CORRESPONDENCE

Variants of innate CD8$^+$ T cells are associated with Grip2 and Klf15 genes

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Lineage commitment of thymocytes can be distinguished and modulated by key factors of transcriptional networks, such as the antagonistic expression and function of Th-POK and Runx3 in the CD4 and CD8 lineages[1] and Foxp3[2] and PLZF[3] in CD4$^+$ regulatory T (Treg) cells and natural killer T (NKT) cells, respectively. Little is known, however, about the transcriptional regulation of the innate T cell lineage, especially innate CD8$^+$ T cells (i8Ts), which were initially defined in genetically manipulated mice[4] and later in BALB/c mice[5] and humans.[6] i8Ts are nonconventional αβT cells that exhibit features of memory phenotype and are dependent on IL-4 produced by NKT cells.[4] Although i8Ts are distinct from conventional memory CD8$^+$ T cells, they do share markers of T cells undergoing homeostatic proliferation and activated CD8$^+$ T cells.[4,7] Thus, the identification of key determinants in i8T development is important to understand the molecular mechanisms underlying their development and responses. In this study, we investigated the genetics of i8T development by determining the frequency of i8Ts and NKT cells among Collaborative Cross (CC) strains. Using CC, a powerful genetic resource designed to facilitate rapid mapping of genes mediating complex traits,[8,9] we identified key genes, *Klf15* and *Grip2*, that are important in the development of i8Ts.

The frequencies of i8Ts and NKT cells were defined in 54 CC strains by flow cytometry (Table S1). Serial phenotypes from low to high frequency of i8Ts (Fig. 1a, S1a, and S1b) were confirmed in the absence of any significant alteration in NKT cell numbers (Fig. 1b and S1c). As the frequency of i8Ts was not correlated with that of NKT cells (Fig. 1c), we could exclude the production of the extrinsic factor, NKT cell-derived IL-4, as influencing the levels of these cells. Thus, this analysis suggests that there might be other genetic variant(s) regulating the variable numbers of i8Ts in these CC strains. Since NKT2 cells, which specifically and mainly produce IL-4,[3] are relatively increased in total NKT cells and promote i8T development in high i8T strains, we further investigated subsets of NKT cells in PEF mice compared to B6 and BALB/c mice (Fig. S1d and S1e). The NKT cell subset was not significantly altered between the CC strains (Fig. 1d), indicating that the high frequency of i8Ts in some strains did not occur because of the alteration of the cytokine environment generated by NKT cells but because of genetic variation between the CC strains.

Next, the frequency of i8Ts was compared with the genotypes of each CC and founder strain. Mapping was performed using a logistic regression model (LRM) fit over the reconstructed haplotype matrix.[8] The resulting genome-wide distribution of –log (*P*) values (ANOVA chi-squared) is shown in Fig. S2a, together with the FDR thresholds. The genome-wide scan for genetic loci associated with changes in i8Ts revealed a major peak on chromosome 6, centered at 93.7 Mb (Fig. S2b). Within the LOD-2 region (89.4–95.6 Mb), *Klf15* was the only gene that had a nonsynonymous SNP in only the WSB and CAST strains, which had the highest i8T levels. The founder strain associated with the lowest levels of i8Ts, PWK, had 20 unique SNPs in the following genes within the peak region: *Uroc1*, *Grip2*, *4930590J08Rik, Fgd5, Adamts9,* and *Magi1* (Fig. S2c).

To further confirm the expression of candidate genes during T cell development, we analyzed the mRNA and protein levels of the candidate genes in sorted CD4$^-$CD8$^-$ (DN), CD4$^+$CD8$^+$ (DP), CD4$^+$CD8$^-$ (CD4SP), and CD4$^-$CD8$^+$ (CD8SP) (which were further sorted into CD44^{lo}CXCR3$^-$ (gate I), CD44^{int}CXCR3$^-$ (gate II) and CD44^{hi}CXCR3$^+$ (gate III)) thymocytes (Fig. S3a and S4a) as well as in sorted NKT, NK, γδT, and B cells from spleen and CD4$^+$ and CD8$^+$ T cells from LN. Consistent with previous studies on *Eomes* and *Tbet* expression in i8Ts[4] we found that *Grip2* and *Klf15* expression in i8Ts (gate III) was markedly higher than that in gate I and gate II CD8$^+$ T cells at both the mRNA (Fig. 1e, S3b, S3c, and S4b) and protein level (Fig. 1f and S4c). Collectively, only *Grip2* and *Klf15* were specifically and highly expressed in i8Ts, indicating that these may be key regulatory factors of i8T development.

Since i8T development depends on IL-4, which upregulates *Eomes* expression,[4] *Grip2* and *Klf15* expression was assessed after stimulation with γc cytokines. Their expression was only IL-4-dependently upregulated, as was *Eomes* (Fig. 1g and S5a). The specific upregulation of Grip2 and Klf15 expression by IL-4 was also confirmed at the protein level (Fig. 1h and S5b). This IL-4-inducible *Grip2* and *Klf15* expression was an unexpected finding. Because there was a wide range of frequencies of i8Ts, and variation in this trait did not correlate with either overall NKT cells or with their particular subsets,[3] it was a reasonable hypothesis that these genes would be heritable intrinsic factors for i8Ts. However, *Klf15* and *Grip2* seem to be inducible factors that are IL-4-dependently and cell-specifically upregulated. This

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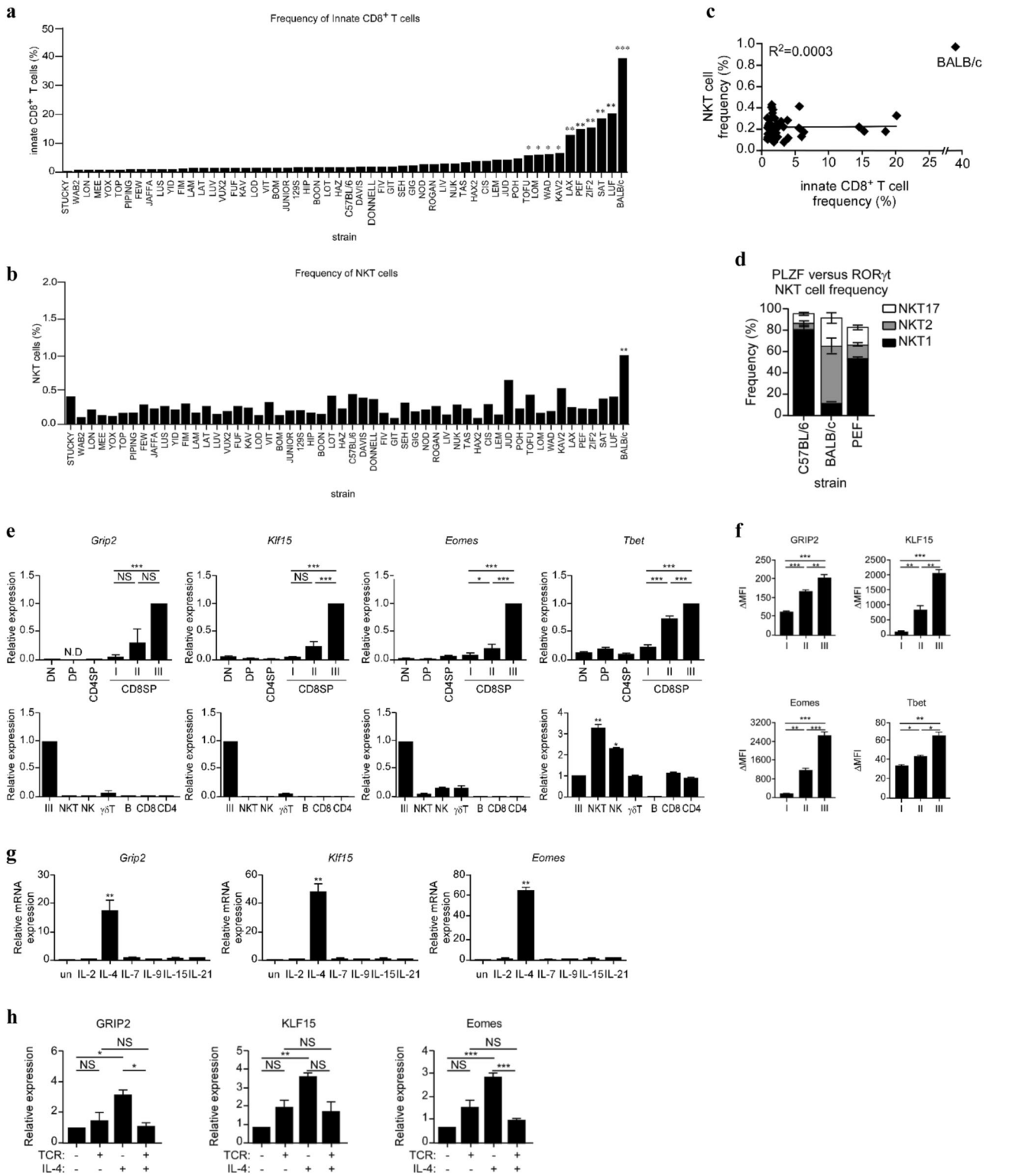

Fig. 1 The identification of *Grip2* and *Klf15* associated with i8Ts in CC strains. a, **b** We analyzed the thymus in inbred mouse strains. Bar graphs show a summary of the frequency of i8Ts (**a**) and NKT cells (**b**). **c** Correlation between strains with a high frequency of i8Ts and those with NKT cells. **d** The bar graph shows a summary of the NKT cell subset according to PLZF and RORγt expression. **e** Specific expression of *Grip2* and *Klf15* in i8Ts. Target gene expression in each sorted cell population from WT B6 mice was determined by qRT-PCR. To determine the cell-specific gene expression levels, mRNA levels were determined in the sorted NKT, NK, γδT, B, CD4$^+$ T, and CD8$^+$ T cells. **f** The GRIP2 and KLF15 protein levels in gated cells were determined by FACS. **g**, **h** IL-4-dependent upregulation of *Grip2* and *Klf15* expression. Naive CD8$^+$ T cells from WT B6 mice were stimulated with γc cytokines, and the mRNA and protein levels of *Grip2*, *Klf15,* and *Eomes* were determined by qRT-PCR (**g**) and FACS (**h**), respectively. Bar graphs show a summary of three independent experiments. Data are represented as the means ± SEMs of at least three independent experiments (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; and NS, not significant).

may be explained by their correlation with IL-4-producing cell populations other than NKT cells. Indeed, PLZF$^+$ T cells are responsible for the high IL-4 in *Itk*-deficient, *id3*-deficient, and *Klf2*-deficient mice[4] and thus for the development of i8Ts.[5]

We showed previously wide heritable variation in lymphocyte subsets between CC strains.[10] Here, the CC strains were exploited to map and identify key responsible genes and markers in i8Ts. We successfully found a locus linked to the proportion of i8Ts in the thymus and identified major effector genes, *Klf15* and *Grip2*, and confirmed their specific expression in developing i8Ts. Finally, although further studies on the role of *Klf15* and *Grip2* in i8Ts are required, we are convinced that these first identified genes will provide pivotal information and clues for future studies on the functions and roles of i8Ts.

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AUTHOR CONTRIBUTIONS

Y.J. and C.H. designed the research, analyzed the data, and wrote the paper. Y.J., L.B., J.A.S., L.A.A. and B.L. performed the experiments and analyzed the data. G.M. designed the research and interpreted the data, and L.B. and G.M. reviewed the paper. All authors have read and approved the final paper.

ADDITIONAL INFORMATION

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Competing interests The authors declare no competing interests.

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