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Upon TLR9 Signaling, CD5+ B Cells Control the IL-12-Dependent Th1-Priming Capacity of Neonatal DCs

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Summary

The susceptibility to infections and the strong Th2 bias observed in neonates are thought to be due to the immaturity of the dendritic cell (DC) compartment. We show that neonatal DCs, like their adult counterparts, elicit Th1 responses. We also demonstrate that during potentially harmful systemic inflammation, after Toll-like receptor (TLR) 9 triggering, neonatal B cells produce high concentrations of IL-10, preventing optimal IL-12 secretion by neonatal DCs and, thus, Th1 priming. Although both CD5+ and CD5− B cell subsets respond to CpG ODN stimulation, we found that only CD5+ B cells produce IL-10. Therefore, these results show the regulatory role of CD5+ B cells on DC activation in vivo for Th1/Th2 polarization and highlight the paradoxical effects of TLR triggering in vivo.

Introduction

Adaptive CD4 T helper cell responses can generally be divided into Th1 and Th2 types based on their cytokine secretion pattern. Th1 responses are characterized by IFN-γ secretion and efficiently eliminate intracellular pathogens and most viral infections, whereas Th2 responses are characterized by IL-4, IL-5, and IL-13 secretion and efficiently eliminate helminthes and are involved in allergy response (O'Garra, 1998). Experimental and clinical observations have shown that neonates exhibit less efficient cellular immune responses to pathogens than adults. Moreover, human and murine neonatal immune responses are polarized mainly towards the Th2 type (for review see Adkins et al., 2004; Siegrist, 2001). These immature or nonprotective immune responses are responsible for the high levels of morbidity and mortality among neonates and young children due to infectious diseases, especially in developing countries (Siegrist, 2001). However, murine and human neonatal CD4 and CD8 T cells can mount adult-like responses when stimulated in appropriate conditions; for example, with appropriate adjuvant such as Freund's complete adjuvant (Forsthuber et al., 1996), with bacterial DNA plasmid encoding microbial antigen (Martinez et al., 1997), with low doses of virus (Sarzotti et al., 1996), or with adult antigen-presenting cells (APCs) (Ridge et al., 1996).

At least two conditions are required to induce efficient CD4 T cell responses: (1) the maturation of APCs, i.e., an increase in the number of MHC molecules and costimulatory molecules on APCs to activate naive T cells (Banchereau and Steinman, 1998), and (2) the secretion of Th1- or Th2-driving cytokines, for example, IL-4 for Th2 cells and IL-12 for Th1 cells (O'Garra, 1998). Microbial products stimulate APCs to produce cytokines that influence the outcome of Th1 or Th2 responses. Bacterial DNA (or its synthetic mimic: synthetic oligonucleotides containing unmethylated CpG motifs, CpG ODN) triggers innate immune responses through TLR9 and induces the secretion of proinflammatory cytokines such as IL-6, IL-12, and TNF-α (Krieg, 2002), in which IL-12 in particular skews immune responses towards Th1 (Trinchieri, 2003). Early studies suggest that human or murine neonatal APCs express lower levels of MHC II than do adult APCs, leading to poor T cell proliferation or IFN-γ secretion (Lu et al., 1979; Taylor and Bryson, 1985). Moreover, neonates would have a very small number of APCs, with a limited capacity to secrete IL-12 (Goriely et al., 2001; Muthukkumar et al., 2000). It is thus generally accepted that the immaturity of neonatal APCs limits T cell responses and is responsible for neonatal immune defects (Adkins et al., 2004). DCs are the only APCs that can activate naive T cells (Banchereau and Steinman, 1998). Thus, we focused on neonatal DCs. In contrast to previous studies, we recently showed that the spleen of neonatal mice contains a substantial number of DCs and that these DCs can secrete IL-12 and type I/II interferons in vitro and can also induce cytotoxic T lymphocyte (CTL) responses when adoptively transferred into adult recipients (Dadaglio et al., 2002; Sun et al., 2003). Here, we investigated the ability of neonatal DCs expressing a high level of CD11c to prime T helper cells in vivo as well as their role in T cell polarization in neonates. We show here that neonatal DCs are as fully competent as adult DCs in priming and polarizing Th1 responses. In addition, our findings identify an unexpected regulatory role of CD5+ B cells in DC activation in vivo for Th1/Th2 polarization through the secretion of IL-10.

Results

The Antigen Presentation and T Cell Stimulatory Abilities of Neonatal DCs Are Similar to Those of Adult DCs

We first evaluated the antigen (Ag) presentation and T cell priming abilities of neonatal DCs in vitro. CD11c+ DCs from 7-day-old neonatal BALB/c mice and from 6- to 10-week-old adult BALB/c mice (Sun et al., 2003) pulsed with peptide, protein, or bacteria; processed, and presented with the same efficiency as these Ag to MHC II restricted, IL-2 secreting T hybridomas. Indeed, DCs from 7-day-old neonatal mice present the PV1

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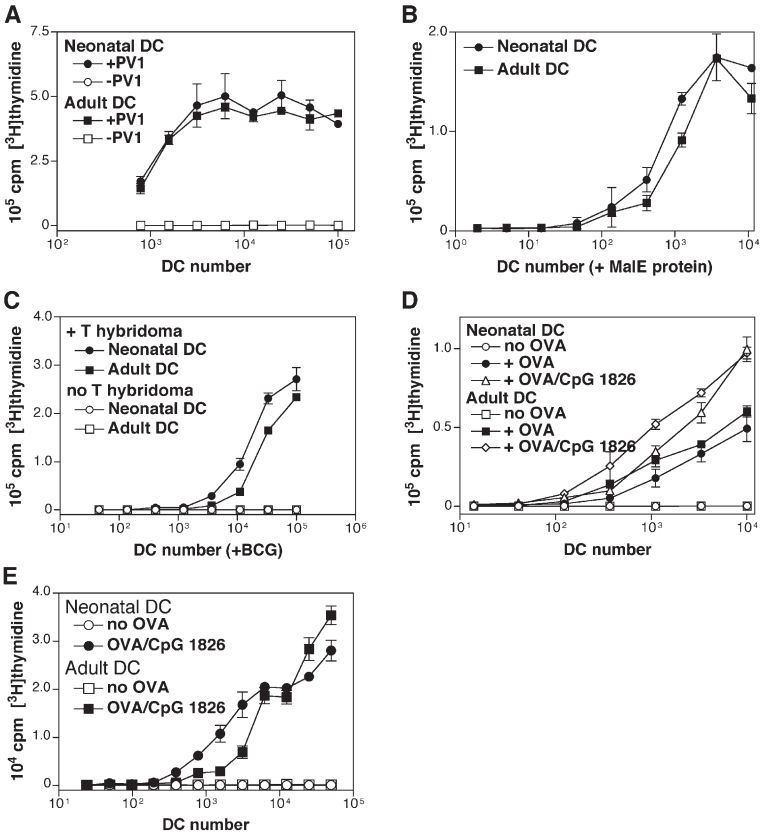

Figure 1. The Antigen Presentation and T Cell Stimulatory Abilities of Neonatal DCs Are Similar to Those of Adult DCs

(A–C) Serial dilutions of neonatal and adult BALB/c CD11c$^+$ DCs were cultured with or without antigens, i.e., 10 μg/ml PV1 peptide (A), 40 μg/ml MalE protein (B), 3 × 10^5 CFU BCG (C), and 10^5 corresponding IL-2-secreting MHC II-restricted T hybridomas. DCs cultured with BCG but without hybridomas were included as a control in (C). The CTL-L cell line was used to measure the IL-2 concentration in the supernatants after 24 hr of culture.

(D and E) Serial dilutions of neonatal and adult C57BL/6 CD11c$^+$ DCs were pulsed with or without 10 μg/ml $OVA_{323–339}$ peptide (D) or OVA protein (E) in the presence or absence of 10 μg/ml CpG 1826 and then cultured with 10^4 OT-II CD4 T cells specific for the $OVA_{323–339}$ peptide for 3 days. The proliferation of cells is expressed in cpm of incorporated [^{3}H]-thymidine.

The results are shown as mean of cpm of replicates (±SD, indicated by error bars), corresponding to incorporated thymidine. Results are representative of at least two similar experiments.

peptide as efficiently as adult DCs to a specific I-E^d-restricted T hybridoma (Figure 1A). Identical results were obtained with different peptides presented by I-A^d and I-A^b molecules to corresponding hybridomas (data not shown). Likewise, neonatal DCs also processed protein Ag or live BCG as efficiently as adult DCs, as shown by MHC II-restricted presentation to specific T cell hybridomas (Figures 1B and 1C). When DCs were stimulated by BCG in the absence of T hybridoma, no IL-2 was induced (Figure 1C). These results clearly show that neonatal DCs are fully competent in Ag processing and MHC II presentation. Because T hybridomas are only weakly dependent on costimulatory molecules, we further evaluated the ability of neonatal DCs pulsed with OVA peptide or protein to stimulate naive OT-II CD4 T cells (Figures 1D and 1E). Neonatal DCs induced OT-II CD4 T cell proliferation as efficiently as adult DCs. Stimulation with CpG 1826 activated both adult and neonatal DCs and increased their T cell priming ability to a similar extent. Thus, neonatal and adult DCs have equal antigen presentation and T cell-stimulating abilities.

Neonatal DCs Induce Th1 Responses In Vivo in Adult Recipients but Th2 Responses in a Neonatal Environment

Because neonatal and adult DCs show the same T cell priming ability in vitro, we thus investigated the capacity of neonatal DCs to prime Th1/Th2 responses in vivo. DCs from 7-day-old mice were purified and loaded with the PV1 peptide, or left unloaded, before being injected i.v. into syngeneic adult mice. Two weeks later, we measured the IFN-γ and IL-5 released by splenocytes upon in vitro restimulation with the PV1 peptide. PV1-loaded neonatal DCs induced a predominant Th1 response in adults that was characterized by strong IFN-γ production and low IL-5 production, whereas no peptide-specific cytokine secretion was observed after injection of unloaded DCs (Figure 2A). No IL-4 was detected under these conditions (data not shown). The same profile and level of T cell cytokine secretion were observed when PV1-loaded adult DCs were injected into adult recipients by the i.p. or the i.v. route (Figure 2A). Next, we asked whether neonatal DCs would also induce such a predominant Th1 response in neonatal recipients. When PV1-loaded DCs from 7-day-old mice were i.p. injected into age-matched syngeneic recipients, the peptide-specific T cell response was characterized by the secretion of large amounts of Th2 (IL-4, IL-5, IL-13) cytokines in the absence of IFN-γ response (Figure 2B). Likewise, PV1-loaded adult DCs also primed a strong Th2 response in neonates. The predominant Th2 response induced was not due to the intrinsic pattern of cytokine production by neonatal T cells, as large amounts of both Th1and Th2 cytokines were produced by these T cells upon concanavalin A stimulation (Figure 2B, ConA control). The same results were also obtained in C57BL/6 mice (data not shown). We conclude that neonatal DCs are fully competent for T cell priming in vivo and do not intrinsically polarize the immune re-

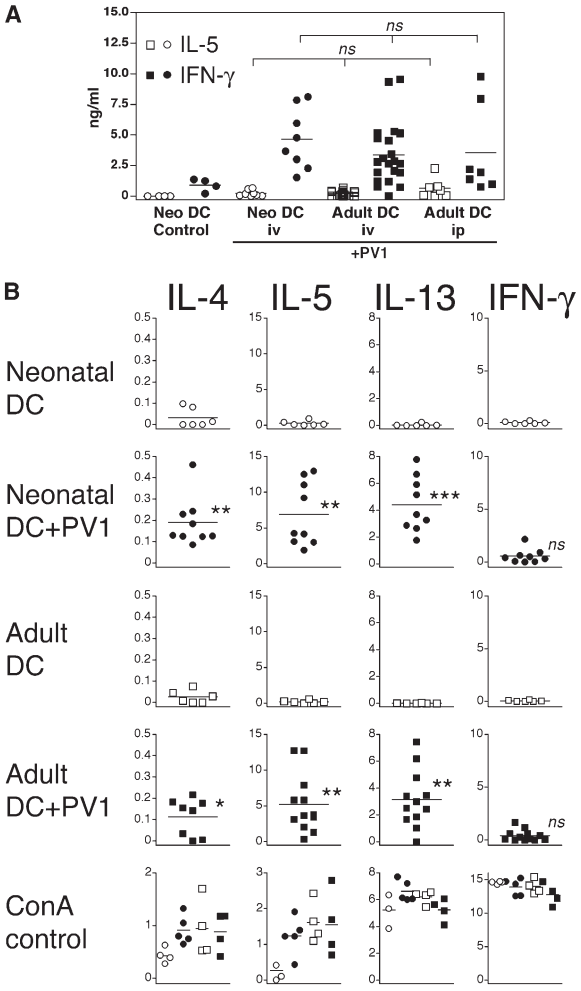

Figure 2. Neonatal DCs Induce Th1 Responses In Vivo in Adult Recipients but Th2 Responses in a Neonatal Environment

Purified neonatal or adult BALB/c CD11c$^+$ DCs (0.5–2 × 10^5) were left untreated or loaded with PV1 and injected i.v. or i.p. into adults (A) or injected i.p. into neonatal syngeneic recipients (B). Two weeks later, splenocytes from immunized and control mice were restimulated in vitro with or without 10 μg/ml PV1 peptide. Cytokines in the culture supernatants were measured by ELISA for IFN-γ IL-5 (A and B), IL-4, and IL-13 contents (B). Results are expressed in ng/ml. In (B), data concerning the splenocyte response to ConA stimulation are shown as a positive control for cytokine production. Each point corresponds to an individual mouse. In (A), statistical analysis with one-way ANOVA is depicted for the IL-5 and IFN-γ secretion between the three groups as indicated, and $p > 0.05$ was considered not significant (*ns*). For (B), the secretion of IL-4, IL-5, IL-13, and IFN-γ of groups immunized with DCs + PV1 were compared with their respective controls (DCs without PV1), and the p values were determined by t test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). Levels of IL-5 and IFN-γ cytokines induced by DCs + PV1 in adult (A) and in neonatal (B) recipients were significantly different ($p < 0.01$).

sponse to Th2, a feature that is therefore under the control of the neonatal microenvironment.

In Vitro, but Not In Vivo, Activation by CpG ODN Enables Neonatal DCs to Induce Th1 Responses

Mature DCs are critical to properly prime T cells (Banchereau and Steinman, 1998). Besides, IL-12 secreted by APCs has been shown to be one of the most important Th1 polarization factors (Trinchieri, 2003). Purified neonatal DCs activated in vitro with CpG ODN produce a high level of IL-12p70 (1–2 ng/ml) and 50- to 100-fold more IL-12p40 (Figure 3A). Similar responses were obtained with different types of CpG ODN (Figure 3A and see Figure S1A in the Supplemental Data available with this article online). To further evaluate the capacity of neonatal DCs to stimulate neonatal Th1 responses, they were first in vitro instructed with CpG 1826. Under these conditions, neonatal DCs upregulated MHC II after 30 min, clearly showing that the activation process was engaged, although the upregulation of CD86 was slightly detectable at 1 hr (Figure 3B). Neonatal and adult DCs cultured with CpG 1826 and loaded with PV1 peptide in vitro induced a Th1 response upon injection into neonatal recipients (Figure 3C, closed symbols). As expected, DCs cultured with control ODN (Ctrl 1982) still primed Th2 responses (Figure 3C, open symbols). Hence, mature neonatal DCs secreting IL-12 can induce Th1 responses in neonates. We then asked whether neonatal DCs would also mature in the same manner in vivo. We injected CpG 1826 into adult or neonatal mice. Three hours later, DCs were purified and loaded with the PV1 peptide. Surprisingly, unlike adult DCs activated in vivo, neonatal DCs activated in vivo failed to induce Th1 responses in neonatal recipients (Figure 4A). Neonatal and adult DCs, purified after administration of Ctrl 1982, both induced a Th2 response. Importantly, CpG 1826 induced upregulation of MHC II, CD40, CD80, and CD86 molecules on neonatal and adult DCs with the same efficiency (Figure 4B and data not shown). In addition, neonatal DCs activated by CpG 1826 in vivo and loaded with a MHC I-restricted CD8$^+$ T cell epitope of lymphocytic choriomeningitis virus (LCMV $NP_{118–126}$) induced a stronger CTL response in neonates, as compared to DCs from mice injected with Ctrl 1982 (Figure 4C).

Neonatal IL-10 Produced by B Cells after CpG ODN Administration Limits IL-12 Production by DCs

The fact that in vivo CpG ODN stimulation instructed adult DCs, but not neonatal DCs, for Th1 priming led us to analyze the amount of IL-12 secreted in neonates and adults. We thus injected neonatal and adult BALB/c mice with CpG 1826, Ctrl 1982, or phosphate-buffered saline. Three hours later, we analyzed the production of IL-12 by splenocytes ex vivo. Administration of CpG 1826 (5 mg per kg body weight) induced a strong IL-12p40 response in adults (2500 pg/ml for 100 μg CpG 1826, Figure 5A) but only a weak IL-12 response in neonates (368 pg/ml for 20 μg CpG 1826, Figure 5A). IL-12p70 was not detectable ex vivo after in vivo injection of CpG ODN (data not shown). Concurrently, CpG 1826 induced noticeable IL-10 secretion in neonates, but not in adults. Injection of neonates with 100 μg of CpG 1826 increased the amount of IL-10, and IL-12 secreted to the same extent (Figure 5A). The different types of CpG ODN led to a balanced production of IL-12p40 and IL-10 in neonates, whereas adult responses were characterized by a predominant IL-12p40 production (Figure 5B). The same results were obtained in C57BL/6 mice (data not shown), indicating that this phenomenon is not limited to BALB/c mice.

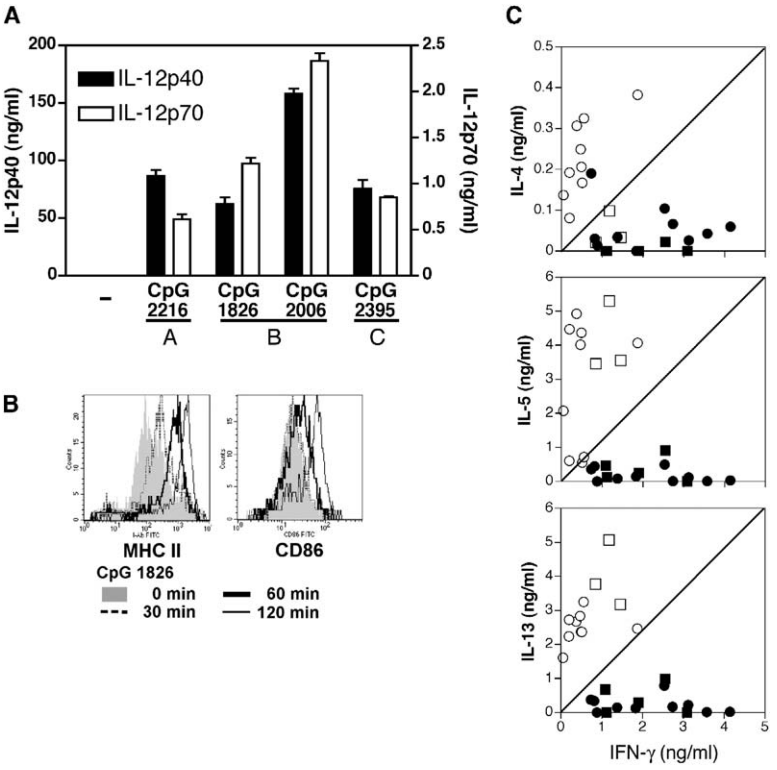

Figure 3. In Vitro Activation by CpG ODN Enables Neonatal DCs to Induce Th1 Responses

(A) 1.4 × 10^5 purified BALB/c neonatal DCs were cultured with or without 10 μg/ml of A, B, or C type of CpG ODN for 2 days, and then IL-12p40 (left y axis) and IL-12p70 (right y axis) secretions were analyzed by ELISA. Results are expressed as the mean ± SD of replicates.

(B) Purified BALB/c $CD11c^+$ neonatal DCs were analyzed for MHC II and CD86 expression after in vitro stimulation with 10 μg/ml CpG 1826 for 30, 60, and 120 min.

(C) 5–10 × 10^4 BALB/c $CD11c^+$ neonatal (circle symbols) or adult (square symbols) DCs were activated with CpG 1826 (closed symbols) or Ctrl 1982 (open symbols) with 10 μg/ml PV1 peptide and injected into neonatal recipients. One to two weeks later, splenocytes were stimulated in vitro with PV1 peptide and cytokines were analyzed by ELISA as in Figure 2. Each point corresponds to the responses obtained from individual mice in at least two independent experiments.

IL-10 is a potent anti-inflammatory mediator, which was first identified as a Th1 inhibitor (Moore et al., 2001) due to its ability to prevent IL-12 secretion by DCs (Koch et al., 1996). To confirm that IL-10 suppresses IL-12 production by neonatal DCs, we injected an anti-IL-10 receptor monoclonal antibody (α-IL-10R Ab [O'Farrell et al., 1998]) or a control immunoglobulin (Ctrl Ig, SFR8-B6) into neonates prior to in vivo administration of CpG 1826. As expected, neonatal DCs secreted more IL-12 after CpG ODN in vivo stimulation when IL-10 signaling was blocked, as compared to DCs from mice injected with Ctrl Ig (Figure 5C). DCs do not appear to be the source of IL-10, as we did not detect IL-10 secretion by neonatal DCs after in vivo or in vitro CpG 1826 stimulation (Figure 5C and data not shown). Therefore, we investigated the source of IL-10 in neonatal spleen cells in vitro after CpG ODN stimulation. FACS analysis of IL-10-secreting cells revealed that $B220^+$ and $CD19^+$ B cells, but not $CD3^+$ T cells or $CD11b^+$ myeloid cells, were mainly responsible for IL-10 production (Figure 6A). This result was confirmed by the low level of IL-10 production observed with neonatal spleen cells from B cell deficient μMT mice (Figure 6B). Importantly, neonatal spleen cells from μMT mice did not differ from wild-type C57BL/6 mice in the percentage of lin^- cells, of $CD3^+$ T cells, and of classical and plasmacytoid $CD11c^+$ DC, but an increase of $CD11b^+$ cells was observed (data not shown). In addition, stimulation with all types of CpG ODN of neonatal $CD19^+$ B cells induced a strong IL-10 response, whereas only low levels of IL-10 were produced by adult B cells (Figure 6C). Under these conditions, B cells produced very low levels of IL-12p40, and IL-12p70 was not detected (Figure S1B). To definitely assess the role of B cells in IL-10 production in vivo after TLR9 triggering, $CD19^+$ B cells were purified from neonatal C57BL/6 neonates or adults injected with CpG 1826. In vivo, 3 hr after CpG administration, neonatal B cells were activated as shown by the upregulation of CD69 (Figure 6D). In these conditions, neonatal $CD19^+$ B cells produced large amounts of IL-10, whereas a low level of IL-10 was secreted by adult B cells (Figure 6E). The lack of IL-10 production by B cells from CpG ODN-treated TLR9-deficient ($TLR9^{-/-}$) neonatal mice assesses the TLR9 dependency of the polyclonal activation of B cells (Figure 6E). It is noteworthy that CpG 1826 similarly stimulated adult and neonatal $CD19^+$ B cells for in vitro cell proliferation and IgM secretion, indicating that the level of TLR9 expression was not responsible for the difference in IL-10 response observed between neonatal and adult B cells (Figure S2).

Furthermore, administration of CpG 1826 to $IL\text{-}10^{-/-}$ neonates leads to the production of a high amount of IL-12 by splenocytes (Figure 6F), confirming the important role of IL-10 on IL-12 production in vivo. To assess the role of B cell-derived IL-10 in vivo, $IL\text{-}10^{-/-}$ neonates were transferred with $IL\text{-}10^{+/+}$ neonatal B cells prior to CpG administration. Under these conditions, the level of IL-12 induced by CpG ODN was dramatically reduced (Figure 6F). Therefore, although in vivo TLR9 triggering efficiently activates DCs for the upregulation of MHC and costimulatory molecules, it also generates different cytokine milieus in neonates and in adults. Thus, IL-10 secretion by neonatal B cells limits the ability of neonatal DCs to produce IL-12 without affecting their capacity to prime CTL.

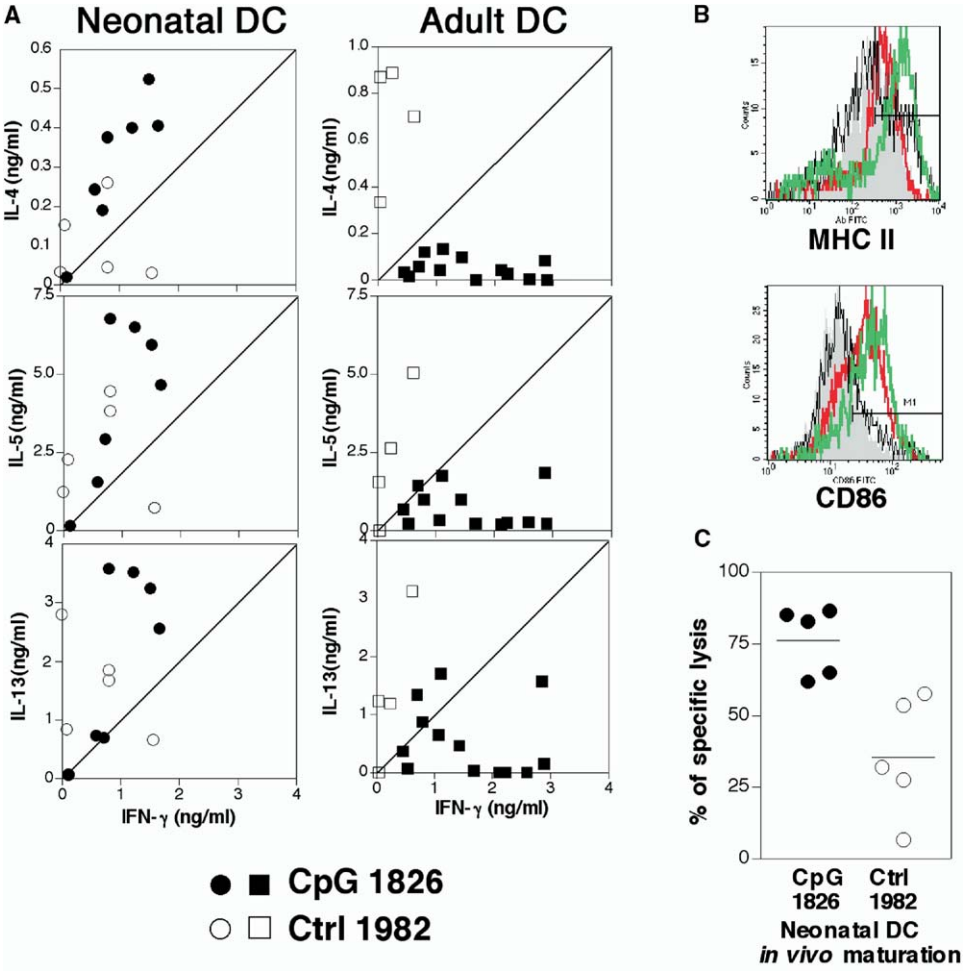

Figure 4. In Vivo Activation by CpG ODN Allows Adult, but Not Neonatal, DCs to Induce Th1 Responses

(A) Neonatal and adult DCs were purified from mice 3 hr after injection of 100 μg CpG 1826 (closed symbols) or Ctrl 1982 (open symbols), then 2–5 × 10^4 $CD11c^+$ neonatal (circle symbols) or adult (square symbols) DCs were loaded with PV1 peptide and injected into neonatal recipients. One to two weeks later, splenocytes were stimulated in vitro with PV1 peptide, and cytokines were analyzed by ELISA as in Figure 2. Each point corresponds to the responses obtained from individual mice in at least two independent experiments.

(B) Flow cytometry analysis showing the maturation profile of neonatal and adult DCs after injection of CpG 1826 (neonate, green line; adult, red line) or Ctrl 1982 (neonate, black line; adult, gray histogram). Three hours after injection, $CD11c^{high}$ DCs were stained for $I\text{-}A^d$ and CD86.

(C) After in vivo activation with CpG 1826 or Ctrl 1982, 1–2 × 10^5 BALB/c neonatal DCs were loaded with LCMV $NP_{118–126}$ peptide and injected into neonatal recipients. Two weeks later, splenocytes were restimulated with LCMV $NP_{118–126}$ and tested for CTL killing of LCMV $NP_{118–126}$-loaded P815 target cells with an effector-to-target ratio of 60:1. Lysis of nonloaded P815 cells was used as a negative control and never exceeded 4%.

In the Absence of IL-10 or of B Cells, In Vivo TLR9 Triggering of Neonatal DCs Allows Th1 Priming

We thus predicted that if neonatal DCs are activated in vivo by CpG ODN in an IL-10-free or in a B cell deficient environment, their Th1 priming ability would be restored. When neonatal DCs were activated in vivo by CpG 1826 in the presence of α-IL-10R antibody, they induced a Th1-biased response (Figure 7A). In contrast, DCs from neonates injected with Ctrl Ig still induced a Th2 response (Figure 7A). To confirm these results, we activated neonatal DCs in IL-10-deficient ($IL\text{-}10^{-/-}$) B6 mice. $IL\text{-}10^{-/-}$ neonatal DCs were stimulated with CpG ODN in vivo for 3 hr and loaded with a MHC II-restricted peptide before being injected into C57BL/6 wild-type neonates. Phenotypic analysis indicated that neonatal DCs were activated by CpG 1826 in vivo with the same efficiency in $IL\text{-}10^{-/-}$ and C57BL/6 wild-type mice (Figure S3). Again, CpG ODN-activated $IL\text{-}10^{-/-}$ neonatal DCs primed a strong Th1 response when injected into wild-type neonatal recipients. This response was marked by high concentrations of IFN-γ and the absence of IL-4, IL-5, and IL-13 (Figure 7B and data not shown). To determine the role of IL-10 produced by B cells in this process, μMT neonates were injected with CpG 1826 to activate DCs. Under these conditions, DCs were able to prime for Th1 responses when transferred to neonatal C57BL/6 recipients (Figure 7B). Neonatal DCs from untreated $IL\text{-}10^{-/-}$ or μMT mice still induced Th2 responses (Figure 7B). These data demonstrate clearly that neonatal DCs are fully capable of

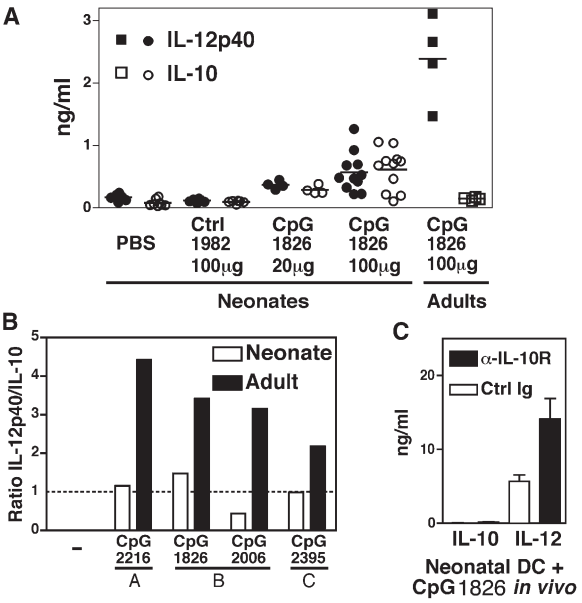

Figure 5. After CpG ODN Administration, Neonates Produce a High Level of IL-10 and a Low Level of IL-12

(A) Adult BALB/c mice were injected with 100 μg CpG 1826 (corresponding to 5 mg/kg of mouse weight, square symbols). Neonates were injected with PBS or with 100 μg Ctrl 1982, CpG 1826, or with 20 μg of CpG 1826 (corresponding to 5 mg/kg, circle symbols). Three hours later, 2 × 10^6 splenocytes were cultured for 72 hr and IL-12p40 and IL-10 secretion were tested by ELISA. Each point represents an individual mouse.

(B) 2.5 × 10^5 neonatal or adult splenocytes were cultured with or without different types of CpG ODN for 48 hr. IL-12p40 and IL-10 secretion was tested by ELISA, and the ratio of IL-12p40/IL-10 is shown.

(C) BALB/c neonates were injected i.p. with 100 μg α-IL-10R Ab or control Ig (Ctrl Ig) plus 100 μg CpG 1826. Three hours later, DCs (10^4) were purified and cultured for 48 hr, and then IL-10 or IL-12p40 secretion was analyzed by ELISA. Results are expressed as the mean ± SD of replicates.

priming Th1 responses; however, upon TLR9 signaling, neonatal B cells secrete IL-10, which decreases IL-12 secretion by DCs and thus limits their Th1 priming ability.

Both CD5$^+$ and CD5$^-$ Neonatal B Cells Respond to TLR9 Triggering but Only the CD5$^+$ B Cell Subset Produces IL-10

The question raised by the above results is why neonatal B cells are much more efficient than adult cells in producing IL-10 in response to CpG ODN. Early studies showed that in contrast to the adult spleen, which possesses only very few B-1 cells, 30% of IgM$^+$ spleen B cells of 5-day-old mice are Ly-1$^+$ (CD5) B-1 cells (Hayakawa et al., 1983) and that B-1 cells can be an important source of IL-10 (O'Garra et al., 1992). Our analysis of CD5 expression on CD19$^+$ B cells shows that indeed CD5$^+$ B cells represent 30% to 40% of spleen B cells during the first 2 weeks of life, whereas they account for less than 5% of B cells in the adult spleen (Figures 8A and 8B). Further analysis of neonatal CD5$^+$ B cells shows that they express a higher level of CD19, IgM, CD54, CD11a, CD44, and TSLP-R as compared to CD5$^-$ B cells (data not shown) but do not express CD11b (Figure 8B). They are also positive for MHC II, CD1d, CD40, and CD43 (data not shown). We then compared the capacity of CD5$^+$ and CD5$^-$ B cells to respond to TLR9 signaling. In response to CpG ODN, both B cell subsets proliferated with similar efficiency (Figure 8C), whereas only CD5$^+$ B cells produced a high level of IL-10 (Figure 8D). These results show that CpG ODN induces a different activation program in CD5$^+$ and CD5$^-$ B cell subsets and that only CD5$^+$ B cells are responsible for the IL-10-mediated control of DC activation following TLR9 triggering.

Discussion

In this report, we have analyzed the in vitro and in vivo T cell priming functions of neonatal DCs. Our results clearly show that neonatal DCs have identical antigen-presenting and T cell-priming abilities as adult DCs and are capable of inducing Th1 responses in an adult microenvironment. Although the development of naive T cells to Th1 or Th2 cells is still not completely understood, it is clear that the cytokine milieu surrounding the emerging T cell responses is mainly responsible for the Th1 or Th2 polarization. Other factors also contribute to this differentiation, such as signal strength or antigen dose (Hosken et al., 1995), genetic background (Guler et al., 1996), the nature of different subsets of DCs (Maldonado-Lopez et al., 1999), or costimulatory molecules (Kuchroo et al., 1995). However, none of these factors explains why the Th1 priming ability of DCs is defective in neonatal recipients but is effective in adults. In contrast to the study of Dakic et al. (2004), we show that neonatal DCs present antigens to T hybridomas or CD4$^+$ T cells as efficiently as adult DCs. Moreover, neonatal DCs induce Th1 responses in adult recipients, indicating that neonatal DCs express a similar level of MHC II and costimulatory molecules and propose similar strength of signal to prime T cells. However, both BALB/c and C57BL/6 neonatal DCs induced Th2 responses in neonatal recipients, showing this is not a mouse-strain-limited default.

Adkins et al. (2002) showed that neonatal T cells primed in an adult microenvironment still show a preferential Th2 expansion over Th1 responses, suggesting that neonatal T cells require a different threshold of stimulation to be polarized. Recently, Li et al. (2004) demonstrated further that in contrast to adult Th1 cells, neonatal Th1 cells express an atypical receptor for IL-4 (IL-13Rα/IL-4Rα) that induces apoptosis of Th1 cells after IL-4 stimulation. These studies indicate that differences exist between neonatal and adult T cells that may influence their capacity to develop Th1 responses. However, these studies did not address the issue of DC maturation in these responses, and we show here that efficient neonatal Th1 responses can be elicited when neonatal DCs are appropriately activated. The maturation or activation of DCs, which are required to initiate T cell immune responses, can be induced by inflammatory signals (upon infection) or physical stress (disruption of cell-cell contact, purification procedure, or in vivo adoptive transfer) (Bousso and Robey, 2003; Pierre et

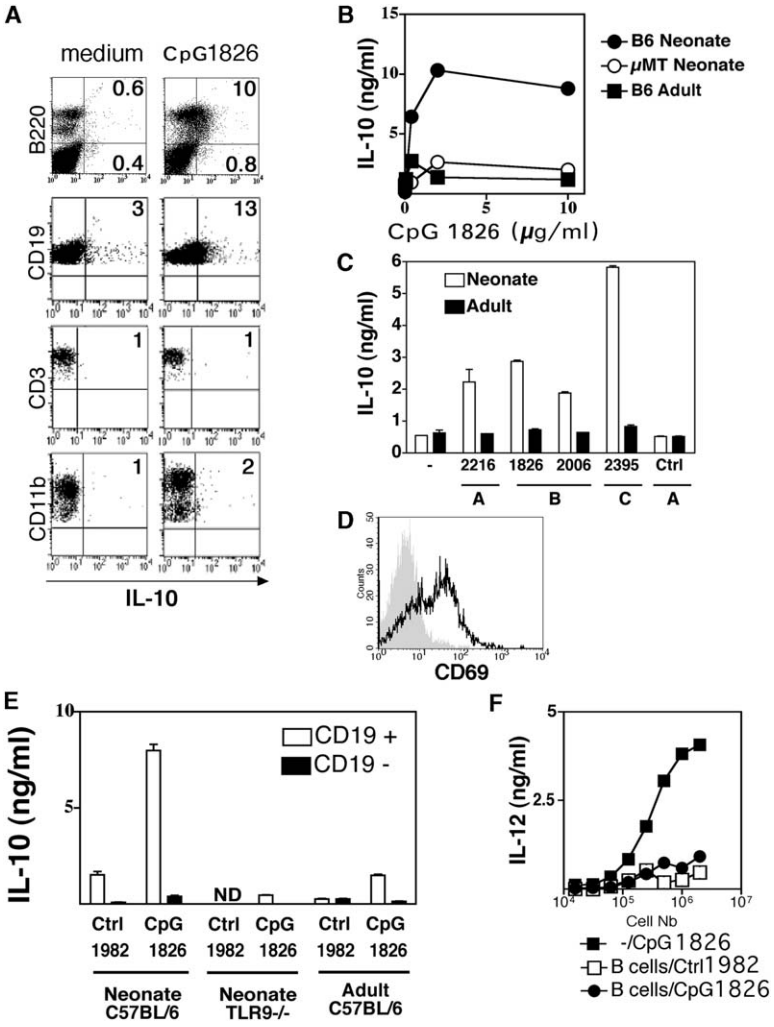

Figure 6. Neonatal B Cells Produce IL-10 in Response to CpG ODN and Limit IL-12 Production by DCs

(A) Spleen cells from neonates were stimulated in vitro with 10 μg/ml CpG ODN for 72 hr, then IL-10-secreting cells were analyzed by FACS for B220-, CD19-, CD3-, or CD11b-positive cells. The percentage of IL-10-secreting cells is indicated in the right quadrants.

(B) Spleen cells from C57BL/6 and μMT neonates and from C57BL/6 adults were stimulated in vitro with CpG 1826 and analyzed for IL-10 production.

(C) 10^6 neonatal or adult C57BL/6 $CD19^+$ B cells were purified and cultured with 10 μg/ml of the different types of CpG ODN for 48 hr, and then IL-10 secretion was analyzed. Results are expressed as the mean ± SD of replicates.

(D) C57BL/6 $CD19^+$ B cells from PBS (grey histogram) and CpG 1826-injected (bold line) neonates were analyzed for CD69 expression 3 hr after injection.

(E) $TLR9^{-/-}$ or wild-type C57BL/6 neonates and adults were injected with 100 μg CpG 1826 or Ctrl 1982, and 3 hr later $CD19^+$ B cells were purified and analyzed for IL-10 production. The IL-10 secretion is also shown for the spleen cell fraction depleted of B cells ($CD19^-$). Results are expressed as the mean ± SD of replicates.

(F) $IL\text{-}10^{-/-}$ neonates were transferred with 2×10^6 neonatal $CD19^+$ B cells from C57BL/6 ($IL\text{-}10^{+/+}$) or left untreated prior to injection of 100 μg CpG 1826. Three hours later, splenocytes from these neonatal mice were cultured for 48 hr and analyzed for IL-12p40 secretion.

al., 1997). These signals may induce different degrees of DC maturation in terms of phenotypical changes and cytokine secretion patterns. In this regard, under our experimental conditions, the purification procedure certainly induced the maturation of DCs without remarkable cytokine secretion but with enough to prime Th1 responses when they were transferred into adult recipients. However, to prime Th1 responses in neonates, full maturation of DCs with stronger IL-12 secretion seems necessary. Therefore, our results show that the key event in neonatal Th1/Th2 polarization is the appropriate activation of DCs.

Strikingly, our results highlight that after TLR9 signaling, B cells tightly control the activation of neonatal DCs for Th1 priming through IL-10. In the conventional sequence of events required to stimulate adaptive immune responses, DCs first activate $CD4^+$ T cells, which may further help B cells for antibody production. DCs may also regulate B responses directly. Some studies demonstrate that with the collaboration of T cells, DCs regulate the activation and proliferation of B cells by the secretion of IL-6 and IL-12 (Dubois et al., 1998, 1997). It has also been shown that human plasmacytoid DCs regulate B cell activation by IFN-α secretion during viral infection (Jego et al., 2003). It has also been suggested that B cells may regulate activation of T cells by steady-state DCs (Moulin et al., 2000). However, to initiate T cell activation, DCs need to also be activated by microbial products through the triggering of sensor molecules such as TLRs. Here, we show that after TLR9 signaling, B cells act upstream on DCs to regulate the T cell immune response. This TLR signaling triggers both B cell and DC activation, but the innate B cell response prevails over DC functions. This takes place in early life and may represent a developmentally regulated mechanism.

Different cells are reported to secrete IL-10, like macrophages, $CD25^+$ T regulatory cells (Shevach, 2002), or Kupffer cells during sepsis (Emmanuilidis et al., 2001). There is little information about the regulatory $CD25^+$ T cells during the neonatal period. Thymectomy of BALB/c mice at day 3 of life demonstrates that elimination of $CD25^+$ T cells in the thymus at this period induces autoimmune gastritis 3 to 4 weeks later (Suri-Payer et al., 1998), indicating that $CD25^+$ T cells may work early in life. However, their regulatory functions during the neonatal period need to be further documented. Importantly, these cells are not involved during

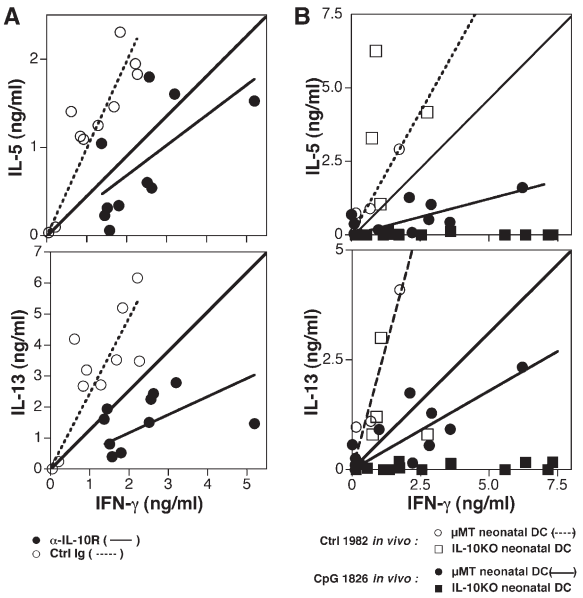

Figure 7. In the Absence of IL-10 or of B cells, In Vivo TLR9 Triggering of Neonatal DCs Allows Th1 Priming

(A) BALB/c neonates were injected i.p. with 100 μg α-IL-10R Ab or control Ig (Ctrl Ig) plus 100 μg CpG 1826. Three hours later, DCs (10^5) were purified, loaded with peptide, and injected into BALB/c neonates. One to two weeks later, splenocytes were stimulated in vitro as in Figure 2. Each point represents an individual mouse. The linear regression line of Th1 cytokine (IFN-γ) versus Th2 cytokines (IL-5 and IL-13) detected by ELISA is shown.

(B) DCs from IL-$10^{-/-}$ (n = 13) or from μMT (n = 11) neonates injected with CpG 1826 were loaded with peptide $Ag85A_{241–260}$ or $MalE_{221–235}$, and 1.4–10 × 10^4 DCs were transferred into C57BL/6 wild-type neonatal recipients. As a control, nonactivated DCs from IL-$10^{-/-}$ (n = 4) or μMT (n = 5) neonates loaded with peptide were injected into C57BL/6 neonates. Th1/Th2 cytokines were determined as in (A).

the cytokine storm induced by CpG ODN. Indeed, after TLR9 triggering, we identified B cells as the main source of IL-10, but not T cells or myeloid cells. The data from μMT splenocytes confirm this observation.

The strong production of IL-10 by neonatal, but not adult, B cells in response to CpG ODN is due to the fact that only $CD5^+$ B cells are able to secrete this cytokine in these conditions and that the $CD5^+$ B cell subset is more abundant in early life. Indeed, the DC:B cell ratio in the spleen is similar (i.e., about 1:25–30) in early life as well as in adults (Dadaglio et al., 2002); in contrast the DC:$CD5^+$ B cell ratio is 1:10 and 1:1 in the neonatal and adult spleen, respectively. We have previously shown that B cells and DCs are the first differentiated cell types to colonize the spleen after birth (Sun et al., 2003). $CD5^+$ B cells are believed to develop mainly from fetal liver (Herzenberg, 2000). However, the neonatal liver, as well as the spleen, is still a source of B cells, whereas the contribution of the bone marrow to the B cell compartment is developing (Hardy, 2003). The high number of $CD5^+$ B cells observed in the spleen for the first 2 weeks of life much likely reflects this hematopoietic scheme. Regarding other B cell subsets, no marginal zone B cells were found during the first week of life (data not shown). It is important to note that TLR9 signaling triggers a different activation program in $CD5^+$ and $CD5^-$ neonatal B cells. Both B cell subsets proliferate and secrete IgM, but only the $CD5^+$ B cell subset is able to produce IL-10. Therefore, differences in subpopulations of B cells early in life are responsible for DCs regulation after TLR9 signaling. Whether such a regulation mechanism takes place in the adult immune system remains to be determined; however, it is likely that the encounter of these two cell types may occur.

The regulatory role of neonatal IL-10 does not impair the capacity of DCs to prime T cells but restrains their capacity to secrete IL-12 and to mount Th1 responses. Therefore, it is possible that this regulation process is important to limit the development of an exacerbated Th1 response. Some studies demonstrate that IL-10 secretion by B cells plays a critical role in controlling different autoimmune diseases like experimental autoimmune encephalomyelitis or arthritis (Fillatreau et al., 2002; Mauri et al., 2003). Of note, administration of CpG ODN in combination with α-IL-10R Ab to neonates or of CpG ODN alone to an IL-$10^{-/-}$ neonate was lethal within 24–48 hr (data not shown). Preliminary observations indicate further that CpG ODN induces some apoptosis and an important extravasation of lymphocytes in different organs of IL-$10^{-/-}$ neonates that is not the case in wild-type neonates (our unpublished data). This phenomenon is observed mainly in the lungs, but also in the liver and spleen. However, CpG ODN was not lethal in μMT neonatal mice, indicating that the control of this lethal inflammation by IL-10 may be compensated by another mechanism in μMT neonates.

Our results elucidate an important mechanism underlying the inefficient induction of Th1 responses in neonatal mice and demonstrate that B cells have a regulatory role over DCs functions. These results also underline that when triggering TLR in vivo, all the different responding cell types should be taken into account. Early release of IL-10 in neonatal $CD5^+$ B cells limits the Th1 priming ability of neonatal DCs by reducing their capacity to produce IL-12. This opens up new perspectives for the treatment of infections during early life and for the prevention of allergic diseases. Moreover, the absence or the poor stimulation of neonatal B cells for IL-10 production may represent an important criterion for the design of future vaccines for neonates.

Experimental Procedures

Mice

BALB/c and C57BL/6 adult mice were purchased originally from Janvier (Le Genest St. Isle, France). C57BL/6-IL-10-deficient (IL-$10^{-/-}$; obtained from A. Bandeira, Institut Pasteur, Paris, France), B cell-deficient C57BL/6 (μMT), TLR9-deficient (TLR$9^{-/-}$; obtained from P. Vieira, Institut Pasteur, Paris, France), and OT-II mice, all on a C57BL/6 background, were bred in our animal facilities. For pups, pregnant females were bred and housed on-site in specific pathogen-free conditions. Neonatal mice are defined as 7 days old and adult mice as 6–10 weeks old. Spleen cells from μMT and IL-$10^{-/-}$ neonatal mice did not differ from C57BL/6 neonatal mice (Sun et al., 2003) in composition of plasmacytoid DC ($B220^+$ $CD11c^{low}$) and of the different $CD11c^{high}$ DC subsets, i.e., 30%–35% of $CD8^+$ DC, 50%–60% of $CD8^-CD4^-$ DC, and about 10% of $CD4^+$ DC.

Culture Medium and Reagents

Complete medium (CM) consisted of RPMI-1640 containing L-alanyl-L-glutamine dipeptide supplemented with 5%–10% fetal calf

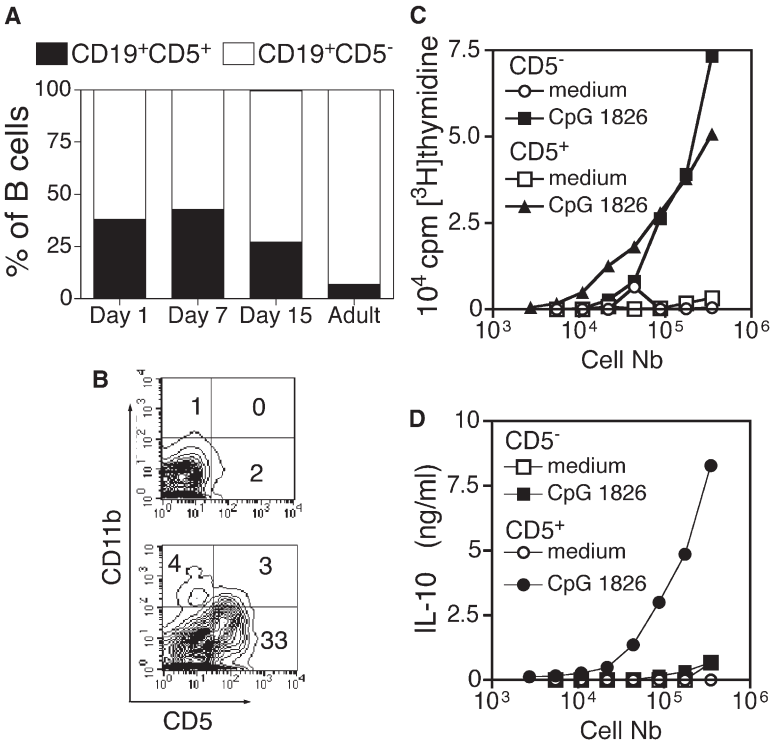

Figure 8. CD5+, but Not CD5−, Neonatal B Cells Are Responsible for IL-10 Production in Response to CpG 1826

(A and B) Spleen B cells of C57BL/6 mice were analyzed for CD5 expression from birth to adulthood. In (B), CD19+ gated cells of adult (top) or neonatal (bottom) spleen were also analyzed for CD11b expression.

(C and D) Purified CD19+CD5+ and CD19+CD5− cells from neonatal C57BL/6 mice were cultured with medium alone or with 10 μg/ml of CpG 1826. After 48 hr, cell proliferation (C) and IL-10 content (D) in the supernatant were determined. Results are representative of four similar experiments.

serum (FCS, ICN Biomedicals, Inc.), 5 × 10^{-5} M of 2-ME (Sigma), and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, GIBCO BRL). In some experiments, cells were cultured in HL-1 serum-free medium (Bio Whittaker). Type A, B, and C of CpG ODN (CpG 1826, 5′-**TCCATGACGTTCCTGACGTT**-3′; CpG 2006, 5′-**TCGTCGTTTTGTCGTTTTGTCGTT**-3′; CpG 2216, 5′-**GGG**GGACGATCGTC**GGGGGG**-3′; CpG 2395, 5′-**TCGTCGTTTTCGGCGCGCGCCG**-3′) and control ODN (Ctrl 1982, 5′-**TCCAGGACTTCTCTCAGGTT**-3′; Ctrl A, 5′-**GGG**GGAGCATGCTG**CGGGGG**-3′) (Klinman et al., 1996; Vollmer et al., 2004) were synthesized by PROLIGO. Boldface nucleotides correspond to phosphorothioate backbone. The following peptides were used: KLFAVWKITYKDTV (PV1), QDA YNAGGGHNGVFDFPDSG (Ag85A$_{241–260}$), ETAMTINGPWAWSNI (MalE$_{221–235}$), ISQAVHAAHAEINEAGR (OVA$_{323–339}$), and RPQASG VYM (LCMV NP$_{118–126}$). All synthetic peptides were purchased from Neosystem. Purified *E. coli* MalE protein provided by J.M. Clement (Institut Pasteur, Paris, France). *M. bovis* BCG 1173P2 was a kind gift from G. Marchal (Institut Pasteur, Paris, France).

Cell Purification and FACS Analysis

Spleens were treated with collagenase D and DNase I (Roche Molecular Biochemicals) and then dissociated in Ca^{2+}-free medium in the presence of EDTA. DCs were positively selected by anti-CD11c MicroBeads (N418), and B cells were selected with anti-CD19 MicroBeads (1D3) (Miltenyi Biotec) and AutoMACs (Miltenyi Biotec). The purification procedure used for DCs led to the selection of classical CD11c+ DCs, but not of plasmacytoid DCs. In some experiments, CD19+ cells were further stained by phycoerythrin (PE)-conjugated anti-CD5 antibody (53–7.3) and sorted as CD5+ and CD5− B cell subsets by flow cytometry on a FACSTARplus (BD). For OT-II CD4 T cell purification, lymph node CD4 T cells from OT-II mice were enriched by immunomagnetic beads (Miltenyi Biotec) of anti-CD45R/B220 (RA3-6B2), anti-CD8α (53-6.7), anti-CD11b (M1/70.15.11.5), anti-CD11c (N418), and anti-CD19 (1D3). Purity of cells was checked by flow cytometry on a FACSCalibur (BD Biosciences) with allophycocyanin (APC) or PE-conjugated anti-CD11c (HL3) for DCs, APC-conjugated anti-CD19 (1D3) for B cells, PE-conjugated anti-CD4 (RM4-5), and fluorescein isothiocyanate (FITC) conjugated CD90.2 (53-2.1) for CD4+ T cells. Purity was usually >95% for adult DCs, T cells, and neonatal B cells and 85%–95% for neonatal DCs. DC maturation or B cell activation was assessed with FITC conjugated anti-I-A^b (25-9-17), anti-I-A^d (AMS-32.1), anti-CD40 (HM40-3), anti-CD80 (16-10A1), anti-CD69 (HI.2F), or anti-CD86 (GL1) mAbs. All mAbs were from BD Pharmingen.

Antigen Presentation Assay

All T hybridomas were generated in our laboratory. The 45G10 T cell hybridoma is specific for the PV1 peptide and is I-E^d restricted, the FBCD1 T cell hybridoma is specific for MalE$_{221–235}$ and is I-A^d restricted, and the CG11 T cell hybridoma is specific for the BCG Ag85A$_{241–260}$ and is I-E^d restricted. For antigen presentation to T cell hybridomas, serial dilutions of purified neonatal or adult BALB/c CD11c+ DCs were pulsed with peptides, proteins, or BCG and then incubated with the corresponding T cell hybridoma for 24 hr. The culture supernatants were frozen for at least 2 hr at −80°C, thawed, tested for IL-2 content by using 10^4 cells/well of the IL-2 dependent CTL-L cell line, and cultured for 3 days. For T cell priming of OT-II CD4 T cells, serial dilutions of purified neonatal or adult C57BL/6 DCs were incubated with OVA$_{323–339}$ alone, or with OVA$_{323–339}$ or OVA protein and 10 μg/ml CpG ODN for 1 hr, then incubated with 10^4 OT-II CD4 T cells for 3 days. For cell proliferation analysis, cells were pulsed with [^{3}H]-thymidine (ICN Biomedicals, Inc.) for the last 6–18 hr of culture and harvested by an automated cell harvester (Skatron), and scintillation was counted. In all experiments, each point was done in at least triplicate. Results are expressed as the mean ± SD of cpm. No proliferation of OT II cells cultured with OVA protein alone was detected.

Immunization Procedure

Purified neonatal or adult DCs were loaded or not with appropriate peptide (5 μg/10^5 cells or 100 μg/ml) in RPMI-1640 medium with 1%–2% mouse serum for 30–60 min at 37°C. In some experiments, DCs were activated in vivo by injection of 100 μg CpG ODN or Ctrl ODN 3 hr before peptide loading, or in vitro with 10 μg/ml CpG ODN, then DCs were loaded with peptides. For blocking IL-10 signal, 100 μg anti-IL-10 receptor mAb (α-IL-10R, clone 1B1.3a, BD Pharmingen) or control immunoglobulin (Ctrl Ig, clone SFR8-B6 [Radka et al., 1982]) was injected 15 min before CpG ODN injection.

After peptide loading, 2–20 × 10^4 DCs were then washed twice with PBS and injected into adult (i.v. or i.p.) or into 7-day-old (i.p.) syngeneic mice. One to two weeks after immunization, splenocytes were isolated for cytotoxicity assay or cytokine secretion assay. The DC number used for injection influenced the intensity of the T response measured but did not modify the cytokine profile of the response.

Cytotoxicity Assay

Two weeks after immunization, splenocytes were isolated and restimulated in vitro with 0.1 μg/ml LCMV $NP_{118–126}$ in the presence of syngeneic irradiated naive splenocytes for 5 days. The cytotoxic activity was determined in a 4 hr, in vitro ^{51}Cr release assay with P815 tumor cells incubated or not with LCMV $NP_{118–126}$ peptide as the target cells. The percentage of specific lysis was calculated as 100× ([experimental release – spontaneous release]/[maximal release – spontaneous release]). Results are expressed as Δ% of specific lysis (percent of lysis in the presence of peptide – percent lysis in the absence of peptide).

Cytokine Analyses

For cytokine secretion, splenocytes from immune mice were restimulated with the appropriate peptide for 72 to 96 hr and culture supernatants were tested for Th1 and Th2 cytokines. 10^6 splenocytes were cultured with 5 × 10^5 irradiated syngeneic adult splenocytes as feeders in CM with or without 10 μg/ml of the corresponding peptide. 72–96 hr later, supernatants were harvested and cytokines were detected. Results are expressed in ng/ml and correspond to the cytokine produced in the presence of peptide after subtraction of cytokine produced in the absence of peptide. In the absence of peptide stimulation, only adult immune spleen cells produced IFN-γ cytokine but at levels between 10%–30% of the response observed in the presence of peptide. Identical responses were observed in the presence or in the absence of irradiated syngeneic adult splenocytes. For in vitro cytokine secretion, whole splenocytes, purified DCs, and B cells were isolated and cultured with CpG ODN in serum-free HL-1 medium for 48 hr. DCs were cultured in the presence of IL-4 and GM-CSF. For ex vivo cytokine secretion, mice were injected with 100 μg CpG ODN or Ctrl ODN then, 3 hr later, cells were directly cultured for 48–72 hr. IL-4, IL-5, IL-10, IL-12p40, IL-12p70, and IFN-γ were measured by standard sandwich ELISA with appropriate Ab pairs (all from BD Pharmingen). IL-13 was captured by purified anti-IL-13 mAb (clone 38213.11; R&D Systems) and detected by biotinylated IL-13 Ab (R&D Systems).

IL-10 Secretion Assay

Characterization of IL-10-secreting cells was performed by stimulating total spleen cells with CpG ODN for 1–3 days. Flow cytometry analysis of IL-10-producing cells was performed with an IL-10 Secretion Assay Detection kit (Miltenyi Biotec) together with FITC-conjugated anti-B220, anti-CD19 (1D3), anti-CD3 (17A2), or anti-CD11b (M1/70) mAbs (BD Pharmingen).

Supplemental Data

Supplemental Data including three figures are available online at http://www.immunity.com/cgi/content/full/22/4/467/DC1/.

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