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Ikaros imposes a barrier to CD8+ T cell differentiation by restricting autocrine IL-2 production¹

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Abstract

Naive CD4+ T cells require signals from the TCR and CD28 to produce IL-2, expand, and differentiate. However, these same signals are not sufficient to induce autocrine IL-2 production by naive CD8+ T cells, which require cytokines provided by other cell types to drive their differentiation. The basis for failed autocrine IL-2 production by activated CD8+ cells is unclear. We find that Ikaros, a transcriptional repressor that silences IL-2 in anergic CD4+ T cells, also restricts autocrine IL-2 production by CD8+ T cells. We find that CD8+ T cell activation *in vitro* in the absence of exogenous cytokines and CD4 help leads to marked induction of Ikaros, a known repressor of the *Il2* gene. Naïve murine CD8 T cells haplo-insufficient for *Ikaros* failed to upregulate Ikaros, produced autocrine IL-2, and differentiated in an IL-2-dependent manner into IFN- γ -producing CTL in response to TCR/CD28 stimulation alone. Furthermore, *Ikaros*-haplo-insufficient CD8+ T cells were more effective at controlling *Listeria* infection and B16 melanoma growth *in vivo*, and could provide help to neighboring, non-IL-2-producing cells to differentiate into IFN- γ -producing effectors. Therefore, by repressing autocrine IL-2 production, Ikaros ensures that naive CD8+ T cells remain dependent upon licensing by antigen presenting cells and CD4+ T cells, and may therefore act as a cell-intrinsic safeguard against inappropriate CTL differentiation and immunopathology.

Introduction

Naive T cell differentiation is a tightly regulated process, as aberrant activation can lead to immunopathology and disease. Naïve CD4+ and CD8+ T cells differ in their requirements for differentiation, as the latter have higher cytotoxicity potential. Naïve CD4+ T cells require TCR recognition of a cognate peptide in a class II MHC molecule and a costimulatory signal from CD28-B7 engagement. Upon receiving these two signals, CD4+ T cells can produce autocrine IL-2 and differentiate (1). In contrast, co-stimulation of naïve

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CD8⁺ T cells through the TCR and CD28 does not result in efficient autocrine IL-2 production (2), and is not sufficient for differentiation into cytolytic effectors. In addition to TCR and CD28, CD8⁺ T cells require a pro-inflammatory cytokines for their differentiation. For instance, IL-12, type I interferons, and IL-21 have been characterized as key inflammatory cytokines that drive naïve CD8⁺ T cells into full-fledged cytotoxic effectors (3). Typically, these cytokines are derived from dendritic cells or CD4⁺ T cells to help promote the appropriate effector immune response.

Another cytokine that strongly influences CD8⁺ T cell responses is IL-2. This cytokine has a pro-survival role through up-regulation of the anti-apoptotic factor Bcl-2, but also can influence CD8⁺ T cell differentiation through affecting the balance of effector versus memory generation. IL-2 during the priming phase is required for effective T cell memory formation, as “unhelped” CD8⁺ T cells fail to generate memory (4–7). However, high levels of IL-2 can promote terminal effector CD8⁺ T cell generation at the expense of memory formation (8–11). Thus, it is critical to regulate IL-2 production during initial phases of an immune response, as means to ensure appropriate CD8⁺ T cell differentiation. Naïve CD8⁺ T cells are highly restricted in their production of autocrine IL-2 (2), and are largely dependent upon IL-2 from CD4⁺ helper T cells (12–14). However, in some systems helper T cells can license CD8⁺ T cells to produce their own IL-2 (15), which is required during initial priming in order to generate robust memory recall responses (16).

Little is known about how autocrine IL-2 is restricted in naïve CD8⁺ T cells. Recently, it was demonstrated that the transcription factor Ikaros, a transcriptional repressor required for lymphocyte development (17, 18), restricts autocrine IL-2 production in mature CD4⁺ T cells (19, 20). We hypothesized that Ikaros may similarly regulate naïve CD8⁺ T cell differentiation through inhibition of autocrine IL-2 production. In this study, we demonstrate that TCR stimulation leads to strong induction of Ikaros unless exogenous cytokines are present, and that naïve CD8⁺ T cells with reduced Ikaros function are able to differentiate into cytolytic effectors in the absence of signal 3 cytokines and CD4 help due to a gain of autocrine IL-2 function. Thus, by restricting autocrine IL-2 production by CD8⁺ T cells, Ikaros ensures that induction of an inflammatory and cytotoxic program only occurs in cells that have been appropriately licensed by a third signal.

Materials and Methods

Mice, Antibodies, Cytokines

Wild-type CD45.2, CD45.1, PMEL, RAG1^{-/-} and OT-1 mice were purchase from JAX. Mice carrying the germline *Ikzf1* null allele were a gift of Dr. K. Georgopoulos, and were backcrossed on a B6 background for greater than 12 generations. RAG1^{-/-} OT-1 *Ikzf1*^{+/-} mice were generated through breeding *Ikzf1*^{+/-} mice onto a RAG1^{-/-} background to obtain RAG1^{-/-} *Ikzf1*^{+/-} mice. These mice were then crossed with the RAG1^{-/-} OT-1 mice to generate the triple cross. Once the triple cross was generated, these mice were maintained by crossing with RAG1^{-/-} or RAG1^{-/-} *Ikzf1*^{+/-} mice. *Ikzf1*^{+/-} PMEL mice were generated by crossing *Ikzf1*^{+/-} mice with PMEL mice. All procedures were approved by The Children's Hospital of Philadelphia Research Institute animal use and care committee. Monoclonal antibodies against CD3 (2C11), CD28 (37.51), CD4 (GK1.4), MHCII (M/5114), FcR

(2.4G2), and IL-2 (JES6-1A12) were purchased from BioXcell and anti-B220 (RA3-6B2), and anti-CD44 (IM7) antibodies were purchased from Biolegend. Mouse IL-2 and IL-12 were purchased from Peprotech and Roche. The SIINFEKL peptide derived from chicken ovalbumin was purchased from Biomatik.

Cell Sorting

Single cell suspensions from spleen and LN of polyclonal mice were sorted for naïve CD8⁺ T cells (CD62L^{hi} CD44⁻) on a MoFlo XDP (Beckman Coulter). Naïve CD8⁺ T cells were at >95% purity. RAG1^{-/-} OT-1 and RAG1^{-/-} *Ikzf1*^{+/-} OT-1 single cell suspensions from spleen and LN were depleted of CD4⁺ T cells, monocytes, MHCII-expressing cells with Qiagen magnetic goat anti-rat IgG beads (#310107). Cells were stained with cocktail of depleting anti-CD4 (GK1.4), MHCII (M/5114), anti-FcR (2.4G2), anti-B220, and anti-CD44 (IM7). Naïve OT-1 (CD62L^{hi} CD44⁻) cells were purified to >90% purity.

Flow cytometry and applications

Fluorochrome conjugated antibodies against anti-mouse IFN- γ (XMG1), APC-Cy7 anti-mouse CD25 (PC61), anti-mouse IL-2 (JES6-1A12), anti-mouse CD8 (53-6.7), anti-mouse CD45.1 (A20), anti-mouse CD62L (MEL-14), anti-mouse CD44 (IM7), anti-mouse CD4 (GK1.5) were purchased from Biolegend. Fixable, Live/Dead Aqua stain (L34957) was purchased from Invitrogen. Fluorochrome antibody to anti-mouse Granzyme B (NGZB), and anti-mouse Eomes (Danmag11) were purchased from eBioscience. Fluorochrome anti-mouse T-Bet (4B10) was purchased from BD Biosciences. CFSE was purchased from Millipore and 7-AAD was ordered from Sigma-Aldrich. Negative gating was based on a 'fluorescence-minus-one' (FMO) strategy. For intracellular cytokine staining, cells were treated with Golgi Stop (BD Biosciences, 0.66 μ g/ml) for 4–6 hours with PMA (30 ng/ml) and ionomycin (1 μ M) or OVA peptide (1 μ M) as indicated. Following harvesting, cells were fixed with 1% PFA for 30 minutes, spun down and washed once with FACS buffer. Cells were then washed with BD Perm Wash (BD Biosciences) 2 times and then stained with cytokine antibodies for 45 minutes at room temperature. Cells were washed 2 times in BD Perm Wash, then re-suspended in FACS Buffer. For transcription factor staining, cells were surfaced stained with fluorochrome-labeled primary antibodies for 20 minutes on ice. After washing in FACS buffer, cells were fixed with Fix/Perm buffer from eBioscience. Following fixation, cells were permeabilized and stained with APC anti-mouse T-bet and PE anti-mouse Eomes. For Ikaros staining, rabbit anti-mouse Ikaros (Abcam, ab26083) was used. Following staining with the Ikaros antibody, cells were washed and then stained with a PE-labeled anti-rabbit secondary antibody. Following completion of stains, cells were processed on a CyanADP (Beckman Coulter) for flow cytometric analysis.

Cell Culture

Naïve sorted CD8⁺ T cells were stimulated in 96-well or 24-well plates, which were coated with anti-CD3/CD28. All T cell cultures were maintained in RPMI supplemented with 10% FBS, L-glutamine, penicillin/streptomycin, and 2- β -mercaptoethanol, and maintained in 37C incubator. EL4 and EL4.ova cells lines were maintained in DMEM, supplemented with 10% FBS, L-glutamine, penicillin/streptomycin and 2- β -mercaptoethanol. EL4.ova cells were

maintained in 400 µg/ml G418 (Invitrogen). All means, measures of variance, and statistical tests are based upon biological replicates, as indicated in the figure legends.

Immunoblot analysis

Immunoblotting was performed for determining the Ikaros isoform expression. For immunoblotting, 0.33×10^6 to 1×10^6 cells were lysed with Lamelli buffer and boiled for 10 minutes to denature the proteins. Total lysates were electrophoresed in Criterion precast 10% Tris-HCl gels (Bio-Rad) and transferred to nitrocellulose membrane using a Trans-Blot™ apparatus (Bio-Rad). The membrane was washed for 10 minutes (3X) with wash buffer (0.1% Tween-20 in 1X PBS). It was then incubated with HRP conjugated anti-goat antibody (1:10,000) for 1 hour at room temperature. The membrane was washed for 10 minutes 3 times with wash buffer and then developed using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific). The image was developed on a Kodak X-ray film. A C-terminus reactive goat anti-mouse antibody to Ikaros (SC-9861) and a goat anti-mouse actin antibody (SC-1615) were purchased from Santa Cruz. β -actin expression level was determined to normalize the differences in loading. Prior to β -actin staining, the membrane was stripped with Restore Plus Western Stripping Buffer (Thermo Scientific) for 10 minutes and then washed for 15 minutes, 3 times. The pre-blocked membrane was stained with goat polyclonal anti-B-actin antibody followed by staining with HRP conjugated secondary antibody. Densitometry was performed using ImageJ software on background-subtracted images derived from the raw immunoblot films.

Immunohistochemistry

50–250 thousand cells were centrifuged onto glass slides and fixed for 20 minutes in 10% neutral buffered formalin. Immunohistochemical stains were performed on a Bond III system (Leica Microsystems, Bannockburn, Ill) with pH6 epitope retrieval solution (Leica), a HRP-conjugated anti-Ikaros primary antibody (ab26083, Abcam, Cambridge, MA) diluted 1:1000 in IHC diluent (Leica), and with nuclear counter stain hematoxylin, following manufacturer's protocol (standard protocol F, Leica) but eliminating steps to de-paraffinize slides. Stained slides were analyzed on a Leica DM 2500 microscope with a 40x HCX PL Fluotar objective ($\infty/0.17/D$). Images were captured using Leica application suite version 2.8.1 (build 1554, 2003–2007).

Cytotoxicity assays

Naïve purified OT-1 CD8+ T cells from *Ikzf1*^{+/+} or *Ikzf1*^{+/-} mice were stimulated with plate bound anti-CD3/CD28 (1.0 µg/ml) for 48 hours and in presence or absence of IL-2 (10 ng/ml). Cells were then harvested, counted and re-suspended at 0.5×10^6 cells/ml in complete RPMI and rested overnight at 37°C. After overnight rest, effectors were mixed at 10:1, 5:1, and 2.5:1 ratio with CFSE-labeled EL4 or EL4.OVA cells and incubated for 3 hours, 37°C. After a 3 hour incubation period, cells were harvested, washed in FACS buffer, stained for CD8 expression and live/dead viability was assessed after addition of 7-AAD (5 µg/ml). Cells were analyzed by flow cytometry and a standard number of flow cytometric beads were collected to standardize the assay. CFSE+ tumor cells were gated on, and 7-AAD gating was measured against EL4 cells not mixed with T cells. Percent killing by CD8+ T

cells was calculated by the following fashion. % Cytotoxicity of CD8+ T cells = (number of 7-AAD- cell counts/ total cell counts) * 100. These numbers were then normalized to the EL4 cell fraction that had no T cells added. Specific lysis = (% cytotoxicity of CD8+ T cells – % cytotoxicity of control tumor cells without CD8+ T cells)/(% maximum cytolysis – % minimum cytolysis).

Recombinant *Listeria monocytogenes* infection model

Purified naive wild-type or *Ikzf1*^{+/-} RAG1^{-/-} OT-I T cells were activated for 48 hours with plate bound anti-CD3/CD28 Ab (1.0 µg/ml each). Cells were harvested and injected *i.v.* (5×10^6) into B6 mice. Two hours following transfer, mice were challenged with 1×10^5 live recombinant *Listeria monocytogenes* expressing the SIINFEKL peptide (LM-OVA). Spleen, LN, and blood were harvested at day 3 post-infection. Bacterial burden in the spleen was determined as previously described (21, 22). Blood and LN were characterized by PE H-2K^b/OVA₂₅₇₋₂₆₄ (K^b/OVA) tetramer staining, and LN cells were assessed for cytokine production upon restimulation with OVA peptide.

B16 melanoma model

Purified naive wild-type or *Ikzf1*^{+/-} PMEL CD8+ T cells were isolated and activated *in vitro* for 3 days with plate bound anti-CD3/CD28 (1.0 µg/ml) in the presence of IL-12 (20 ng/ml) and/or IL-2 (10 U/ml). Cells were harvested and injected *i.v.* into sex-matched B6 mice (1×10^6 cells/mouse). Twenty four hours later, mice were challenged with 1×10^5 cells B16 melanoma cells *s.c.* on each flank. Length and width of tumor was recorded and tumor volume was calculated by $(L * W^2) / 2$.

Results

IL-2 opposes Ikaros induction and promotes CD8+ T cell differentiation

To study the differentiation of naïve CD8+ T cells into effectors in a reductionist system, we utilized agonistic anti-CD3 and anti-CD28 antibodies in an *in vitro* culture system devoid of other cell types or exogenous cytokines. To control for previous antigen exposure, we used polyclonal CD44^{lo}CD62L^{hi} CD8+ T cells enriched by flow sorting, or monoclonal CD8+ T cells developed in RAG1^{-/-}OT-1 mice. Naïve CD8+ T cells express the full length DNA binding isoforms of Ikaros, with no evidence for expression of the smaller isoforms that lack the DNA binding domain (Fig. 1A). Ikaros exhibited a characteristic, punctate nuclear pattern by immunohistochemical staining (Fig. 1B), as observed in previous studies (23). When costimulated through the TCR and CD28 in the absence of exogenous cytokines, naïve cells showed marked upregulation of Ik1 and Ik2 (Fig. 1A and B). The Ik2 band may contain the Ik3 isoform, which also binds DNA and can co-migrate with Ik2 (24). TCR/CD28-activated cells also consistently exhibited higher molecular weight species that most likely represent post-translationally modified (*e.g.*, sumoylated, ubiquitinated, and/or phosphorylated) forms that have been detected in thymocytes and other cell lines (51). In some experiments, a minor low molecular weight form that is smaller than the Ik6 and Ik7 splicing isoforms is detected (see Fig. 2), which may represent an N-terminal degradation product that we have detected previously in Th1 cells (19). These modified forms were only variably detected in naive cells. The increase in Ikaros protein expression upon TCR/CD28

stimulation correlated with failure to differentiate into IFN- γ producing effector cells (Fig. 1D, left panel). The addition of exogenous IL-2 opposed the accumulation of Ikaros (Fig. 1A and C), and promoted differentiation of naïve CD8⁺ T cells into IFN- γ producing effectors (Fig. 1D, right panel). These results suggest that Ikaros, a known repressor of CD4⁺ T cell differentiation (19, 25) may also be involved in cytokine-regulated CD8⁺ T cell differentiation.

Ikaros restricts CD8⁺ T cell differentiation in the absence of signal 3 cytokines

To determine whether Ikaros imposes a direct barrier to CD8⁺ T cell differentiation, we utilized mice that carry one null and one wild-type allele of *Ikzf1*, the gene encoding Ikaros. This model offers significant advantages over nullizygous mice, which do not develop an intact lymphoid immune system (26), and over mice expressing a dominant-negative mutant of Ikaros (18), which develop fatal lymphomas early in life (27), in that *Ikzf1*^{+/-} mice show normal hematopoietic development and do not develop cancer (23). We find that naïve CD8⁺ T cells with only one functional allele of *Ikzf1* exhibit a ~50% reduction in Ikaros protein, as measured by both immunoblot and flow cytometric analyses (Fig. 2A). *Ikzf1*^{+/-} CD8⁺ T cells exhibited only modest induction of Ikaros in response to TCR/CD28 stimulation, achieving levels approximately 5-fold less than wild-type cells (Fig 2A–C). This failure was not due to under-stimulation, as wild-type and *Ikzf1*^{+/-} CD8⁺ T cells up-regulated the activation markers CD69 and CD25 to a similar degree following TCR and CD28 stimulation (Fig. 2D). Consistent with their reduced Ikaros protein levels, *Ikzf1*^{+/-} CD8⁺ T cells were able to upregulate CD25 and differentiate in response to TCR/CD28 co-stimulation alone (Fig. 3A), giving rise to frequencies of IFN- γ producers comparable to that observed in wild-type cultures given exogenous IL-2 (Fig. 3B). The addition of exogenous IL-2 blunted the induction of Ikaros in wild-type cells, but led to nearly complete loss of Ikaros in the *Ikzf1*^{+/-} cells (Fig. 2B and C). Addition of exogenous IL-12 also led to reduced levels of Ikaros in wild-type CD8⁺ T cells, which was also more pronounced in *Ikzf1*^{+/-} cells (data not shown), and this was associated with increased responsiveness of *Ikzf1*^{+/-} cells to IL-12 at the level of IFN- γ production (Fig. 3C). Expression of the high affinity IL-2 receptor by activated T cells is amplified and stabilized by IL-2, as IL-2-induced STAT5 drives transcription of the *cd25* gene in a feed-forward loop(28, 29). The high expression of CD25 therefore suggested the presence of IL-2 in TCR/CD28 costimulated *Ikzf1*^{+/-} cultures. Consistent with this, neutralization of IL-2 completely blocked TCR/CD28-mediated CD25 and IFN- γ expression (Fig. 3D) and prevented down-regulation of Ikaros by *Ikzf1*^{+/-} cells (data not shown), indicating that the gain of function exhibited by these cells is entirely dependent upon IL-2.

We also assessed expression of the T-box transcription factors T-bet and Eomes (30, 31), as IL-2 induces Eomes expression (8) and Ikaros has been shown to regulate T-bet during CD4⁺ T cell differentiation (25). TCR and CD28 signals induced T-bet expression by wild-type cells (32), which was augmented by exogenous IL-2 (Fig. 3E, left panel). In comparison, TCR/CD28-stimulated *Ikzf1*^{+/-} CD8⁺ T cells induced more T-bet than wild-type cells, and also exhibited increased T-bet expression in response to IL-2 (Fig 3E, left panel). While wild-type CD8⁺ T cells required exogenous IL-2 for the induction of Eomes (Fig. 3E, right panel), *Ikzf1*^{+/-} CD8⁺ T cells were able to induce Eomes when stimulated

without additional cytokines, to levels comparable to that in wild-type cells primed in the presence of IL-2 (Fig. 3E, right panel). These data indicate that Ikaros influences the expression of T-bet and Eomes, key factors for CD8⁺ T cell effector differentiation (30, 31).

Ikaros influences CD8 differentiation *via* control of autocrine IL-2

The requirement for IL-2 in the differentiation of naïve *Ikzf1*^{+/-} CD8⁺ T cells indicated that loss of Ikaros function is accompanied by a gain of autocrine IL-2 production. To test this, we measured IL-2 levels in the supernatants of wild-type and *Ikzf1*^{+/-} cultures over 48 hours of stimulation. Consistent with previous studies (2), wild-type naïve CD8 cells produced very little autocrine IL-2 in response to TCR/CD28 costimulation (Fig. 4A). However, CD8⁺ T cells lacking a single copy of *Ikzf1* secreted significant levels of IL-2, and this was observed in both polyclonal cells and monoclonal OT-I cells (Fig. 4A). This gain of IL-2 production could also be observed at the single-cell level immediately after TCR and CD28 stimulation (Fig. 4B).

To determine whether this enhanced IL-2 production is due to a reduced signaling threshold, or is the result of an absolute gain of autocrine function by *Ikzf1*^{+/-} cells, we varied the strength of TCR or costimulatory signal received by the naïve cells in this system and measured T cell activation and IL-2 production. Naïve, wild-type CD8⁺ T cells produced very little IL-2, and did so only at supra-maximal concentrations of α TCR antibody (5–10 μ g/mL, Fig. 4C), even though wild-type and *Ikzf1*^{+/-} cells were equally able to induce the CD69 and CD25 activation markers (Fig. 2D). Similarly, increasing the strength of CD28 costimulation at a fixed, high concentration of α TCR antibody did not result in significant IL-2 production by wild-type cells, but *Ikzf1*^{+/-} cells showed a strong, dose-dependent increase in IL-2 production under these conditions (Fig. 4C). Therefore, increasing TCR/CD28 signal strength could not raise the wild-type level of autocrine IL-2 production to that of the *Ikzf1*^{+/-} cells, indicating that a loss of Ikaros function does not merely shift the T cell activation threshold, but results in an absolute gain of autocrine IL-2 function by naïve CD8⁺ T cells.

Loss of Ikaros function leads to differentiation of a relatively high frequency of CD8 T cells in this system, as measured by IFN- γ secretion at the single-cell level (Fig. 3A). While this is clearly due to a large increase in IL-2 secretion by *Ikzf1*^{+/-} CD8 cultures (Fig. 4A), our ICS data indicate that this IL-2 is produced from relatively few cells at any one time (Fig. 4B). To determine if Ikaros-regulated autocrine IL-2 can also drive the differentiation of neighboring, non-IL-2 producers in a paracrine manner, we utilized a mixed culture experiment. We mixed naïve-sorted, wild-type CD45.1⁺ CD8⁺ T cells with naïve CD45.2⁺ *Ikzf1*^{+/-} CD8⁺ T cells and activated them *in vitro* in the presence or absence of IL-2. This would test if the increased autocrine IL-2 from naïve *Ikzf1*^{+/-} CD8 cells could act in a paracrine fashion on the wild-type cells to promote their differentiation. We also used a suboptimal dose of anti-CD3/28 to increase dependency on cytokine signals for their differentiation. As before, priming of *Ikzf1*^{+/-} cells with anti-CD3/CD28 alone resulted in differentiated CD25^{hi}, IFN- γ producing cells (Fig. 5A and F), while wild-type cells failed to differentiate under these conditions (Fig. 5A and D). However, wild-type CD8⁺ T cells in the presence of *Ikzf1*^{+/-} CD8⁺ T cells became CD25^{hi}, and a significant frequency was able

to produce IFN- γ as measured by intracellular staining (Fig. 5B) and ELISA (Fig. 5E). To test if this was due to the increased autocrine IL-2 production from the CD8+ T cells with reduced Ikaros, cultures were also stimulated in the presence of a neutralizing anti-IL-2 antibody. Blockade of IL-2 resulted in a failure to differentiate into IFN- γ producing cells in these mixed cultures (Fig. 5C), indicating that the IL-2 from the *Ikzf1*^{+/-} CD8+ T cells was necessary for both the autocrine and paracrine effects on differentiation. These data show that Ikaros controls not only the capacity of an activated CD8+ T cell to produce autocrine IL-2, but also its ability to 'help' other activated CD8+ T cells to differentiate by providing paracrine IL-2 signals.

Ikaros controls CD8+ T cell cytotoxicity

To address how a loss of Ikaros function influences the secondary effector function of CD8+ T cells, we assessed granzyme B expression and cytotoxic activity upon restimulation, functions that are driven by IL-2 (1, 8, 9, 33, 34, 35). Wild-type CD8+ T cells primed through the TCR and CD28 and re-challenged with PMA and ionomycin expressed low levels of granzyme B (Fig. 6A, left panel), and required the addition of exogenous IL-2 for high-level expression (Fig. 6A, middle panel). However, *Ikzf1*^{+/-} effector cells were able to induce granzyme B to high levels in the absence of exogenous cytokines (Fig 6A), and this gain of granzyme B expression was completely dependent upon IL-2 (Fig 6A, right panel).

To assess cytolytic activity, OVA-specific wild-type and *Ikzf1*^{+/-} OT-I cells were challenged *in vitro* with EL4 thymoma targets engineered to express OVA (EL4.OVA). TCR/CD28-primed OT-I cells exhibited relatively low killing of EL4.OVA cells unless exogenous IL-2 was included in the primary cultures (Fig. 6B, dark gray bars). However, consistent with their increased IL-2 and granzyme B expression, TCR/CD28 primed *Ikzf1*^{+/-} OT-I cells exhibited a cytotoxic capacity similar to that observed in wild-type cells stimulated in the presence of IL-2 (Fig 6B, light gray bars). To gain more insight into the increased killing in *Ikzf1*^{+/-} co-cultures, we focused on the conjugates formed between CD8+ cells and tumor cells (Fig. 6C). The frequency of CD8+ T cells engaged in conjugates with tumor cells was comparable between wild-type and *Ikzf1*^{+/-} co-cultures (data not shown), suggesting that the increased tumor cell killing was not due to enhanced antigen recognition by the *Ikzf1*^{+/-} CTL. However, we found that tumor cells actively engaged in a conjugate with *Ikzf1*^{+/-} CTL were killed more efficiently than tumor cells engaged in a conjugate with wild-type CTL (Fig. 6D, left panel). Again, the efficiency of wild-type cell-mediated cytotoxicity was increased by exogenous IL-2, reaching levels comparable to that of the *Ikzf1*^{+/-} CTL (Fig. 6D, right panel). These data indicate that *Ikzf1*^{+/-} cells are more efficient at killing tumor cells in an antigen-specific manner once the cells have already engaged their targets. Together, these results demonstrate that the autocrine IL-2 that is normally repressed by Ikaros is sufficient to drive naïve CD8+ T cells to differentiate into cytotoxic, IFN- γ -producing effector cells, even in the absence of CD4+ T cell help and/or exogenous cytokines like IL-2 or IL-12.

Ikaros regulates anti-bacterial and anti-tumor CD8+ effector function *in vivo*

To test whether the enhanced *in vitro* differentiation and cytotoxicity of *Ikzf1*^{+/-} CD8+ T cells translated to enhanced *in vivo* effector function, we utilized an IFN γ -dependent

bacterial clearance model using recombinant *Listeria monocytogenes* expressing OVA (LM-OVA) in the context of the OVA-specific OT-I TCR, and a IFN γ -dependent tumor rejection model using the B16 melanoma in the context of a transgenic, melanocyte/melanoma antigen-specific TCR (PMEL). CD8⁺ T cells are crucial for the control of *Listeria* infection, and clear this bacterium by secreting IFN- γ , which activates macrophages to kill intracellular bacilli, and by direct killing of infected macrophages in a perforin/granzyme-dependent manner (36, 37). We primed naive OT-I or *Ikaros*^{+/-} OT-I cells through the TCR and CD28 for 48 hours and adoptively transferred these cells into B6 mice. Transfer of OT-I cells into LM-OVA-infected B6 mice resulted in a 5-fold reduction in the number of live LM-OVA in the spleen at day 3 post-infection, as compared to control animals that received no OT-I cells (Fig. 7A). However, LM-OVA replication was reduced by greater than 10-fold by day 3 in animals that received the same number of OT-I cells that lacked one copy of the *Ikaros* gene (Fig. 7A). The enhanced capacity of *Ikaros*^{+/-} CD8⁺ T cells to control *Listeria* infection was associated with a moderate (but not statistically significant) increase in the number of OVA peptide-specific CD8⁺ T cells in the blood and lymph nodes (Fig. 7B), and with a highly significant increase in number of antigen-specific IFN γ - and TNF α -producing cells in the LN (Fig. 7C). These cytokines have both been linked to bacterial clearance in this model (38–40). These data demonstrate that *Ikaros* restricts the inflammatory response of CD8⁺ T cells in a cell-intrinsic manner during acute bacterial infection, which has potential implications for immunity and the control of immunopathology.

To further evaluate *in vivo* CD8⁺ T cell effector function, we utilized the poorly immunogenic B16 melanoma model, together with TCR-transgenic PMEL-1 T cells that recognize the melanocyte/melanoma antigen gp100 (41). Effective cellular immunotherapy of established B16 tumors in this stringent model normally requires the administration of exogenous IL-2 (42) or IL-12 (43, 44), and requires IFN- γ (45). Since our *Ikaros*^{+/-} CD8⁺ T cells produce more IFN- γ upon stimulation with IL-12, and IL-12 also improves IL-2 signaling (46, 47), we primed PMEL or *Ikaros*^{+/-} PMEL cells through the TCR and CD28, plus either IL-12 or the combination of IL-12 and IL-2. To test the fitness of these cells *in vivo*, the CD8⁺ T cells from these primary cultures were adoptively transferred into B6 mice and injected with highly aggressive B16 melanoma cells 24 hours later. As the endogenous T cells in the B6 host animals are ignorant of this tumor, this approach measures the *in vivo* efficacy of the transferred PMEL cells in absence of tumor-specific CD4⁺ helper T cells. Transfer of PMEL cells that were primed through the TCR and CD28 alone (Fig. 7D, dark grey line) resulted in a modest delay of tumor growth as compared to mice receiving no PMEL CD8⁺ T cells (Fig. 7D, dashed line). Addition of exogenous IL-12 or the combination of IL-2 and IL-12 to the *in vitro* priming cultures resulted in some early control of tumor growth by the adoptively transferred PMEL CD8⁺ T cells (*e.g.*, days 17–19), but by day 22 tumor growth reached that observed in control mice (Fig. 7E and F, dark grey lines). Mice that received the TCR/CD28-primed *Ikaros*^{+/-} PMEL CD8⁺ T cells (Fig. 7A, black line) demonstrated a trend toward decreased tumor growth in comparison to the wild-type effectors, but this difference was not statistically significant. However, priming these cells in the presence of IL-12 resulted in more pronounced and significant tumor delay in comparison to *Ikaros*-sufficient PMEL cells (Fig. 7E, black line). This is consistent with the fact that *Ikaros*^{+/-} CD8⁺ T cells produce more IFN- γ in response to IL-12 than wild-type

cells (Fig. 3C), and with a recent study showing that CD8⁺ T cells expressing a dominant-negative mutant of Ikaros also exhibit increased sensitivity to IL-12 (48). The combination of IL-2 and IL-12 resulted in the most significant tumor delay in recipients of *Ikzf1*^{+/-} PMEL cells compared to tumors growing in recipients of wild-type PMEL cells (Fig. 7F, black line). These results indicate that autocrine IL-2 contributes to the enhanced responsiveness of *Ikzf1*^{+/-} CD8⁺ T cells to IL-12, but is still limiting and can be enhanced by exogenous IL-2. Interestingly, these tumor studies show that CD8⁺ T cells with autoreactive TCR specificity can be driven to differentiate into functional effector CTL by inflammatory cues (*i.e.*, IL-2 and IL-12) if activated without benefit of full Ikaros activity. This suggests that Ikaros may normally function to protect naive CD8⁺ T cells from a loss of self tolerance during infection or other inflammatory situations.

Discussion

In this report we demonstrate a novel role for Ikaros in the regulation of naïve CD8⁺ T cell differentiation, at least in part through the control of autocrine IL-2. TCR signals in the absence of cytokines result in accumulation of Ikaros at the protein level, imposing a barrier to IL-2 production and effector differentiation. Extrinsic signals from IL-2 or IL-12 oppose the accumulation of Ikaros, and drive differentiation into IFN- γ producing CTL. Naïve CD8⁺ T cells with only one functional copy of the *Ikzf1* gene, which could not induce Ikaros to a significant degree, were able to differentiate in the absence of CD4 help or exogenous cytokines. Neutralization of IL-2 blocked the capacity of both wild-type and *Ikzf1*^{+/-} cells to differentiate. Thus, the repressive activity of Ikaros renders CD8⁺ T cells dependent upon environmental cues such as IL-12 from activated dendritic cells, or IL-2 from activated CD4⁺ T cells, to license their differentiation into effector CTL. Ikaros is also a potent repressor of IL-2 and differentiation in CD4⁺ T helper cells (19, 49). Importantly, we find that endogenous CD44^{hi} memory CD8⁺ T cells, which have been previously licensed and can rapidly produce high levels of IFN- γ and IL-2 in response to antigenic stimulation, express lower levels of Ikaros than naïve phenotype cells (our unpublished observations), a finding predicted by our model in which Ikaros appears to integrate TCR, costimulatory and cytokine signals, and mans a global checkpoint for T cell differentiation.

While some aspects of the hematopoietic lineage-restricted control of Ikaros expression are understood, the type of dynamic 'tuning' of Ikaros levels by TCR vs. cytokine signals reported in this study has not been previously observed. The TCR-induced increase in Ikaros protein levels could result from increased transcription of the *Ikzf1* gene and/or stability of the *Ikzf1* transcript *via* the same mechanisms that increase expression of thousands of other genes during T cell activation. Interestingly, the larger-scale transcriptional architecture of the *Ikzf1* locus includes a T cell lineage-specific enhancer region (50), which may potentially be involved in inducible expression in CD8⁺ T cells. Similarly, the mechanism by which IL-2 blocks induction of Ikaros in CD8⁺ T cells is not clear, and could either be due to transcriptional repression or post-translational regulation of Ikaros protein translation or stability. Ikaros is a known substrate of CK2 (51), Syk (52) and BTK (53), and hyper-phosphorylation of Ikaros can result in its degradation (51). IL-2 can also repress expression of Ikaros at the mRNA level (54), but the mechanism by which this occurs is unclear. It is

possible that cytokine signals may induce the expression of miR-27a and miR-92, micro-RNAs known to target Ikaros expression in tumor cells (55).

IL-2 has important roles in CD8⁺ T cell effector differentiation and memory. IL-2 drives CD8 effector differentiation, in part by enhancing cell survival, but also by inducing the T-box transcription factor Eomes (8), which cooperates with its family member T-bet to transactivate IFN- γ , perforin, and granzyme B gene expression (30, 31). STAT5, a transcription factor activated by IL-2R signaling, has been shown to promote accessibility of the *ifn γ* promoter to binding by T-bet (56), and drives feed-forward expression of the high affinity IL-2 receptor chain, CD25 (29). Indeed, expression of CD25 can delineate effector from memory precursor cells. CD25 was shown to mark a terminally differentiated population of highly cytolytic KLRG1⁺ effector cells during acute LCMV infection (9), and high concentrations of exogenous IL-2 during CD8⁺ T cell activation *in vitro* results in CTL with limited survival and homeostatic capacity (8). In contrast, low levels of IL-2 promote the generation of effectors with reduced cytotoxicity and increased memory potential (8), resembling the CD25^{lo} memory precursor cells that arise during LCMV infection (9). While IL-2 is crucial for tuning effector CD8 differentiation, it is also required for CD8⁺ T cell memory. CD8⁺ T cells primed in the absence of IL-2 signaling generate a blunted memory pool with poor recall responses (4, 57–59). Through the use of mixed bone marrow chimeras, it was demonstrated that CD25-deficient memory CD8⁺ T cells were defective in IFN- γ and IL-2 production, and exhibited poor cytotoxicity upon re-challenge with antigen. The use of IL-2/anti-IL-2 complexes to mimic strong IL-2 activity during primary immune responses also resulted in increased recall responses (60) or converted IL-2R α ^{-/-} CD8⁺ T cells into competent memory CD8⁺ T cells (4). Adoptive transfer studies with naïve IL-2^{-/-} cells into B6 mice also demonstrated that these cells fail to generate an effective memory recall response to *Listeria* challenge (16). These studies all demonstrate that failure to produce and/or respond to IL-2 during the primary response has a profound impact on CD8⁺ T cell memory.

The main cellular source of IL-2 is CD4⁺ T cells, and along with CD40-mediated licensing of dendritic cell maturation (61–63), IL-2 represents a major paracrine mechanism for CD4 help for CD8⁺ T cell responses (12–14). CD8⁺ T cells primed in the absence of CD4 help can differentiate into effectors if other signal 3 cytokines are present, but are defective in homeostasis, and exhibit markedly reduced proliferation, cytolytic capacity and cytokine production when challenged with antigen during the memory phase (4, 57, 64). Consistent with this, the *Il2* and *Ifn γ* loci are epigenetically silenced in unhelped CD8⁺ T cells, at the level of both DNA methylation and chromatin structure (6, 7). The phenotype of IL-2-deprived CD8⁺ T cells is highly similar to that of memory CD8⁺ T cells generated in the absence of CD4 help, suggesting a common molecular basis for the functional defect. Interestingly, it was recently shown that CD4⁺ T cells can license CD8⁺ effector cells to produce their own IL-2 (15, 16), implicating autocrine IL-2 as an important regulatory node in the development of effective CD8⁺ T cell memory. Our current studies demonstrate a previously unappreciated role for the transcriptional repressor Ikaros in the control of autocrine IL-2 production by CD8⁺ T cells.

Ikaros interacts with the NURD, Sin3a and CtBP transcriptional co-repressor complexes (65–67), and is a potent regulator of chromatin structure and DNA methylation at its target genes (49). The repressive activity of Ikaros is required to silence gene expression programs, ensuring that only those T cells that have received the appropriate instructive signals can develop and differentiate. For example, Ikaros binds to the *Il2* promoter in naïve CD4⁺ T cells, keeping the chromatin in this region in a ‘closed’ conformation unless signals from CD28 are received (19). Ikaros is also required to epigenetically silence the genes encoding T-bet and IFN- γ in CD4⁺ T cells that fail to receive Th1-promoting signals from IL-12 or IFN- γ , and cells with a loss of Ikaros function exhibit poly-lineage cytokine expression patterns upon differentiation (49), and are resistant to anergy induction (19, 20). These studies suggest that instructive signals from cytokines must oppose Ikaros-mediated repression at lineage-specific effector genes. Indeed, IL-12 inhibits the binding of Ikaros to the endogenous *Tbx21* promoter in differentiating Th1 cells (49), and we show in this current study that IL-2 (Fig. 1, Fig. 2) and IL-12 (data not shown) can downregulate Ikaros in activated CD8⁺ T cells. These results also provide an explanation for recent finding that CD8⁺ T cells transduced with a dominant-negative Ikaros transgene are more responsive to IL-12 signaling (68). This suggests a model in which Ikaros integrates signals from the TCR, CD28 and the IL-2 receptor to regulate CD8⁺ T cell differentiation. We show that Ikaros is expressed in naïve CD8⁺ T cells, and is highly induced upon TCR/CD28 costimulation. Our previous studies indicate that targets such as *Il2*, *T-box* genes, *Gzmb*, and *Ifn γ* would be subject to strong Ikaros occupancy and repressive activity under these circumstances, and the chromatin at these genes would be inaccessible to transcriptional activators induced during T cell activation. However, if a naïve CD8⁺ T cell receives antigenic stimulation in the presence of paracrine IL-2 from a CD4⁺ T cell, or IL-12 from the dendritic cell, our results show that Ikaros does not accumulate, and T-bet and Eomes are induced instead. In the absence of the repressive activity of Ikaros, these factors would then be free to bind to and transactivate the accessible *Ifn γ* , *Gzmb* and other loci to drive effector differentiation.

Our studies also reinforce the importance of restricting autocrine IL-2 production by CD8⁺ T cells. We show that naïve CD8⁺ T cells that do not express the appropriate level of Ikaros are able to produce IL-2 without the need for CD4 help, driving their own differentiation into IFN γ -producing, granzyme B^{hi} CTL that can efficiently kill target cells *in vitro* and control *Listeria* infection *in vivo*. Moreover, these cells are able to help neighboring, non-IL-2 producing CD8 cells in a paracrine manner to differentiate into CD25^{hi} IFN γ -producing effectors. From a clinical perspective, inhibition of Ikaros activity could improve vaccination, where the lack of inflammation or IL-2 derived from CD4⁺ T cells can limit CD8 responses, as our studies show that inhibition of Ikaros expression improves the anti-bacterial efficacy of pathogen-specific CD8⁺ T cells when primed in the absence of inflammatory stimuli. Similarly, barriers to anti-tumor immunity include immunologic tolerance to self/tumor antigens and the immuno-suppressive tumor microenvironment (69). Indeed, our experiments utilizing the poorly immunogenic B16 melanoma demonstrate that inhibition of Ikaros activity can cooperate with inflammatory cytokines to break tolerance to a self antigen, leading to effective control of tumor growth. On the other hand, the ability of Ikaros to control autoreactive T cell responses has important implications for autoimmunity.

The *IKZF1* gene has been implicated in the susceptibility to human autoimmune diseases such as systemic lupus erythematosus (SLE), type 1 diabetes (T1D) and Crohn's/ inflammatory bowel disease (IBD) in genome-wide association studies (70–72), indicating that proper Ikaros gene regulation is crucial for the maintaining self tolerance, and suggesting that interventional strategies that promote Ikaros function could lead to novel treatments for autoimmune disease or organ transplant rejection. Together, our results show that Ikaros regulates CD8+ T cell differentiation by restricting autocrine IL-2 production and enforcing dependence on paracrine signals from other cells.

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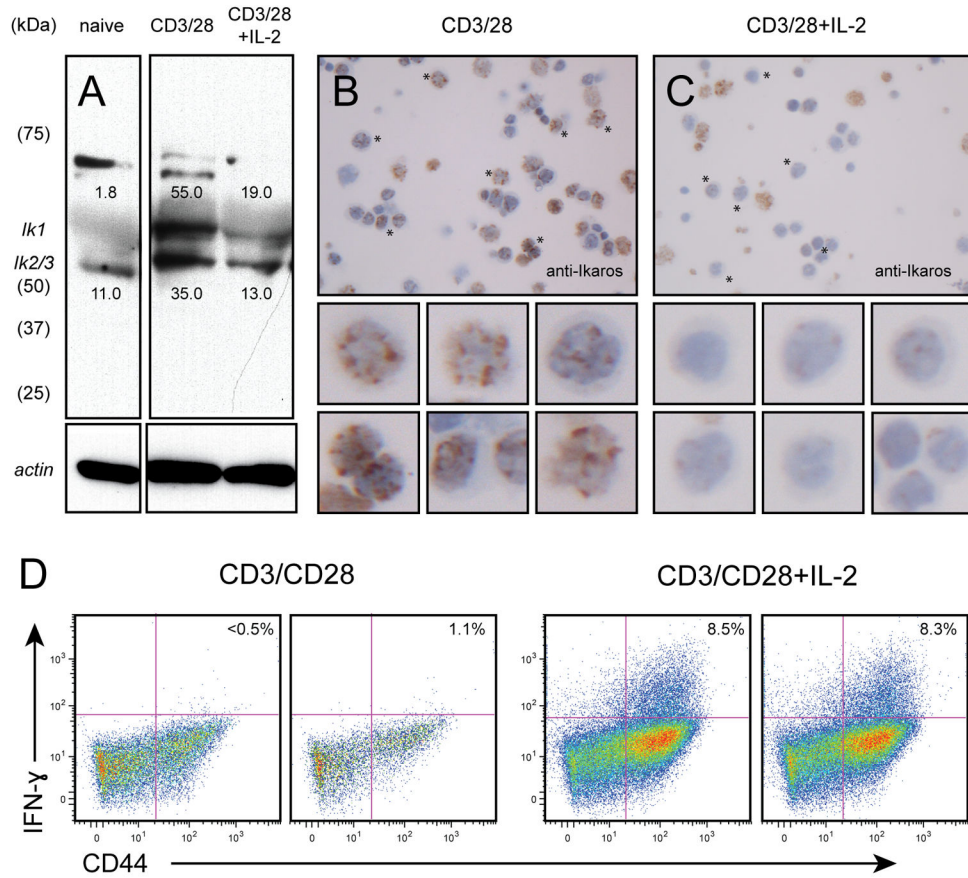


Fig. 1. Activation- and cytokine-dependent Ikaros expression in CD8+ T cells
 Cells from RAG1^{-/-} OT-1 mice were stimulated with soluble CD3 and CD28 antibodies (0.5 μ g/ml each) for 24 hours and in the presence (lane 3) or absence (lane 2) of IL-2 (10 ng/ml). Resting (lane 1) or stimulated (lanes 2 and 3) cells were washed in PBS, immunoblotted (A, 0.33×10^6 cell equivalents), and probed with antisera against Ikaros (top panels) or actin (bottom panels). The predominant Ik1, Ik2 and Ik3 isoforms are indicated. Numbers above the Ik1 band and below the Ik2/3 band indicate pixel density (x1000). Alternatively, stimulated cells were centrifuged onto glass slides and subjected to immunohistochemical staining for Ikaros (B). Arrows in B indicate intact cells with characteristic positive, punctate staining for Ikaros. Asterisks indicate intact cells that are negative or dim for Ikaros. Anucleate, apoptotic cells show a low level of background reactivity. Data are representative of 3 independent experiments. C. Naïve-enriched RAG1^{-/-} OT-I CD8+ T cells were stimulated with plate bound anti-CD3 and anti-CD28 (1.0 μ g/ml each) in the presence (right panel) or absence (left panel) of IL-2 (10 ng/ml) for 48 hours, and Golgi Stop was added for last 4 hours of stimulation. Cells were harvested, stained for CD8, CD44 and IFN- γ , and subjected to flow cytometric analysis. Gates were set using a fluorescence-minus-one (FMO) approach, and plots depict IFN- γ expression by activated (CD44^{hi}) cells from duplicate cultures, with numbers indicating % IFN- γ + cells. Data are representative of at least 3 independent experiments.

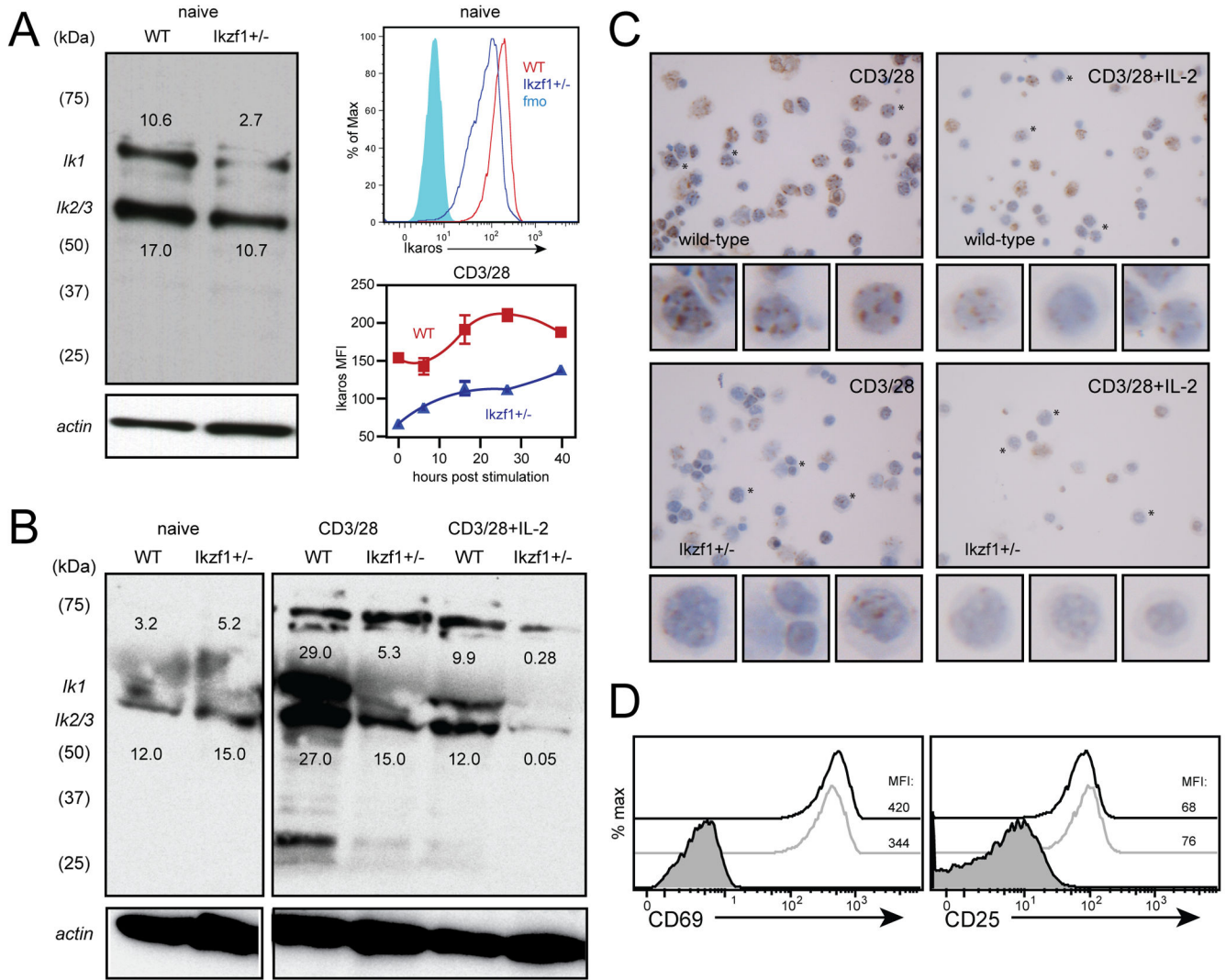


Fig. 2. CD8⁺ T cells hemizygous for the *Ikzf1* gene exhibit reduced Ikaros protein expression
 Polyclonal naive CD8⁺ T cells sorted by CD62L+CD44⁻ from wild-type or *Ikzf1*^{+/-} mice (1×10⁶ cell equivalents) were subjected to immunoblot analysis of Ikaros protein expression as in Fig. 1 (A, left panel). Ikaros expression by naive RAG1^{-/-} OT-1 (WT, red) or RAG1^{-/-} OT-1 *Ikzf1*^{+/-} CD8⁺ T cells (blue) was measured by flow cytometry (A, right panels, gated on CD62L+CD44-CD8⁺), immunoblot analysis (B), or immunohistochemistry (C) following stimulation with plate-bound anti-CD3/CD28 in the presence or absence of IL-2 (10 ng/ml). D. CD69 and CD25 expression by 12 hour anti-CD3/CD28-stimulated RAG1^{-/-} OT-1 (black) or RAG1^{-/-} OT-1 *Ikzf1*^{+/-} (grey) CD8⁺ T cells was measured by flow cytometry (gated on live CD8⁺ T cells). Numbers above the *Ik1* band and below the *Ik2/3* band indicate pixel density (x1000). Data are representative of two independent experiments.

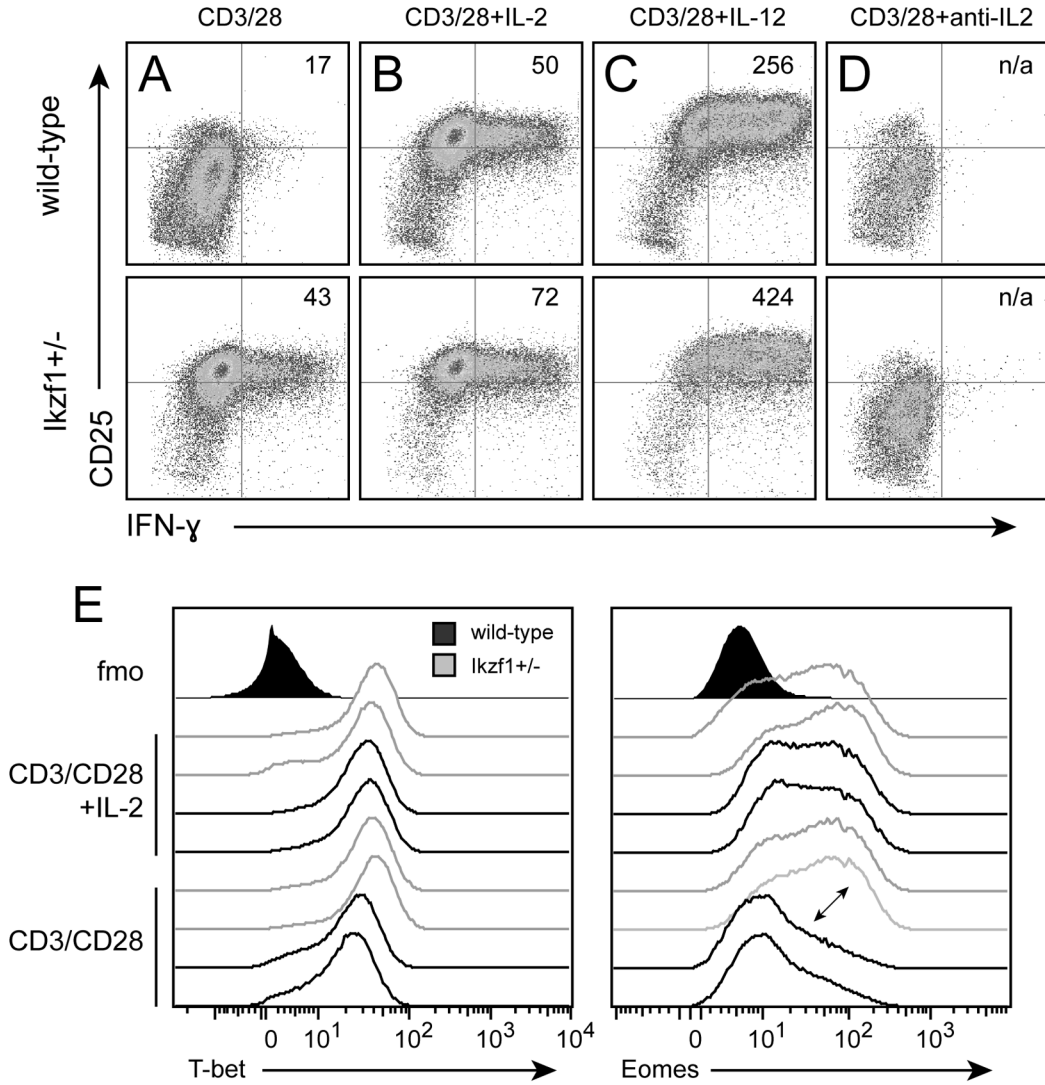


Fig. 3. CD8⁺ T cells hemizygous for the *Ikzf1* gene exhibit enhanced effector differentiation
 CD8⁺ T cells from RAG1^{-/-} OT-1 (black) or RAG1^{-/-} OT-1 *Ikzf1*^{+/-} (grey) mice were sorted by naïve (CD62L^{hi}CD44^{lo}) phenotype, stimulated with plate bound anti-CD3/CD28 (A) with exogenous IL-2, IL-12 or anti-IL-2 for 48 hours, and analyzed for expression of CD25 and IFN γ (MFI is depicted in upper right quadrant), or for expression of Eomes and T-bet (B) by flow cytometry. Two biological replicates are shown for each group, and data are representative of two independent experiments.

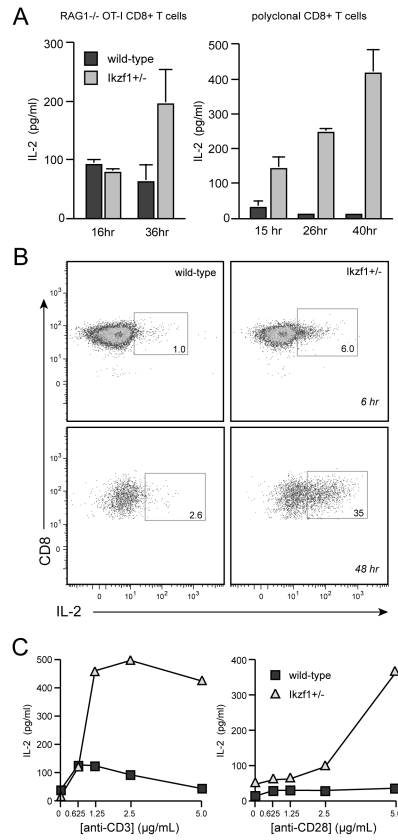


Fig. 4. *Izkf1*^{+/-} CD8⁺ T cells produce autocrine IL-2

Naïve-enriched CD8⁺ T cells from *Izkf1*^{+/+} or *Izkf1*^{+/-} B6 or RAG1^{-/-} OT-I mice were stimulated as in Fig. 2, and culture supernatants were assayed for IL-2 production by ELISA at the indicated time points (A) or by intracellular staining at 6 and 48 hours (B). Plots in B are gated on CD69⁺ cells. In C, naïve-enriched *Izkf1*^{+/+} or *Izkf1*^{+/-} OT-I CD8⁺ T cells were stimulated for 24 hours with either a fixed concentration of plate-bound anti-CD28 and a titration of anti-CD3 (left panel), or a fixed concentration of anti-CD3 and a titration of anti-CD28 (right panel). Values plotted in C represent the mean \pm SEM of duplicate cultures and are representative of 2 independent experiments.

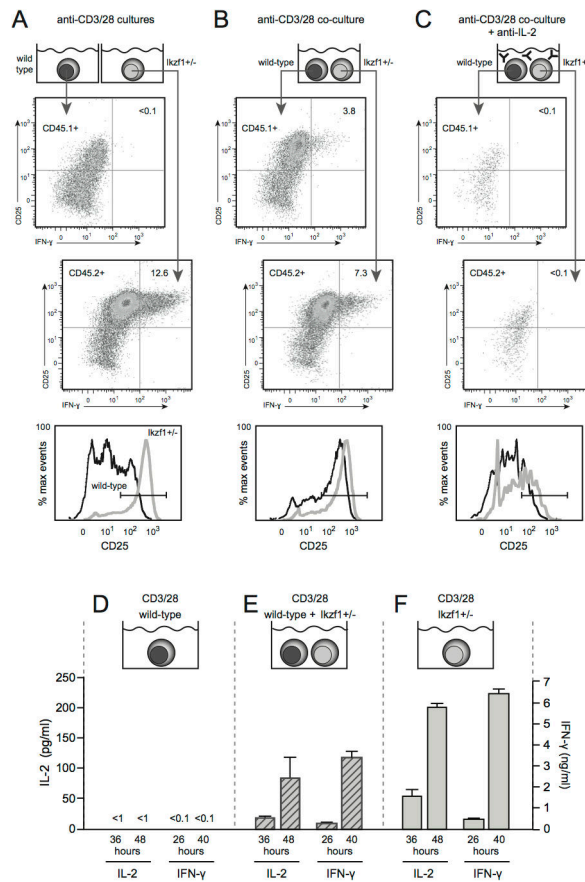


Fig. 5. IL-2 produced by *Ikzf1*^{+/-} CD8⁺ T cells can act in a paracrine fashion to induce differentiation of wild-type CD8⁺ T cells

Naïve-enriched CD8⁺ T cells from *Ikzf1*^{+/+} (CD45.1⁺) or *Ikzf1*^{+/-} (CD45.2⁺) mice were stimulated in individual cultures as in Fig. 1 (A), or in co-cultures at a 1:1 ratio for 48 hours in the absence (B) or presence (C) of anti-IL-2 neutralizing antibody. Expression of CD25 and IFN-γ were assessed by flow cytometric analysis, and numbers represent % IFN-γ⁺CD25⁺ cells. CD25 expression in *Ikzf1*^{+/+} (black) and *Ikzf1*^{+/-} (gray) cultures is also illustrated in histogram overlays (bottom panels in A–C). Supernatants from these cultures were assessed for IL-2 and IFN-γ production by ELISA (D, E and F). Data are representative of 3 independent experiments.

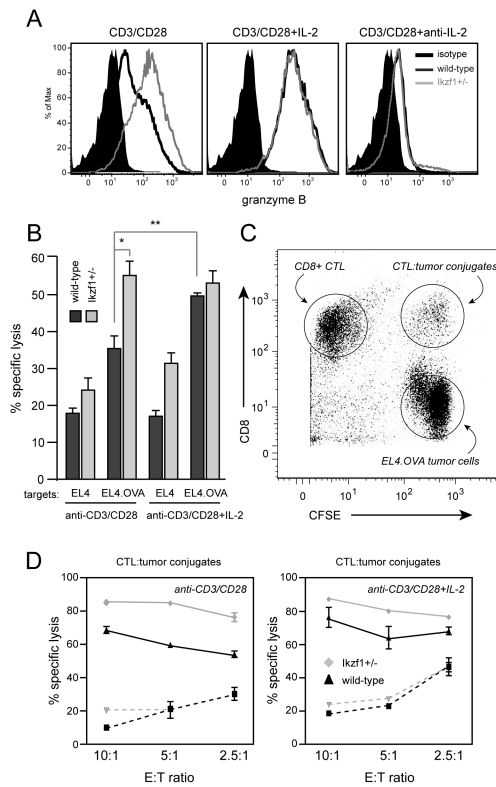


Fig. 6. Loss of Ikaros function leads to enhanced cytolytic capacity by CD8+ T cells

A. Naïve-enriched RAG1^{-/-} OT-I (dark gray) or *Izkf1*^{+/-} OT-I (light gray) CD8+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 for 48 hours in the presence or absence of IL-2 or anti-IL-2. PMA (30 ng/ml) and ionomycin (1 μ M) were added for the last four hours of culture. Expression of granzyme B was assessed by flow cytometry. Data are representative of two independent experiments. Filled black histograms - granzyme B FMO negative control. OT-I cells (dark gray) or *Izkf1*^{+/-} OT-I cells (light gray) were stimulated for 48 hours as in **A**, rested in medium overnight, then mixed at a 10:1, 5:1 and 2.5:1 ratio with CFSE-labeled EL4 or EL4.OVA targets for 3 hours. Viability of bulk CFSE+ EL4 or EL4.OVA targets at a 10:1 E:T ratio is shown in **(B)** or in conjugates with CD8+ T cells (CFSE+CD8+ gate in **C**) at all ratios in **(D)**. Dashed lines indicate response to EL4. Data are representative of 3 independent experiments. Statistical significance was determined by Student's T-test for triplicate values/group in **B** - * p<0.05, ** p<0.001, *** p<0.0001. Values plotted in **D** represent mean \pm SEM of duplicate cultures.

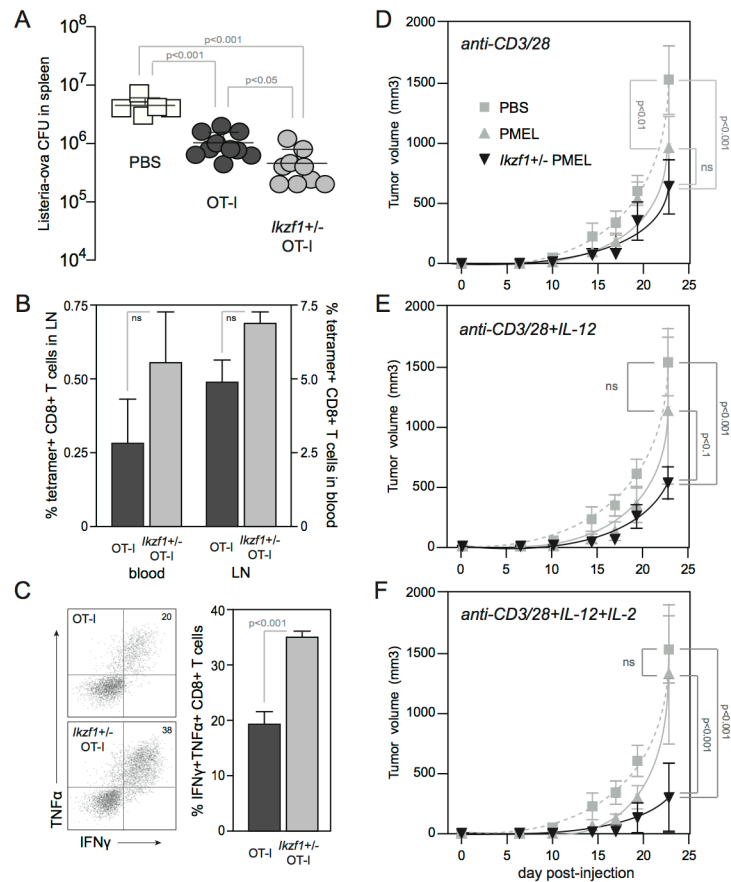


Figure 7. Enhanced *in vivo* effector function by *Ikzf1*^{+/-} CD8⁺ T cells

Naive RAG1^{-/-} OT-1 (dark grey) and *Ikzf1*^{+/-} RAG1^{-/-} OT-1 (light grey) CD8⁺ T cells were purified, primed with plate-bound anti-CD3/28 Ab for 48 hours, and 5×10^6 cells were adoptively transferred into B6 mice. Two hours later, mice were challenged with 1×10^5 LM-OVA, and blood, LN and spleens were harvested at day 3 post-infection. Live LM-OVA in the spleen (A) and MHC-OVA tetramer⁺ CD8⁺ T cells in blood and LN (B) were enumerated. LN cells were restimulated *ex vivo* with OVA peptide (1 μ M) and analyzed by flow cytometry for production of IFN- γ and TNF α (C). Recipients: PBS, n=3; RAG1^{-/-} OT-1, n=4; *Ikzf1*^{+/-} RAG1^{-/-} OT-1, n=3. Statistical significance was determined by Student's T-test (A) and one-way ANOVA (B, C) and p values are indicated. For tumor studies, naive PMEL (grey) or *Ikzf1*^{+/-} PMEL (black) CD8⁺ T cells were purified, stimulated *in vitro* with plate-bound anti-CD3/28 Ab (D), or with the addition of IL-12 (E) or IL-2 and IL-12 (F), and 1×10^6 cells were adoptively transferred into B6 mice. Mice were challenged subcutaneously 24 hours later with 1×10^5 B16 melanoma cells. Tumors: PBS, n=6; PMEL, n=6; *Ikzf1*^{+/-} PMEL, n=6. Statistical significance was determined by two-way ANOVA and p values are indicated. * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$.