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Most NK1.1+ T (NKT) cells express a biased TCRαβ repertoire that is positively selected by the monomorphic MHC class I-like molecule CD1d. The development of CD1d-dependent NKT cells is thymus dependent but, in contrast to conventional T cells, requires positive selection by cells of hemopoietic origin. Here, we show that the Src protein tyrosine kinase Fyn is required for development of CD1d-dependent NKT cells but not for the development of conventional T cells. In contrast, another Src kinase, Lck, is required for the development of both NKT and T cells. Impaired NKT cell development in Fyn-deficient mice cannot be rescued by transgenic expression of CD8, which is believed to increase the avidity of CD1d recognition by NKT cells. Taken together, our data reveal a selective and non-redundant role for Fyn in NKT cell development. The Journal of Immunology, 1999, 163: 4091–4094.

Natural killer T (NKT) cells express a TCRαβ as well as phenotypic markers common to the NK cell lineage, including NK1.1 and IL-2Rβ (CD122) (1, 2). Two types of NKT cells can be distinguished on the basis of their requirement for the monomorphic MHC class I-like molecule CD1d during development (3). The majority of NKT cells are CD1d dependent (4–7), express a TCR repertoire biased to Vε14 (8) and Vβ8.2 (9–11), and segregate preferentially in thymus and liver (3). A second type of NKT cells is CD1d independent, expresses a diverse TCR repertoire, and is found mainly in spleen and bone marrow (3). A significant proportion of CD1d-independent NKT cells develop in the absence of the thymus, suggesting that the two types of NKT cells may belong to distinct cell lineages.

Protein tyrosine kinases of the Src family, including Fyn (p59fyn) and Lck (p62lck), are important components of proximal TCR/CD3 signaling (12). Fyn noncovalently associates with CD3ζ, but is not required for normal development of thymocytes and peripheral T cells (13, 14). In contrast, Lck associates with the cytoplasmic domains of CD4 and CD8α and is critical for both pre-TCR (15) and TCR (12) signaling. Indeed, Lck-deficient mice show a dramatic reduction in immature CD4+CD8+ thymocytes, no detectable single positive thymocytes, and a low number of peripheral T cells (16).

While the signaling properties of Fyn and Lck have been extensively studied in conventional T cells, their role in NKT cell development and function is not clear. Interestingly, it has been reported that NKT cells do not develop in Fyn- and Lck-double deficient mice (17). Here, we tested whether Fyn and Lck, individually, are required for normal NKT cell development. Surprisingly, we find that the number of CD1d-dependent NKT cells is severely reduced in Fyn-deficient mice, whereas CD1d-independent NKT cells and conventional T cells are virtually unaffected. In Lck-deficient mice, only low numbers of both NKT and T cells can be found. These results demonstrate that Fyn plays a selective and non-redundant role in NKT cell development.

Materials and Methods
Mice
C57BL/6, Lck-deficient (Lck−/−), and CD8αβ-transgenic mice were purchased from Harlan/Netherlands (Zeist, The Netherlands), The Jackson Laboratory (Bar Harbor, ME), and Taconic (Germantown, NY), respectively. Fyn-deficient (Fyn−/−) mice were provided by Dr. Roger Perlmutter (Merck Research Laboratories, Rahway, NJ). All mutant mice had been back-crossed to C57BL/6 mice for several generations. CD8αβ-transgenic Fyn−/− or Lck−/− mice were derived at the Ludwig Institute from F2 intercrosses between CD8αβ-transgenic and Fyn−/− or Lck−/− mice or back-crosses between CD8αβ-transgenic Fyn−/− or Lck−/− F1 mice and Fyn+/− or Lck+/− mice. Fyn typing was performed by PCR as described elsewhere (13), whereas Lck−/− mice were identified based on the severely decreased number of T cells in PBL as a consequence of Lck deficiency (16). All mice were used at 8–12 wk of age.

Cell preparation
Single-cell suspensions were prepared from liver, spleen, thymus, and bone marrow. Total liver cells were resuspended in a 40% isotonic Percoll solution (Pharmacia, Uppsala, Sweden) and underlaid with an 80% isotonic Percoll solution. Centrifugation for 20 min at 2000 rpm isolated the mononuclear cells at the 40–80% interface. The cells were washed twice with PBS containing 2% FCS. Spleen cells and bone marrow (femur, tibia) cells were resuspended in DMEM medium supplemented with 5% FCS and 1% HEPES and loaded onto 10-ml nylon wool columns that were preincubated overnight at 37°C with supplemented medium. The columns were incubated 45 min at 37°C and the cells, purified of B cells and monocytes, were harvested by washing the columns with 20 ml of supplemented medium. Thymocytes were resuspended in PBS containing 2% FCS together with a 1:10 dilution of B2A2 (anti-heat-stable Ag) hybridoma culture supernatants. After an incubation of 45 min at 4°C, the cells were washed and incubated for another 45 min at 37°C with an appropriate dilution of rabbit complement. The live mature (heat stable Ag+) thymocytes were isolated and washed twice.
Flow cytometry

A maximum of 1 × 10^6 cells were preincubated with 50 μl of 2.4G2 culture supernatant to block Fεy receptors. Cells were washed and incubated with the indicated mAb conjugates for 40 min in a total volume of 100 μl of PBS containing 2% FCS. Cells were washed and, if required, incubated with streptavidin conjugates for 20 min. After a further wash, cells were resuspended in PBS containing 2% FCS and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA) for 3-color stainings or on a FACScalibur flow cytometer (Becton Dickinson) for 4-color stainings.

Antibodies

The following mAbs were purchased from PharMingen (San Diego, CA): FITC-, APC-, or biotin-conjugated anti-TCRβ (H57-597), PE-conjugated anti-NK1.1 (PK136), FITC- or CyChrome-conjugated anti-CD4 (H129.19), and CyChrome-conjugated anti-CD8α (53-6.7). APC-conjugated streptavidin was purchased from Molecular Probes Europe (Leiden, The Netherlands). FITC-conjugated TCR Vβ8.2 (F23.2) was prepared at the Ludwig Institute.

Results and Discussion

We first determined the proportions of NKT (TCRαβ^+ NK1.1^+ ) and conventional T (TCRαβ^- NK1.1^- ) cells in several organs of C57BL/6 (wild-type), Fyn-deficient (Fyn^-/^-), and Lck-deficient (Lck^-/^-) mice. As reported previously (13, 14), the proportion of conventional T cells is similar in wild-type and Fyn^-/^- mice (Fig. 1A and data not shown). Surprisingly, the proportion of NKT cells is severely decreased in Fyn^-/^- mice, in particular in thymus and liver, and to a lesser extent in spleen and bone marrow (Fig. 1, A and C). The decrease of NKT cells in Fyn^-/^- mice is not the consequence of a lack of NK1.1 expression, because T cells found in Fyn^-/^- mice do not express a biased TCR repertoire (data not shown) as is the case for NKT cells in wild-type mice (9–11). In Lck^-/^- mice, the development of both NKT and conventional T cells is severely impaired (16, 17) (Fig. 1, A and C). These data indicate that Fyn plays a nonredundant role in NKT cell development, whereas Lck can apparently substitute for Fyn in T cell development (17, 18).

Three NKT cell subsets can be defined on the basis of CD4/CD8 coreceptor expression, namely CD4^+, CD4^-CD8^- (DN), and CD4^-CD8^+ NKT cells (1, 2). We have shown recently that most CD4^- NKT cells are CD1d dependent, whereas a significant proportion of DN NKT cells and most CD8^+ NKT cells develop in the absence of CD1d (3). Moreover, CD1d-dependent NKT cells, and in particular CD4^- NKT cells, express a biased TCR repertoire, including a preferential usage of the Vβ8.2 chain (3). In wild-type animals, a majority (~70%) of liver and thymus NKT cells express CD4 and very few express CD8 (~2%), whereas higher proportions of spleen and bone marrow NKT cells express CD8 (20–25%) or are DN (60–65%) (Fig. 1, B and C). (3). Interestingly, the subset composition of NKT cells is profoundly altered in Fyn^-/^- mice (Fig. 1, B and C). Indeed, the absolute number of CD4^- NKT cell is reduced ~20-fold in thymus and liver and 2- to 6-fold in spleen and bone marrow of Fyn^-/^- mice, whereas the number of CD8^- NKT cells is only marginally affected. Moreover, the overall proportion of Vβ8.2^+ NKT cells is significantly reduced in Fyn^-/^- mice (Fig. 2), even though residual CD4^- NKT cells still express a fully Vβ8.2-biased TCR repertoire (Fig. 2B).

<FIGURE 1. Impaired development of CD1d-dependent NKT cells in Fyn^- or Lck-deficient mice. Thymus, liver, spleen, and bone marrow cells isolated from C57BL/6, Fyn^- , or Lck-deficient mice were stained for TCRαβ, NK1.1, CD4, and CD8. A, Numbers indicate the proportion of NKT and T cells in liver. The experiment shown is representative of three independent experiments. B, Expression of CD4 and CD8 by liver NKT cells as gated in A. Numbers indicate the proportion of CD4^+ or CD8^- NKT cells. The experiment shown is representative of three independent experiments. C, Absolute numbers of total NKT cells, CD4^- NKT cells, and CD8^- NKT cells in different organs of C57BL/6 ( ), Fyn-deficient ( ), and Lck-deficient ( ) mice. Data are the mean ± SD of three to five independent experiments.>

<FIGURE 2. The TCR Vβ repertoire of NKT cells in Fyn^- or Lck-deficient mice. Thymus, liver, spleen, and bone marrow cells isolated from C57BL/6, Fyn^- , or Lck-deficient mice were stained for TCRαβ, NK1.1, CD4, or CD8, and Vβ8.2. A, Vβ8.2 expression by liver NKT cells. Numbers indicate the proportion of Vβ8.2^- NKT cells among total NKT cells. The experiment shown is representative of three independent experiments. B, Percentage of Vβ8.2^- NKT cells among total NKT cells, CD4^- NKT cells, and CD8^- NKT cells in different organs of C57BL/6 ( ), Fyn-deficient ( ), and Lck-deficient ( ) mice. Numbers are the mean ± SD of three to five independent experiments. Low numbers of thymus CD8^- NKT cells and of CD4^- and CD8^- NKT cells in all organs of Lck-deficient mice did not permit an accurate estimation of Vβ8.2 expression.>
Fyn<sup>−/−</sup> mice express normal levels of CD1d (data not shown), it is excluded that the absence of Fyn affects the development of CD1d-dependent NKT cells indirectly by regulating expression of CD1d. Together, these results show that the absence of Fyn selectively and severely impairs the development of CD1d-dependent (CD4<sup>+</sup>) NKT cells, but only marginally affects the development of CD1d-independent (CD8<sup>+</sup>) NKT cells. In Lck<sup>−/−</sup> mice, the few remaining NKT cells, found essentially in spleen and bone marrow, express CD8 (~40%) or are DN (50–60%) (Fig. 1, B and C) and do not express a biased TCR repertoire (Fig. 2).

T cells from Fyn<sup>−/−</sup> mice have been shown in several instances to be hyporesponsive to Ag-, mitogen-, or Ab-mediated activation. Indeed, whereas peripheral T cells isolated from Fyn<sup>−/−</sup> mice respond normally to TCR-mediated activation, single positive (CD4<sup>+</sup> or CD8<sup>+</sup>) thymocytes are refractile to stimulation (13, 14). Moreover, Utting et al. reported that single positive thymocytes and lymph node T cells isolated from Fyn<sup>−/−</sup> mice are hyporesponsive to stimulation by low-affinity or low-density cognate ligands (19). Finally, T cell lymphoproliferation and autoimmunity in lpr/lpr mice are suppressed in the absence of Fyn (20, 21). Taken together, these observations suggest that the absence of Fyn increases the avidity threshold required for T cell activation via the TCR. Therefore, it is reasonable to propose that Fyn may also increase the avidity threshold required for the development of CD1d-dependent NKT cells. As a consequence, NKT cell precursors expressing the seminvariant TCR would fail to undergo positive selection in the absence of Fyn. Interestingly, Bendelac et al. have reported that a CD8αβ-transgene, expressed under the control of the CD2 promoter, prevents the development of CD1d-dependent NKT cells (defined as V<sub>α8.2</sub>-biased CD4<sup>44hi</sup> thymocytes) (22). Therefore, it was suggested that CD8 expression is incompatible with the normal development of CD1d-dependent NKT cells, because CD8 interaction with CD1d (23) would lead to increased avidity of the seminvariant TCR on NKT cell precursors for CD1d, ultimately resulting in negative selection (22). According to such a model, the absence of Fyn might be compensated by the presence of CD8 during development, leading to the appearance of a novel subset of CD8<sup>+</sup> CD1d-dependent NKT cells in Fyn<sup>−/−</sup> mice. However, the absolute number of CD8<sup>+</sup> NKT cells is not increased in Fyn<sup>−/−</sup> mice as compared with wild-type mice (Fig. 1C), and these cells do not express a V<sub>β8.2</sub>-biased TCR repertoire in the absence of Fyn (Fig. 2B), as would be expected for CD1d-dependent NKT cells (3). Moreover, forced overexpression of a CD8αβ transgene in Fyn<sup>−/−</sup> mice does not rescue development of CD1d-dependent NKT cells (Fig. 3), and even inhibits development of the small number of CD1d-dependent NKT cells usually present in Fyn<sup>−/−</sup> mice (Fig. 3). Taken together, these data imply that physiological (or even elevated) levels of CD8 expression on NKT cells or their precursors does not set an avidity threshold appropriate for the development of CD1d-dependent NKT cells in the absence of Fyn. Clearly, other scenarios in which the expression of CD8 on NKT cell precursors may be qualitatively incompatible with the development of CD1d-dependent NKT cells remain to be tested.

In addition to TCR-mediated signaling, Fyn is involved in signaling mediated by integrins, Thy-1, CD2, and IL receptors. It has been shown recently that ligation of β<sub>1</sub>-integrins by components of the extracellular matrix induces association of Fyn with the integrin α subunit (24, 25) and with the focal adhesion kinase (26, 27), leading to activation of the Ras/Raf/mitogen-activated protein (MAP)/extracellular signal-related kinase (MEK)/MAP pathway and regulation of cell cycle progression. Nevertheless, NKT cells develop normally in mice expressing dominant negative mutants of Ras and/or MEK (28). Other surface receptors, including Thy-1 (29), the adhesion molecule CD2 (or LFA-2) (30, 31), and the IL receptors IL-2R/IL-15Rβ (32, 33), IL-3R (34), IL-5R (35), and IL-7R (36, 37), activate and/or associate with Fyn upon binding of their cognate ligand. Several of these molecules have been shown to play a role in NKT cell development. Indeed, mice deficient for the common γ-chain, a critical component of IL-2R, IL-4R, IL-7R, IL-9R, and IL-15R, have Var14<sup>+</sup> (but NK1.1<sup>+</sup>) T cells in the thymus, but not in periphery, showing that development of CD1d-dependent NKT cells is incomplete (38). Moreover, IL-2R/IL-15Rβ<sup>−/−</sup> mice have a marked defect in NKT cell development (39). Finally, even though the proportions of NKT cells are normal in IL-7−/− mice (but reduced 10-fold in terms of absolute numbers), they fail to produce cytokines upon stimulation (40). Therefore, the impaired development of CD1d-dependent NKT cells in Fyn<sup>−/−</sup> mice may reflect the requirement for TCR-independent pathways of activation mediated by adhesion molecules and/or IL receptors.

In conclusion, we have shown that the development of CD1d-dependent NKT cells is selectively impaired in Fyn<sup>−/−</sup> mice. Hence, Fyn may contribute to the maintenance of a signaling threshold that is critical for TCR-mediated positive selection of CD1d-dependent NKT cells, but not for selection of CD1d-independent NKT cells and conventional T cells. Alternatively (or in addition), Fyn may be involved in integrin, Thy-1, CD2, or IL receptor-mediated signaling that is critical for the development of CD1d-dependent NKT cells, but not for other subsets of T cells. Whatever the explanation, our data provide the first example of a signaling molecule that is required for NKT cell development, yet dispensable for development of both NK cells and conventional T cells.

**Note added in proof.** Data complementary to those reported here have been obtained independently by Gadue et al. (41).

### References

