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Development of the Dendritic Cell System during Mouse Ontogeny

Aleksandar Dakic,* Qi-xiang Shao,† Angela D’Amico,* Meredith O’Keeffe,* Wei-feng Chen,† Ken Shortman,* and Li Wu*#†

Based on the view that the efficacy of the immune system is associated with the maturation state of the immune cells, including dendritic cells (DC), we investigated the development and functional potential of conventional DC and plasmacytoid pre-DC (p-preDC) in spleen, thymus, and lymph nodes during mouse development. Both CD11c+ DC and CD45RA+ p-preDC were detected in small numbers in the thymus as early as embryonic day 17. The ratio of DC to thymocytes reached adult levels by 1 wk, although the normal CD8α+ phenotype was not acquired until later. Significant, but low, numbers of DC and p-preDC were present in the spleen of day 1 newborn mice. The full complement of DC and p-preDC was not acquired until 5 wk of age. The composition of DC populations in the spleen of young mice differed significantly from that found in adult mice, with a much higher percentage (50–60% compared with 20–25%) of the CD4−CD8α+ DC population and a much lower percentage (10–20% compared with 50–60%) of the CD4+CD8α− DC population. Although the p-preDC of young mice showed a capacity to produce IFN-α comparable with that of adult mice, the conventional DC of young mice were less efficient than those of their adult counterparts in IL-12p70 and IFN-γ production and in Ag presentation. These results suggest that the neonatal DC system is not fully developed, and innate immunity is the dominant form of response. The complete DC system required for adaptive immunity in the mouse is not fully developed until 5 wk of age. The Journal of Immunology, 2004, 172: 1018–1027.

The maturation state of dendritic cells (DC) has been suggested to be a determining factor for the induction of immune tolerance or immunity (1). It is well known that neonatal mice mount a limited immune response to infection and are more susceptible to adults to the induction of immunological tolerance after Ag exposure (2, 3), but the mechanisms responsible are not yet fully elucidated. Understanding these mechanisms is crucial in developing strategies for either enhancing the immune responses in a situation of microbial infection or suppressing it in the case of autoimmunity. Studies of the possible reasons for the incompetence of the neonatal immune system have focused mostly on neonatal T cells and their qualitative and quantitative differences from adult T cells (4). The potential influence of DC, the major inducers of effector T cell responses or tolerance (1, 5), on the functional state of the immune system at this early developmental stage has not been fully assessed. In contrast to the earlier view that neonatal T cells were more susceptible to tolerance induction, later studies showed that the nature of the APCs determines whether the outcome is neonatal tolerance or immunity (6). A recent study by Vollstedt et al. (7) has also shown that the relative incompetence of immune responses to microbial infection in the neonatal mouse is due to a low frequency of functionally mature DC in mouse lymphoid tissues.

There had been no thorough study on the development of DC in different lymphoid organs during mouse ontogeny, and the time of their first appearance and their functional capabilities remain unclear. In light of the identification of mouse plasmacytoid pre-DC (p-preDC) and their distinct functions in the innate and adaptive immune responses (8–11), it was also important to determine when these p-preDC first develop and to assess their functional potential during ontogeny.

In this study we demonstrate that DC are present in lower numbers overall in the lymphoid tissues of neonatal mice and that the composition of DC populations in neonatal mice is different from that in adult mice, with a much higher percentage of the splenic CD4−CD8α+ DC population and a lower percentage of CD4+CD8α− DC population. In addition, neonatal DC display a lower capacity for IL-12p70 and IFN-γ production and a lower efficiency in Ag presentation by newborn DC compared with their adult counterparts, but their capacity to stimulate allogeneic T cell proliferation is comparable to that of adult DC. These quantitative and qualitative differences contribute to the limited immune responsiveness of younger mice and suggest that a fully developed DC system is a prerequisite for the establishment of an efficient adaptive immune system.

Materials and Methods

Mice

C57BL/6j mice, from embryonic day 14 to 6 wk of age, and BALB/c mice, from 1–6 wk of age, were used for surface phenotype and functional studies of DC populations during mouse ontogeny. CBA/CaH mice at 6–8 wk of age were used to provide T cells in allo-MLR assays. All mice were bred under specific pathogen-free conditions in the animal facility of The Walter and Eliza Hall Institute.
Fluorescence-conjugated mAbs used for flow cytometric analysis

The FITC-conjugated Abs were anti-CD11c (N418), anti-MHC class II (M5/114), anti-CD8 (5075-3.6), anti-CD4 (GK1.5), anti-B220 (RA3-6B2). The allophycocyanin-conjugated Abs were anti-CD45RA (14.8, anti-MHC class II (M5/114), anti-CD11b (M1/70), anti-CD8a (YTS156), anti-CD3 (KT3-1.1). The Alexa 594-conjugated Abs were anti-CD11c (N418), anti-CD4 (Gk1.5), and anti-CD3 (KT3-1.1). The biotinylated Abs were anti-CD19 (ID3), anti-CD40 (GK45.45), anti-CD80 (16-10A1), and anti-CD86 (GL-1). The second-stage stain was streptavidin-PE or streptavidin-allophycocyanin.

The mAbs used for immunomagnetic bead depletion

The mAb supernatants used for depletion in splenic DC and splenic plasmacytoid p-pDC preparations were anti-CD3 (KT3-1.1), anti-CD19 (ID3), anti-CD90 (T24/31.7), anti-Gr-1 (RA6-8C5), and anti-erythrocyte (TER-119). For thymic DC and thymic p-pDC preparations, anti-CD11b (M1/70) and anti-macrophage Ag F4/80 (F4/80) were also added to the Abs used for spleen preparations. Note that the use of anti-Gr-1 did not cause any depletion of plasmacytoid cells in our C57BL/6 mice.

Isolation and surface phenotype analysis of DC and p-pDC populations in lymphoid tissues during mouse ontogeny

The level and surface phenotype of DC and p-pDC populations in mouse thymus (from embryonic day 14 (E14), E17, day 1 newborn, and 1–6 wk of age), spleen (from day 1 newborn to 6 wk of age), and lymph nodes (LN; from 1–6 wk of age) were examined using the DC and p-pDC enrichment procedures described previously (11), followed by flow cytometric analysis. The DC and p-pDC enrichment procedures involved three major steps: (1) collagenase and DNase digestion of the tissue fragments to release DC and p-pDC, (2) collection of low density cells by centrifugation of the digested cells through a density medium Nycodenz (Nycopharma Oslo, Norway) with a density 1.076 g/cm³ (4°C, mouse osmolality) for thymic DC and p-pDC, 1.077 g/cm³ for splenic DC and p-pDC, and 1.082 g/cm³ for LN DC and p-pDC; and (3) immunomagnetic bead depletion of non-DC from the selected low density cells. To enrich DC and p-pDC from fetal thymuses on E14 and E17, the density separation step was omitted, because of the low cell numbers and to avoid possible loss of DC that might be of different density in this early developmental stage. To ensure that there was no selective loss of DC populations during the enrichment procedures, the number of viable nucleated cells was determined after each enrichment step, and a sample of cells (5–10 x 10⁶) was taken for immunofluorescent staining and flow cytometric analysis. The cell numbers obtained after collagenase digestion were used to calculate total DC and p-pDC numbers in each lymphoid organ.

No significant loss of particular DC populations was observed after each DC enrichment step. The cells taken after each enrichment step were then stained for DC and p-pDC surface markers using fluorescence-conjugated Abs and were analyzed on a FACSStar Plus instrument (BD Immunocytometry Systems, San Jose, CA). The conventional DC were identified as CD11c+CD45RA−, and the p-pDC was identified as CD11c+CD45RA−. Subsets of the conventional DC were further defined by the expression of CD4, CD8α, CD205, and CD86. The p-pDC were also subdivided into subsets based on the expression of CD4 and CD8α.

Cytokine production by DC and p-pDC populations

For functional studies of DC or p-pDC populations, DC and p-pDC populations were first enriched and stained using the procedures described above. Each DC or p-pDC population was then purified by flow cytometric sorting using the MoFlo instrument (Cytomation, Fort Collins, CO). The purity of the sorted DC populations was checked by reanalyzing a small sample on the MoFlo and was usually >97%. The purified p-pDC and DC were then activated in vitro with the stimuli that were previously used for each enrichment step, and a sample of cells (5–10 x 10⁶) was cultured in 96-well, U-bottom tissue culture plates in 10% CO₂-in-air incubator at 37°C for 18 h of incubation. The culture supernatants were collected after 18 h of incubation and stored at −20°C for later testing. For measuring IFN-γ production, culture supernatants were collected after 60 h of incubation.

The analysis of IL-12 and IFN-γ in the culture supernatants was conducted by ELISA as previously described (12). The production of IFN-α was assayed via a two-site ELISA, using as a capture Ab anti-mouse IFN-α (clone RMMA-1; PBL Biomedical Laboratories, New Brunswick, NJ) and as a detection Ab, a rabbit anti-mouse IFN-α polyclonal (PBL Biomedical Laboratories), followed by an (F(ab')₂) donkey anti-rabbit HRP conjugate (Jackson ImmunoResearch Laboratories, West Grove, PA), with final readout as described for IL-12. The IFN-α standard used was recombinant mouse IFN-α A (PBL Biomedical Laboratories).

Allogenic MLR

To test and compare T cell stimulatory capacities of DC at various stages of mouse ontogeny, purified CD8α and CD8α− conventional DC subsets were analyzed in an allogenic MLR as described previously (13). Briefly, the CD4+ or CD8+ T cells purified from the LN of CBA/CaH mice were added (2 x 10⁶ in 50 μl) to each well of a 96-well, U-bottom plate containing the splenic CD8α or CD8α− DC from C57BL/6 mice of various ages (day 200, 300, and 1000 cells/well in 1 ml of RPMI 1640 medium). Each culture condition was set up in triplicate and incubated at 37°C in 10% CO₂ in air. The wells containing only T cells and only DC served as background controls. On days 2.5, 3.5, 4.5, 5.5, and 6.5, the cultures were pulsed with [³²P]H3Tdr (1 μCi/well) for 6 h. The cells were then harvested, and T cell proliferation was measured by liquid scintillation counting of the incorporation of [³²P]H3Tdr into cellular DNA.

Induction of OT-II T cell proliferation in vitro

DC were incubated with the indicated concentration of OVA protein (Sigma-Aldrich, St. Louis, MO) for 45 min at 37°C in culture medium, washed three times, and then plated in round-bottom, 96-well plates at 5 x 10⁴ cells/well. The naive OT-II cells were purified from transgenic mice and labeled with CFSE. The CFSE-labeled OT-II cells were added to the DC at 5 x 10⁴ cells/well. The plates were incubated for 2.5 days, and then the cells were harvested and resuspended in 200 μl of balanced salt solution/EDTA/3% FCS containing 2.5 x 10⁴ Calibrite beads (BD Immunocytometry Systems). Samples were analyzed by FACS until 1 x 10⁵ beads were collected. The number of dividing (propidium iodide−, CFSElow) OT-II cells was then determined.

Results

Ontogeny of thymic DC and p-pDC

The appearance of thymic DC and p-pDC was examined in mice starting on E14. At this time point, the majority of thymocytes were at the CD3−CD4+CD8− immature stage, and no CD4+CD8+ double-positive (DP) and CD4+CD8− or CD4−CD8+ single-positive thymocytes had developed. Hence, the majority of thymocytes on E14 were at the preselection (positive and negative selection) stage. Flow cytometric analysis did not detect any cells with the phenotype characteristics of DC or p-pDC in the thymus at this early stage (Fig. 1A). By the E17 stage, a high proportion of thymocytes became CD3+CD4+CD8− at this early stage (Fig. 1A). By the E17 stage, a high proportion of thymocytes became CD3+CD4+CD8− immature stage, and no CD4+CD8+ double-positive (DP) and CD4+CD8− or CD4−CD8+ single-positive thymocytes had developed. Hence, the majority of thymocytes on E14 were at the preselection (positive and negative selection) stage. Flow cytometric analysis did not detect any cells with the phenotype characteristics of DC or p-pDC in the thymus at this early stage (Fig. 1A). By the E17 stage, a high proportion of thymocytes became CD3+CD4+CD8−, and a small proportion of these DP also expressed CD3 (data not shown), an indication that some thymocytes had gone through the selection processes. At this time, a population of CD11c+CD45RA− CD4+CD8− DC could readily be detected and represented 0.2% of the total thymic cells (Fig. 1A). A small number of CD45RA−CD11c− p-pDC-like cells could also be detected (Fig. 1A). In the thymus of day 1 newborn mice, both DC and p-pDC populations were apparent and represented 0.2 and 0.04% of the total thymic cells, respectively (Fig. 1A). The total numbers of thymic DC and p-pDC increased with age thereafter until ~5 wk of age. The increase in the number of DC and p-pDC paralleled the increase in the number of total thymocytes, as the percentage of thymic DC and p-pDC was maintained constant from 1–6 wk of age (Fig. 1B).
The analysis of thymic DC for surface expression of CD8α, CD205, and MHC class II molecules revealed that thymic DC from E17 and 1-day-old mice were mostly CD8α−CD205−DC. CD8α+ DC appeared in the thymus of 1-wk-old mice (~30% DC) and became the majority of thymic DC (70–80%) by 2 wk of age, at which time the phenotype resembled that of thymic DC in adult mice. At all stages, all thymic DC were CD205+ (data not shown) and CD4− (14).

Ontogeny of splenic DC and p-pre-DC

Although the spleen is a rich source of DC in adult mice, the number and nature of splenic DC populations and their potential influence on the state of the immune system during ontogeny were not known. Accordingly, we examined the levels and surface phenotype of splenic DC populations in mice from 1 day after birth to 6 wk of age. Although the numbers of splenic DC and p-preDC detected in 1-day-old newborn mice was very low, they increased rapidly after this stage. The total splenic cell numbers, the percentage of DC and p-preDC, and the absolute number of DC and p-preDC during ontogeny are shown in Figs. 2A. The percentage of DC increased from around 0.2% on day 1 to a final value of 2.4% at wk 6. The total number of DC at 6 wk of age reached 5 × 10^6/spleen, a >600-fold increase from 1 day to 6 wk of age, and remained constant thereafter. From 3–5 wk DC cell numbers increased more rapidly than those of total spleen cells, but the ratio of DC to splenic T cells remained at 1/10 during this time, consistent with that reported previously (15). Similarly, the percentage of p-preDC increased from 0.1% on day 1 to the peak of ~0.7% at wk 5 (Fig. 2A).

Although the numbers of both splenic DC and p-preDC continually increased in the first 5 wk of ontogeny, a difference in the kinetics of their increase was revealed when p-preDC vs DC ratios were compared. The percentage of p-preDC was ~45% of all DC family cells (including DC and p-preDC) at wk 1, but it decreased with age because of the more rapid increase in DC numbers. The p-preDC and DC represented around 20 and 80% of all DC family cells, respectively, in the spleens of 6-wk-old adult mice.

The adult mouse spleen contains three major DC populations, namely CD8α−CD4−CD205+, CD8α−CD4+CD205+, and CD8α+CD4−CD205−, representing 25, 55, and 20% of the total splenic DC respectively (14). In contrast to the adult mouse, DC detected in the spleen of day 1 newborn mice were negative for both CD8α and CD4 markers, but expressed a low level of CD205 (Fig. 2C). A special feature of the composition of splenic DC populations in younger mice was the dominance of the CD8α−CD205+ DC subset (50–60%) and the presence of a distinct DC population that was absent in adult mouse spleen, namely the CD8α+CD205+CD11b+ DC subset in 1- to 2-wk-old mice (Fig. 2C). The percentage of the CD4−CD8α+CD205+ DC population decreased from 2 wk onward, accompanied by an increase in the percentage of the CD4+CD8α−CD205− DC population (Fig. 2C). The proportions of all three splenic DC populations reached the adult level by 5 wk of age. The expression of MHC class II on DC at different stages was also examined. MHC class II expression was low on splenic DC at day 1, but it reached the levels seen in DC of adult mice by 2 wk of age (data not shown).

To confirm that these phenotypic changes in DC populations represented a general trend of DC ontogeny rather than a strain-specific phenomenon, DC populations of BALB/c mice at different ages were also examined. Similar phenotypic changes in splenic DC populations were also seen in BALB/c mice (data not shown), indicating that this was a general process during DC ontogeny.

Surface phenotype changes were also observed in splenic p-preDC during ontogeny. There was a transition from the dominant CD4− p-preDC in neonatal mice to the dominant CD4+ p-preDC.
representative of two or three experiments for each age group. The data presented are gate 1) populations based on CD4 and CD8 expression. The data presented are gate 2) populations were defined by the expression of CD4 vs CD8α and of CD205 vs CD8α, as shown by the contour plots. D. Changes in the surface phenotype of splenic p-preDC (CD11c<sup>hi</sup>CD45RA<sup>−</sup>, gate 1) populations based on CD4 and CD8 expression. The data presented are representative of two or three experiments for each age group.

FIGURE 2. Splenic DC and p-preDC populations during mouse ontogeny. A, DC and p-preDC populations in the spleens of mice at different ages. The DC-enriched cells from mouse spleen were stained as described in Fig. 1A. DC and p-preDC were identified as CD11c<sup>hi</sup>CD45RA<sup>−</sup> (gate 2) and CD11c<sup>dim</sup>CD45RA<sup>−</sup> (gate 1), respectively. B, The total cell numbers (top panel), the number of DC and p-preDC (middle panel), and the percentages of DC and p-preDC (bottom panel) per spleen in mice at different ages. The absolute numbers of total cells and the numbers of DC and p-preDC per thymus are presented as solid lines. The values represent the average cell numbers obtained from two to four experiments. Error bars represent the ranges of cell numbers. Each experiment included 4 to 35 mice. The percentages of DC and p-preDC are presented as bars. A large portion of these CD11c<sup>dim</sup>MHC-II<sup>hi</sup> DC in SLN expressed the highest level of CD8α<sup>+</sup>DC205<sup>+</sup> DC with age. The CD11c<sup>dim</sup> MHC-II<sup>hi</sup> DC population consisted mostly of CD8α<sup>−</sup>CD205<sup>+</sup> cells. A large portion of these CD11c<sup>dim</sup>MHC-II<sup>hi</sup> DC in SLN were tested after culture with a mixture of microbial stimuli used for induction of the bioactive form of IL-12 p70 by CD8α<sup>−</sup> DC were CpG, GM-CSF, IFN-γ, and IL-4. The factors used for induction of IFN-γ production by CD8α<sup>−</sup> DC were IL-12p70 and IL-18. As IL-4 has been shown to drastically up-regulate IL-12p70, but to down-regulate IL-12p40 (17), it was excluded from the stimuli used when IL-12p40 production was measured. Whenever possible, cytokine production was tested from sorted CD8α<sup>−</sup> and CD8α<sup>+</sup> DC subsets separately. DC from day 1 newborn mice, however, do not express either CD8α or CD4 markers and so they were tested unseparated.

The production of IL-12 by CD8α<sup>−</sup> and CD8α<sup>+</sup> DC from mice of different age groups is shown in Fig. 4A. In the case of day 1 newborn mice, in which the majority of splenic DC were in adult mice (Fig. 2D). This was consistent with our previous findings that showed upon in vivo transfer, the adult splenic CD4<sup>+</sup> p-preDC gave rise to CD4<sup>+</sup> p-preDC (11), suggesting that the CD4<sup>+</sup> p-preDC were more mature than the CD4<sup>+</sup> p-preDC. However, no functional difference has been demonstrated between the two populations.

Ontogeny of LN DC populations

The DC populations in the s.c. LN (SLN; a pool of axillary, brachial, and inguinal LN) and mesenteric LN (MLN) were examined in parallel from 1-, 3-, and 6-wk-old mice. DC were present at higher percentages in the very small LN of younger mice. Although representing 3.8% of SLN cells and 3.6% of MLN cells at wk 1, the proportion of DC declined to 1.2–1.5% by 3 wk (Fig. 3A). However, similar to the DC in thymus and spleen, the total numbers of DC in LN increased with age (Fig. 3A).

Based on the expression of CD11c and MHC class II molecules, LN DC could be divided into two major populations: CD11c<sup>hi</sup>MHC-II<sup>int</sup> and CD11c<sup>dim</sup>MHC-II<sup>high</sup> (Fig. 3B). The CD11c<sup>hi</sup>MHC-II<sup>int</sup> DC population of both SLN and MLN contained CD8α<sup>−</sup>CD205<sup>+</sup> DC and CD8α<sup>+</sup>CD205<sup>low</sup> DC, resembling the splenic DC populations (Fig. 3B). There was a slight increase in the proportion of CD8α<sup>−</sup>CD205<sup>−</sup> DC with age. The CD11c<sup>dim</sup> MHC-II<sup>high</sup> DC population consisted mostly of CD8α<sup>−</sup>CD205<sup>+</sup> cells. A large portion of these CD11c<sup>dim</sup>MHC-II<sup>high</sup> DC in SLN were found when the majority of splenic DC were in adult mice (Fig. 2D). This was consistent with our previous findings that showed upon in vivo transfer, the adult splenic CD4<sup>+</sup> p-preDC gave rise to CD4<sup>+</sup> p-preDC (11), suggesting that the CD4<sup>+</sup> p-preDC were more mature than the CD4<sup>+</sup> p-preDC. However, no functional difference has been demonstrated between the two populations.

The capacities of neonatal and adult DC to produce IL-12p70 and IFN-γ in addition to the changes in the number and surface phenotype of DC populations during mouse ontogeny described above, differences in functional potential of the individual cells at different developmental stages might be responsible for the immature status of the immune system in neonatal mice. To determine the functional potentials of DC throughout mouse ontogeny, splenic DC from neonatal mice at 1 day, 1 wk, and 2 wk of age were compared with DC from adult mice at 6 wk of age for their ability to produce functionally important cytokines, namely IL-12p70 and IFN-γ.

The capacities of neonatal and adult DC to produce IL-12 and IFN-γ were tested after culture with a mixture of microbial stimuli and cytokines previously reported to induce maximal production of these cytokines from DC in adult mice (12, 17). The factors used for induction of the bioactive form of IL-12 p70 by CD8α<sup>−</sup> DC were CpG, GM-CSF, IFN-γ, and IL-4. The factors used for induction of IFN-γ production by CD8α<sup>−</sup> DC were IL-12p70 and IL-18. As IL-4 has been shown to drastically up-regulate IL-12p70, but to down-regulate IL-12p40 (17), it was excluded from the stimuli used when IL-12p40 production was measured. Whenever possible, cytokine production was tested from sorted CD8α<sup>−</sup> and CD8α<sup>+</sup> DC subsets separately. DC from day 1 newborn mice, however, do not express either CD8α or CD4 markers and so they were tested unseparated.

The production of IL-12 by CD8α<sup>−</sup> and CD8α<sup>+</sup> DC from mice of different age groups is shown in Fig. 4A. In the case of day 1 newborn mice, in which the majority of splenic DC were
CD8α−CD205+; the production of IL-12 was measured from the whole DC population. Interestingly, although they were CD8α−, these DC produced a significant amount of IL-12p70 upon stimulation (Fig. 4A). Although the 1 wk neonatal CD8α+ DC produced 2- to 3-fold less IL-12p70 than adult CD8α+ DC, the CD8α− DC produced similar amounts of IL-12p70. The characteristic pattern of CD8α+ DC as the major producers of IL-12p70, as found in adult mice, was established by 2 wk of age. However, the total amount of IL-12p70 produced by CD8α+ DC at this stage was still 2-fold less than that of adult CD8α+ DC. Because the majority of the splenic CD8α+ DC in day 1 and 1-wk-old mice was CD8α−CD205+, and they could produce similar amounts of IL-12p70 as that by CD8α+ DC (Figs. 2C and 4A), it is likely that these CD8α−CD205+ cells represented the immature stage of the CD8α+CD205+ DC. Similar results were obtained from the purified CD8α+CD205+ DC isolated from 2-wk-old mice (data not shown).

As reported previously, the CD8α− splenic DC of adult mice produced higher amounts of IL-12p40 than the CD8α− DC upon activation in the absence of IL-4. When the DC populations of mice at different ages were tested for their potential to produce IL-12p40, the CD8α− DC population from all age groups showed similar levels of IL-12p40 production (Fig. 4B). Interestingly, the CD8α− DC population from day 1 and 1-wk-old mice again produced similar amounts of IL-12p40 as CD8α+ DC (Fig. 4B), whereas the CD8α− DC population of adult mice produced much less IL-12p40 than CD8α+ DC. These results indicated that these CD8α−CD205+ DC in the neonatal stage shared similar properties with CD8α−CD205+ DC. This again suggested that the neonatal CD8α−CD205+ cells might represent the immature stage of the CD8α+CD205+ DC. Overall, the above results demonstrated that DC populations from neonatal mice could produce relatively higher levels of IL-12p40, but lower levels of IL-12p70 than their DC counterparts in adult mice.

One of the properties of adult CD8α− (CD4−) DC is to produce significant amounts of IFN-γ upon activation with IL-12p70 and IL-18. We also tested the neonatal and adult DC for their capacities...
influenza virus or bacterial CpG motifs. This type of response is important for limiting early bacterial and especially viral infections. In addition, a role for the linking of innate and adaptive immunity by p-preDC has been suggested (18). To test whether p-preDC from neonates are as efficient as p-preDC from adult mice in IFN-α production, the amount of IFN-α produced by p-preDC from mice of different ages was determined by specific ELISA after 18 h of culture with GM-CSF and CpG. Interestingly, although the splenic p-preDC of newborn mice had a reduced capacity to produce IFN-α, those of young (1- to 2-wk-old) mice displayed a higher capacity than those from adult mice (Fig. 5). This result suggested that p-preDC might play a more important role in the innate immune response during the early development of mice.

**Capacity of DC populations from mice at different ages to stimulate allogenic T cell proliferation**

One of the important functions of DC is to activate and stimulate naive T cells to proliferate. To compare DC of different ages for their abilities to stimulate allogenic CD4^+ and CD8^+ T cell proliferation, purified splenic CD8α^- and CD8α^+ DC from 1-, 2-, and 6-wk-old mice were tested in an allogenic MLR. When different numbers of purified DC from different ages of C57BL/6 mice were cocultured with 2 × 10^6 CD4^+ LN T cells purified from CBA/CaH mice, comparable intensities and kinetics of T cell proliferative responses were seen with DC from all age groups tested (Fig. 6A). Similar results were obtained for CD8^+ allogenic T cell proliferation (data not shown). Therefore, the DC from neonatal mice and those from adult mice showed comparable ability to stimulate allogenic T cell proliferation, although it was not clear whether they could be equally efficient in inducing effector T cells.

**Capacity of DC populations from mice at different ages to stimulate Ag-specific OT-II T cell proliferation**

The presentation of Ag to naive T cells and activation of Ag-specific T cells are major functions of DC. We therefore compared the capacity of DC populations from mice at different ages to present Ag to naive Ag-specific T cells and to induce the proliferation of these T cells. As shown in Fig. 6B, when pulsed with OVA Ag, newborn splenic DC showed a lower capacity to induce

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**FIGURE 4.** Cytokine production by DC populations from mice of different ages. A and B, IL-12p70 and p40 production by DC populations from mice of different ages. Total splenic DC from 1-day-old mice (all were CD4^+CD8α^- double negative (DN)) and CD8α^- and CD8α^+ splenic DC populations from mice of 1, 2, and 6 wk of age were purified. The purified DC were stimulated in culture for 18 h with GM-CSF, IFN-γ, IL-4, and CpG for IL-12p70 production or with GM-CSF, IFN-γ, and CpG for IL-12p40 production. The amounts of IL-12p70 and IL-12p40 produced by these DC in the culture supernatants were measured by the specific ELISAs. C, IFN-γ production by DC populations from mice of different ages. The same DC populations as those described above were cultured for 48–60 h with IL-12p70 and IL-18 for IFN-γ production. The amount of IFN-γ produced in the culture supernatants was measured by a specific ELISA. The values shown are the average of triplicate samples. The error bars represent the ranges.

**FIGURE 5.** IFN-α production by p-preDC from mice of different ages. The p-preDC were purified from the spleens of mice on day 1 and weeks 1, 2, and 6 of age. The purified p-preDC were activated in the culture for 18 h with GM-CSF, IFN-γ, IL-4, and CpG for IFN-α production. The amount of IFN-α produced by p-preDC in the culture supernatant was measured by ELISA. The values shown are the average of triplicate samples. The error bars represent the ranges.
FIGURE 6. Stimulation of allogenic T cell or OVA-specific OT-II T cell proliferation by DC populations of neonatal and adult mice. A, Stimulation of allogenic T cell proliferation. Splenic CD8\textsuperscript{+} and CD8\textsuperscript{−} DC were purified from the spleens of 1- and 6-wk-old C57BL/6 mice, respectively. Different numbers of purified DC (1,000–4,000) were cocultured with 20,000 CD4\textsuperscript{+} T cells isolated from LN of CBA/CaH mice for the indicated times. T cell proliferation was determined by the measurement of the incorporation of \[ \text{H} \] TdR. The values shown are the average of triplicate samples. The error bars represent the SDs.

B, Stimulation of OVA Ag-specific OT-II T cell proliferation. Total splenic DC from 1-day-, 1-wk-, and 6-wk-old mice were pulsed with OVA Ag and incubated with CFSE-labeled OT-II T cells for 2.5 days. The number of proliferating OT-II T cells was measured and calculated by FACS analysis for viable propidium iodide \textsuperscript{−} CFSE\textsuperscript{−} cells. The data presented are from one of two such experiments with similar results.
the proliferation of OVA-specific OT-II T cells than their adult counterparts. Because CD8α− DC represent the majority of 1 wk neonatal splenic DC in contrast to 20% in adult mice, purified CD8α+ neonatal DC were also compared with adult splenic CD8α+ DC for their capacity to present Ag and to stimulate Ag-specific T cell proliferation. The OVA-pulsed 1 wk neonatal CD8α+ DC showed a lower capacity than their adult counterpart to stimulate OT-II T cell proliferation (data not shown). These results suggested that neonatal DC were less effective in Ag uptake/processing/presentation than adult DC.

Discussion

It is well known that neonates have an immature immune system and are more susceptible to microbial infections. It has been shown that T cells from neonatal mice can be primed equally well as their adult counterparts (6). The contribution of neonatal T cells to the immaturity of the neonatal immune system was therefore considered not crucial, and the maturation status of DC was suggested as a determining factor (6). A large number of studies of neonatal DC function have emerged recently (7, 15, 19–22). However, the results of these studies varied considerably, mainly due to the differences in the DC populations examined, the types of Ags tested, and the assay systems used. In an attempt to understand the possible cellular basis of the incompetence of the neonatal immune system, we examined the levels of different DC populations in mouse lymphoid organs throughout mouse ontogeny and compared their functional potentials.

Our studies demonstrated that DC (CD11c+ MHC class II+) and p-preDC (CD45RA+/CD11c++)-like cells could be detected in mouse thymus as early as E17, which coincided with the appearance of CD4−CD8+ thymocytes and the beginning of thymic selection processes. We also found that the percentage of DC among thymocytes remained constant after birth, i.e., the increase in numbers of thymic DC paralleled the increase in total number of thymocytes. These findings support the view that thymic DC play a role in thymocyte development, probably through involvement in the thymic negative selection processes (23–26).

We have also found that both conventional DC and p-preDC were present in the lymphoid organs of newborn mice, although in low numbers. The absolute and per cell levels of DC and p-preDC in the spleen did not reach adult levels until ~5 wk of age. The ratio of p-preDC to DC in the spleen was higher in the younger mice (~1:1) than that in adult mice (1:4). The change in the ratio of p-preDC to DC was due to a dramatic increase in conventional DC numbers after 3 wk of age. The p-preDC of younger mice were able to produce even higher levels of IFN-α than those of adult mice, indicating that they were functionally mature and might play a crucial role in the innate immunity to microbial pathogens at this early age. These findings suggested a transition from a dominant innate immunity at the neonatal stage to the establishment of a more efficient adaptive immune system in the adult mice.

It is interesting that marked changes in the composition of the conventional DC populations in mouse spleen were observed during ontogeny. The CD4−CD8α−CD205+ DC was the major DC population in the spleen of mice at 1–2 wk of age. It is this CD4−CD8α−CD205+ DC population that has been shown to selectively capture apoptotic cells and induce immunological tolerance in the steady state (27). Our data therefore suggest that the domination of the CD4−CD8α−CD205+ DC population at an early age might be responsible for the increased susceptibility of neonatal mice to the induction of immunological tolerance. Our results also demonstrate that different DC populations develop with different kinetics, with CD4−CD8α−CD205+ DC developing earlier than CD4−CD8α−CD205− and CD4−CD8α−CD205− DC during ontogeny. It is of interest that our previous studies also showed similar hierarchy in the developmental kinetics of the three splenic DC populations when irradiated mice were reconstituted with adult mouse bone marrow precursor cells (28, 29).

One apparent exception, however, was observed in the spleen of 1-day newborn mice, in which only a CD4−CD8α− DC population was present. However, unlike the CD4−CD8α− DC population in adult mouse spleen, which is mainly CD205+, the CD4−CD8α− DC in the newborn mouse spleen was mainly CD205−. A significant proportion of the CD4−CD8α− DC population in the spleen of 1-wk-old mice was also CD205−. This CD8α−CD205− population became undetectable in the spleen after 3 wk of age. Similar findings have been reported recently by another group (22). Functional studies revealed that these neonatal CD4−CD8α−CD205− DC had similar properties as CD8α−CD205+ DC in IL-12p70 and IL-12p40 production. These findings suggested that neonatal CD8α−CD205− cells could represent an immature stage of CD8α−CD205+ DC. However, these CD8α−CD205− DC failed to up-regulate the expression of CD8α after 3 days in culture in the presence of various cytokines and stimuli (GM-CSF, IL-4, IFN-γ, and CpG) that induce DC functional maturation (data not shown). In vivo studies are required for further investigation of the relationship of these two splenic DC populations in neonatal mice.

An important measure of the functional capacity of DC is their ability to produce certain cytokines on stimulation. IL-12 is an immunoregulatory cytokine that favors the differentiation of Th1 cells and is crucial for the activation of naive CD8+ T cells and the production of cytolytic effector T cells (30, 31). DC are a major source of IL-12 in response to microbial infections. The capacity to produce IL-12 is considered one of the major functions of mouse splenic CD8α+ DC (12). IFN-γ has an essential role in resistance to microbial and intracellular pathogens, and it can be produced by different immune cell types, including T cells, NK cells, DC (CD4+CD8α− DC in mouse), and some B cells (12, 32–34). When activated with the stimuli able to reveal their full capacity to produce cytokines, the splenic CD8α+ DC of young mice produced lower levels of IL-12p70 than their adult counterparts. Similarly, the CD8α− DC of neonatal and young mice showed lower potential for producing IFN-γ than the adult CD8α− DC. In these respects the DC from neonatal and young mice were functionally less potent than their adult DC counterparts. These results appear to be different from those reported by Sun et al. (22), in which neonatal DC were shown to produce higher amount of IL-12p70 and IFN-γ than adult DC. These differences could be due to the different stimuli used in these two studies. In our study, instead of using an individual stimulus, we used a combination of cytokines and stimuli (CpG1668, GM-CSF, IFN-γ, and IL-4 for IL-12p70 production, or IL-12 and IL-18 for IFN-γ production), which had been shown to stimulate the maximum amount of cytokine production by adult DC. We therefore measured the full capacity of cytokine production by each DC population. However, the actual amount of this capacity that is revealed will depend on the particular stimulus used.

In contrast to the IL-12p70 results, we found that the overall amount of IL-12p40 produced by neonatal DC was higher than that produced by adult DC. Both CD8α+ and CD8α− DC populations of neonatal mice produced a large amount of IL-12p40, in contrast to the adult mice in which only the CD8α− DC produced a large amount of IL-12p40. Because IL-12p70 is the bioactive form of IL-12 and is required for the induction of Th1-type immune responses, whereas IL-12p40, when it forms homodimers, may have an inhibitory effect on IL-12 signaling (30, 35, 36), the cytokine production profile of neonatal DC therefore suggests that they
would not be as efficient as adult DC in the induction of a Th1-type of immune response and the generation of cytolytic CD8+ effector T cells, which are crucial for specific resistance to microbial and intracellular pathogens. These findings are in line with the view that there is a bias to the Th2-type responses in neonatal mice (2, 19, 37, 38). However, it is possible that the IL-12p40, produced in larger amounts by neonatal DC, forms dimers with a p19 subunit to form IL-23. IL-23 may provide some new insights.

In addition to the lower efficiency in cytokine production by the neonatal DC, a lower capacity to process/present Ag to Ag-specific T cells by neonatal DC was observed in the in vitro OT-II T cell proliferation assays. These results again suggested that the neonatal DC are functionally less mature than the adult DC.

It is also interesting to note that although neonatal DC showed lower efficiency in cytokine production and in Ag processing/presentation, they had a comparable capacity to stimulate allogenic T cell proliferation as their adult counterparts. This suggests that the capacity of stimulating T cell proliferation by a DC subset is not always correlated with its capacity for cytokine production or induction of effector T cells. This is consistent with the fact that T cell proliferation can equally be followed by activation-induced cell death, development of regulatory T cells, or development of effector T cells. In addition, it has been suggested that the induction of immunological tolerance is an active process, which also requires activation of T cells by DC (41, 42). Therefore, the comparable T cell proliferation responses induced by neonatal and adult DC in allogenic MLR shown by this study do not necessarily reflect an equal capacity of neonatal and adult DC to induce an effector T cell response.

Overall, our data suggested that the lower number of DC in the lymphoid tissues of neonatal mice and their lower capacity to produce IL-12p70 and IFN-γ and to induce Ag-specific T cell proliferation would contribute to the lower efficiency in eliciting specific immune responses in neonatal and young mice. This view is consistent with a recent report (7) that showed enhanced immunity against intracellular pathogens and virus infection when neonatal mice were treated with Flt3L. The Flt3L treatment increased the number of p-preDC and DC, and the effect involved IFN-α- and IL-12-associated immune responses. Although the immaturity of neonatal T cells may also contribute to the incompetence of the neonatal immune system, our study did not attempt to address this issue. The findings from this study demonstrated that although innate immunity may be sufficient for the resistance to certain pathogens in neonatal mice, the full control of a variety of microbial infections can only be achieved when adaptive immunity is established. A prerequisite for this is the gradual development of a functionally mature DC system.

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References


