CORRESPONDENCE

Ets1 regulates the differentiation and function of iNKT cells through both Pointed domain-dependent and domain-independent mechanisms

Tzong-Shyuan Tai[1], Hsiao-Wei Tsao[2,3,4], Wan-Chu Chuang[5], Chih-Chun Liu[5], Yu-Wen Huang[5], Peter Oettgen[3,6], Ya-Ting Chuang[5] and I-Cheng Ho[2,3]

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The transcription factor Ets1 is essential for the development of invariant natural killer T (iNKT) cells; however, its detailed role and mechanism of action are unknown. Here, we show that Ets1 is dispensable for CD1d-mediated selection, but is essential for subsequent differentiation of post-selected iNKT cells. This function is partly compensated by forced expression of a transgenic Vα14 Jα18 TCR and is independent of its N-terminal Pointed (PNT) domain. Ets1 is also required for optimal expression of cytokines by differentiated iNKT1 and iNKT2 cells, but inhibits the differentiation/function of iNKT17 in vitro and in vivo. These functions, however, depend on the PNT domain. Taken together, our results indicate that Ets1 regulates the differentiation/function of iNKT cells at multiple steps through both PNT domain-dependent and PNT domain-independent mechanisms.

Ets1 is the prototype of the ETS family of transcription factors and is expressed at comparably high levels in all stages of iNKT cells[1, 2], which express Vα14 Jα18 TCR. Germline deficiency of Ets1 leads to a near absence of iNKT cells (Supplementary Fig. 1a), making it challenging to study the roles and mechanism of action of Ets1 in iNKT cells. We found that the number of thymic iNKT cells was fully restored by a CD4 promoter-driven Vα14 Jα18 TCR transgene (TG) (Fig. 1a and Supplementary 1b). Since the positive selection of TG iNKT cells also requires CD1d-mediated selection[3], these data demonstrate that Ets1 is dispensable for CD1d-mediated selection of TG iNKT cells. Nearly 50% of the TG/Het thymic iNKT cells were stage 0 ($CD24^+$), and <10% were $CD44^+NK1.1^+$ stage 3 cells (Fig. 1b, c). In the absence of Ets1, the number and percentage of stage 0 cells further increased, and nearly all of the non-stage 0 TG/KO iNKT cells were $CD44^-NK1.1^-$ stage 1 cells (Fig. 1c, d), whose number was comparable to that of their TG/Het counterparts. Thus, the Vα14 Jα18 transgene was able to rescue the differentiation of stage 1 cells, but not stage 2 or stage 3 cells.

The PNT domain of Ets1 serves as a protein-interacting domain[4]. Interaction with other proteins through the PNT domain critically influences the function of Ets1 in many in vitro systems[5–7]. It is unknown whether the PNT domain is required for Ets1 to regulate the differentiation and function of iNKT cells. We therefore created a mouse strain (FFcre) expressing Ets1 lacking the PNT domain (amino acid residues 28–134) in a T cell-specific manner (Supplementary Fig. 2a). The level of the truncated protein, called PNTless Ets1, in FFcre thymocytes was comparable to that of the full-length Ets1 in control cells (FF) (Supplementary Fig. 2b). FFcre mice were born according to the Mendelian ratio and were grossly healthy up to at least 6 months of age.

The number of iNKT cells in the thymus was similar between FFcre mice and FF mice (Supplementary Fig. 2c). Thymic iNKT cells of FFcre mice matured normally into stage 1, stage 2, and stage 3 (Supplementary Fig. 2d). Normal numbers of iNKT cells were also detected in various peripheral organs of FFcre mice (Supplementary Fig. 2e); however, there was a significant increase in the percentage of the $CD4^+$ subset of FFcre iNKT cells compared to that of FF counterparts (Fig. 1e, f). Thus, the PNT domain is dispensable for the differentiation and maturation of iNKT cells, but is critical for maintaining the ratio between $CD4^+$ and $CD4^-$ subsets of iNKT cells.

Emerging data have suggested that $CD24^+$ iNKT precursors differentiate directly into three functional subsets: IFN-γ-producing iNKT1 ($T\text{-}bet^+PLZF^{low}$), IL-4-producing iNKT2 ($GATA3^+$, $PLZF^{high}$), and IL-17-producing iNKT17 ($ROR\gamma t^+PLZF^{medium}$). Typically, iNKT1 cells fall into the stage 3 population, iNKT2 cells into the stage 1 and stage 2 populations, and iNKT17 cells into the stage 2 population[8]. We found that the percentage of iNKT1 and iNKT2 was comparable between FF and FFcre mice, except for a slight decrease in the iNKT2 population in the lymph nodes (LNs) of FFcre mice (Fig. 1g, h and Supplementary 3a). Despite the normal or near-normal percentage of iNKT2 cells in FFcre mice, there was a 20 to 50% reduction in the percentage of $IL\text{-}4^+$ and $IL\text{-}13^+$ iNKT cells within a single-cell suspension obtained from the lymphoid organs of FFcre mice in response to in vitro stimulation with phorbol myristate acetate (PMA)/ionomycin (Fig. 1i). Similarly, the percentage of $IFN\text{-}\gamma^+$ and $IL\text{-}2^+$ iNKT cells was also reduced in the thymus and LNs of FFcre mice even though the percentage of iNKT1 cells was normal (Fig. 1i). Surprisingly, no significant reduction in the staining of IL-2 (a type 1 cytokine) or type 2 cytokines was detected in the liver iNKT cells of FFcre mice (Supplementary Fig. 3b), probably because the cytokine staining of in vitro stimulated liver iNKT cells was weak.

[1]Department of Medical Research, E-Da Hospital, I-Shou University, Kaohsiung, Taipei, China; [2]Department of Medicine, Division of Rheumatology, Inflammation, and Immunity, Brigham and Women's Hospital, Boston, MA, USA; [3]Harvard Medical School, Boston, MA, USA; [4]Broad Institute of Massachusetts Institute of Technology and Harvard, Cambridge, MA, USA; [5]Department of Medical Research, National Taiwan University Hospital, Taipei 10002, China and [6]Department of Medicine, Division of Cardiology, Beth Israel Deaconess Medical Center, Boston, MA, USA
Correspondence: Ya-Ting Chuang (ytchuang1976@ntuh.gov.tw) or I-Cheng Ho (iho@bwh.harvard.edu)

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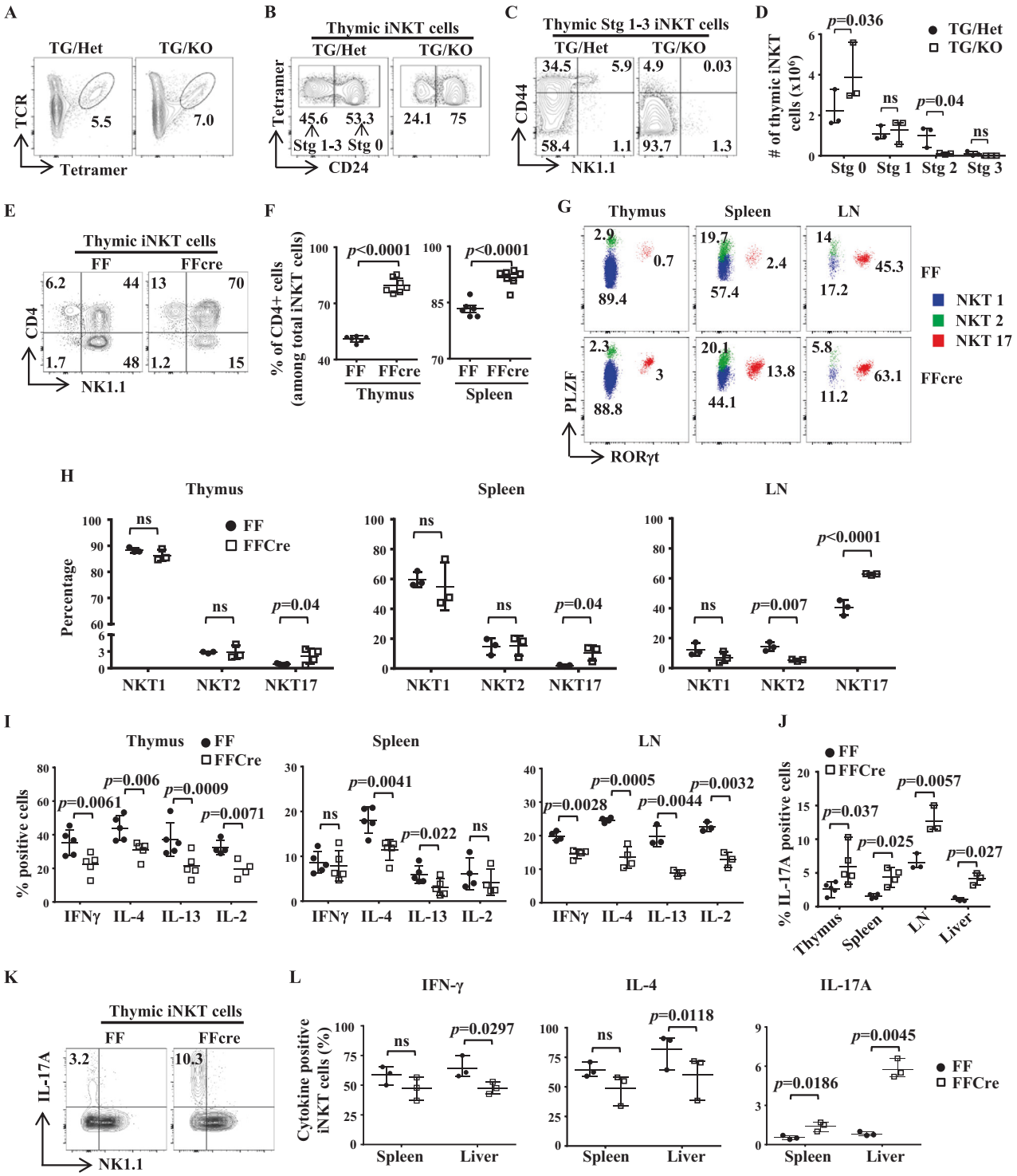

Fig. 1 PNT domain-dependent and -independent functions of Ets1 in iNKT cells. **a**–**d** Thymic iNKT cells of the indicated genotypes were identified with TCR and loaded CD1d tetramer. A representative FACS plot is shown in **a** Thymic iNKT cells were further divided into various stages based on the expression of CD24 (**b**), CD44 and NK1.1 (**c**). The absolute numbers of iNKT cells at each stage are shown in **d**. **e**, **f** The expression of CD4 and NK1.1 in thymic iNKT cells was analyzed with FACS. A representative FACS plot is shown in **e**, and the percentage of $CD4^+$ cells is shown in **f**. **g**, **h** iNKT cells from the indicated organs were stained for RORγt and PLZF. Representative FACS plots are shown in **g**, and the percentages of iNKT1, iNKT2, and iNKT17 cells are shown in **h**. **i**–**k** A single-cell suspension was prepared from the indicated organs, stimulated with PMA/ionomycin, and subjected to intracellular cytokine staining along with surface staining for NK1.1. The percentage of cytokine-positive cells within the iNKT population is shown in **i**. The percentage of IL-$17A^+$ cells within the iNKT population is shown in **j**. A representative IL-17A/NK1.1 FACS plot is shown in **k**. **l** Mice were injected intraperitoneally with αGC. Single-cell suspensions from the indicated organs were stained for the indicated cytokines. The percentage of cytokine-positive cells within the iNKT population is shown

In contrast, the percentage of iNKT17 cells was increased in FFcre mice in the thymus, spleen, and LN (Fig. 1g, h and Supplementary 3a). The percentage of IL-17A$^+$ iNKT cells in response to in vitro PMA/ionomycin stimulation was also elevated in cell suspensions from various organs of FFcre mice (Fig. 1j). The IL-17A$^+$FFcre iNKT cells were still restricted to the NK1.1$^-$ population (Fig. 1k). We subsequently injected α-galactosylceramide intraperitoneally into FF and FFcre mice and examined the production of cytokines ex vivo 2 h after the injection. We found that FFcre iNKT cells produced less IFN-γ and IL-4 but, more IL-17A than FF iNKT cells (Fig. 1l). These differences were statistically significant in liver iNKT cells. Therefore, Ets1 regulates the cytokine production of iNKT cells through a PNT domain-dependent mechanism.

One picture that emerged from this study is that Ets1 is a multifunctional transcription factor in iNKT cells (Supplementary Fig. 4). Systemic comparison of the transcription profiles of wild-type and knockout iNKT cells at each developmental and activation stage will shed more light on the mechanism of action of Ets1. The genetic approaches described above have made such an endeavor technically possible.

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AUTHOR CONTRIBUTIONS

T.-S.T. designed the study, performed most experiments, analyzed data, and co-wrote the manuscript. Y.-T.C. performed the experiments, analyzed the data, and co-wrote the manuscript. H.-W.T., W.-C.C., C.-C.L., and Y.-W.H. performed the experiments. P.O. designed and developed the FF mice. I.-C.H. conceived and designed the study, analyzed the data, and co-wrote the manuscript.

ADDITIONAL INFORMATION

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