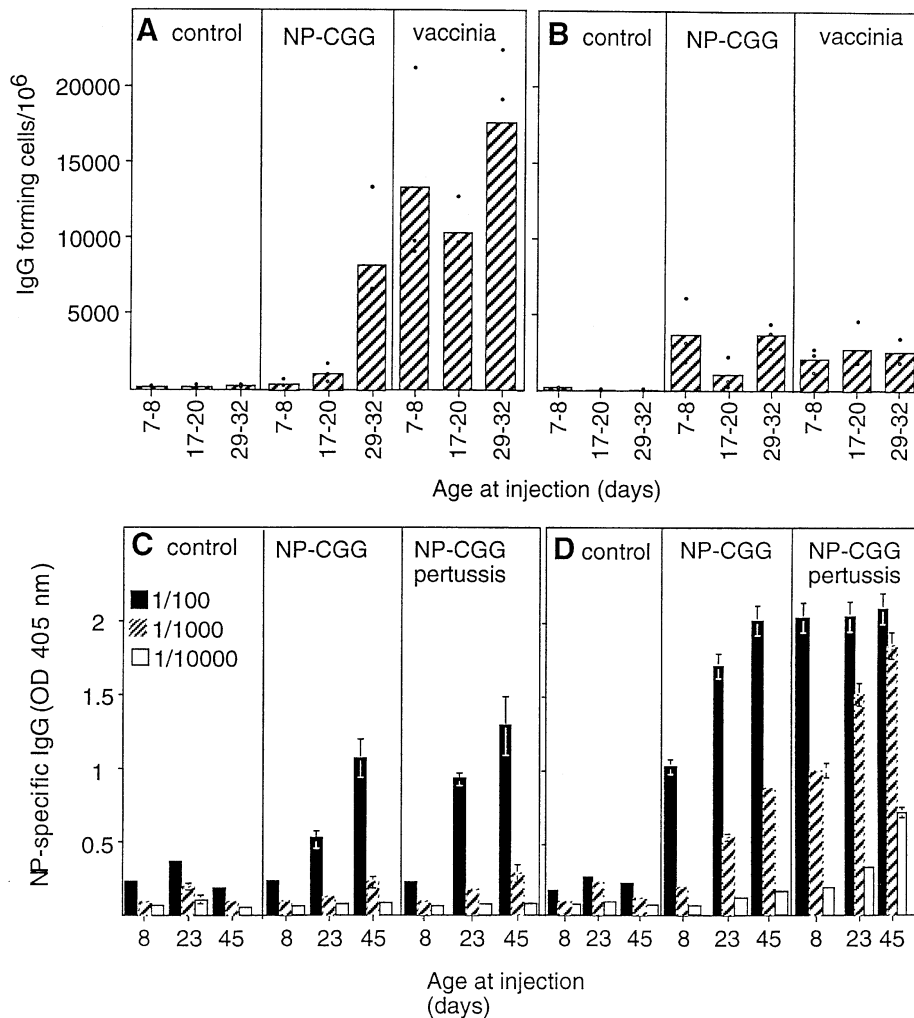
### *ELISA*

Flat-bottom 96-well microtiter plates were coated overnight at  $4^{\circ}\text{C}$  with 50  $\mu\text{l}$  of either NP-BSA (5  $\mu\text{g}/\text{ml}$ ) or anti-murine IgG + IgM (2  $\mu\text{g}/\text{ml}$ ) (Tago). After washing with 0.05% Tween 20 in PBS, the plates were blocked with 1% BSA. Diluted test sera were added after 1 h and incubated overnight at  $4^{\circ}\text{C}$ . A high titer anti-NP immune serum of BALB/c mice or murine IgG and IgM mAb were used as positive controls. Pre-immune serum of BALB/c mice served as a negative control. Bound antibodies were detected using biotinylated goat anti-murine IgM (Caltag) or anti-murine IgG (Amersham Rahn, Zurich, Switzerland) followed by streptavidin-conjugated alkaline phosphatase (Boehringer). The absorbance at 405 nm of *p*-nitrophenyl phosphate (Sigma) filled wells was determined with a Titertek Multiscan spectrophotometer.

## Results

### *Induction of a polyclonal IgG response by vaccinia virus in neonatal mice*

Since vaccinia virus is a potent inducer of cytokines and of T and B cell responses, we injected the virus into the hind



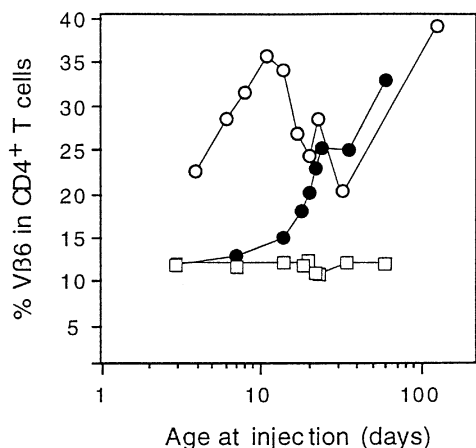
**Fig. 1.** Antibody response to vaccinia virus and NP-CGG in neonatal and adult mice. Mice at the indicated ages were injected into the hind footpad with either vaccinia virus or NP-CGG in Alum. When indicated NP-CGG was injected together with killed *B. pertussis* bacteria. Either 6 or 10 days later, the number of IgG-secreting cells in the popliteal draining lymph node was determined by ELISPOT (A and B). The specific IgG response towards NP-CGG in serum is shown in (C; day 6) and (D; day 10) as determined by ELISA. Controls are non-injected littermates.

footpad of mice of different ages and studied the early polyclonal B cell activation in the draining popliteal lymph nodes. Mice aged 7–8, 17–20 and 29–32 days produced a strong polyclonal B cell activation and polyclonal IgG secretion within 6 days after injection of vaccinia virus as shown by ELISPOT (see Fig. 1A). Ten days after injection, this response decreased in all animals (see Fig. 1B). At these early time points no vaccinia-virus specific antibodies were detectable in the serum (data not shown). These results show that neonatal mice are already able to produce a comparable polyclonal T cell-dependent antibody response to that observed in adult mice in the lymph node draining the site of injection.

#### *Development of the antibody response to hapten-protein complexes*

NP-CGG in alum, with or without the addition of killed *B. pertussis* bacteria, was used to investigate a classical hapten antigen response in neonatal mice. NP-specific IgG serum

levels were measured by ELISA at 6 and 10 days after immunization (Fig. 1C and D). Six days after immunization, no anti-NP antibody responses were detected in 8-day-old mice. Although the response to NP-CGG became detectable 10 days after injection it was still 10-fold lower than that of adult animals. In mice of all ages, at this later time point *B. pertussis* increased the specific IgG responses. The numbers of IgG-secreting B cells in the draining popliteal lymph nodes were determined using an ELISPOT assay (Fig. 1A and B). Six days after injection, very few Ig-secreting cells were found in 8-day-old mice compared to high levels in adult mice. However, 10 days after immunization, neonatal and adult mice had intermediate levels of antibody-secreting cells. Polyclonal activation followed similar kinetics to the specific antibody response. These results show that neonatal mice are capable of producing T cell-dependent antibody responses to soluble antigens; however, the responses are weaker and delayed when compared to adult mice. To address the question whether reduced frequency of antigen-reactive



**Fig. 2.** Kinetics of sAg-induced T cell activation. BALB/c mice were injected into the footpad at different ages after birth with MMTV(SW)-infected milk (●) or lymph node cells from adult BALB.D2 mice (containing  $0.9 \times 10^6$  B cells) (○). Control un.injected mice are represented by (□). T cell-depleted or total lymph node cells gave similar results provided that the number of injected B cells was kept constant. Six days after injection, the increase in  $V\beta 6^+ CD4^+$  T cells was measured by FACS analysis. Each time point shows the age of mice at the time of the injection and represents an experiment with a pool of three to six mice. The kinetics were repeated 3–4 times in separate experiments and similar results were obtained.

B cells or immaturity of the immune response were responsible for this reduced and delayed response, we used MMTV sAg.

#### Development of the *Mtv-7* sAg-induced neonatal peripheral T cell response

To analyze the development of the sAg-mediated T cell response, we injected BALB/c mice with lymph node cells from *Mtv-7*-congenic mice. Lymph node cells ( $3 \times 10^6$ ) from adult or 10-day-old *Mtv-7*-congenic BALB.D2 mice were injected into the footpad of recipient BALB/c mice of different ages—ranging from 3 to 60 days. To ensure that cells transferred to the neonatal or adult footpad reached the lymph node efficiently, adult lymph node cells were labeled with an aliphatic fluorescent chromophore before injection. Fluorescence microscopy analysis on frozen sections showed that 3 h after injection, large numbers of adult cells had reached the subcapsular sinus of the lymph node and had colonized the body of the node at 24 h (data not shown).

As BALB.D2 B lymphocytes express the *Mtv-7*sAg constitutively, these mice are almost completely devoid of  $V\beta 6^+ CD4^+$  T cells due to clonal deletion. T cells from neonatal BALB/c mice receiving lymph node cells from adult BALB.D2 mice responded maximally in the peripheral draining lymph node independently of the age of the recipient (Fig. 2). The reduced response in 30-day-old mice receiving BALB.D2 lymph node cells was not seen in other experiments. To ensure that neonatal T cells can respond to sAg in the absence of adult T cells, we depleted donor BALB.D2 T cells prior to transfer. The sAg response remained unchanged (data not shown).

To test whether neonatal B cells also have the ability to induce a sAg response in neonatal BALB/c mice, *Mtv-7*-congenic B cells from lymph node of 12- to 18-day-old mice or as a control B cells from adult mice were injected into

**Table 1.** Adult and 12-day-old BALB/c mice were injected with lymph node cells from 12-day-old, 18-day-old or adult *Mtv-7* expressing BALB.D2 mice

Age of donor BALB.D2 mice (days)	Recipient BALB/c	Percent $V\beta 6^+ CD4^+$ T cells	s.f.c./ $10^6$ cells IgG
–	90	13.7	0
90	90	36.7	6080
18	90	28.2	280
12	90	38.1	170
–	12	13.6	0
90	12	31.2	7200
18	12	26.9	320

Lymph node cells corresponding to  $9 \times 10^5$  B cells were injected. Uninjected mice were used as controls. The sAg-induced T cell response (by FACS analysis) and the antibody secretion patterns (by ELISPOT assay) were measured 6 days after footpad injection. Each value represents an experiment with a pool of three to four mice. The experiment was performed 4 times. Antibody secretion by B cells was always very low when B cells from young mice were injected and was high when B cells from adult mice were injected. In three out of four experiments we obtained T cell stimulations as shown in the table, whereas in the fourth, the increase in  $V\beta 6$  T cells was weaker in newborn mice than in adult mice.

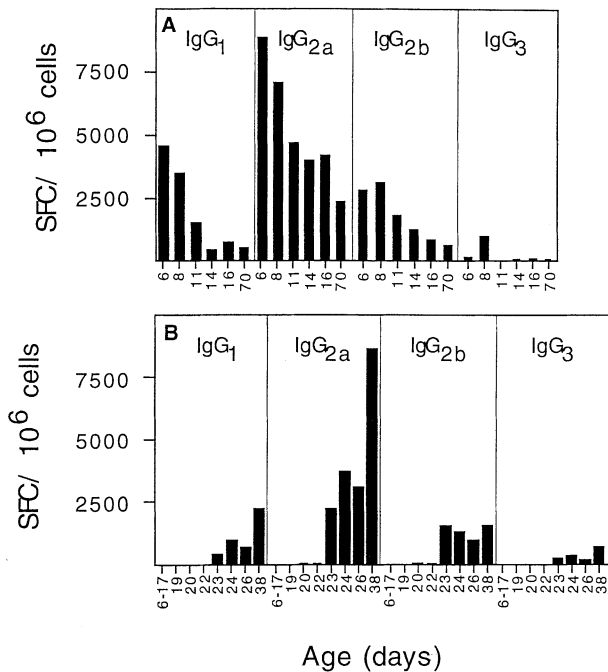
mice of different ages. A clear and strong activation was reproducibly seen in all combinations (Table 1). Injection of splenocytes instead of lymph node cells from young BALB.D2 mice (12–18 days) induced only a weak sAg response (data not shown, see Discussion).

These results indicate that neonatal T cells are sufficiently mature to mount a strong sAg response.

#### Antibody secretion after adoptive transfer of *Mtv-7* sAg-expressing B cells

Having shown that neonatal T cells respond to sAg, we asked whether these T cells could help B cells to differentiate into antibody-secreting cells. To answer this question, *Mtv-7*-congenic B cells from lymph nodes of 12-, 18- or 90-day-old mice were injected into neonatal or adult BALB/c mice. Six days after footpad injection, popliteal lymph nodes were removed and the number of antibody-secreting B cells quantified by ELISPOT assay (Table 1). Despite the comparable T cell response in all combinations, strong antibody secretion was only observed when B cells from adult BALB.D2 mice were injected into neonatal or adult mice. Injection of B cells from neonatal mice did not lead to strong antibody secretion even in adult recipients. To see whether the B cell response was delayed in neonatal mice, we analyzed antibody secretion 10 days after injection of neonatal BALB.D2 B cells into neonatal or adult BALB/c recipients. No antibody response was detectable in ELISPOT assays (data not shown).

To determine the Ig isotypes induced by the sAg response, we used four groups of mice of different ages. Two groups were injected with BALB.D2 lymphocytes from adult or neonatal (day 10) mice. The third was injected with MMTV(SW)-infected milk and the fourth remained unimmunized. When neonates or adult mice were injected with lymph node cells from adult BALB.D2 mice, a strong IgG response was found.



**Fig. 3.** Kinetics of IgG isotype secretion after MMTV(SW) or *Mtv-7* cell injection. At different ages after birth, BALB/c mice were injected into the footpad with adult *Mtv-7* BALB.D2 cells ( $3 \times 10^6$ ) (A) or MMTV(SW)-infected milk (B). The results for total lymph node cells were similar to T cell depleted lymph node cells. Six days post-injection, cells from the draining popliteal lymph node were isolated and the number of B cells secreting different isotypes (s.f.c.) was assessed by ELISPOT assay. Injection of neonatal *Mtv-7* congenic lymphocytes induced no significant Ig secretion (data not shown). Each point represents the mean of three to six mice and each experiment was repeated 3 times with similar results. In (B) day 6–17 are the results from mice injected at 6, 8, 15 or 17 days which all were negative.

The numbers of IgG2a-secreting cells were predominant (Fig. 3A). This initial IgG response was reduced between the ages of 17 and 34 days, and the response in neonatal mice was always stronger than that in adult mice. The transfer of T cell-depleted BALB.D2 lymphocytes from adult mice showed a similar antibody response. Therefore we concluded that by the age of 6 days, neonatal T cells are mature enough to help to establish a sAg-induced antibody response, provided efficient antigen presentation occurs.

Injection with infectious MMTV(SW) does not lead to a significant sAg response before 10 days of birth (Fig. 2). The absence of T cell stimulation following MMTV(SW)-injection prior to 10 days of age is due to a lower level of infection of sAg-presenting cells (data not shown). Between day 20–25, however, sAg-induced T cell stimulation was comparable (Fig. 2 and data not shown). Neonates injected with MMTV(SW)-infected milk did not show a humoral immune response before 20 days of age (Fig. 3B). From 20 to 22 days only a few IgM- and IgG-secreting cells were detected. Thereafter, on days 23, 24 and 26 the number of antibody-secreting cells reached levels comparable to those observed in adult mice, and were mainly of IgG2a isotype. These results show that despite normal T cell responses, B cells from 20-

to 22-day-old mice are not induced to secrete antibodies after MMTV injection.

We also analyzed the antibody-secretory capacity of the neonatal Peyer's patches which, as established previously, develop a sAg-dependent T cell response to MMTV shortly after birth (33). Peyer's patches chronically infected by the oral route in BALB/c neonates showed a full capacity to secrete IgA and IgG isotypes within 9 days of birth (data not shown).

## Discussion

In this study we investigated the reduced immune response of neonatal mice. Using vaccinia virus infection and adjuvant-antigen injections we confirmed earlier observations indicating that neonatal mice were able to mount a strong immune response after viral infection but showed reduced antibody responses after antigen application. Taking advantage of endogenous sAg expressed by B cells we were able to show that this reduced immune response of the neonate was due to an immaturity of the B cell *in vivo*. Neonatal T cells as well as the environment of the neonatal lymph node were not responsible for this reduced responsiveness.

The capacity of mice to induce a specific antibody response to T-dependent antigens develops gradually during the neonatal period. At this early stage of life, the development of the immune response depends on the type of antigen, adjuvant and the mouse strain used (3,4,34). A strong increase in the number of B and T cells bearing antigen receptors is observed in peripheral lymph nodes during the first week of life. Thereafter, the increase in lymphocyte number is only minor (34,35), suggesting that the lack of antigen-specific immune responses for most antigens in neonatal mice is not due to the absence of specific lymphocytes in the lymph nodes.

Several receptor-ligand pairs, either expressed by antigen-presenting cells (CD80/86, OX-40L and CD40) or by T cells (CD28, OX40 and CD40L), are crucial for antibody responses (for review see 36–38). It has been argued that one reason for the inability of neonates to mount an efficient antibody response is the low level of CD40L expression on T cells as this is one of the key co-stimulatory molecules responsible for inducing B cell activation and differentiation (39 and references therein). Here we show that neonatal T cells are fully capable of providing help to adult B cells *in vivo*. Therefore it seems unlikely that the low levels of cytokines secreted by or of co-stimulatory molecules expressed on neonatal T cells are responsible for the reduced antibody production.

Neonatal B cells in contrast to those of adult mice show a reduced proliferative response after cross-linking of surface Ig with anti-IgM, anti-IgD dextran or anti-IgD plus IL-4 (40–42). Recent evidence suggests different signal transduction patterns in neonatal B cells after Ig cross-linking (43,44). Utilization of  $T_H2$  clones, however, showed that neonatal B cells were able to elicit IgG and IgM responses *in vitro* (45). These results suggest that although neonatal B cells can be stimulated to secrete antibodies and to proliferate, they require stronger stimulation than B cells of adult mice.

Injection of vaccinia virus induced a strong T-dependent

polyclonal antibody response early after infection. A comparable polyclonal IgG response was found in mice of all ages tested. This virus is known to induce high levels of cytokines and type I and II IFN, as well as giving efficient co-stimulation and bystander activation (46). The strong immune stimulation induced by vaccinia virus might be required for induction of an IgG response in a neonatal environment where memory T and B cells are absent. It has been shown that polyclonal B cell activation by lipopolysaccharide can help specific antibody production in newborn mice (35). In addition, killed *B. pertussis* bacteria have been shown to induce strong IFN production, and enhanced the neonatal and adult immune response (47). This confirms the absence of an intrinsic defect in the generation of a T cell-dependent antibody response when additional stimulation is provided. Similarly utilization of strong adjuvants allows induction of antibody responses in neonatal mice (15).

To analyze the role of B or T cell maturity in this delayed antibody response, we used MMTV sAg. This system offers the following advantages: (i) the sAg is presented by B cells, (ii) T-B cell interaction is comparable to classical antigen systems (16), (iii) sAg-reactive T cells can be easily quantitated and (iv) MMTV sAg induce a strong T-dependent polyclonal IgG response in the absence of adjuvants (25,26). sAg therefore can be used as tools to determine the role of T and B cells during an immune response in neonatal and adult mice. A study using a similar approach suggested that neonatal T cells respond poorly to sAg-specific T cell stimulation (48). These results contradict our study and are most likely due to the use of neonatal *Mtv*-congenic splenocytes instead of lymph node cells. We also have repeatedly observed this lack of T cell stimulation when injecting BALB.D2 splenocytes into newborn mice (data not shown). In contrast to neonatal lymph nodes, neonatal spleen is a hematopoietic organ and contains only a few mature B cells. In this study we have clearly shown that neonatal T cells can respond to *Mtv-7* sAg expressed by B cells derived from adult or neonatal mice, but only induce B cell differentiation when B cells from adult mice were injected.

Taken together, our results show an inefficient differentiation of neonatal B cells into antibody-secreting plasma cells after interaction with neonatal or adult T cells. B cells are clearly responsible for the reduced antibody production. This inefficient B cell differentiation can be due to an inability of neonatal B cells to receive or stimulate T cell help.

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### Abbreviations

MMTV mouse mammary tumor virus  
NP-CGG (4-hydroxy-3-nitrophenyl) acetyl conjugated to chicken gammaglobulin

PE phycoerythrin  
sAg superantigen

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