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Invariant NKT Cell Development Requires a Full Complement of Functional CD3 ζ Immunoreceptor Tyrosine-Based Activation Motifs

Amy M. Becker,* Jon S. Blevins,† Farol L. Tomson,‡ Jennifer L. Eitson,* Jennifer J. Medeiros,* Felix Yarovinsky,* Michael V. Norgard,† and Nicolai S. C. van Oers* ,†

Invariant NKT (iNKT) cells regulate early immune responses to infections, in part because of their rapid release of IFN-γ and IL-4. iNKT cells are proposed to reduce the severity of Lyme disease following Borrelia burgdorferi infection. Unlike conventional T cells, iNKT cells express an invariant αβ TCR that recognizes lipids bound to the MHC class I-like molecule, CD1d. Furthermore, these cells are positively selected following TCR interactions with glycolipid/CD1d complexes expressed on CD4+CD8+ thymocytes. Whereas conventional T cell development can proceed with as few as 4/10 CD3 immunoreceptor tyrosine-based activation motifs (ITAMs), little is known about the ITAM requirements for iNKT cell selection and expansion. We analyzed iNKT cell development in CD3 ζ transgenic lines with various tyrosine-to-phenylalanine substitutions (YF) that eliminated the functions of the first (YF1,2), third (YF5,6), or all three (YF1–6) CD3 ζ ITAMs. iNKT cell numbers were significantly reduced in the thymus, spleen, and liver of all YF mice compared with wild type mice. The reduced numbers of iNKT cells resulted from significant reductions in the expression of the early growth response 2 and promyelocytic leukemia zinc finger transcription factors. In the mice with few to no iNKT cells, there was no difference in the severity of Lyme arthritis compared with wild type controls, following infections with the spirochete B. burgdorferi. These findings indicate that a full complement of functional CD3 ζ ITAMs is required for effective iNKT cell development. The Journal of Immunology, 2010, 184: 6822–6832.

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iNKT cells in the thymus and periphery (28). Moreover, the absence of protein kinase C θ, a serine/threonine kinase involved in calcium mobilization and NF-AT activation, results in impaired iNKT cell development (29). Invariant NKT cell development involves a large number of transcription factors, including NF-κB, Ets-1, Runx, GATA-3, promyelocytic leukemia zinc finger (PLZF), IRF-1, and T-bet (8). These diverse transcription factors regulate iNKT cell commitment, differentiation, expansion, and distribution.

To determine whether iNKT cell development and function are dependent on ITAM number, we analyzed these cells in a set of CD3-ε transgenic mice in which selected tyrosine-to-phenylalanine substitutions were introduced to eliminate the functions of the first (YF1,2), third (YF5,6), or all three CD3-ε (YF1–6) ITAMs. In this study, we show that iNKT cell development is almost completely abrogated in mice lacking as few as two of six functional CD3-ε ITAMs. The developmental block occurred at an early iNKT cell developmental stage, and this was correlated with increased cell death in developing iNKT cells. The few iNKT cells that formed in the CD3-ε ITAM-substituted lines contained more CD4-CD8- (DN) subsets relative to the CD4+CD8+ (CD4+), opposite to that observed in wild type mice. Despite a near absence of iNKT cells, there was no difference in the onset of inflammatory response against Borrelia burgdorferi infection with the spirochete causing Lyme disease, iNKT cells, there was no difference in the onset of inflammatory response against that observed in wild type mice. Despite a near absence of iNKT cells, there was no difference in the onset of inflammatory response against Borrelia burgdorferi infection with the spirochete causing Lyme disease, iNKT cells, there was no difference in the onset of inflammatory response against that observed in wild type mice.

Materials and Methods

CD3-ε transgenic mice

The CD3-ε transgenic mice, containing selected tyrosine-to-phenylalanine substitutions in the CD3-ε ITAMs, are termed YF1,2, YF5,6, and YF1–6 (Supplemental Fig. 1) (20, 21, 39, 40). The mice were generated directly onto the C57BL/6 background using the VA-CD2 transgenic cassette that drives expression in T cells, NKT cells, and NK cells (41). All these lines were maintained on a CD3-ε-deficient background, eliminating contributions from the wild type endogenous gene. At least five different transgenic founders or constructs were established, with those that were matched for TCR expression levels being similar to wild type mice selected founders or constructs were established, with those that were matched for TCR expression levels being similar to wild type mice selected.

iNKT cell isolation

Lymphocytes were isolated from the thymus, lymph node, and spleen as previously described (45). Bone marrow was isolated from the spinal column, femur, and tibia. To isolate intrahepatic lymphocytes (IHLs), livers were obtained after perfusion with 10 ml cold DPBS with Ca2+-Mg2+ chelators. Livers were isolated by Percoll gradient centrifugation using standard procedures (GE Healthcare, Piscataway, NJ). Bone marrow and IHL populations were washed in HBSS prior to use (Mediatech, Herndon, VA).

Flow cytometric analyses

Staining buffer consisted of 1% FBS in DPBS with Ca2+-Mg2+ (Mediatech, Herndon, VA). Between 1 and 2.5 × 10^6 cells were pretreated for 10–30 min at 4˚C with the 2.4G2 mAb to block nonspecific Fc receptor binding. Cells were then stained with the various Abs, and/or tetraters (diluted in FACs buffer) for 30 min to 1 h at 4˚C followed. Thirty thousand to 1 × 10^5 cells per sample were acquired on the LSRII (BD Biosciences, San Jose, CA). Data were analyzed using FlowJo software (Tree Star, Ashland, OR). iNKT cells were isolated from pooled spleens with a combination of magnetic bead depletion and cell sorting. Between three and five spleens per YF line or wild type controls were isolated, prepared as a single cell suspension, and depleted of RBCs. The remaining cells were depleted of CD4-CD8+ T cells and B cells with a combination of anti-CD8- and anti-B220-conjugated magnetic beads, according to the manufacturers’ instructions (Invitrogen, Carlsbad, CA). The remaining cells were stained with a mixture of mAbs including anti-CD3 e-FITC, PE-labeled murine PBS57-loaded CD1d tetramer, and allopregoycin-conjugated to anti-B220 and anti-CD11b mAbs. These cells were sorted for CD3+tetramer+B220+CD11b− cells. From two independent sorts, the number of iNKT cells isolated from wild type mice was >5 × 10^5. The YF1,2 lines had >40,000 cells per sort, and ~1 × 10^5 cells were obtained from the YF5,6 and YF1–6 lines. It should be noted that the YF1,2 cells exhibited low cell-surface expression of CD3ε and the tetramerε, suggesting an abnormal development (Fig. 3A).

Real-time PCR

RNA was isolated using standard Trizol extraction procedures (Invitrogen). Containing genomic DNA was removed with Turbo DNA-free according to the manufacturer’s suggestions (Ambion, Austin, TX). cDNA was synthesized using the Oligo(dT)15 Primer (Promega, Madison, WI) and the SMARTScribe Reverse Transcriptase kit (Clontech, Mountain View, CA). SYBR Advantage qPCR Premix (Clontech) was used in all reactions. Real-time PCR was performed on the MX3000P (Agilent Technologies, Santa Clara, CA). The primers used in the assays are included in Supplemental Table I.

Borreliaburgdorferi infection studies

Six-week-old mice were injected intradermally with 1.2–1.5 × 10^6 B. burgdorferi spirochaetes (strain 297) or with media alone (46). In certain experiments, preimmune and postimmunization blood samples were taken. Whole bacterial extracts (2.5 μg/ml), generated from B. burgdorferi using sonication procedures, were coated onto ELISA plates and used as the immunogen. ELISA assays were performed on serial dilutions of the sera from media control and B. burgdorferi-infected mice. Ab binding was detected by incubating the plates with HRP-conjugated goat anti-mouse IgG Abs. Reactions were developed using standard colorimetric assays. Ear punch biopsies were taken from the mice on day 14 postinfection, were incubated in Barbour–Stoerner–Kelly II media for 6 d, and the presence or absence of bacteria was confirmed by visualization of outgrowth spirochetes using dark phase microscopy. Two or 6 wk postinfection, mice were sacrificed. Hind limbs and cardiac tissues were isolated and placed in 10% buffered formalin for 7 d at room temperature in the dark. Hearts, knees, and tibiotarsal joints were submitted for histologic analysis as described below.

Histologic analysis and tissue scoring

Hearts, knees, and tibiotarsal joints were sent to the University of Texas Southwestern Pathology Core (Dallas, TX) for processing, paraffin embedding, and sectioning. Seven-micrometer sections were cut, processed, and stained with H&E. Sections were then sent to the University of Texas Southwestern ARC Diagnostic Unit (Dallas, TX) for blinded scoring. Arthritis scores were scored on a scale of 0–3 by the amount of lymphocyte infiltration into the tissue: 0 = no inflammation; 1 = mild; 2 = moderate; and 3 = highly inflamed joints as evidenced by the amount of lymphocyte infiltration into the tissue. Carditis and aortitis were scored using a similar

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Results

CD3 ε ITAMs are required for iNKT cell development

The development of αβ T cells can occur in mice containing as few as four functional CD3-encoded ITAMs (47). Thus, thymocyte development is relatively normal in mice containing tyrosine-to-phenylalanine substitutions in the first (YF1,2), third (YF5,6), or all three CD3 ε ITAMs (YF1–6), eliminating either 2 or 6 of the 10 functional ITAMs in the TCR complex (Fig. 1D; Supplemental Figs. 1 and 3A–D) (20, 21, 39, 40). Much less is known about the number of functional ITAMs required for iNKT cell development. To determine whether the development of iNKT cells was also permissive with reduced numbers of functional ITAMs, we analyzed the YF series of mice for the presence of these cells. These mice retain 8/10 (YF1,2 and YF5,6) or 4/10 (YF1–6) functional ITAMs, with the YF1,2 and YF5,6 differing only in the presence or absence of the constitutively phosphorylated 21-kDa form of CD3 ε (Supplemental Fig. 1) (20, 21, 39, 40). The iNKT cells were detected with glycolipid-loaded CD1d tetramers (PBS57) combined with anti-CD3 ε mAbs. The percentage of iNKT cells, defined as CD3intermediate and CD1d tetramer+, was significantly reduced in the thymus, spleen, and liver of the YF1,2 (0.02, 0.02, and 1.17%), YF5,6 (0.02, 0.05, and 0.30%), and YF1–6 (0.05, 0.07, and 0.59%) mice, compared with wild type controls (0.35, 0.66, and 15.6%; Fig. 1A, 1B). The lack of iNKT cells was also reflected in a statistically significant decrease in absolute number of these cells in the thymus and spleen of YF1,2, YF5,6, and YF1–6 lines compared with the wild type controls (Fig. 1C). Furthermore, iNKT cells from all the YF series of mice were reduced in the bone marrow (Supplemental Fig. 2). The total thymic cellularity was similar in all mice analyzed (Fig. 1D). Furthermore, there were similar percentages and absolute numbers of DP thymocytes in the all the mice analyzed (Supplemental Fig. 3B). These DP cells function as the precursor population from which iNKT cells are derived. In addition, DP thymocytes express the selecting ligands required for iNKT cell development. Given the equal numbers and equivalent CD1d cell surface expression in the DP thymocytes suggested that the failure of iNKT cells to develop in the YF series of mice is intrinsic to weakened TCR signaling in these cells. Interestingly, the mice that lacked only two of the six CD3 ε-encoded ITAMs (YF1,2 and YF5,6) had a more severe reduction in iNKT cell numbers, compared with those missing all CD3 ε ITAMs (YF1–6) (Fig. 1A). This finding is likely a consequence of the increased TCR density in the YF1–6 lines, which occurs because of decreased CD3 ε ubiquitination and degradation (Supplemental Fig. 3D) (43, 44). Regardless of these effects, our results demonstrate that normal iNKT cell development requires a full complement of CD3 ε ITAMs.

iNKT cells from mice lacking two or more functional CD3 ε ITAMs have increased cell death

Mice deficient in the protein tyrosine kinase Itk and the transcription factor Egr2 have reduced iNKT cell numbers, in part because of increased cell death in the thymus (27, 28). To examine whether a similar mechanism occurred in the YF sets of mice, the CD3intermediate CD1d tetramer+ iNKT cells were also stained with annexin V and 7AAD to detect dead cells. Consistent with the results in Fig. 1, the percentage of these iNKT cells was significantly reduced in the YF series of mice (Fig. 3A, lower panel). The iNKT cells from of the thymus of the YF1,2 (43%) and the YF5,6 (13%) mice had a statistically significant increase in the percentage of annexin V+ 7AAD+ cells compared with the wild type controls (3%; Fig. 3A, B). Interestingly, despite an equivalent TCR density and functional ITAM number, the YF1,2 line exhibited much greater cell death than did the YF5,6 line. For conventional T cells, the difference between these two lines is the presence of the constitutively phosphorylated CD3 ε subunit of 21 kDa, or p21, in the YF1,2 mice (40). Notably, the constitutive presence of p21, when expressed at high levels and, in the absence of other phospho-ζ intermediates, can attenuate all T cell development (49). Although there was a higher rate of cell death in the YF1–6 mice relative to C57BL/6 controls, it did not reach significance, consistent with the presence of more iNKT cells in the YF1–6 relative to the YF1,2 and YF5,6 lines.
We have performed proliferation assays with α-galactosylceramide, a potent agonist of iNKT cells, and measured cell expansion by CFSE dilution in the YF1,2, YF5,6, and wild type mice. The CSFE-labeled iNKT cells from the YF1–6 line demonstrated the most rapid proliferative response by 72 h, which was reflected in a decreased CSFE intensity. By 96 h, the wild type and YF1–6 iNKT cells had a similar proliferative response. Contrasting this, iNKT cells from YF1,2 mice exhibited a limited proliferative response at both time points, with a large percentage of cells blocked after only one or two rounds of cell division (Fig. 3 C).

These data suggest that the reduction in the iNKT cell compartment in YF1,2 mice could also be due to an inability of these cells to undergo proliferative expansion in vivo in response to the endogenous isoglobotrihexosylceramide-selecting ligand.

**FIGURE 1.** iNKT cell development requires a full complement of functional CD3ζ ITAMs. A, The percentage of iNKT cells in the thymus, spleens, and livers of the indicated mice were identified by staining cells with anti-CD3ε-Pacific Blue in combination with PBS57-loaded CD1d tetramer-PE and then analyzing the cells by flow cytometry. iNKT cells are represented in oval gates. The percentage (B) and absolute number (C) of iNKT cells from WT, YF1,2, YF5,6, and YF1–6 mice were compared using at least five mice per group. D, The absolute number of thymocytes and splenocytes from at least five mice per group are shown. Data are consistent from four independent experiments. *p < 0.001; **pp = 0.0001; values were generated by Kruskal–Wallis test.

iNKT cells from mice lacking two or more functional CD3ζ ITAMs have a substantially reduced expression of several transcription factors

The most likely reason for the significantly reduced iNKT cell numbers in the YF mice was a reduced TCR signaling intensity that limited the induction of key transcription factors required for iNKT cell development. However, the lack of iNKT cells could have resulted from an inability of CD3ζ transgenic mice to express the canonical TCR. Consequently, we analyzed the rearrangement of the Vα14Jα18 TCR by real-time PCR. All the CD3ζ transgenic lines (YF1,2, YF5,6, and YF1–6) expressed the Vα14Jα18 TCR, albeit at levels 5-fold less than in wild type mice (Fig. 4A). The most likely reason for the reduced expression was the decreased
Alternatively, there could be a quantitative difference in the number of preselected cells in the YF lines versus wild type mice. This could relate to differences in the time the thymocytes spend at the DP stage (7, 50). As expected, there were no Vα14Jα18 TCR gene rearrangements detected in the Jα18 knockout mice (Fig. 4A).

**FIGURE 2.** iNKT cell development is blocked at an early stage of thymopoiesis in mice lacking one or more functional CD3ζ ITAMs. Thymocytes or splenocytes from wild type, YF1,2, YF5,6, and YF1–6 mice were stained with anti-CD3ε-Pacific Blue, PBS57-CD1d tetramer-PE, anti-HSA FITC, -CD44 PerCp-Cy5.5, and –NK1.1 allophycocyanin. A. The percentage of HSA^lo^ iNKT cells was analyzed by gating on HSA^lo^ PBS57-CD1d tetramer^+^ lymphocytes (left gate). The forward scatter and side scatter profiles of the cells analyzed in the upper panel are shown in the lower panel. B. Stage I, II, and III iNKT cells were analyzed in the thymus and spleen of indicated mice, as indicated in corresponding quadrants labeled I, II, and III. Quadrants represent CD3^intermediate^ CD1d tetramer^+^ HSA^lo^ cells. C. The percentage of CD4 SP and DN iNKT cells was analyzed on CD3^intermediate^ CD1d tetramer^+^ HSA^lo^ cells. Graph represents the ratio of CD4 to DN iNKT cells. Three to seven mice were analyzed per group. Data are representative of three independent experiments. Statistics were generated by Kruskal–Wallis test. ***p < 0.0001; **p < 0.001.
The expression of Ca, used as an internal control, was detected in thymocytes from all the mouse lines tested (Fig. 4A). These findings suggested that the limited number of iNKT cells in the YF lines using the Vα14Jα18 TCR α-chain resulted from an inability of TCR signals to drive their development or expansion, rather than an inability of precursor cells to express the canonical TCR. This developmental block could have resulted from a reduced expression of one or more transcription factors implicated in iNKT cell development. For example, the transcription factor Egr2, induced following TCR-driven calcium signals, is required for iNKT cell development and expansion (28, 32, 36). PLZF is expressed at high levels in iNKT cells and is also necessary for early iNKT cell development. c-Myc has been shown to drive iNKT cell expansion in vivo. Thus, to account for iNKT cell deficiencies in the YF series of mice, we used quantitative real-time PCR reactions to analyze the expression of Egr2, c-Myc, and PLZF in iNKT cells sorted from the spleens of wild type, YF1, YF5, and YF1–6 mice using the CD1d tetramer. Early growth response 1 (Egr1) and early growth response 3 (Egr3) were also included in these comparisons; however, these factors have not been shown to play a role in iNKT cell development.

**FIGURE 3.** Mice lacking two or more CD3ζ ITAMs have an increase in cell death compared with controls. A, Thymic iNKT cells from the indicated mice were stained with anti-CD3ε Pacific Blue, PBS57-loaded CD1d tetramer PE, annexin V allophycocyanin, and 7AAD and then analyzed by flow cytometry. Annexin V and 7AAD staining was analyzed on CD3intCD1d tetramer+ iNKT cells. Numbers represent the percentage of cells in each quadrant. B, Graph indicates the percentage or absolute numbers of iNKT cells that are annexin V+ 7AAD+ using at least six mice per group pooled from three independent experiments. Statistics were generated by Kruskal–Wallis test. ***p < 0.0001; **p < 0.001. C, Proliferative responses of iNKT cells. Cells from the various mice were labeled with CFSE and stimulated with α-galactosylceramide for the indicated times. The cells were subsequently washed and analyzed by flow cytometry. Data are representative of three independent experiments.
The yield of the iNKT cells sorted from the YF1,2 and YF5,6 lines was consistently low (three sorts). Furthermore, many of the sorted iNKT cells from the YF1,2 line had a reduced cell surface expression of both CD3 and the TCR (tetramer positive). This finding suggested that the sorted iNKT cells were blocked in their development or proliferation, or both. This was evident with the reduced levels of Egr2, Egr3, and PLZF detected in sorted cells from the YF1,2 and YF5,6 lines (Fig. 4B). We were unable to amplify Egr2 in four of five different real-time PCR reactions using sorted cells from the YF1,2 and YF5,6 lines. PLZF was not successfully amplified in three of these five reactions; however, other transcription factors were detected. In cases in which amplification was successful, the relative expression of each transcription factor was calculated from five independent real-time experiments using two or three independent cell sorts. The statistical analyses were not included in the YF1,2 and YF5,6 samples, because the amplification for Egr2 and PLZF was below the critical threshold in three and four reactions, respectively. In those samples in which the Ct values were detected, the values were normalized to GAPDH. Data represent the relative expression of the transcription factors in the YF line compared with wild type mice. n is the number of successful PCR reactions from a total of five or six attempts.

C. Thymocytes were obtained from the indicated mice and aliquots were stained with fluorescently labeled mAbs against CD4 PerCy5.5, CD8 PerCy7, CD1d, Ly108, or CD150 PE. Histograms represent D thymocytes. Data are representative of two independent experiments.

**FIGURE 4.** iNKT cells from mice lacking one or more functional CD3 ζ ITAMs have reduced expression of multiple transcription factors. A, Total RNA was isolated from thymocytes obtained from wild type, YF1,2, YF5,6, YF1–6, and Jα18 knockout mice (Jα18KO). Equivalent amounts of RNA were used for real-time RT-PCR reactions with primers specific for Vα14Jα18 rearrangements. Primers for Cα were used as controls. Samples were run in triplicate, with GAPDH included as an internal control. Vα14Jα18 and Cα expression was first normalized to GAPDH. Data represent relative expression compared with wild type controls. B, iNKT cells were isolated from pooled splenocytes using magnetic bead depletion and cell sorting with a high-speed cell sorter. Total RNA was isolated from the purified iNKT cells obtained from wild type, YF1,2, YF5,6, and YF1–6 mice. The RNA was used in real-time RT-PCR reactions with primers specific for Egr1, Egr2, Egr3, c-Myc, and PLZF, with GAPDH included as a control for the RT-PCR reactions. The relative expression of each transcription factor was calculated from five independent real-time experiments using two or three independent cell sorts. The statistical analyses were not included in the YF1,2 and YF5,6 samples, because the amplification for Egr2 and PLZF was below the critical threshold in three and four reactions, respectively. In those samples in which the Ct values were detected, the values were normalized to GAPDH. Data represent the relative expression of the transcription factors in the YF line compared with wild type mice. n is the number of successful PCR reactions from a total of five or six attempts. C, Thymocytes were obtained from the indicated mice and aliquots were stained with fluorescently labeled mAbs against CD4 PerCy5.5, CD8 PerCy7, CD1d, Ly108, or CD150 PE. Histograms represent D thymocytes. Data are representative of two independent experiments.
expression is critically dependent on a full complement of functional CD3ζ ITAMs. A reduced expression of PLZF likely accounts for some of the iNKT cell developmental defects, because this factor is necessary for early iNKT cell development. One final explanation to account for the reduced iNKT cell numbers is a change in the cell surface expression of CD1d, LY108, and SLAM on DP thymocytes. Thus, a reduction in any of these cell surface molecules could limit the development of iNKT cells. Thymocytes from all the mice were analyzed for the expression of CD1d, LY108, and SLAM (Fig. 4C). There was no difference in the expression of any of these three proteins on DP thymocytes among the various mice. These findings suggest that iNKT cell development requires a full complement of functional CD3ζ ITAMs to allow sufficient TCR signaling intensities that promote efficient Egr2 and PLZF expression.

**iNKT cells are not necessary to prevent inflammatory arthritis following *Borrelia burgdorferi* infection**

All the YF series of mice had severe reductions in iNKT cell numbers. Because iNKT cells are proposed as necessary for controlling inflammatory arthritis in mice infected with the spirochete *B. burgdorferi*, we assessed whether the arthritis in the YF sets of mice was more severe compared with that in wild type mice (2, 5). Wild type, YF1,2, and YF1–6 lines were inoculated intradermally with 1.2–1.5 × 10^6* B. burgdorferi* spirochetes. At 2 and 6 wk postinfection, the knees and tibiotarsal joints were sectioned and stained with H&E. Tissues were then analyzed for arthritis severity using a scale from 0–3, indicating no, slight, moderate, or severe inflammation, respectively. All images were acquired with a ×4 objective magnification.

**FIGURE 5.** iNKT cells are not necessary to prevent inflammatory arthritis following *B. burgdorferi* infection. Six-week-old mice were inoculated intradermally with 1.2–1.5 × 10^6* B. burgdorferi* spirochetes. Right and left tibiotarsal joints were isolated and paraffin embedded, and 7-μm sections were cut and stained with H&E. A and B. Digital pictures taken of tibiotarsal joints from indicated mice. C and D. Joints were scored for arthritis on a scale 0–3 indicating no, slight, moderate, or severe inflammation, respectively. Bar graphs indicated the mean arthritis score at 2 or 6 wk postinfection. Three sham and six to nine infected mice were analyzed per group at each time point. All images were acquired with a ×4 objective magnification.

**Discussion**

Although conventional αβ T cells can develop with as few as four functional CD3-encoded ITAMs, our study shows that iNKT cell development requires a complete complement of CD3ζ ITAMs. Thus, mice lacking only one functional CD3ζ ITAM, effectively eliminating two of the 10 TCR/CD3 ITAMs, had a severe block in iNKT cell maturation or expansion, or both. These findings suggest that the TCR signaling strength, necessary for iNKT cell development following TCR interactions with glycolipid/CD1d complexes, requires all available CD3 encoded ITAMs and connected signaling proteins. This interpretation is consistent with previous studies showing that conventional αβ T cells expressing low-avidity TCRs require a full complement of CD3ζ ITAMs for effective positive and negative selection (21, 23). The fact that some iNKT cells develop normally in the YF sets of mice described herein could be explained in one of two ways. First, a subset of iNKT cells might express a TCR with sufficient affinity for the selecting glycolipids expressed in the thymus (52–55). For
example, Vβ7-expressing iNKT cells are preferentially selected by isoglobotrihexosylceramide in an in vitro culture system, because the Vβ7 is a higher affinity receptor (56, 57). However, YF1–6 mice exhibited a similar Vβ profile compared with wild type mice, suggesting that this is not the likely explanation. Second, a full complement of TCR ITAMs might actually be required for the proper expansion and survival of the iNKT cells once they are positively selected. Comparing the wild type and YF sets of mice, the developmental patterns are not dramatically different when analyzing the three maturation stages defined as stages I, II, and III. Rather, all the iNKT cells in the YF lines exhibited a statistically significant increase in the number of cells undergoing cell death. In particular, the YF1,2 lines had the most appreciable extent of cell death, with the residual iNKT cells detected in the thymus of these mice expressing relatively low TCR densities. The fact that the YF1,2 line differs from the YF5,6, despite having an equivalent TCR density and ITAM number, is consistent with our early studies comparing conventional T cell development in an equivalent TCR density and ITAM number, is consistent with the thymus of these mice expressing relatively low TCR densities.

Mechanistically, the TCR–ITAM signaling pathway involves the activation of downstream PTKs, such as ZAP-70 and Itk (26, 59). These kinases are required for TCR-mediated elevations in intracellular calcium levels. Notably, both ZAP-70 and Itk-deficient mice have a block in iNKT cell development (27, 60). Recent experiments have revealed a direct requirement for TCR-mediated calcium responses in the activation of calcineurin, leading to iNKT cell selection and/or expansion (28). Itk functions downstream of the TCR by amplifying calcium signaling pathways through phospholipase C-γ induction (26). The calcium–calcineurin pathway contributes to the expression of the Egr2, a transcription factor required for iNKT cell development. Impressively, the developmental arrest of the iNKT cells in the Itk- and Egr2-deficient mice phenocopy that noted in our YF series of mice. Thus, the reason for these differences in iNKT cell development in the YF series of mice compared with controls is likely due to the reduced expression of various transcription factors required for iNKT cell development and expansion. This finding is consistent with the almost undetectable expression of Egr2, Egr3, and PLZF in the YF1,2 iNKT cells. Interestingly, the YF5,6 line has slightly higher numbers of iNKT cells in both the thymus and spleen compared with the YF1,2 mice. Although we could detect Egr1 and c-Myc in the iNKT cells isolated from the YF5,6 mice, there was almost no detectable expression of Egr2, Egr3, and PLZF (Fig. 4). These experiments are again consistent with our observations that the YF5,6 line has a slightly more efficient signaling capacity than the YF1,2 line, especially in the setting of a low-avidity TCR (20, 21, 58). Our additional observation that the elimination of 6 of 10 functional CD3 ITAMs (YF1–6 line) had an iNKT cell selection–maturation defect less severe than the YF1,2 or YF5,6 lines is explained by the increased TCR density previously described in these mice, owing to the lack of ubiquitination and protein degradation (43, 44, 61). Strong TCR-driven intracellular signals, regulated by both ITAM numbers and TCR densities, are required for efficient iNKT cell development through the induction of the Egr and PLZF transcription factors.

iNKT cells are reported as necessary for preventing the arthritis that develops in mice following infections with the spirochete, B. burgdorferi (2, 5, 6). These findings would suggest that a more severe arthritis should develop in mice deficient in iNKT cells. However, in our experimental system involving direct bacterial infections, the YF1,2 and YF1–6 mice exhibited similar inflammatory pathology in the tibiotarsal and knee joints at 2 and 6 wk postinfection compared with wild type mice. In addition, we could not detect differences in cardiac pathologies, even in mice lacking all iNKT cells (Jox18−/− mice). We considered several explanations to account for the differences between our results and earlier publications. First, the haplotype of the mice studied might be important for pathogenesis. It is known that BALB/c mice are tolerant only to low doses of B. burgdorferi, whereas C57BL/6 mice are tolerant even at high doses of infection (62). Consistent with this fact, RAG−/− C57BL/6 mice maintain resistance to arthritis, unlike RAG−/− BALB/c mice, despite the lack of T and B cells and a similar bacterial burden (63). All mice in our experiments were maintained on a C57BL/6 background; therefore, it is possible that, in more susceptible strains of mice, iNKT cells could provide efficient protection from or immunoregulation of the disease. This protection could be mediated by early cytokine production by iNKT cells, because early IL-4 production correlates with decreased susceptibility to arthritis, whereas IFN-γ production has been associated with increased severity of arthritis (63–66). Second, iNKT cells might regulate arthritis following exposure to B. burgdorferi in conjunction with other T cell populations. These cell populations, perhaps expressing a distinct TCR, might have been increased in numbers.
in the YF series of mice. Studies have shown that *B. burgdorferi*-specific Ab production by B cells is critical for mediating protection, because serum transfer from infected to uninfected animals can ameliorate arthritis (67). Similar levels of Abs directed against *B. burgdorferi* were observed in all the YF lines and in mice lacking all iNKT cells (Fig. 6A). Again, this finding might indicate a secondary role for iNKT cells in disease pathogenesis that is only apparent in certain genetic backgrounds. A third explanation involves the nature of the infection. Previous work using a tick-based infection model revealed differences in tibiotarsal swelling when iNKT cells were absent (6). The tick model could contribute to an enhanced innate inflammatory response, which is worse in the absence of iNKT cells, causing more damaging tissue damage in our experiments, a direct bacterial infection was used. Using this infection strategy, we were unable to detect a statistically significant difference in carditis, aortitis, or pericarditis in the YF or Ja18 knockout mice. Contrasting this finding, a recent report suggested that iNKT cells, from a C57BL/6 background strain of mice, modulate acute Lyme carditis with direct bacterial infections (51). Interestingly, three of the Ja18-null mice in our experiments had a more severe aortitis, although this did not reach statistical significance (Fig. 6B). A similar split was noted in the published results and did reach significance (51). Because our mice are maintained in a specific pathogen-free facility, it is possible that the lack of innate inflammatory responses in these mice limited the bacteria-induced pathogenesis, even in the absence of iNKT cells. Further work will be necessary to uncover the genetic modifiers that are likely causing the split in disease pathogenesis, but these are likely related to those contributing to systemic lupus erythematosus (68, 69).

Our data show that early iNKT cell development is dependent on signaling through a full complement of CD3 ζ ITAMs. Whereas iNKT cells can contribute to disease severity in *B. burgdorferi* infections, they are not essential for the prevention of Lyme arthritis or carditis in certain murine disease models.

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Disclosure

The authors have no financial conflicts of interest.

References


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In iNKT cell development, multiple CD3 ε ITAMs are involved in the selection process. This is evidenced by the role of ITAM multiplicity and thymocyte selection, as described in the work of Pellicci et al. (2000), and the ITAM multiplicity and thymocyte selection study by Bezbradica et al. (2005).

The Ets transcription factor is required for the development of NKT cells in mice, as demonstrated by the work of Lin et al. (2002), indicating the importance of Ets transcription factors in the development of NKT cells.

The 21- and 23-kDa forms of TCR zeta are generated by alternative splicing and lead to the generation of functionally uncoupled TCR zeta subunits. This is supported by the findings of Myers et al. (2005).

The cross-linking of CD3 ε by the TCR alpha-β chain is critical for the activation of the T cell. This is supported by the work of Myers et al. (2005) and the findings of van Oers et al. (2003).

The role of the TCR γδ chain in the development of NKT cells is supported by the findings of Myers et al. (2005) and the work of Myers et al. (2005).