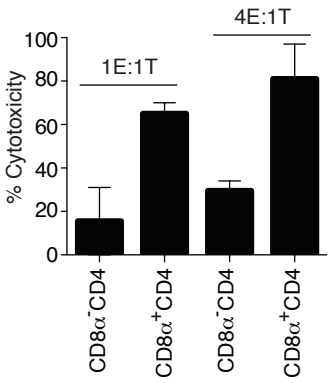
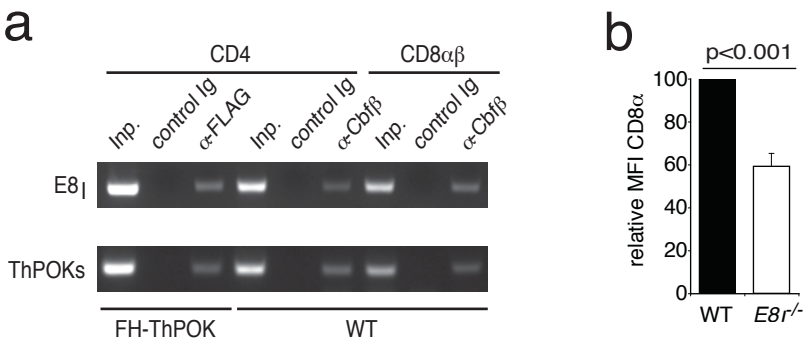
Transcriptional Reprogramming of Mature CD4$^+$ T helper Cells generates distinct MHC class II-restricted Cytotoxic T Lymphocytes

Daniel Mucida[1,2,14], Mohammad Mushtaq Husain[1,14], Sawako Muroi[3,14], Femke van Wijk[1,4,14], Ryo Shinnakasu[1,5], Yoshinori Naoe[3], Bernardo Sgarbi Reis[2], Yujun Huang[1], Florence Lambolez[1], Michael Docherty[1,6], Antoine Attinger[1,7], Jr-Wen Shui[1], Gisen Kim[1], Christopher J. Lena[1], Shinya Sakaguchi[8], Chizuko Miyamoto[3], Peng Wang[9,10], Koji Atarashi[11], Yunji Park[1,12], Toshinori Nakayama[13], Kenya Honda[11], Wilfried Ellmeier[8], Mitchell Kronenberg[1], Ichiro Taniuchi[3*] & Hilde Cheroutre[1*]

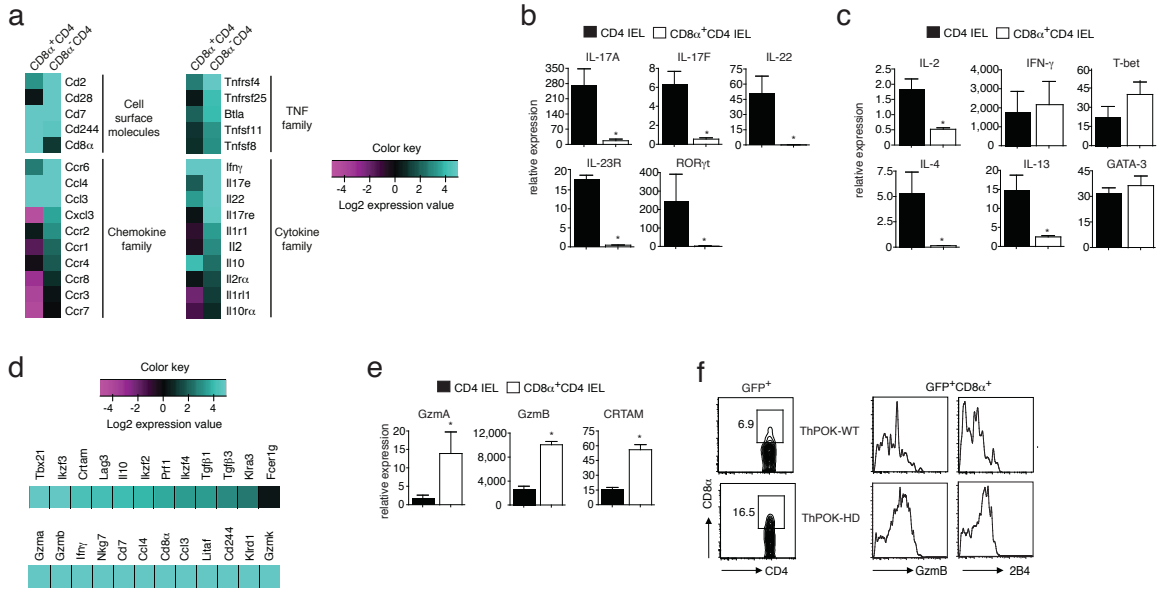
Nature Immunology doi:10.1038/ni.2523



Supplementary Figure 1. CD4 CTLs are cytotoxic. % cytotoxicity (anti-TCRβ redirected killing of P815 target cells) of respective lymphocyte subsets as measured by LDH release assay. Target killing was done in two effectors (E) to targets (T) ratios, two times each. Each bar shows standard error of the mean (SEM; n=2).



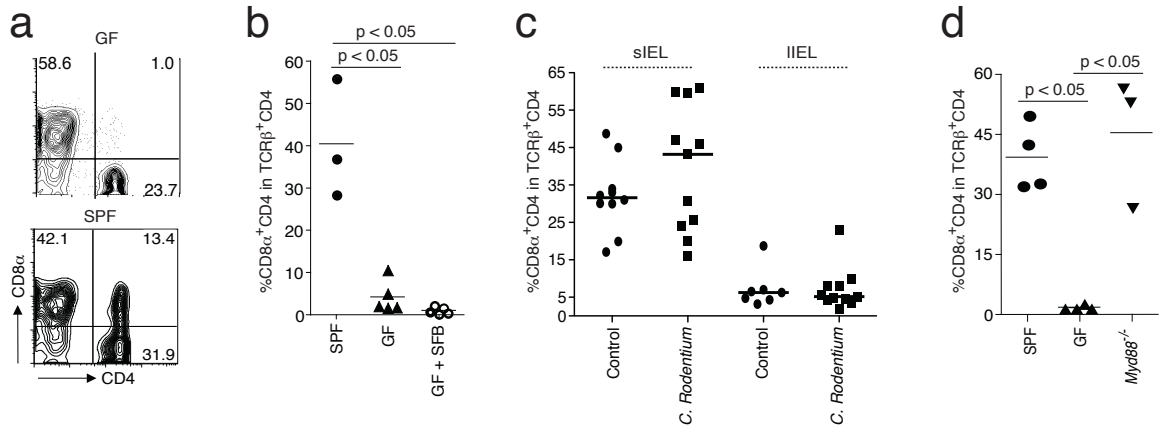
Supplementary Figure 2. ThPOK binds to $E8_I$ and prevents CD8α expression in mature CD4 T cells. **(a)** Chromatin immunoprecipitation (ChIP) assay using CD4 T cells from FLAG-hemagglutinin epitope-tagged ThPOK protein (FH-ThPOK) knock-in mice and WT mice. *Thpok* silencer locus was used for positive control for ThPOK binding (anti-FLAG antibodies) and wild type CD8 T cells were used as positive control for the Cbfb binding. **(b)** ThPOK-negative CD4 effector cells lost ThPOK as mature cells in the periphery. Relative MFI of CD8α expression on $CD45^+TCR\beta^+CD4^+$ sIELs from *Rag1*$^{-/-}$ recipient mice, 6-8 weeks after adoptive transfer of sorted $TCR\beta^+CD8\alpha^-CD45RB^{high}CD25^-CD4^+$ spleen T cells from naïve WT or $E8_I^{-/-}$ donor mice. Data are representative of 4 independent experiments, n = 4 or 5 mice.



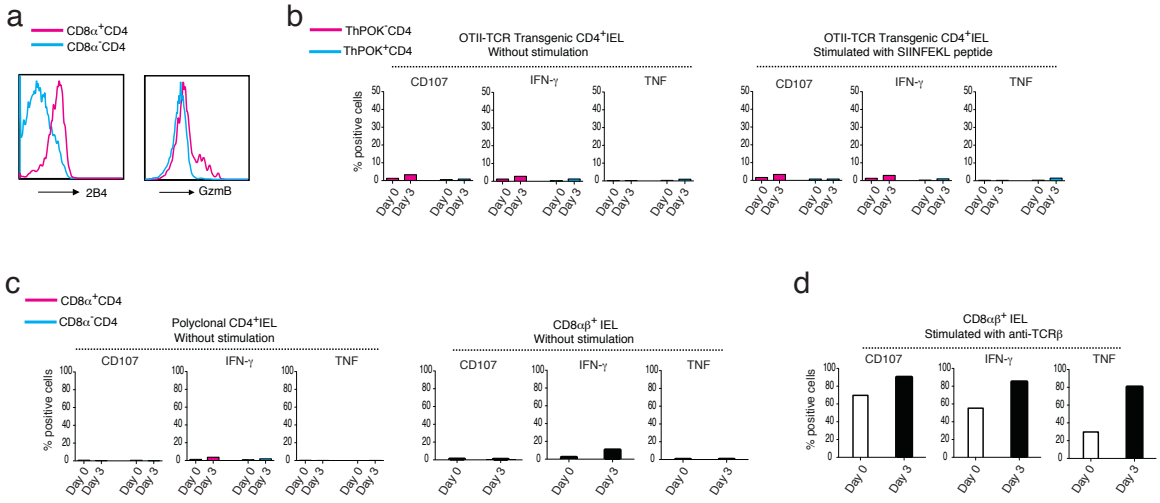
Supplementary Figure 3. Activated CD4 T_H cells that lose ThPOK expression differentiate to CTL. (**a**) Log2 transformed normalized signal intensity heatmap for selected genes related to CD4 T_H gene expression profiles (rows) expressed in sorted $TCR\beta^+CD4^+$ and $TCR\beta^+CD8\alpha^+CD4^+$ IELs (columns) isolated from *Rag1*$^{-/-}$ recipient mice that previously received sorted naïve $TCR\beta^+CD8\alpha^-CD45RB^{high}CD25^-CD4^+$ spleen T cells from wild type B6 donor mice. (**b**) Relative mRNA levels of T_H17-associated genes in IELs as in (**a**). Values are from individual mice and without further activation and expressed relative to L32 as an internal standard. Representative data (as mean +/− SEM) are shown for one of at least three independent experiments. (**c**) Relative mRNA levels of T_H1 and T_H2 associated genes as in (**a**). Representative data (as mean +/− SEM) are shown for one of at least three independent experiments. (**d**) Heat map of normalized gene expression as in (**a**) for selected genes enriched in the $TCR\beta^+CD8\alpha^+CD4^+$ subset are shown. (**e**) Relative mRNA expression of *Granzyme A* and *B* and *crtam* as in (**a**). Pooled data of five mice are shown from one of three independent experiments. * $P < 0.05$ (unpaired, two-tailed Student *t* test). **(f)** Staining for CD4 and CD8α (left panels) or Granzyme B or 2B4 (right panels) of gated $TCR\beta^+CD4^+$

GFP^{+} sIELs isolated from *Rag1*$^{-/-}$ recipients 4-5 weeks after adoptive transfer of ThPOK-*gfp* or ThPOK-HD mutant-*gfp*-transfected TCRβ^{+}CD4 primary cells. Data are representative of 3 independent experiments, n = 3 mice.

Nature Immunology doi:10.1038/ni.2523



Supplementary Figure 4. The ThPOK loss and reprogramming of CD4 CTL is an antigen-driven process *in vivo*. (**a**) Staining for CD8α expression on $CD45^+TCR\beta^+$ IELs of GF (upper panel) or SPF (lower panel) B6 mice. Data are representative of 2 independent experiments, n = 4 or 5**.** (**b**) Frequency of $CD8\alpha^+CD4^+$ T cells in gated $CD45^+TCR\beta^+CD8\beta^-CD4^+$ lymphocytes from Swiss-Webster SPF, GF mice or GF mice reconstituted for 4 weeks with SFB. Data are representative of 2 independent experiments, n = 4 or 5. ANOVA and Bonferroni as post-test were used for statistics. (**c**) Frequency of $CD8\alpha^+CD4^+$ T cells in gated $CD45^+TCR\beta^+CD4^+$ sIELs and lIELs of ThPOK-*gfp* mice 14 days post *C. rodentium* infection. Data are representative of 2 independent experiments, n = 3 or 4 mice. (**d**) Frequency of $CD8\alpha^+CD4^+$ cells as in (**b**) from conventionally raised specific-pathogen-free (SPF) or germ-free (GF) or *Myd88*$^{-/-}$ C57Bl6 mice. Data are representative of 2 independent experiments, n = 4 or 5 mice. ANOVA and Bonferroni as post-test were used for statistics.



Supplementary Figure 5. CD4 CTL function as potent antigen-specific and IL-15-sensitive effector cells. (**a**) 2B4 and Granzyme B expression on OT-II TCR transgenic $CD8\alpha^{-}CD4^{+}$ and $CD8\alpha^{+}CD4^{+}$ sIELs isolated from *Rag1*$^{-/-}$ recipient mice after 4 weeks of OVA-diet feeding. Data representative of 2 independent experiments. (**b**) Frequency of CD107, IFNγ and TNFα positive cells among unstimulated (left panels) or SIINFEKL stimulated (right panels) ThPOK $^{-}$ (red) and $ThPOK^{+}$ (blue) gated $TCR\beta^{+}CD4^{+}$ OT-II TCR transgenic sIELs isolated from *Rag1*$^{-/-}$ recipient mice after 4 weeks of OVA-diet feeding and analyzed at day 0 without- or after 3 days culturing with rIL-15. (**c**) Frequency of CD107, IFNγ and TNFα positive cells among unstimulated gated $CD4^{+}CD45^{+}TCR\alpha\beta^{+}$- or $CD8\alpha\beta^{+}CD45^{+}TCR\beta^{+}$ polyclonal sIELs analyzed at day 0 (open bars) or after culturing in rIL-15 (solid bars). (**d**) Frequency of CD107, IFNγ and TNF positive cells among gated $CD8\alpha\beta^{+}CD45^{+}TCR\beta^{+}$ polyclonal sIELs analyzed at day 0 (open bars) or after culturing in rIL-15 (solid bars) prior to anti-TCRβ stimulation *in vitro.* Data are representative of 3 independent experiments.