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Epigenetic Reduction in Invariant NKT Cells following In Utero Vitamin D Deficiency in Mice

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Vitamin D status changes with season, but the effect of these changes on immune function is not clear. In this study, we show that in utero vitamin D deficiency in mice results in a significant reduction in invariant NKT (iNKT) cell numbers that could not be corrected by later intervention with vitamin D or 1,25-dihydroxy vitamin D$_3$ (active form of the vitamin). Furthermore, this was intrinsic to hematopoietic cells, as vitamin D-deficient bone marrow is specifically defective in generating iNKT cells in wild-type recipients. This vitamin D deficiency-induced reduction in iNKT cells is due to increased apoptosis of early iNKT cell precursors in the thymus. Whereas both the vitamin D receptor and vitamin D regulate iNKT cells, the vitamin D receptor is required for both iNKT cell function and number, and vitamin D (the ligand) only controls the number of iNKT cells. Given the importance of proper iNKT cell function in health and disease, this prenatal requirement for vitamin D suggests that in humans, the amount of vitamin D available in the environment during prenatal development may dictate the number of iNKT cells and potential risk of autoimmunity. The Journal of Immunology, 2011, 186: 1384–1390.

Vitamin D is produced in the skin following sunlight exposure, and, as a result, there are seasonal changes in vitamin D that occur. Infants born in the winter start out with low levels of vitamin D that rise in the summer, and infants born in the summer start out with higher levels of vitamin D that dip in the winter (1, 2). The effects of changing levels of vitamin D on immune function are not known.

Vitamin D that is either ingested or made in the skin following sunlight exposure is inactive and transported to the liver where it is converted to 25-hydroxy vitamin D$_3$ (25(OH)D$_3$), the major circulating form of the vitamin. The active form of vitamin D (1,25-dihydroxy vitamin D$_3$, 1,25D$_3$) is produced from the hydroxylation of the precursor 25(OH)D$_3$ by the enzyme 1α-hydroxylase (CYP27B1 gene) (3–6). Vitamin D status and 1,25D$_3$ treatments have been shown to regulate immune function and suppress experimental autoimmunity, including experimental autoimmune encephalomyelitis (EAE) (7).

Activation of mature NKT cells delays the onset and reduces the symptoms of experimental autoimmune diseases such as EAE.

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Abbreviations used in this article: BM, bone marrow; Cyp, Cyp27B1; 1,25D$_3$, 1,25-dihydroxy vitamin D$_3$; D$, vitamin D-sufficient; D$, vitamin D-deficient; DP, double-positive; DP$^{ab}$, CD24$^+$CD4$^{ab}$CD8$^{ab}$; EAE, experimental autoimmune encephalomyelitis; Egr2, early growth response 2; αGalCer, α-galactosylceramide; iNKT, invariant NKT; KO, knockout; 25(OH)D$_3$, 25-hydroxy vitamin D$_3$; RXR, retinoid X receptor; VDR, vitamin D receptor; WT, wild-type.

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iNKT cell precursors in the thymus of D− mice. Vitamin D supplementation of D− WT and D− CypKO mice beginning at 3 wks of age and continuing until 8 wk failed to increase iNKT cell numbers. 1,25D3 supplementation of either D− WT or D− CypKO mice during the same time frame increased iNKT cell numbers but not to the level present in vitamin D-sufficient (D+) WT mice. Earlier 1,25D3 supplementation was also ineffective at increasing iNKT cell numbers to those present in the D+ WT mice. The data support a differential role for vitamin D and the VDR in controlling the development of iNKT cells. Additionally, there is an intrinsic requirement for vitamin D and 1,25D3 for optimal iNKT cell numbers to develop. Later alterations in vitamin D and/or 1,25D3 fail to recover iNKT cells.

Materials and Methods

Mice

Age- and sex-matched CypKO and WT C57BL/6 mice were produced at the Pennsylvania State University (University Park, PA). CypKO mice were a gift from Dr. Hector DeLuca (University of Wisconsin, Madison, WI). For all experiments, heterozygous (Cyp ko+/−) mice were used as breeders so that the WT and CypKO littermates would be fed the same diets throughout the experiment. For D− mice, heterozygous breeders were fed synthetic diets that do not contain vitamin D, as described previously (28). For some experiments mice were continued on the D− diet following weaning and until sacrifice (week 8). D− mice had undetectable serum 25(OH)D levels (<5.3 nMl). Another experimental design used the D− littermates and fed diets that contained vitamin D or 1,25D3 (25–50 ng/day/mouse) from 3 wk age until 8 wk age. The 1,25D3 dose was increased from 25 to 50 ng/day when the mice weighed 18 g or more. In one experimental design, pregnant mothers were switched to diets that included 50 ng/day/mouse at embryonic day 20 or day 13 and continued on the diets. In another design, the breeders were fed 50 ng/day/mouse 1,25D3 throughout pregnancy. The weaning mice were fed diets that contained 1,25D3 (25–50 ng/day/mouse) from 3 wk age until 8 wk age. The 1,25D3 dose was increased from 25 to 50 ng/day when the mice weighed 18 g or more. All experimental procedures received approval from the Office of Research Protection’s Institutional Animal Care and Use Committee (Pennsylvania State University, University Park, PA).

αGalCer stimulation

αGalCer (Axxora, San Diego, CA) was dissolved in PBS containing 0.5% Tween 20, heated to 80°C for 10 min, and sonicated for 5 min on ice. Mice were i.p. injected with 2 μg αGalCer or vehicle. Blood was collected from the retro-orbital plexus for serum isolation.

Flow cytometry

Single-cell suspensions of thymus, spleen, and liver were isolated. Mononuclear cells from liver were prepared as described previously (27). Cells were stained with PE-labeled CD1d-αGalCer tetramers (gift of the National Institutes of Health Tetramer Facility, Atlanta, GA). mAbs used in this study for flow cytometry include FITC-labeled anti-CD4 (L3T4), PE-labeled anti-NK1.1 (PK136), PE-Cy5–labeled anti-TCRβ (H57-597), FITC-labeled anti-B220 (RA3-6B2), PE-labeled anti-CD11b (M1/70), PE-

![FIGURE 1. Reduced numbers of iNKT cells in 1,25D3-deficient mice. A]() In all cases, iNKT cells are defined as being TCRβ+ and αGalCer-CD1d tetramer+. The empty tetramer and isotype control staining can be found in Supplemental Fig. 1. The frequency of iNKT cells in the thymus, spleen, and liver from WT, VDR KO, and CypKO mice is shown. Values are the mean ± SEM of n = 10 mice/group. The percentage of iNKT cells in VDR KO and CypKO thymus and liver are significantly different from those in WT mice (p < 0.01). B, Mice were injected with αGalCer in vivo followed by intracellular staining ex vivo as described in Materials and Methods. Histograms show production of IL-4 by iNKT cells from one representative mouse. IL-4 isotype control staining is shown in Supplemental Fig. 1. Mean ± SEM values of n = 10/group. The percentage of IL-4–producing iNKT cells in VDR KO mice is significantly lower than that in WT and CypKO mice (p < 0.01). C, Thymocytes from WT and 1,25D3-deficient mice were incubated with or without (control, Ctrl) the CD1d-restricted NKT cell hybridoma, and IL-2 production was measured. No IL-2 was produced from the WT and 1,25D3-deficient thymocytes cultured alone (Ctrl). Results shown are from one representative of three independent experiments with thymocytes from n = 3 mice/group.

![FIGURE 2. Decreased iNKT cell numbers in vitamin D-deficient mice. A]() Percentage of iNKT cells in thymus and liver from D− WT, D− CypKO, D− WT, and D− CypKO mice (n = 10–15 mice/group). Values are the mean ± SEM. B, Serum cytokine production in D− WT and D− mice induced by systemic administration of αGalCer. The values from the D− CypKO and D− WT mice overlap. Levels of IFN-γ and IL-4 in the serum were determined at different times following injection (n = 9/group). Values are mean ± SEM. **p < 0.001; *p < 0.05. C, Frequency of cytokine-producing iNKT cells from D− WT and D− livers. A representative histogram from each group shows production of IFN-γ by iNKT cells. IFN-γ isotype control staining is shown in Supplemental Fig. 1. Values are mean ± SEM of 10 mice/group. D, Dot plots showing expression of CD44 and NK1.1 on TCRβ+ CD1d-αGalCer tetramer DP thymocytes. The percentage of CD44+ NK1.1− iNKT cells and CD44+NK1.1+ iNKT cells in D− WT or D− CypKO mice are significantly different from D− WT mice (p < 0.05). Values are mean ± SEM (n = 8/group).
BM transplantation

BM cells were harvested from D\textsuperscript{2}, 1,25D\textsubscript{3} D\textsuperscript{3}, 1,25D\textsubscript{3} CypKO, D\textsuperscript{−}, and D\textsuperscript{−} CypKO (CD45.2) mice and transferred into sublethally (950 rad) irradiated WT and D\textsuperscript{2} KO mice. The frequency and number of CD8\textsuperscript{+} T cells in the D\textsuperscript{2} KO spleen. However, when the absolute numbers of CD4\textsuperscript{+} T cells were calculated, the D\textsuperscript{2} KO mice had normal numbers of other leukocyte populations in the thymus, spleen, and liver. One million thymocytes from WT and CypKO mice were incubated with 5 \times 10\textsuperscript{4} DN32D3 NKT cell hybrids (gift of Dr. Albert Bendelac, University of Chicago, Chicago, IL) for 24 h, and IL-2 in the supernatant was measured by ELISA (BD Pharmingen).

Ag presentation assay

One million thymocytes from WT and CypKO mice were incubated with 5 \times 10\textsuperscript{4} DN32D3 NKT cell hybrids (gift of Dr. Albert Bendelac, University of Chicago, Chicago, IL) for 24 h, and IL-2 in the supernatant was measured by ELISA (BD Pharmingen).

Statistical analysis

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, La Jolla, CA). Cell percentage and numbers were compared by ANOVA. A p value \leq 0.05 was considered statistically significant.

| Table I. Specific iNKT cell defects as a consequence of vitamin D deficiency |
|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Mice                        | D\textsuperscript{2} WT      | D\textsuperscript{3} WT      | D\textsuperscript{3} KO      |
| Cell Type                   | Frequency (%) No. Cells      | Frequency (%) No. Cells      | Frequency (%) No. Cells      |
| Spleen                      |                             |                             |                             |
| iNKT                        | 1.14 \pm 0.20 596,220        | 0.5 \pm 0.01\textsuperscript{a} 325,000                   | 0.4 \pm 0.0\textsuperscript{a} 244,400                   |
| NK                          | 1.6 \pm 0.1 836,800          | 12 \pm 0.2 780,000          | 13 \pm 0.1 794,300          |
| T                           | 46 \pm 1.4 24,058,000        | 46 \pm 2.4 29,900,000       | 47 \pm 0.7 28,717,000       |
| NK                          | 5 \pm 0.5 2,615,000          | 5 \pm 0.7 3,250,000         | 6 \pm 0.5 5,094,000         |
| CD4\textsuperscript{+} T    | 57 \pm 0.4 13,713,060        | 50 \pm 1.3\textsuperscript{a} 14,950,000                     | 48 \pm 1.3\textsuperscript{a} 13,784,160                     |
| CD8\textsuperscript{+} T    | 36 \pm 0.4 8,660,880         | 39 \pm 1.8 11,661,000       | 42 \pm 1.3\textsuperscript{a} 12,061,140                     |
| B                           | 45 \pm 2 23,535,000          | 41 \pm 2 26,650,000        | 36 \pm 2.5 21,996,000       |
| Macrophage                  | 12 \pm 2 6,276,000          | 14 \pm 2 9,100,000         | 15 \pm 1 9,165,000         |
| Thymus                      |                             |                             |                             |
| iNKT                        | 0.49 \pm 0.03 247,940        | 0.07 \pm 0.005\textsuperscript{a} 31,360                      | 0.06 \pm 0.01\textsuperscript{a} 28,140                      |
| NK                          | 0.56 \pm 0.06 283,360        | 0.13 \pm 0.02\textsuperscript{a} 58,240                      | 0.12 \pm 0.01\textsuperscript{a} 56,280                      |
| CD4\textsuperscript{+} T    | 12 \pm 1 6,072,000          | 10 \pm 2 4,480,000         | 10 \pm 1 4,690,000         |
| CD8\textsuperscript{+} T    | 6 \pm 0.8 3,036,000         | 7 \pm 0.5 3,136,000        | 4 \pm 0.6 1,876,000        |
| DP                          | 80 \pm 4 40,480,000         | 79 \pm 2 35,392,000        | 81 \pm 3 37,989,000        |
| Liver                       |                             |                             |                             |
| iNKT                        | 32 \pm 2 1,024,000          | 2 \pm 0.3\textsuperscript{a} 6,200                           | 1.8 \pm 0.2\textsuperscript{a} 5,940                           |
| NKT                        | 32 \pm 2 1,024,000          | 18 \pm 3\textsuperscript{a} 558,000                         | 16 \pm 1\textsuperscript{a} 528,000                         |
| NK                          | 14 \pm 2 448,000           | 11 \pm 1.5 341,000        | 13 \pm 1 429,000           |
| T                           | 28 \pm 3 896,000           | 30 \pm 4 930,000         | 31 \pm 2 1,023,000         |

Values are the frequencies expressed as percentiles and the mean \pm SEM of four mice per group and one representative of nine individual experiments. Markers for different cell types are as follows: iNKT, CD1D16-galCer tetramer "TCRb; NK, CD3\textsuperscript{+}NK1.1; T, CD3\textsuperscript{+}NK1.1; CD4, percentage of CD4\textsuperscript{+} cells among CD3\textsuperscript{+} cells; CD8, percentage of CD8\textsuperscript{+} cells among CD3\textsuperscript{+} cells; B, B220\textsuperscript{+}; macrophage, F4/80\textsuperscript{+}; Th, 3.1-3.3 \times 10\textsuperscript{6} cells isolated) of any mice. In the experiment shown, the frequency of CD4\textsuperscript{+} T cells was lower in the D\textsuperscript{2} WT and D\textsuperscript{3} KO spleen. However, when the absolute numbers of CD4\textsuperscript{+} T cells were calculated, the values were higher in the D\textsuperscript{2} WT and D\textsuperscript{3} KO than in the D\textsuperscript{2} WT mice. The frequency and number of CD8\textsuperscript{+} T cells in the D\textsuperscript{3} KO mice were higher than in the D\textsuperscript{2} WT mice. A change in the CD4 and/or CD8 cell frequencies was not reproducible in D\textsuperscript{2} mice. Conversely, the alterations in iNKT cell frequencies occurred in every experiment.

*p < 0.05; **p < 0.001, values significantly different from D\textsuperscript{2} WT mice.

Results

Reduced numbers of iNKT cells from 1,25D\textsubscript{3} deficiency

The role of 1,25D\textsubscript{3} in iNKT cell development was probed. CypKO mice are not able to produce 1,25D\textsubscript{3} from 25(OH)D\textsubscript{3} and therefore are 1,25D\textsubscript{3}-deficient mice. The 1,25D\textsubscript{3}-deficient mice had significantly lower frequencies of iNKT cells in the thymus and liver compared with WT littermates (Fig. 1A). The percentages present in 1,25D\textsubscript{3}-deficient mice were comparable to the percentages present in the VDR KO mice (Fig. 1A). Consistent with the decreased percentage of iNKT cells in the 1,25D\textsubscript{3}-deficient mice, the absolute numbers of iNKT cells were significantly reduced compared with WT littermates (Supplemental Fig. 2). This was a selective defect in iNKT cells since the 1,25D\textsubscript{3}-deficient mice had normal numbers of other leukocyte populations in the thymus, spleen, and liver, including T cells, B cells, NK cells, and macrophages (data not shown).

Previously, we have shown that VDR KO iNKT cells are functionally defective (27). To look at the function of iNKT cells in the absence of 1,25D\textsubscript{3}, WT and 1,25D\textsubscript{3}-deficient mice were injected with galCer, and production of IL-4 by liver iNKT cells was analyzed ex vivo. The frequencies of IL-4- and IFN-\textgamma-producing iNKT cells were similar in 1,25D\textsubscript{3}-deficient compared with WT mice (Fig. 1B and IFN-\textgamma data not shown). Sixty-one \pm 5\% of WT iNKT cells and 61 \pm 3\% of 1,25D\textsubscript{3}-deficient iNKT cells produced IL-4 (Fig. 1B). This is in contrast to the failure of iNKT cells from VDR KO mice to make either IL-4 or IFN-\textgamma (Fig. 1B) (27). The VDR KO mice have significantly fewer IFN-\textgamma and IL-4-producing iNKT cells than do WT or 1,25D\textsubscript{3}-deficient mice (Fig. 1B) (27). The results indicate a differential role for the VDR and 1,25D\textsubscript{3} ligand in regulating iNKT cell function versus numbers.
Previous work showed that VDR KO thymocytes were poor stimulators of the NKTC cell hybridoma (27). As shown for VDR KO thymocytes, 1,25D3-deficient mice expressed lower levels of CD1d than did WT mice (mean fluorescence intensity, 56 ± 3 1,25D3-deficient mice, 86 ± 4 WT mice) (27). Thymocytes from WT and 1,25D3-deficient mice were used to activate the CD1d-restricted NKTC cell hybridoma. 1,25D3-deficient thymocytes were as potent as WT thymocytes in stimulating the NKTC cell hybridoma to produce IL-2 (Fig. 1C). The results suggest that the VDR and 1,25D3 regulate iNKT cell development through different mechanisms.

**The effect of vitamin D deficiency on iNKT cells**

Because of the failure of CypKO mice to convert 25(OH)D3 to 1,25D3, 25(OH)D3 might accumulate in the CypKO mice at concentrations high enough to bind and activate the VDR (29). To eliminate 25(OH)D3, production, D− diets were fed to breeding and experimental mice. D− mice were confirmed to have no detectable 25(OH)D3 in circulation. The D+ WT thymus contained only 0.08% of iNKT cells as compared with 0.6% in the D+ WT thymus (8-fold reduction, Fig. 2A). The D− CypKO mice were indistinguishable from the D+ WT mice (Fig. 2A). Similar results were found in the periphery with the liver of D− WT and D− CypKO mice showing only 1.65% iNKT cells compared with 27% in the D+ WT mice (15-fold reduction, Fig. 2A). This was a selective defect in iNKT cells from the D− mice since the normal numbers of T cells, B cells, NK cells, and macrophages were present in the spleen, thymus, and liver of D− mice (Table I). The numbers and percentages of iNKT cells recoverable in the VDR KO and 1,25D3-deficient mice were significantly higher than those found from D+ mice (Figs. 1A, 2A).

To rule out the possibility that the D− iNKT cells homed to other tissues, D− WT and D− CypKO littermates were injected with αGalCer and serum was collected for cytokine analysis. IFN-γ production by D− WT mice peaked at 6 h after injection and then disappeared by 48 h (Fig. 2B). The kinetics of IL-4 production were different from those of IFN-γ since IL-4 peaked earlier (2 h) in D+ WT mice (Fig. 2B). D− CypKO and D− WT mice made very low amounts of both IL-4 and IFN-γ at all time points tested (Fig. 2B). As observed for the 1,25D3-deficient iNKT cells, remaining D− iNKT cells were functionally normal and contained equal percentages of both IFN-γ- and IL-4-producing liver iNKT cells compared with their D+ WT counterparts (Fig 2C and IL-4 data not shown). The very low cytokine response to αGalCer injection confirms the data that show very low numbers of iNKT cells in the D− mice.

To identify what stages of iNKT cell development are affected by vitamin D deficiency, CD44 and NK1.1 expression on thymic iNKT cells were measured. The DP CD44/NK1.1 fully mature iNKT cells made up most cells in the D− and D− mice (Fig. 2D). However, the percentage of CD44/NK1.1 DP iNKT cells was significantly lower in the D− host compared with the D+ mice (Fig. 2D). The mature CD44/NK1.1 DP iNKT cells in D− mice likely accounts for the ability of these iNKT cells to produce cytokines. All three stages of CD44/NK1.1-expressing iNKT cells were significantly reduced when absolute numbers were calculated in thymocytes from D− mice. The data show that D− mice contain fewer iNKT cells, but that unlike the VDR KO iNKT cells, the remaining iNKT cells function normally.

**Requirement of early exposure to vitamin D for iNKT cell development**

To determine whether feeding mice D+ diets could recover iNKT cell development from D− mice, D− CypKO and D− WT mice littermates from D− Cyp ko/+ breeders were fed D+ diets from 3 to 8 wk of age (Fig. 3A). The numbers of iNKT cells in the thymus and liver were determined. Surprisingly, vitamin D interventions that started at 3 wk age were unable to recover iNKT cells in either the D− CypKO or D− WT mice (Fig. 3A). The D+ feeding did result in an increase in 25(OH)D3 levels that confirmed the effectiveness of the diets (data not shown). The only mice that contain normal numbers of iNKT cells are the D+ WT mice that were exposed to vitamin D throughout development (Fig. 3A). The extremely low levels of iNKT cells in D− WT mice were not changed by vitamin D intervention.

To test whether feeding D− mice the active form of vitamin D would rescue iNKT cell numbers, D− mice were fed 1,25D3 from the ages of 3 to 8 wk (1,25D3 late, Fig. 3B). The frequency of iNKT cells in D− WT and D− CypKO mice increased significantly with 1,25D3 treatment from 0.05–0.07% to 0.17–0.18% in thymus, and from 1.3–1.6% to 4–7% in liver (Fig. 3B). However, the percentage of iNKT cells in 1,25D3 fed D− mice was still significantly lower than that of D+ WT mice. To investigate whether feeding D− mice with 1,25D3 for longer and starting earlier would rescue iNKT cell numbers, mothers of D− mice were fed 1,25D3 beginning just before birth (embryonic day 20) and continuing during lactation (1,25D3 early, Fig. 3C). At 3 wk age, these mice were fed D+ diets and continued until 8 wk age (1,25D3 late). Values are mean ± SEM of $n=5$ mice/group. B, Effect of 1,25D3 intervention on iNKT cell numbers. Cyp ko/+ breeders started on D+ diets at 3 wk age and continued until 8 wk age (D+). Values are mean ± SEM of $n=5$ mice/group. C, Effect of early 1,25D3 intervention on iNKT cell numbers. Cyp ko/+ breeders started on D− diets, and littersmates were switched to D+ diet at 3 wk age and continued until 8 wk age (D+). Values are mean ± SEM of $n=8$ mice/group. C, Effect of early 1,25D3 intervention on iNKT cell numbers. Cyp ko/+ breeders started on D− diets, and littersmates were switched to 1,25D3 at 3 wk age and continued until 8 wk age (1,25D3 late). Values are mean ± SEM of $n=12–15$ mice/group. D, Effect of continuous supplementation of 1,25D3 on iNKT cell numbers. Breeders and offspring were fed 1,25D3 throughout lactation (1,25D3 late). Continuous supplementation of 1,25D3 resulted in normalization (D+ WT numbers) of the iNKT cell numbers in both the WT and CypKO mice. Values are mean ± SEM of $n=6–8$ mice/group. *$p<0.001$; **$p<0.05$.

**FIGURE 3.** Epigenetic effects of vitamin D deficiency on iNKT cell numbers. A, Effect of vitamin D intervention on iNKT cells. Cyp ko/+ breeders started on D− diets, and littersmates were switched to D+ diet at 3 wk age and continued until 8 wk age (D+). Values are mean ± SEM of $n=5$ mice/group. B, Effect of 1,25D3 intervention on iNKT cell numbers. Cyp ko/+ breeders started on D− diets, and littersmates were switched to 1,25D3 at 3 wk age and continued until 8 wk age (1,25D3 late). Values are mean ± SEM of $n=8$ mice/group. C, Effect of early 1,25D3 intervention on iNKT cell numbers. Cyp ko/+ breeders started on D− diets, and intervention with 1,25D3 started just before birth (embryonic day 20) and continuing during lactation (1,25D3 early, Fig. 3C). At 3 wk age, these mice were fed D+ diets and continued until 8 wk age (1,25D3 late). Values are mean ± SEM of $n=5$ mice/group. B, Effect of 1,25D3 intervention on iNKT cell numbers. Cyp ko/+ breeders started on D− diets, and littersmates were switched to 1,25D3 at 3 wk age and continued until 8 wk age (1,25D3 late). Values are mean ± SEM of $n=12–15$ mice/group. D, Effect of continuous supplementation of 1,25D3 on iNKT cell numbers. Breeders and offspring were fed 1,25D3 throughout lactation (1,25D3 late). Continuous supplementation of 1,25D3 resulted in normalization (D+ WT numbers) of the iNKT cell numbers in both the WT and CypKO mice. Values are mean ± SEM of $n=6–8$ mice/group. *$p<0.001$; **$p<0.05$. The Journal of Immunology 1387
weaning, the mice were continued on the 1,25D3 until sacrifice (8 wk) and iNKT cells were analyzed. Even with early 1,25D3 intervention the frequency of iNKT cells in D− mice was significantly lower than that in D+ WT mice (Fig. 3C). Even before the appearance of DP thymocytes (embryonic day 13), 1,25D3 intervention of D− mice failed to fully recover iNKT cell numbers (Supplemental Fig. 3). Feeding Cypko/+ breeders 1,25D3 throughout (1,25D3; Fig. 3D) resulted in normal numbers of iNKT cells in both the WT and CypKO mice (Fig. 3D). The data demonstrate an early requirement for vitamin D and 1,25D3 for normal numbers of iNKT cells to develop.

Intrinsic defect of iNKT cell precursors in the absence of 1,25D3

To study whether the defective iNKT cell numbers in vitamin D deficiency were cell intrinsic, BM transplants were done. Reconstitution of the thymus was 95–97% and the spleen was 85–87%, and regardless of the treatment or source of BM cells there were no differences in the ability of the donor cells to reconstitute WT mice (Fig. 4A). Donor-derived BM from CypKO and WT mice exposed to 1,25D3 throughout gestation (1,25D3 and 1,25D3 KO) repopulated the iNKT cell numbers to the same level of the untreated WT reconstitution (~0.35% donor iNKT cells; Fig. 4B). BM from D− mice (D− WT or D− KO) failed to repopulate iNKT cells to the WT levels (Fig. 4B). Half as many iNKT cells developed when the BM was from a D− donor as compared with when the donor was 1,25D3 treated. The frequency of iNKT cells that developed from D− WT and D− CypKO BM was the same (Fig. 4B).

Because iNKT cells use the donor-derived DP thymocytes both as APCs and iNKT cell precursors, mixed chimeras were done using a 1:1 mix of D+ WT (CD45.1) and CypKO BM (CD45.2) cells. The thymus, liver, and spleen were repopulated with about half of the cells from each donor (Fig. 5A). There was a selective reduction in iNKT cells derived from CypKO BM in the thymus, spleen, and liver of recipients (Fig. 5B). The thymus had 28%, the spleen had 37%, and the liver had 41% of the iNKT cells derived from CypKO BM. Vitamin D and 1,25D3 deficiency results in a cell-intrinsic iNKT cell defect.

Increased apoptosis of early iNKT cell precursors in the thymus of D− mice

To determine whether the mechanisms underlying the low numbers of iNKT cells in D− mice were due to a reduction in homeostatic proliferation or increased apoptosis, the percentage incorporation of BrdU or the annexin V staining was measured. There were no differences in the rates of BrdU incorporation of iNKT cells in the thymus and liver of D− and D+ WT mice (Supplemental Fig. 4). Comparison of the percentage of annexin V+ iNKT cells in the thymus of D+ and D− mice showed that a higher percentage of the D− iNKT cells (both WT and CypKO) than D+ iNKT cells were annexin V+ undergoing apoptosis (45–47% D− versus 30% D+) (Fig. 6A). Early iNKT cell precursors were gated on by identifying the DP+ cell population and tetramer+ cells (Fig. 6B). These cells were further evaluated for CD24 and annexin V expression. In D− mice most of the iNKT cells are of the more mature CD24+ phenotype, and most of the apoptosis occurs in the CD24+ less mature iNKT cells (Fig. 6C). There are significantly lower percentages of the more mature CD24+ iNKT cells in the D− compared with D+ hosts (60% D− versus 91% D+; Fig. 6C). Additionally, a significantly higher percentage of apoptosis was observed in the CD24+ iNKT cell precursor populations from D− than D+ WT mice (62% versus 15%), while CD24+ subpopulations exhibited similar percentages (60% versus 60%) of apoptosis (Fig. 6C).

Discussion

iNKT cells have an early requirement for vitamin D and 1,25D3. Based on the inability of early 1,25D3 treatment to recover iNKT cell numbers in the D− mice, we conclude that vitamin D is required before the appearance of the first tetramer+ cells in the thymus (day 5). Vitamin D deficiency results in a cell-intrinsic defect in iNKT cells. Our data suggest that a vitamin D-regulated committed precursor for iNKT cells may be present in the BM. Timed pregnancies show that vitamin D is required before embryonic day 13 for normal numbers of iNKT cells to develop.

Factors that affect very early iNKT cell development are beginning to be identified. iNKT cell development requires rearrangement of the Vα14Jα18 TCRα-chain. The Jα18 rearrange-

FIGURE 4. Intrinsic defect of iNKT cell precursors in the absence of 1,25D3. BM transplants were done using D+, 1,25D3, and D− WT donor mice into WT recipients (donor BM → recipient). A. Reconstitution of the thymus and spleen of WT (CD45.1) recipients with donor BM (CD45.2) of WT, 1,25D3, D−, 1,25D3KO, and D− KO mice. Values are mean ± SEM of n = 5 mice/group. B. Percentages of donor CD45.2-gated iNKT cells in the thymus and spleen are shown for the same groups of mice shown in A. The results from the 1,25D3 WT mice were identical to those from 1,25D3 KO mice, and the results from the D− WT mice were identical to those from the D− KO mice. Data shown are one representative of n = 5 mice/group, and the mean ± SEM is given for all 5 mice.
ment is a secondary rearrangement that occurs only if the primary rearrangements fail to generate a productive TCRα-chain. Because the Jα18 rearrangement takes longer than primary TCRα rearrangements, factors that prolong DP cell survival have been shown to be required for iNKT cell development (30). Several transcription factors have been shown to affect DP thymocyte survival and, in particular, HEB has been shown to be important in the development of iNKT cells (31). Interestingly, HEB-deficient thymocytes had reduced DP thymocyte survival, defective iNKT cell development (31). Interestingly, HEB-deficient thymocytes had reduced DP thymocyte survival, defective iNKT cell development (31). Interestingly, HEB-deficient thymocytes had reduced DP thymocyte survival, defective iNKT cell development (31). Interestingly, HEB-deficient thymocytes had reduced DP thymocyte survival, defective iNKT cell development (31). Interestingly, HEB-deficient thymocytes had reduced DP thymocyte survival, defective iNKT cell development (31). Interestingly, HEB-deficient thymocytes had reduced DP thymocyte survival, defective iNKT cell development (31). Interestingly, HEB-deficient thymocytes had reduced DP thymocyte survival, defective iNKT cell development (31). Interestingly, HEB-deficient thymocytes had reduced DP thymocyte survival, defective iNKT cell development (31). Interestingly, HEB-deficient thymocytes had reduced DP thymocyte survival. HEB is a transcription factor that controls expansion of CD24+ iNKT cells (34). The Jα18 rearrangement allows the primary TCRα chain to be generated in the thymus, spleen, liver, and lymph nodes. Half of the cells are of WT CD45.1 origin and half of the cells are of CypKO CD45.2 origin. B, Dot plots showing percentage of donor-derived iNKT cells in the thymus, spleen, liver, and lymph nodes. Data shown are from n = 8 mice (mean ± SEM).

FIGURE 5. 1,25D3 deficiency results in an intrinsic defect of iNKT cells. Competitive BM chimeras were generated using a 1:1 ratio of WT CD45.1 and CypKO CD45.2 BM into WT CD45.1 recipients. A, Lymphocyte chimerism was checked by flow cytometry in the thymus, spleen, liver, and lymph nodes (LN). Half of the cells are of WT CD45.1 origin and half of the cells are of CypKO CD45.2 origin. B, Dot plots showing percentage of donor-derived iNKT cells in the thymus, spleen, liver, and lymph nodes. Data shown are from n = 8 mice (mean ± SEM).

FIGURE 6. Increased cell apoptosis in D− iNKT cell precursors. A, Annexin V staining of iNKT cells in the thymus from D+ and D− mice. TCRβ and βGalCer-CD1d tetramer+ cells were gated. Data shown are representative of n = 8 mice/group. Values are mean ± SEM. The percentage of annexin V+ iNKT cells is significantly different between D− WT and D− WT or D− CypKO mice (p < 0.05). B, Gating strategy for identifying DP dull iNKT cell precursors. The DP dull cells in the top panel are gated and evaluated for tetramer+ iNKT cells (bottom panel). The DP dull /tetramer+ cells are then gated. C, CD24 and annexin V staining of iNKT cell precursors gated in B. Negative control staining for annexin V is shown in Supplemental Fig. 1. Data represent n = 8 mice/group. The percentage of CD24+/DP dull tetramer+ cells is significantly different between D− WT and D− WT mice (p < 0.01). The apoptosis is significantly higher in the D− iNKT cell precursors (CD24+/DP dull tetramer+ cells, p < 0.001).

The VDR KO mice have a block in iNKT cell development. The failure of VDR KO iNKT cells to develop normally is reflected in the poor activation of the iNKT cell hybridoma by VDR KO DP thymocytes but not by 1,25D3-deficient or D−-derived DP thymocytes (27). There are other examples in the literature where VDR and vitamin D deficiency result in disparate results. Vitamin D deficiency has been shown to increase susceptibility to EAE whereas VDR deficiency makes the animals more resistant (7, 36). In experimental allergic asthma, vitamin D deficiency has no effect whereas VDR-deficient mice fail to develop allergic asthma (37). The VDR is a nuclear receptor that regulates transcription, and there are at least two possibilities of how the unliganded VDR regulates iNKT development/function. Unliganded VDR might bind to other proteins (nuclear receptor corepressor is a possibility) that normally inhibit the induction of iNKT cells by DP thymocytes. The other possibility is that VDR functions as a heterodimer with the retinoid X receptor (RXR). In the absence of the VDR excess, RXR would be available to dimerize with one of the many other nuclear receptors that require RXR for activity. This other nuclear receptor (estrogen receptor, retinoic acid receptor, glucocorticoid receptor) would then be an inhibitor of the induction of iNKT cells by the DP thymocytes.

Early exposure of neonatal mice to vitamin D is required for mice to develop optimal numbers of iNKT cells. Vitamin D
deficiency results in epigenetic changes in iNKT cells that cannot be rescued by later exposure to vitamin D or 1,25D3. The reduced numbers of iNKT cells is a result of increased apoptosis of early iNKT cell precursors in the thymus of the D-/- host. Expression of the VDR is required for normal Ag presentation by DP thymocytes whereas ligand deficiency has no effect on DP thymocyte development. The amount of vitamin D available in the environment early during development of iNKT cells dictates the number of iNKT cells. The reduced numbers of iNKT cells is a result of increased apoptosis of early CD4+/CD8- NK1.1+ NKT cell precursors whereas ligand deficiency has no effect on DP thymocyte development.

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Disclosures
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