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Differential Requirements for IRF-2 in Generation of CD1d-Independent T Cells Bearing NK Cell Receptors

Tsu Yoshi Notake,*† Shin Horisawa,* Hideki Sanjo,* Shin-ichi Miyagawa,† Shigeaki Hida,* and Shinsuke Taki*

NK cell receptors (NKRs) such as NK1.1, NKG2D, and Ly49s are expressed on subsets of CD1d-independent memory phenotype CD8+ and CD4+CD8− T cells. However, the mechanism for the generation and functions of these NKR+ T cells remained elusive. In this study, we found that CD1d-independent Ly49+ T cells were reduced severely in the spleen, bone marrow, and liver, but not thymus, in mice doubly deficient for IFN regulatory factor-2 (IRF-2) and CD1d, in which the overall memory phenotype T cell population was contrastingly enlarged. Because a large fraction of Ly49+ T cells coexpressed NK1.1 or NKG2D, the reduction of Ly49+ T cells resulted indirectly in underrepresentation of NK1.1+ or NKG2D+ cells. Ly49+ T cell deficiency was observed in IRF-2−/− mice additionally lacking IFN-α/βR α-chain (IFNAR1) as severely as in IRF-2−/− mice, arguing against the involvement of the accelerated IFN-α/β signals due to IRF-2 deficiency. Rather, mice lacking IFN-α/βR alone also exhibited relatively milder Ly49+ T cell reduction, and IL-2 could expand Ly49+ T cells from IFNAR1−/−, but not from IRF-2−/−, spleen cells in vitro. These results together indicated that IRF-2 acted in Ly49+ T cell development in a manner distinct from that of IFN-α/β signals. The influence of IRF-2 deficiency on Ly49+ memory phenotype T cells observed in this study suggested a unique transcriptional program for this T cell population among other NKR+ T and memory phenotype T cells. The Journal of Immunology, 2012, 188: 4838–4845.

It has been well documented that a small fraction of CD8+ T cells in mice and in humans bear surface molecules that were originally described as the receptors instrumental for NK cells in recognizing foreign molecules and self MHC molecules (1–4). Those receptors, termed NK cell receptors (NKRs), include both activating and inactivating receptors, such as NKR-P1C (NK1.1), NKG2s, and Ly49s. NKR+CD8+ T cells differ from CD1d-dependent NKT cell expressing invariant TCRs (iNKT cells), as the latter are mainly CD4+ or CD4−CD8+ (5–7). Based on the common memory-like CD44hiCD122+ phenotype and the observation that both NKR+ and CD8+ memory phenotype T (TMP) cells arose naturally in mice without deliberate infection and immunization (8), a lineage relationship was pointed out, yet not proved, between these two CD8+ T cell subpopulations.

Activating receptors such as NKG2D were shown to exhibit costimulatory functions in T cell activation (9, 10) and to be potentially important in anti-tumor immunity (11), whereas the role for inhibitory NKRs remains controversial (8, 12, 13). It is also not clear if T cells expressing inhibitory NKRs have physiological functions unique to this T cell subpopulation. In this regard, CD8+ TMP cells expressing inhibitory Ly49s have very recently been shown to exhibit regulatory T cell (Treg) activity suppressing follicular Th cell-mediated Ab responses (14). Despite the potential importance of NKR+ T cells in immune regulation, it remains largely unknown how these Ly49+ and other NKR+ T cells are generated, except for the common dependence of NKR+ T cells and CD8+ TMP cells in vivo on the cytokine IL-15 (15, 16).

IFN regulatory factor-2 (IRF-2) is a transcription factor that plays multiple roles in the development and regulation of the immune system (17). Thus, IRF-2-deficient (IRF-2−/−) mice exhibited various abnormalities in the immune system, including defective development of CD4+ dendritic cells (18, 19), impaired NK cell maturation (20, 21), exhaustive proliferation of hematopoietic stem cells (22), and spontaneous basophil expansion leading to Th2-biased immune responses (23). In addition, CD8+ TMP cells were reported to be more abundant in these mice than in wild-type mice (24). Among these phenotypes, the abnormalities in hematopoietic and CD4+ dendritic cells were attributed to the absence of a function of IRF-2 to attenuate physiological responses to IFN-α/β (18, 19, 22). In contrast, the absence of IFN-α/β-independent functions of IRF-2 was shown to be responsible for the impaired NK cell maturation and excess basophil expansion in the same mice (21, 23).

In this study, we found that the development of CD1d-independent Ly49+ TMP cells was impaired severely in naive IRF-2−/− mice and such impairment resulted indirectly in the reduction of T cells bearing other NKRs, an unexpected observation in light of the enlarged CD8+ TMP cell population in the same mice. Failure of Ly49+ T cell generation to be restored in mice lacking both IRF-2 and IFN-α/βR disfavored the involvement of the attenuator function of IRF-2 for IFN-α/β signals in the phenotype. Rather, IFN-α/β signals were also, yet presumably through a mechanism distinct from that for

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IRF-2, required for the efficient generation of Ly49+ T cells. The opposite functions of IRF-2 shown here for Ly49+ and Ly49− TMP cells highlighted the distinct transcriptional programs operating in these TMP cell subpopulations. IRF-2−/− mice will provide us with a useful experimental tool to study how the development of this poorly understood T cell subpopulation is regulated.

Materials and Methods

**Mice**

IRF-2−/− mice (25), back-crossed to C57BL/6 (B6) mice at least 10 times, and IRF-2+/− mice lacking IFN-α/β receptor (IFNAR1) were described previously (18). CD1d−/− mice (26), kindly provided by Dr. L. Van Kaer (Vanderbilt University, Nashville, TN) and Dr. K. Takeda (Juntendo University, Tokyo, Japan), were crossed to IRF-2−/− mice to generate IRF-2−/−CD1d−/− mice. CD1d+/− (Ly5.1) and CD1d−/− (Ly5.1/5.2) mice on the wild-type or RAG-1−/− background were also generated by successive crossing of CD1d−/− mice with B6.Ly5.1 (Sankyo Labo) and RAG-1−/− mice. All mice were maintained in the animal facility of the Research Center for Human and Environmental Science, Shinshu University, under specific pathogen-free condition and used at 8–12 wk of age. All animal experiments were preapproved by the Division of Laboratory Animal Research of Shinshu University and performed in accordance with the Regulation for Animal Experimentation of Shinshu University.

**Flow cytometry**

The following fluorochrome-conjugated Abs were used for flow cytometry: FITC- and allophycocyanin–anti-Ly49G2 (4D11), FITC–anti-Ly49D (4E5), allophycocyanin–anti-CD4 (RM4-5), biotin–anti-CD11b (M1/70), allophycocyanin–anti-CD44 (IM7), PE–anti-CD62L (MEL-14), PE–anti-CD122 (TM-β1), PE–anti-Ly5.1 (A20), and FITC– and biotin–anti-Ly6C (AL-21) purchased from BD Pharmingen; FITC– and PE–anti-TCRβ (5H5, 5F5), allophycocyanin– and biotin–anti-NK1.1 (PK136), FITC– and biotin–anti-Ly49CH/I/F (14B11), biotin–anti-NKG2D (CX5), biotin–anti-NKG2A/C/E (20D5), allophycocyanin–anti-KLRG1 (2F1), allophycocyanin– and PE–Cy7–anti-CD8 (53-67), FITC–anti-NKP46 (29A14), PE–anti-CD127 (A7/R34), biotin–anti-CCR7 (4B12), biotin–anti-B220 (RA3-6B2), and biotin–anti-Ter119 (TER-119) from eBioscience. Cells were stained on ice in the presence of anti-CD16/32 Ab (culture supernatant from 2J4G hybridoma) to block Fc-mediated nonspecific staining. PE–Cy7–streptavidin (BioLegend) was used to develop biotin-conjugated Abs. To identify iNKT cells, dimeric mouse CD1d-IgG fusion protein (DimerX I; BD Pharmingen) was loaded with α-galactosylceramide (KRN7000, provided by Dr. A. Kriftzer, Golden, CO) for staining, and developed with PE-labeled anti-mouse IgG1 Ab (BD Pharmingen) according to the manufacturer’s instructions. Staining with anti-CCR7 Ab (eBioscience) was carried out at 37°C as instructed by the manufacturer. Stained cells were analyzed on the Cytomics FC500 flow cytometer (Beckman Coulter). Data analysis was carried out with the RXP software (Beckman Coulter). Intracellular Bcl-2 was stained with FITC–anti–Bcl-2 (clone 10C4; eBioscience) in cells fixed and made permeable with Cytofix/Cytoperm Plus kit (BD Biosciences) as instructed by the manufacturer. Annexin V staining was done with annexin V–PE apoptosis detection kit I (BD Pharmingen) and Alexa Flour 647–Annexin V (BioLegend) as instructed. Preparation of hepatic lymphocytes was done as described before (21).

**Cell culture and real-time PCR**

Spleen cells (1 × 10^6) were cultured in RPMI 1640 medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol, and 2 mM L-Gln in the presence of 150 ng/ml recombinant human IL-2 (provided by Ajinomoto Company) in 24-well plates for 6 d. In some experiments, NK1.1−, NKG2D−, and Ly49− cells were stained with biotin–anti-NK1.1, biotin–anti-NKG2D, or a mixture of FITC–anti-Ly49G2 and anti-Ly49CH/I/F Abs followed by anti-biotin or anti-FITC magnetic beads and depleted on the AutoMACS (Miltenyi Biotech) before culture. Cultured T cells were sorted into Ly49 and Ly49− T cells on the FACSAria (BD Biosciences) and subjected to real-time PCR. Real-time PCR was performed as described (27) with the following PCR primers: IRF-2 forward, 5′-CGATTATCTAACGCTAGGGCCTTTC-3′; IRF-2 reverse, 5′-GCATTCGATCCGCTTAC-3′. Primers for β-actin were described previously (27).

**Radiation bone marrow chimeras**

Radiation bone marrow (BM) chimeras were established as previously described (18) by reconstituting irradiated (9 Gy) CD1d−/− (Ly5.1) mice with RBC-lysed BM cells (1 × 10^6) prepared from IRF-2−/−CD1d−/− or IRF-2−/−CD1d+/− mice on the Ly5.2 background and analyzed 8–9 wk later. For creation of mixed BM chimeras, donor BM (Ly5.2) were mixed with those from CD1d−/− mice on the Ly5.1 background, followed by transfer into irradiated (9 Gy) CD1d−/− mice (Ly5.1/5.2). Because of the inferior capacity of IRF-2−/− BM cells in reconstituting peripheral hematopoietic cells (18, 19, 22), IRF-2−/−CD1d−/− BM cells (1 × 10^6) and IRF-2−/−CD1d−/− BM cells (4 × 10^5) were mixed with 0.4 × 10^7 and 1 × 10^7 CD1d−/− (Ly5.1) BM cells, respectively.

**Homeostatic proliferation**

Spleenic T cells were purified by depleting B220+, CD11b+, and TER119+ cells from IRF-2−/−CD1d−/− or IRF-2−/−CD1d+/− mice and labeled with 2 μM CFSE (Molecular Probes) for 8 min. CD1d−/−RAG−/− mice were injected i.v. with 2 × 10^6 labeled T cells in 200 μl PBS. Spleen cells from the recipients were analyzed 6–7 d later.

**Statistical analysis**

Data were analyzed for statistical significance by Student t test.

**Results**

**Reduction of CD1d-independent NK1.1+ and other NKR+ T cells in IRF-2−/− mice**

In contrast to the substantial reduction of NK (NK1.1+"TCRβ") cells as previously reported (20, 21), NK1.1+ T cells were unaffected at a glance in the spleen in IRF-2−/− mice (Fig. 1A). However, when stained with CD1d dimers loaded with α-galactosylceramide (αG/CD1d), a reagent that identifies CD1d-dependent iNKT cells, IRF-2−/− splenic NK1.1+ T cells were found to contain more αG/CD1d+ cells than did wild-type NK1.1+ T cells (Fig. 1B). αG/CD1d+ cells including both NK1.1+ and NK1.1− cells were not severely affected in IRF-2−/− mice; 5.7 × 10^5 and 5.4 × 10^5 on average in the spleen in IRF-2−/− and IRF-2−/− mice, respectively (n = 8 for each), suggesting a selective decrease of CD1d-independent NK1.1+ T cells. Consistently, inspection in mice on the CD1d−/− background showed that the frequencies and absolute numbers of NK1.1+ T cells were much lower in the spleen and BM in IRF-2−/−CD1d−/− mice than in IRF-2−/−CD1d+/− mice (Fig. 1C, 1D). In addition to NK1.1+ T cells, T cells expressing Ly49G2 or Ly49C/H/I/F (termed Ly49+ T cells in this study) and NKG2D+ T cells were also less frequent and reduced in absolute numbers in the spleen and BM in IRF-2−/−CD1d−/− mice compared with those in control mice (Fig. 1C, 1D). The reduction of these NKR+ T cells was much more prominent than that of CD4+ and CD8+ T cells (Supplemental Fig. 1). Similar reduction was observed for T cells bearing Ly49G2, A, I, or F (Supplemental Fig. 2), whereas NKG2A/C/E+ and KLRG1+ T cells were not affected, except for moderate reduction of NKG2A/C/E+ T cells in BM (Fig. 1C, 1D). NKP46+ or Ly49D+ T cells were virtually undetectable (<0.1%) in the spleen even in CD1d−/− mice (data not shown). Ly49+ T cells were rarely CD4+CD8− and either CD4+CD8+ or CD4−CD8+, both of which were affected (Supplemental Fig. 3). NKR+ T cells, particularly those expressing Ly49 or NKG2D, were reduced also in the liver in IRF-2−/−CD1d−/− mice (Fig. 1E). These results indicated that the generation of NKR+ T cells, particularly those expressing NK1.1, Ly49s, and NKG2D, required IRF-2.

**Paucity of Ly49+ T cells accounted largely for the reduction of NKG2D+ and NK1.1+ T cells in IRF-2−/−CD1d−/− mice**

To examine the relationships among various NKR+ T cell populations in BM, we analyzed the BM with combinations of NKR Abs. As seen in Fig. 2, a substantial fraction of Ly49+ T cells also expressed NK1.1, NKG2D+, and NKG2A/C/E+ T cells coexpressing Ly49s and NKG2D, whereas NKG2A/C/E+ T cells were virtually undetectable in the spleen in IRF-2−/− mice (Fig. 1A). How-
were decreased prominently in IRF-2−/− CD1d−/− mice, whereas the Ly49+ fraction of those T cells was reduced less severely (Fig. 2). KLRG1+ T cells were largely Ly49+ and barely affected in IRF-2−/−CD1d−/− mice. On the basis of these double stainings, we considered that Ly49+ T cells were primarily affected, and the paucity of Ly49+ T cells resulted indirectly, to substantial extents, in the reduction of T cells expressing NK1.1, NKG2D, or NKG2A/C/E in IRF-2−/−CD1d−/− mice. Ly49+ T cells were not expanded from IRF-2−/− spleen cells in IL-2–supplemented cultures in vitro

Ly49+, NK1.1+, and NKG2D+ T cells could be expanded from control spleen cells efficiently in vitro in the presence of IL-2 (Fig. 3A). T cells reactive to αGc/CD1d were virtually absent in these cultures (<1% in cultured TCRβ+ cells; T. Notake, unpublished observations). In contrast, spleen cells from IRF-2−/− mice yielded greatly reduced numbers of NK1.1+ and Ly49+ T cells, whereas substantial numbers of NKG2D+ T cells were recovered, and the expansion of NKG2A/C/E+ T cells occurred almost normally even from IRF-2−/− spleen cells (Fig. 3A, 3B). Furthermore, IRF-2 messages were expressed at comparable levels irrespective of the expression of Ly49s on T cells sorted from IL-2–propagated control spleen cells (Fig. 3C). These results indicated that the presence of IRF-2 within T cells alone did not determine whether Ly49s was expressed or not and suggested rather that IRF-2 acted at a certain stage during Ly49+ T cell generation in vivo.

Influence of IRF-2 deficiency on the induction of NKG2D and NK1.1 expression in vitro and in vivo

NKG2D expression could be induced on NKG2D+ T cells de novo, as NKG2D+ T cells were recovered from both control and IRF-2−/− spleen cells depleted of NKG2D+ cells prior to the culture (Fig. 4A). In addition, NKG2D+, but not NK1.1+ and Ly49+, T cells were generated from IRF-2−/− splenic T cells under the condition of homeostatic proliferation in the wild-type environment in vivo (Fig. 4B). These observations indicated that IRF-2 deficiency did not affect the potential of T cells to express NKG2D. Notably, NK1.1+ T cells were newly generated efficiently from control, but not IRF-2−/−, spleen cells depleted of NK1.1+ cells (Fig. 4C). NK1.1+ T cells could be generated not only from Ly49+ NK1.1+ but also from Ly49− NK1.1− T cells in control mice (Fig. 4D). Nevertheless, Ly49− NK1.1− T cells in IRF-2−/− mice failed to generate NK1.1+ T cells (Fig. 4C). Thus, the almost complete inability of IRF-2−/− T cells to generate NK1.1+ T cells in vitro (see Fig. 3A, 3B) seemed to be due partly to the near absence of Ly49+ T cells in the starting cell preparation that otherwise expanded in response to IL-2 to yield the majority of NK1.1+ T cells and partly to the impaired potential of NK1.1+ Ly49+ T cells to express NK1.1 in response to IL-2. Ly49 expression appeared to

FIGURE 1. Reduction of CD1d-independent NKR+ T cells in IRF-2−/− mice. (A) Splenic NK1.1+ T cells in control (IRF-2+/+) and IRF-2−/− mice. Numbers represent the means ± SD of the percentages of cells within the square region of at least eight mice of each genotype. The absolute numbers of TCRβ+ T cells expressing the indicated NKRs (as defined with smaller-square regions) within total TCRβ+ cells (larger-square regions). Representative flow cytometric profiles for two mice of each genotype. The absolute numbers of NK1.1+ and Ly49+ T cells in spleen and BM in IRF-2−/−CD1d−/− (C) or IRF-2−/−CD1d−/− (D) mice. For BM, the numbers are for one side of inferior limbs (femur and tibia) from each animal. Each symbol represents the value for an individual mouse. Horizontal bars are the means. Statistically significant differences are marked: *p < 0.01, **p < 0.0001. (E) The percentages of TCRβ+ cells expressing each NKR in mononuclear cells isolated from the liver are indicated as in (C). Representative flow cytometric profiles for two mice of each genotype. The absolute numbers of TCRβ+ cells were 6.3 × 105 and 3.2 × 105 in IRF-2−/−CD1d−/− and IRF-2+/−CD1d−/− mice, respectively (means of two mice).
IRF-2 deficiency affected Ly49+ and total CD8+ TMP cells under these conditions.

Cell-intrinsic requirement for IRF-2 in the generation of Ly49+ T cells

We created BM chimeras using CD1d−/− mice (Ly5.1) as recipients for BM cells from IRF-2+/−CD1d−/− and IRF-2−/−CD1d−/− mice (Ly5.2). As shown in Fig. 5A, IRF-2−/−CD1d−/− BM donor cells generated NK1.1+, Ly49+, and NKG2D+ T cells less efficiently than did control donor cells. An insignificant effect of IRF-2 deficiency on splenic NK1.1+ T cells in these chimeras seemed to mirror the less severe reduction of these cells in nonchimeric animals (see Fig. 1D). Even when allowed to differentiate in the presence of “bystander” CD1d−/− BM cells, IRF-2−/−CD1d−/− hematopoietic cells failed to generate NK1.1+, Ly49+, and NKG2D+ T cells efficiently (Fig. 5B). Both CD8 positive (CD4−CD8+) and negative (CD4−CD8−) fractions of Ly49+ T cells were affected by the cell-intrinsic IRF-2 deficiency (Fig. 5B).

IRF-2 deficiency affected Ly49+ and total CD8+ T_Mp cells oppositely

Ly49+ T cells in CD1d−/− mice and those residual in IRF-2−/−CD1d−/− mice were indistinguishable in exhibiting memory phenotypes; the great majority of them were CD44+CD122−CD127+CD62L+ and lacked KLRG1 and CCR7 expression (Fig. 6A; see also Fig. 2). Despite the reduction of these Ly49+ T_Mp cells as well as total numbers of splenic CD8+ T cells (Supplemental Fig. 1), CD44+CD8+ or CD44+CD62L+CD8+ T_Mp cells were more frequent in IRF-2−/−CD1d−/− mice than in CD1d−/− mice (Fig. 6B). These observations, extending a previous examination in mice on the CD1d+− background (24), indicated that IRF-2 acted negatively on the homeostatic expansion of CD8+ T_Mp cells lacking Ly49 expression but positively for Ly49+ T_Mp cell generation.

Expansion of NKR+ T cells in vitro. (A) The percentages of cells expressing the indicated NRks within TCRβ+ spleen cells from IRF-2−/−CD1d−/− (open bars) and IRF-2−/−CD1d−/− (black bars) mice cultured with IL-2 in vitro. Data are the means ± SDs of four independent cultures. Statistically significant differences are marked: *p < 0.05, **p < 0.005. (B) Double staining of IL-2–propagated T (TCRβ+) cells for NK1.1+ and Ly49+ thymocytes (TCRβ+) cells from wild-type spleen cells and IRF-2−/− mice were stained as in (A). Numbers are the percentage of cells in each quadrant. (C) The numbers of IRF-2 messages relative to those of β-actin are shown for Ly49+ (98% pure, open bar) and Ly49− (95% pure, black bar) TCRβ+ cells sorted from wild-type spleen cells and cultured in vitro as in (A).

Potential requirement for IRF-2 in postthymic development of Ly49+ T cells

Notably, NK1.1+ and Ly49+ thymocytes (TCRβ+) were observed at similar frequencies in IRF-2+/−CD1d−/− and IRF-2−/−CD1d−/− mice (Fig. 7A), suggesting a possibility that the inefficient generation of Ly49+ T cells in IRF-2−/−CD1d−/− mice was due to a defect in further maturation and expansion of these thymocytes in the periphery. Because the levels of Ly49+ and NK1.1+ expression on these thymocytes in IRF-2−/−CD1d−/− and control mice were equivalent, as seen also for splenic and hepatic NK cells (see TCRβ+ cells in Fig. 1C, 1E), it was unlikely that IRF-2 deficiency resulted simply in inefficient NK1.1+ and Ly49 expression in general. Consistently, forced expression of IRF-2 via retroviral transduction failed to restore NK1.1+ and Ly49 expression in IL-2– propagated IRF-2−/− splenic T cells (T. Notake, unpublished observation). Cells expressing relatively low amounts of the antiapoptotic protein bcl-2 were slightly more frequent among residual Ly49+ T cells in IRF-2−/−CD1d−/− mice than in CD1d−/− mice (Fig. 7B). However, the amounts of bcl-2 in these Ly49+ T cells were as high as those observed in Ly49− T cells. Ly49+ T cells in IRF-2−/−CD1d−/− mice were stained with annexin V at only marginally elevated frequencies, substantially lower in comparison with those in NK (Ly49+TCRβ+) cells in the same mice (Fig. 7C).
**Discussion**

We showed here that the transcription factor IRF-2 played important cell-intrinsic roles in the homeostatic regulation of CD1d-independent NKR+ T cells. Generation of Ly49+ T cells, the large fraction of which concomitantly expressed NK1.1, NK2D, and NK2A/C/E, but not KLRG1, was impaired in IRF-2−/− mice, whereas Ly49+ T cells expressing NK1.1, NK2D, NK2A/C/E, or KLRG1 could be generated rather normally. Because the ability to express NK2G2D upon culture in IL-2 or during homeostatic proliferation was obviously unaffected in IRF-2−/− T cells, the reduction of the NK2G2D+ T cell population in IRF-2−/− mice seemed to be accounted for by the near absence of its major subset Ly49+NK2G2D+ T cells. The majority of NK1.1+ T cells also expressed Ly49s, and Ly49+ T cells were the predominant source of NK1.1+ T cells propagated with IL-2 in vitro. Thus, the inefficient IL-2-mediated expansion in vitro and reduction in vivo of NK1.1+ T cells in the absence of IRF-2 seemed to result secondarily, at least in part, from the severe reduction of Ly49+ T cells. In addition, however, impaired IL-2-inducible NK1.1 expression in

**FIGURE 4.** Induction of NKG2D+ and NK1.1+ T cells in cultures in vitro and during lymphopenia-induced proliferation. (A) NKG2D and NK1.1 expression on TCRβ cells of each genotype depleted of NKG2D+ or NK1.1+ cells before culture. NKG2D+ cells had been <1% in each depleted cell preparation. (B) Phenotypic T cells were enriched from IRF-2−/−CD1d−/− (CD1d−/−) and IRF-2−/−CD1d+−/− mice, labeled with CFSE, and transferred into RAG−/−CD1d−/− mice. Flow cytometry profiles for NKG2D, NK1.1, and Ly49 (G2+C/H/I/F) on splenic TCRβ cells recovered from the transferred mice are shown together with CFSE fluorescence. Representative of two independent transfers. Numbers are for the percentages of cells within the square regions. Similar expansion of NKG2D+, but not NK1.1+ and Ly49+, T cells were observed in three pairs of RAG−/− mice transferred with unlabeled IRF-2−/− or IRF-2−/− T cells (T. Notake, unpublished observation). (C) NK expression on TCRβ+ cells expanded with IL-2 from NK1.1-depleted spleen cells. NK1.1−/− cells had been <1% in each depleted cell preparation. (D) NK expression on TCRβ+ cells before (“Before culture”) and after (“Cultured”) expansion from wild-type spleen cells depleted of NK1.1+ (upper plots) or both NK1.1+ and Ly49 (G2+C/H/I/F) (lower plots). In (A), (C), and (D), numbers are for the percentages of cells within each quadrant. Representative data of at least four (A, C) and two (D) independent experiments.

**FIGURE 5.** Generation of NK1.1−/− T cells in radiation BM chimeras. (A) Radiation BM chimeras established by reconstituting irradiated CD1d−/− (Ly5.1) mice with BM cells from IRF-2−/−CD1d−/− (CD1d−/−) or CD1d+−/−IRF-2−/− (CD1d−/−) mice on the Ly5.2 background were analyzed 2 mo later. Shown are the percentages of cells expressing the indicated NRks within TCRβ+Ly5.1+ cells. Each symbol represents the value for an individual chimeric mouse. Horizontal bars are the means. Statistically significant differences are marked: *p < 0.05, **p < 0.005. (B) Mixed BM chimeras were established by reconstituting irradiated CD1d−/− (Ly5.1/5.2) mice with BM cells from IRF-2−/−CD1d−/− (CD1d−/−) or CD1d−/−IRF-2−/− mice (both were Ly5.2), together with BM cells from CD1d−/− mice (Ly5.1). Representative plots are shown for Ly5.2 donor-derived T cells (TCRβ+Ly5.1−/− cells) in BM of three chimeras for each donor cell combination. Anti-Ly49G2 and anti-Ly49C/H/I/F Abs were mixed and used to reveal Ly49+ T cells. Numbers represent the percentage of cells in each quadrant. (Supplemental Fig. 4). Importantly, Ly49+CD8+ T cells were significantly less frequent in IRF-2−/−IFNAR1−/− mice than in IFNAR1−/− mice (Fig. 8B, Supplemental Fig. 4), revealing an IFN-α/β-independent function of IRF-2. Consistent with this, substantial numbers of Ly49+ T cells could be expanded from IFNAR1−/−spleen cells but hardly from IRF-2−/− or IRF-2−/−IFNAR1−/− spleen cells upon culture with IL-2 in vitro (Fig. 8C).

**IRF-2 and IFN-α/β signals were both required for efficient generation of Ly49+ T cells but acted in distinct manners**

One of the functions of IRF-2 known to date is to attenuate IFN-α/β signals (24), pointing to a possibility that the Ly49+ T cell deficiency in IRF-2−/− mice was due to the uncontrolled IFN-α/β signals. However, Ly49+ T cells remained reduced in IRF-2−/− mice additionally lacking IFNAR1 (Fig. 8A), arguing against the possibility. Rather, we noticed a similar, yet slightly less severe, reduction of Ly49+ T cells in mice lacking IFNAR1 alone (Fig. 8A). Because these mice were on the CD1d−/− background, we focused only on CD8+ T cells to rule out the contribution of iNKT cells and found that Ly49+CD8+ T cells were less frequent in IFNAR1−/− mice than in control mice (Fig. 8B) similar to what was observed for whole Ly49+ T cells. The absolute cell numbers of Ly49+CD8+ T cells were also reduced in IFNAR1−/− mice.
IRF-2\textsuperscript{+/−} T cells might also underlie the reduction of NK1.1\textsuperscript{+} T cells in vivo, although it is not clear to what extent such an NK1.1 induction contributed to NK1.1\textsuperscript{+} T cells present in naive animals. How IRF-2 controls NK1.1 expression in T cells, but not splenic and hepatic NK cells or thymocytes, is not clear at this moment and is a subject for future studies. Our current study revealed at least that the requirements for the generation of NKG2D\textsuperscript{+}, NK1.1\textsuperscript{+}, and Ly49\textsuperscript{+} T cells were not identical.

To define Ly49\textsuperscript{+} T cells in this study, we used a mixture of Abs recognizing Ly49G2, C, H, I, and F, as well as those recognizing Ly49A, F, I, or G2 individually, and it remains possible that T cells expressing none of those Ly49s but other Ly49s would fall in the Ly49\textsuperscript{−} T cell fraction. However, activating Ly49s such as Ly49D and H and inhibitory Ly49E were reported to be absent on peripheral αβT cells (8, 29), and we also failed to detect Ly49D\textsuperscript{+} T cells in CD1d\textsuperscript{+/−} mice. Hence the “Ly49\textsuperscript{−}” T cells in IRF-2\textsuperscript{+/−}CD1d\textsuperscript{+/−} mice would have contained few, if any, T cells expressing Ly49s. Because normal Ly49\textsuperscript{+} T cells bore IL-2/IL-15R β-chains (CD122) and responded well to IL-2 or IL-15 in vitro (Ref. 28 and this study), the failure of Ly49\textsuperscript{+} T cells to expand from IRF-2\textsuperscript{+/−} spleen cells also supported the absence of these cells. On the basis of these observations, we concluded that IRF-2 played indispensable roles in the development of the T cell subset expressing one or more Ly49s.

Unlike CD1d-independent NKR\textsuperscript{+} T cells, CD1d-dependent iNKT cells in spleen were hardly affected in these mice. Past studies pointed out the similarity in the transcriptional program for NK and iNKT cell development/maturaiton, where multiple transcription factors, such as GATA3, T-bet, Ets-1, and Id2, were involved commonly (30, 31). In this regard, this is the first example, to our knowledge, of the common requirement of transcription factors in NK and Ly49\textsuperscript{+} T but not iNKT cells.

The practical inability to “induce” Ly49\textsuperscript{+} T cells from mature Ly49\textsuperscript{−} T cells in vivo and in vitro observed in this study was consistent with a previous report (28). Ly49\textsuperscript{−} T cell “induction” reported in the past, for example via chronic autoantigen stimulation (28), could result from mere expansion of preexisting Ly49\textsuperscript{+} T cells as seen in IL-2–supplemented cultures (this study) and in mice infected with lymphocytic choriomeningitis viruses (32). Such a difficulty to convert Ly49\textsuperscript{−} to Ly49\textsuperscript{+} T cells as well as the Ly49\textsuperscript{+} T cell deficiency in IRF-2\textsuperscript{−/−} mice reminded us of an interesting hypothesis that Ly49\textsuperscript{+} T cells constituted a lineage of T cells separated developmentally from conventional Ly49\textsuperscript{−} T cells (1). Whether the hypothesis is correct or not, it is unlikely that IRF-2 expression per se is the master switch to define the Ly49\textsuperscript{+} T cell subset or to support Ly49 gene expression, as IRF-2 was expressed comparably in CD1d\textsuperscript{−} lymphocytes (33), which contained many fewer Ly49\textsuperscript{+} T cells than those in the spleen (28). The inability of the retrovirally transduced IRF-2 to restore Ly49 expression in IL-2–propagated IRF-2\textsuperscript{−/−} splenic T cells (T. Notake, unpublished observation) added further support to the conclusion. The opposite influence of IRF-2 deficiency on Ly49\textsuperscript{+} and Ly49\textsuperscript{−} T cells observed in this study rather suggested the distinct transcriptional programs involving IRF-2 for these two T\textsubscript{MP} cell populations. In line with this prediction, Ly49\textsuperscript{−}CD8\textsuperscript{+} and CD44\textsuperscript{+}CD8\textsuperscript{+} T cells were reported previously to be dissimilar in the in vivo requirements for their appearance (8).

As to the function of IRF-2 in Ly49\textsuperscript{+} T cell development, we showed here that the frequencies of Ly49\textsuperscript{+} as well as NK1.1\textsuperscript{+} thymocytes were comparable in CD1d\textsuperscript{−/−} and IRF-2\textsuperscript{−/−}CD1d\textsuperscript{−/−} mice. Although evidence is to be obtained that these T cells in the thymus are the direct precursors of peripheral counterparts, these observa-

FIGURE 6. Increase of CD8\textsuperscript{+} T\textsubscript{MP} cells despite the reduction of the Ly49\textsuperscript{+} fraction in IRF-2\textsuperscript{−/−}CD1d\textsuperscript{−/−} mice. (A) Memory markers on Ly49\textsuperscript{+} T cells in IRF-2\textsuperscript{−/−}CD1d\textsuperscript{−/−} (CD1d\textsuperscript{−/−}) and IRF-2\textsuperscript{−/−}CD1d\textsuperscript{−/−} mice. Profiles for splenic TCR\textsuperscript{β} cells are shown. Numbers represent the percentages of cells in corresponding quadrants. Representative of at least three pairs of mice. (B) The absolute numbers of CD44\textsuperscript{+}CD8\textsuperscript{+} (CD44\textsuperscript{hi}) and CD44\textsuperscript{−}Ly6C\textsuperscript{+}CD8\textsuperscript{+} (CD44\textsuperscript{lo}Ly6C\textsuperscript{+}) T\textsubscript{MP} cells in the spleen in IRF-2\textsuperscript{−/−}CD1d\textsuperscript{−/−} (CD1d\textsuperscript{−/−}; ○) and IRF-2\textsuperscript{−/−}CD1d\textsuperscript{−/−} (●) mice. Each symbol represents the value for an individual mouse. Horizontal bars are the means. Statistically significant differences are marked: *p < 0.01, **p < 0.001.

FIGURE 7. Potential requirement for IRF-2 in postthymic Ly49\textsuperscript{+} T cell development. (A) Profiles for Ly49s (G2 +C/H/I/F) and NK1.1 expression on TCR\textsuperscript{β} cells in the thymus from IRF-2\textsuperscript{−/−}CD1d\textsuperscript{−/−} (CD1d\textsuperscript{−/−}) and IRF-2\textsuperscript{−/−}CD1d\textsuperscript{−/−} mice. Profiles for splenic TCR\textsuperscript{β} cells are shown. Numbers indicate the percentages of cells in each quadrant. (B) Intracellular staining for Bcl-2 in Ly49\textsuperscript{+} T cell (solid line) and Ly49\textsuperscript{−} T cell (dotted line) from spleen. Shaded histograms indicate total T cells stained with an isotype control. (C) Annexin V binding profiles for Ly49\textsuperscript{+} T cells (Ly49\textsuperscript{+}TCR\textsuperscript{β}) or Ly49\textsuperscript{−} NK cells (Ly49\textsuperscript{−}TCR\textsuperscript{β}) in spleen. Numbers indicate the percentages of annexin V\textsuperscript{+} cells as defined by the regions. Representative data for three (A, C) or two (B) mice of each genotype.
functions in Ly49 + T cell generation through the same mechanism as those seen previously in NK cell maturation and basophil expansion (21, 23), although the function of IRF-2 to attenuate physiological IFN-α/β signals (18, 19, 22, 24) was irrelevant commonly.

Another cytokine shown to be critical in generating and expanding Ly49 + T cells and other NKR + T cells was IL-15 (15, 34). However, the enlarged NKR - CD8 + Treg population in IRF-2 -/CD1d -/ mice, which was perhaps dependent on IL-15 (16, 35, 36), did not support the contribution of IRF-2 to IL-15 production, responses, and/or presentation. In contrast, because IFN-α/β induced “bystander” proliferation of CD44 hiCD8 + T cells, at least in part, via IL-15 (34), so might it be in Ly49 + T cell generation. Consistent with this prediction, substantial numbers of Ly49 + T cells could be recovered from IFNAR1 -/-, but not IRF-2 -/IFNAR1 -/ mice, spleen cells upon culture in vitro with high doses of IL-2 that signaled through the receptor common to that for IL-15. A hypothesis drawn from these observations would be that Ly49 + T cells require IRF-2 for development and IL-15–IFN-α/β for postdevelopmental expansion.

Killer cell Ig-like receptors, functionally equivalent to murine Ly49s, were expressed on human CD8 + T cells (2–4), but it remained unclear if these human CD8 + T cells as well as murine Ly49 + T cells have discrete functions unique to them. A very recent report by Kim et al. (14) showed that Ly49 +CD122 + CD8 + T cells contained virtually all Qa-1-restricted Tregs, the functional impairment of which in C57BL/6-Yaa mice led to lupus-like autoimmunity. IRF-2 -/ mice are also prone to an autoimmune-like cutaneous disease (24, 27). However, Ly49 + T cell deficiency alone appears to be insufficient for that form of autoimmunity, as the cutaneous disease developed no longer in IRF-2 -/IFNAR1 -/ mice (24) where Ly49 + T cells were still severely reduced as shown in this study. Nevertheless, IRF-2 -/CD1d -/ mice may provide a unique experimental system with which to investigate not only how Ly49 + T cells develop but also the relationship between Ly49 + T and CD8 + Tregs without influence of iNKT cells that are also known to have a versatile immunoregulatory activity (37). Finally, the requirement for IFN-α/β signals in the expansion of Ly49 + T cells pointed out an interesting possibility that Ly49 + T cells mediate a fraction, if not all, of immunomodulating functions of IFN-α/β (38).

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