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Cutting Edge

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Cutting Edge: Lymphoproliferation Caused by Fas Deficiency Is Dependent on the Transcription Factor Eomesodermin

Ichiko Kinjyo,[*,†] Scott M. Gordon,[*,†] Andrew M. Intlekofer,[*,†] Kennichi Dowdell,[‡] Erin C. Mooney,[*,†] Roberto Caricchio,[§] Stephan A. Grupp,[¶,‖] David T. Teachey,[¶,‖] V. Koneti Rao,[‡] Tullia Lindsten,[*,#] and Steven L. Reiner[*,†]

A hallmark of autoimmune lymphoproliferative syndrome (ALPS), caused by mutation of the Fas death receptor, is massive lymphadenopathy from aberrant expansion of $CD4^-CD8^-$ (double-negative [DN]) T cells. Eomesodermin (Eomes) is a member of the T-box family of transcription factors and plays critical roles in effector cell function and memory cell fitness of $CD8^+$ T lymphocytes. We provide evidence in this study that DN T cells exhibit dysregulated expression of Eomes in humans and mice with ALPS. We also find that T cell-specific deletion of Eomes prevents lymphoid hypertrophy and accumulation of DN T cells in Fas-mutant mice. Although Eomes has critical physiological roles in the function and homeostasis of $CD8^+$ T cells, overexpression of Eomes appears to enable pathological induction or expansion of unusual CD8-related T cell subsets. Thus, antagonism of Eomes emerges as a therapeutic target for DN T cell ablation in ALPS. *The Journal of Immunology*, **2010, 185: 7151–7155.**

Mice and humans with mutations in the death domain-containing receptor, Fas, suffer from dysregulated homeostasis of lymphocyte populations, often leading to massive lymphadenopathy and splenomegaly primarily composed of αβ TCR-bearing $CD4^-CD8^-$ (double-negative [DN]) T cells. Autoimmune manifestations, including severe cytopenias, and heightened risk of lymphoma are also features of autoimmune lymphoproliferative syndrome (ALPS) (1, 2). Accumulation of DN T cells was recently described as a highly predictive biomarker of ALPS (2). This hallmark expansion of the DN subset and the subsequent enlargement of secondary lymphoid organs in ALPS lead to significant morbidity for affected patients.

The signaling and transcriptional events that induce the abnormal DN T cell fate, as well as the precise ontogeny of the DN T cells, are unknown. Previous reports suggest that DN T cell expansion requires deficiency of Fas only within the T cell compartment, as mice with B cell- or dendritic cell (DC)-specific deletions of *Fas* do not amass the anomalous T cells (3, 4). DN T cells in ALPS are thought to arise from $CD8^+$ T cells (3, 5–7), though there has been some debate as to whether the formerly $CD8^+$ lymphocytes were self-reactive (3) or bore hyporeactive TCRs (6, 7). Both models postulate that aberrantly selected $CD8^+$ T cells are cleared via a peripheral, Fas-dependent quality control mechanism (6–8). In the absence of Fas, it is presumed that the improperly selected $CD8^+$ T cell has an opportunity to become a DN T cell and proliferate uncontrollably by an as yet unknown mechanism (3–7).

Eomesodermin (Eomes), a paralog of T-bet, is expressed in effector/memory $CD8^+$ T cells and NK cells and plays redundant roles with T-bet in the induction of cytokine secretion and cytotoxic capacity of $CD8^+$ T lymphocytes (9–11). Eomes expression is also a hallmark of noncanonical, IL-15–responsive, innate-like $CD8^+$ T cells that acquire functions, such as IFN-γ production and cytolytic potential, during their development in the thymus (12–16). Previous studies suggested that ALPS DN T cells exhibit heightened sensitivity to the cytokine IL-15 (7). Additionally, Fas-mutant T cells were found to produce IFN-γ independently of the T-box transcription factor T-bet (17). Responsiveness to IL-15 and T-bet–independent induction of IFN-γ are both characteristics controlled by the transcription factor Eomes (11, 18). In this study, we report that Eomes dysregulation defines the DN T cells in *lpr/lpr* animals and in humans with

*Abramson Family Cancer Research Institute, †Division of Infectious Diseases, Department of Medicine, ‖Department of Pediatrics, and #Department of Pathology, University of Pennsylvania, Philadelphia, PA 19104; ‡National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; §Division of Rheumatology, Department of Medicine, Temple University, Philadelphia, PA 19122; and ¶Department of Pediatrics, The Children's Hospital of Philadelphia, Philadelphia, PA 19104

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Address correspondence and reprint requests to Dr. Steven L. Reiner, University of Pennsylvania, 421 Curie Boulevard, BRB II/III, Room 414, Philadelphia, PA 19104. E-mail address: sreiner@mail.med.upenn.edu

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Abbreviations used in this paper: ALPS, autoimmune lymphoproliferative syndrome; del, deletion; DC, dendritic cell; DN, double-negative; Eomes, eomesodermin; *Eomes F/F*, mice harboring floxed alleles of *Eomesodermin*; F, female; fs, frameshift mutation; hEomes, human eomesodermin; hNK, human NK; LAD, lymphadenopathy; M, male; MMF, mycophenolate mofetil; qRT-PCR, quantitative real-time RT-PCR.

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ALPS. We sought to investigate the effects of T cell-specific deletion of Eomes on the abnormal T cells of Fas deficiency, and our results suggest that Eomes is essential for the development or maintenance of this population.

Materials and Methods

Mice

All animals were housed at the University of Pennsylvania (Philadelphia, PA) in specific pathogen-free conditions, and all experiments were performed in accordance with approved protocols by the University of Pennsylvania Institutional Animal Care and Use Committee. Mice harboring floxed alleles of *Eomes* (*Eomes F/F*) mated to mice expressing Cre-recombinase, driven by the *Cd4* promoter (*Cd4:Cre*$^+$), have been previously described (10). To study Fas-deficient, Eomes-deficient T cells, *lpr/lpr* mice were mated to *Eomes F/F, Cd4:Cre*$^+$ mice. To study Fas-deficient, T-bet–deficient T cells, *lpr/lpr* mice were mated to *Tbx21*$^{-/-}$ mice. To study Fas-deficient, IL-15–deficient mice, *lpr/lpr* animals were bred to *Il15*$^{-/-}$ animals.

Human samples

Human cells were obtained with informed consent and in accordance with the Institutional Review Boards of the Children's Hospital of Philadelphia (Philadelphia, PA) and the National Institutes of Health (Bethesda, MD).

Quantitative RT-PCR, cell sorting, and flow cytometry

Sorting indicated populations for quantitative real-time RT-PCR (qRT-PCR) on murine cells was carried out on a BD FACSAria (BD Biosciences, San Jose, CA). qRT-PCR was carried out as previously described (10). Target gene probes were purchased from Applied Biosystems (Foster City, CA). The following Abs (from BD Pharmingen, San Diego, CA, unless otherwise indicated) were used for FACS staining: TCRβ allophycocyanin or PE-Cy5, CD4 FITC or PE-Cy7, CD8α PerCP-Cy-5.5 or Alexa Fluor 700 (Biolegend, San Diego, CA), B220 PE or PE-Texas Red (Caltag Laboratories, Burlingame, CA), CD19 allophycocyanin Cy7, and Eomes PE (eBioscience, San Diego, CA). Data were collected on a BD FACSCalibur, BD FACSAria, or BD LSRII (BD Biosciences). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

Autoantibody detection

Anti-nuclear Abs were detected with an anti-nuclear Ab test kit (Antibodies Incorporated, Davis, CA). Briefly, slides precoated with fixed, mitotic HEp-2 cells were exposed to sera from the indicated mice, followed by detection of anti-nuclear Abs with FITC-labeled goat anti-mouse IgG. Slides were later mounted and fluorescent microscopy was performed.

Results and Discussion

We examined the expression of Eomes in the T cell subsets of Fas-mutant *lpr/lpr* mice (from the nonautoimmune-prone C57BL/6 background) and found that Eomes mRNA (Fig. 1*A*) and protein (Fig. 1*B*) levels were substantially higher in Fas-mutant T cells compared with cells of wild-type mice. Expression of *Eomes* was most dysregulated in the DN T cell subset. Comparable results were obtained from the PBMCs of ALPS-FAS patients, who harbor confirmed mutations in the *Fas* gene (Fig. 1*C*, 1*D*, Table I). Each patient exhibited elevated levels of Eomes in DN T cells (Fig. 1*C*). The patient with highest levels of *Eomes* in DN T cells (Pt5) had notably early and aggressive onset of disease (Fig. 1*C*, Table I). As in *lpr/lpr* mice (Fig. 1*A*, 1*B*), expression of *Eomes* in ALPS patients was most dysregulated in the DN T cell subset (Fig. 1*D*). Levels of Eomes protein and mRNA in murine and human Fas-mutant DN T cells appeared comparable to or greater than levels found in NK cells and effector/memory CD8$^+$ T cells (Fig. 1*B*, 1*C*).

In view of the dysregulated expression of Eomes in DN T cells from patients and mice with ALPS, we took advantage of Cre-Lox technology to achieve a conditional knockout of the murine *Eomes* locus (*Eomes F/F, Cd4:Cre*$^+$) to ask whether Eomes expression in T cells plays a causal role in the pathogenesis of ALPS (10). Deletion of *Eomes* in the T cell lineage of Fas-mutant mice resulted in substantial amelioration of the hallmark T cell dysregulation and lymphoproliferation of ALPS syndrome. In *lpr/lpr*, *Eomes F/F*, *Cd4:Cre*$^+$ mice, accumulation of DN T cells was reduced to the percentage found in wild-type mice (Fig. 2*A*). The mass and cellularity of lymphoid tissue in *lpr/lpr*, *Eomes F/F*, *Cd4:Cre*$^+$ mice was also substantially reduced compared with Eomes-proficient *lpr/lpr* mutants (Fig. 2*B–D*). Residual increase in mass and cellularity of *lpr/lpr*, *Eomes F/F*, *Cd4:Cre*$^+$ spleens relative to wild-type mice (together with a similar trend in the lymph nodes) suggests an additional, Eomes-independent phenotype of cellular excess in *lpr/lpr* mice. This Fas-dependent, Eomes-independent abnormality is likely to be affecting apoptosis or proliferation of conventional leukocytes, in general, because

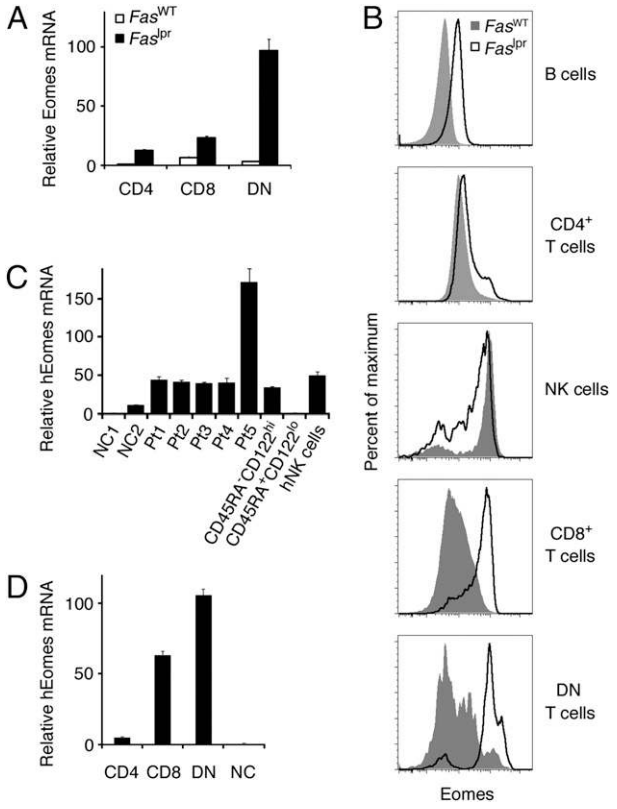

FIGURE 1. Dysregulated expression of Eomes is a hallmark of DN T cells in both human and murine ALPS. *A*, CD4$^+$, CD8$^+$, and CD4$^-$CD8$^-$ (DN) T cells were sorted from wild-type and *lpr/lpr* mice (*Fas*^{WT} and *Fas*^{lpr}, respectively), and murine *Eomes* mRNA levels were analyzed by qRT-PCR. Values represent the mean ± SEM of triplicate determinations normalized to hypoxanthine-guanine phosphoribosyltransferase. Results are representative of at least three independent experiments. *B*, Intranuclear Eomes protein expression assessed by mAb and flow cytometry of indicated subpopulations within the freshly isolated splenocytes of wild-type and *lpr/lpr* mice. Results are representative of three independent experiments. *C*, qRT-PCR analysis of human Eomes (hEomes) mRNA in the sorted DN T cells of five patients with ALPS (Pt1–Pt5) that have been diagnosed clinically and confirmed with genetic testing for *Fas* mutation (see Table I for additional clinical information). Comparison is made to bulk PBMCs from two normal controls (NC1, NC2), sorted memory (CD45RA$^-$CD122^{hi}), and naive (CD45RA$^+$CD122^{lo}) phenotype CD8$^+$ T cells from normal controls, and sorted human NK (hNK) cells. *D*, CD4$^+$, CD8$^+$, and DN T cells were fractionated from the PBMCs of three ALPS patients (Pt1, Pt4, Pt5), and hEomes mRNA levels were compared with bulk PBMCs from healthy donors. Values represent the mean ± SEM. Results are representative of two independent experiments.

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Table I. *Patient characteristics*

Subject No.	Age (y)/Sex	Identified Fas Mutation	Diagnosis	Percent DN	LAD	Splenomegaly	IgG (mg/dl)	Autoimmune Manifestations	Therapy
NC1	40/F	None	Healthy volunteer	<1	−	−	−	−	None
NC2	47/M	None	Healthy volunteer	<1	−	−	−	−	None
Pt1	10/M	942C→T, p.R234 stop (exon 9)	ALPS-FAS	8	++	+	1300–1910	Autoimmune cytopenia	MMF
Pt2	10/M	952G→T, p.G237V (exon 9)	ALPS-FAS	16	+++	Asplenic	2540–3600	Autoimmune cytopenia	MMF
Pt3	11/F	952G→T, p.G237V (exon 9)	ALPS-FAS	7	++	+	507–1270	No cytopenia	None
Pt4	12/M	430del AAG, p.E63fs (exon 3)	ALPS-FAS	10	+++	+	300–1000	Autoimmune cytopenia	MMF
Pt5	4/M	383T→A, p.C47X (exon 2)	ALPS-FAS	8	++++	+	2230–2520	Autoimmune cytopenia, Guillain-Barré	None

Percent DN denotes percent DN T cells among PBMCs. LAD denotes grades of lymphadenopathy: +, shotty nodes; ++, multiple nodes up to 2 cm; +++, many nodes >2 cm; ++++, visible lymphadenopathy; −, within normal limits. IgG denotes serum IgG concentration. Therapy denotes chronic immunosuppressive regimen. Pt1, Pt2, and Pt3 have previously been referred to in publication as NIH 080.8, NIH 128.1, and NIH 128.4, respectively.

del, deletion; F, female; fs, frameshift mutation; LAD, lymphadenopathy; M, male; MMF, mycophenolate mofetil.

the percentage of $CD4^+$ T cells, $CD8^+$ T cells, B cells, and non-B/non-T cells was not reproducibly different between wild-type and Eomes-deficient *lpr/lpr* mice (not shown).

Consistent with the finding that Fas deficiency might also alter homeostasis of conventional immune cell lineages independently of Eomes, we found that the autoimmune mani-

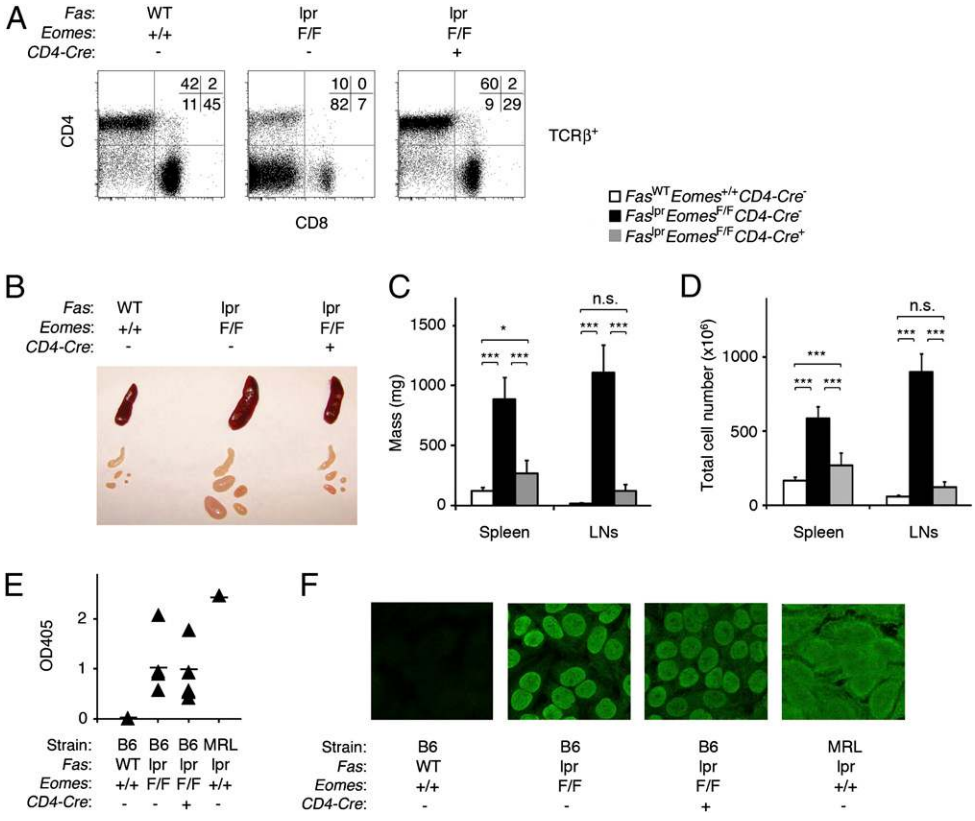

FIGURE 2. Eomes is required for the lymphoproliferative, but not autoimmune, component of murine ALPS. *A*, Flow cytometry of freshly isolated, $TCR\beta^+$ lymph node cells from wild-type, *lpr/lpr* (with homozygous floxed alleles of *Eomes* but no Cre-recombinase transgene), and *lpr/lpr* mice with T cell-specific deletion of *Eomes* (homozygous floxed alleles of *Eomes* and transgenic Cre-recombinase driven by the *Cd4* promoter). Results are representative of >10 independent experiments. Of note, the percentage of $CD4^+$ T cells, $CD8^+$ T cells, B cells, and non-B/non-T populations was not reproducibly different between wild-type and Eomes-deficient *lpr/lpr* mice. *B*, Photograph of spleens and lymph nodes from mice with the indicated genotypes. Results are representative of eight independent experiments. Mass of spleens and pooled lymph nodes (*C*) and total cell number contained therein from mice of the indicated genotypes (*D*). Bar graphs indicate mean values, and error bars represent SEM; $n = 8$ mice per group. A one-way ANOVA with Tukey's postcomparison test was performed using Prism software (GraphPad, San Diego, CA). *E* and *F*, Autoantibody production in 12–16-mo-old female mice of the indicated genotypes. Results are representative of three independent experiments. *E*, Sera were isolated and analyzed for anti-dsDNA Abs by ELISA. Lupus-prone MRL-*lpr/lpr* mice serve as positive control for severe autoantibody titers. *F*, Anti-nuclear Abs were detected by exposing HEp-2 cells to sera of indicated mice, followed by staining with a secondary, FITC-conjugated Ab reactive to mouse IgG. Original magnification ×60. NS, $p > 0.05$; $^*p < 0.05$; $^{***}p < 0.001$.

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festations of ALPS were not affected by the T cell-specific deletion of *Eomes* (Fig. 2*E*, 2*F*). This result was not unexpected because independent lines of evidence have uncoupled DN T cell accumulation from the pathogenic autoantibody production of ALPS (3, 4, 9). Whereas B cell- or DC-specific deficiency of Fas is not sufficient to drive DN T cell expansion, either is sufficient to recapitulate the autoantibody production of ALPS (3, 4). $CD4^+$ T cells, DCs, and B cells are still present and Fas-deficient in *lpr/lpr*, *Eomes F/F*, *Cd4:Cre*$^+$ mice, providing a sufficient cellular network for the elaboration of pathogenic autoantibodies. Taken together, these data suggest that the Eomes-dependent DN T cell population is responsible for the lymphoproliferative phenotype but does not appear to be required for the humoral autoimmunity characteristic of ALPS.

Previous evidence implicated T-bet in the pathogenic autoantibody production in Fas-mutant mice, owing to the B cell-autonomous role of T-bet in class switch recombination. However, germline deletion of *Tbx21*, the gene encoding T-bet, had little impact on the course of the T cell-mediated pathology of murine ALPS (17). Consistent with the prior result, there was only moderate dysregulation of T-bet mRNA expression in the cells of Fas-mutant mice (Supplemental Fig. 1*A*), and deletion of *Tbx21* did not substantially affect the accumulation of DN T cells (Supplemental Fig. 1*B*). These data support a nonessential role for T-bet and an essential, nonredundant role for Eomes in driving DN T cell expansion and lymphoproliferation in ALPS.

In addition to their role in conferring functional competence to killer lymphocytes, Eomes and T-bet are responsible for enhancing expression of CD122, the receptor that confers responsiveness to IL-15 (11). IL-15 serves as a critical growth factor in the maturation and maintenance of memory $CD8^+$ T cells, NK cells, and some atypical T cell subsets (19, 20). It was previously suggested that DN T cells from ALPS mice are more sensitive to IL-15 (7), an effect that might be mediated by Eomes. We, therefore, intercrossed *Il15*$^{-/-}$ mice with *lpr/lpr* mice. In contrast to the substantial protection associated with T cell-specific deletion of *Eomes*, deficiency of IL-15 afforded limited protection to *lpr/lpr* mice against accumulation of DN T cells and lymphadenopathy (Supplemental Fig. 2*A*, 2*B*). These data suggest that Eomes may direct other proliferative or survival mechanisms in DN T cells that transcend its effect on IL-15 responsiveness. Additionally, we found that IL-15 is not responsible for driving dysregulated Eomes expression in DN T cells of *lpr/lpr* mice (Supplemental Fig. 2*C*).

The events leading to expression of Eomes in DN T cells remain to be investigated. DN T cells are thought to arise from $CD8^+$ T cells (5). A self-reactive T cell might undergo self-antigenic activation (3), leading to induction of Eomes as part of an incipient program of effector differentiation (11). Alternatively, a $CD8^+$ T cell unfit to be engaged by self-peptide/MHC might degenerate into a state of autonomous survival (6), somewhat akin to Eomes-expressing central memory T cells, which survive independently of self-peptide/MHC (21). It is also possible that cytokine secretion during the pathogenesis of ALPS acts to induce *Eomes* (22), as IL-4 was recently found to drive expression of Eomes in a population of noncanonical, innate-like $CD8^+$ T lymphocytes (14, 15). Although previous data argue against an absolute requirement for IL-4 in the accumulation of DN T cells in *lpr/lpr* mice (22), we cannot rule out a role for other cytokines or soluble factors in inducing and enhancing Eomes expression in Fas-mutant $CD8^+$ and DN T cells.

It has recently been suggested that $CD8^+$ T cells deficient in Eomes may fail to effectively compete for the memory T cell niche (23). The present results raise the possibility that gain-of-function of Eomes in Fas-deficient DN T cells promotes their preferential proliferation in lymphoid tissues. This hypothesis is consistent with both the prior evidence of increased proliferation and defective apoptosis of Fas-deficient DN T cells (24) as well as the enhanced proliferation and Bcl-2 expression of Eomes-proficient compared with Eomes-deficient memory $CD8^+$ T cells (23). Future studies will be designed to identify the target genes of Eomes that are involved in ALPS DN T cell homeostasis, because it appears to be more complex a matter than Eomes simply regulating responsiveness to IL-15 (Supplemental Fig. 2*A*, 2*B*). Despite uncertainties surrounding the ontogeny of DN T cells, our finding that Eomes is essential and nonredundant for DN T cell development or maintenance offers a therapeutic target to reduce the DN T cell compartment and alleviate a major source of morbidity in children with ALPS.

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Disclosures

The authors have no financial conflicts of interest.

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