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Ikaros Imposes a Barrier to CD8+ T Cell Differentiation by Restricting Autocrine IL-2 Production

Shaun O’Brien,* Rajan M. Thomas,† Gerald B. Wertheim,*† Fuqin Zhang,‡ Hao Shen,‡ and Andrew D. Wells*†

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 I12 gene. Naive murine CD8 T cells haplo-insufficient for Ikzf1 failed to upregulate Ikaros, produced autocrine IL-2, and differentiated in an IL-2-dependent manner into IFN-γ–producing CTLs in response to TCR/CD28 stimulation alone. Furthermore, Ikzf1 haplo-insufficient CD8+ T cells were more effective at controlling Listeria infection and B16 melanoma growth in vivo, and they 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 on licensing by APCs and CD4+ T cells, and it may therefore act as a cell-intrinsic safeguard against inappropriate CTL differentiation and immunopathology. The Journal of Immunology, 2014, 192: 5118–5129.

Naive T cell differentiation is a tightly regulated process, as aberrant activation can lead to immunopathology and disease. Naive CD4+ and CD8+ T cells differ in their requirements for differentiation, as the latter have higher cytotoxicity potential. Naive 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, costimulation of naive 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 proinflammatory cytokines for their differentiation. For instance, IL-12, type I IFNs, and IL-21 have been characterized as key inflammatory cytokines that drive naive 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 prosurvival role through upregulation of the antiapoptotic factor Bcl-2, but it 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 a means to ensure appropriate CD8+ T cell differentiation. Naive CD8+ T cells are highly restricted in their production of autocrine IL-2 (2) and are largely dependent on IL-2 from CD4+ helper T cells (12–14). However, in some systems Th cells can license CD8+ T cells to produce their own IL-2 (15), which is required during initial priming to generate robust memory recall responses (16).

Little is known about how autocrine IL-2 is restricted in naive 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 naive 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 naive 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, Abs, and cytokines

Wild-type CD45.2, CD45.1, PMEL, RAG1−/−, and OT-I mice were purchased from The Jackson Laboratory. Mice carrying the germline Ikzf1-null allele were a gift of Dr. K. Georgopoulos and were backcrossed on a C57BL/6 (B6) background for >12 generations. RAG1−/−OT-I Ikzf1+/− mice were generated through breeding Ikzf1+/− mice onto a RAG1−/− background to obtain RAG1−/− Ikzf1−/− mice. These mice were then crossed

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; B6, C57BL/6; Eomes, eomesoderm; FMO, fluorescence minus one; LM-OVA, recombinant Listeria monocytogenes expressing OVA; LN, lymph node.

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with the RAG1−/− OT-I 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. mAbs against CD3 (2C11), CD28 (37.51), CD4 (GK1.4), MHC class II (M5/114), FcR (2.4G2), and IL-2 (JES6-1A12) were purchased from Bio X Cell, and anti-B220 (RA3-6B2) and anti-CD44 (IM7) Abs were purchased from BioLegend. Mouse IL-2 and IL-12 were purchased from PeproTech and Roche. The SiNIPEKL peptide derived from chicken OVA was purchased from BioBath.

Cell sorting

Single-cell suspensions from spleen and lymph nodes (LNs) of polyclonal mice were sorted for naive CD8+ T cells (CD62LhiCD44−) on a MoFlo XDP (Beckman Coulter). Naive CD8+ T cells were at ~95% purity. RAG1−/− OT-I and RAG1−/−Ikzf1−/− OT-I single-cell suspensions from spleen and LNs were depleted of CD4+ T cells, monocytes, and MHC class II-expressing cells with QiaGen magnetic goat anti-rat IgG beads (catalog no. 310107). Cells were stained with mixture of depleting anti-CD4 (GK1.4), MHC class II (M5/114), anti-CD4, anti-B220, and anti-CD44 (IM7). Naive OT-I (CD62LhiCD44−) cells were purified to ~90% purity.

Flow cytometry and applications

Fluorochrome-conjugated Abs, including anti-mouse IFN-γ (XMGI1), allophycocyanin-Cy7 anti-mouse CD25 (PC61), anti-mouse IL-2 (JES6-1A12), anti-mouse CD68 (53-6.7), anti-mouse CD44 (IM7), anti-mouse CD26 (MEL-14), anti-mouse CD44 (IM7), and anti-mouse CD4 (GK1.5), were purchased from BioLegend. Fixable, Live/Dead aqua stain (L34957) was purchased from Invitrogen. Fluorochrome Ab to anti-mouse granzyme B (NGZB) and anti-mouse eomesodermal (Eomes; Danmag11) were purchased from ebioscience. Fluorochrome anti-mouse T-Bet (4B10) was purchased from BD Biosciences. CFSE was purchased from Millipore, and 7-aminoactinomycin D (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 GolgiStop (BD Biosciences, 0.66 μg/ml) for 4–6 h with PMA (30 ng/ml) and ionomycin (1 μM) or OVA peptide (1 μM) as indicated. Following harvesting, cells were fixed with 1% PFA for 30 min, spun down, and washed once on FACS buffer. Cells were then washed with BD Perm/Wash (BD Biosciences) twice and then stained with cytokine Ab for 45 min at room temperature. Cells were washed twice in BD Perm/Wash and then resuspended in FACS buffer. For transcription factor staining, cells were surface stained with fluorochrome-labeled primary Abs for 20 min on ice. After washing in FACS buffer, cells were fixed with Fix/Perm buffer from eBioscience. Following fixation, cells were permeabilized and stained with allophycocyanin anti-mouse T-Bet and PE anti-mouse Eomes. For Ikaros staining, rabbit anti-mouse Ikaros (ab20603, Abcam, Cambridge, MA) was used. Following staining with the Ikaros Abs, cells were washed and then stained with a PE-labeled anti-rabbit secondary Ab. Following completion of stains, cells were processed on a CyanADP (Beckman Coulter) for flow cytometric analysis.

Cell culture

Naive sorted CD8+ T cells were stimulated in 96- or 24-well plates, which were coated with anti-CD3/CD28. All T cell cultures were maintained in RPMI 1640 supplemented with 10% FBS, l-glutamine, penicillin/streptomycin, and 2-ME and maintained in a 37˚C incubator. EL4 and EL4.ova cells lines were maintained in DMEM, supplemented with 10% FBS, L-glutamine, penicillin/streptomycin, and 2-ME. EL4.ova cells were maintained in 400 μg/ml G418 (Invitrogen). All means, measures of variance, and statistical tests are based on biological replicates, as indicated in the figure legends.

Immunoblot analysis

Immunoblotting was performed for determining the Ikaros isoform expression. For immunoblotting, 0.33 × 10⁶ to 1 × 10⁶ cells were lysed with Laemmli buffer and boiled for 10 min 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 min (3×) with wash buffer (0.1% Tween 20 in 1× PBS). It was then incubated with HRP-conjugated anti-goat Ab (1:10,000) for 1 h at room temperature. The membrane was washed for 10 min three times with wash buffer and then developed using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific). The image was developed on a Kodak x-ray film. A C terminus–reactive goat anti-mouse Ab to Ikzf1 (SC-9861) and a goat anti-mouse actin Ab (SC-1615) were purchased from Santa Cruz Biotechnology. 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 blot stripping buffer (Thermo Scientific) for 10 min and then washed three times for 15 min. The preblocked membrane was stained with goat polyclonal anti-β-actin Ab followed by staining with HRP-conjugated secondary Ab. Densitometry was performed using ImageJ software on background-subtracted images derived from the raw immunoblot films.

Immunohistochemistry

Fifty thousand to 250,000 thousand cells were centrifuged on glass slides and fixed for 20 min in 10% neutral buffered formalin. Immunohistochemical stains were performed on a Bond III system (Leica Microsystems, Bannockburn, IL) with pH6 epitope retrieval solution (Leica), an HRP-conjugated anti-Ikzf1 primary Ab (ab25083, Abcam) diluted 1:1000 in IHC diluent (Leica), and with nuclear counter stain hematoxylin, following the manufacturer’s protocol (standard protocol F; Leica) but eliminating steps to deparaffinize slides. Stained slides were analyzed on a Leica DM 2500 microscope with a ×40 HXCL PL Fluorotar objective (×0.17/D). Images were captured using Leica application suite version 2.8.1 (build 1554, 2003–2007).

Cytotoxicity assays

Naive purified OT-I CD8+ T cells from Ikzf1−/− or Ikzf1+/- mice were stimulated with plate-bound anti-CD3/CD28 (1.0 μg/ml) for 48 h and in the presence or absence of IL-2 (10 ng/ml). Cells were then harvested, counted, and resuspended at 0.5 × 10⁶ cells/ml in complete RPMI 1640 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 h at 37˚C. After a 3-h incubation period, cells were harvested, washed in FACS buffer, stained for CD8 expression, and live/dead viability was assessed after addition of 7-AAD (3 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. Percentage killing by CD8+ T cells was calculated as follows: Percentage cytotoxicity of CD8+ T cells = (no. 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 was determined as (% cytophcticity 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 h with plate-bound anti-CD3/CD28 Ab (1.0 μg/ml each). Cells were harvested and injected i.v. (5 × 10⁶) into B6 mice. Two hours following transfer, mice were challenged with 1 × 10⁶ live recombinant Listeria monocytogenes expressing OVA (i.e., the SiNIPEKL peptide, LM-OVA). Spleens, LNs, and blood were harvested at day 3 postinfection. Bacterial burden in the spleen was determined as previously described (21, 22). Blood and LNs were characterized by PE H-2Kk/OVA57-245 (Kk/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 d 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 × 10⁶ cells/mouse). Twenty-four hours later, mice were challenged with 1 × 10⁶ B16 melanoma cells s.c. on each flank. Length and width of tumor was recorded and tumor volume was calculated by length × width²/2.

Results

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

To study the differentiation of naive CD8+ T cells into effectors in a reductionist system, we used agonistic anti-CD3 and anti-CD28 Abs in an in vitro culture system devoid of other cell types or exogenous cytokines. To control for previous Ag exposure, we used polyconal CD44+CD62L+CD8+ T cells enriched by flow sorting, or monoclonal CD8+ T cells developed in RAG1−/− OT-I mice. Naive 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, naive cells showed marked upregulation of Ik1 and Ik2 (Fig. 1A, 1B). The Ik2 band may contain the Ik3 isoform, which also binds DNA and can comigrate with Ik2 (24). TCR/CD28-activated cells also consistently exhibited higher molecular mass species that most likely represent posttranslationally modified (e.g., sumoylated, ubiquitinylated, and/or phosphorylated) forms that have been detected in thymocytes and other cell lines (25). In some experiments, a minor low–molecular mass 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 (26). 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 effectors cells (Fig. 1D, left panel). The addition of exogenous IL-2 opposed the accumulation of Ikaros (Fig. 1A, 1C) and promoted differentiation of naive 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, 26), 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 used 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 (27), and over mice expressing a dominant-negative mutant of Ikaros (18), which develop fatal lymphomas early in life (28), in that Ikzf1+/− mice show normal hematopoietic development and do not develop cancer (23). We find that naive CD8+ T cells with only one functional allele of Ikzf1 exhibit an ∼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 ∼5-fold less than in wild-type cells (Fig. 2A–C). This failure was not due to understimulation, as wild-type and Ikzf1−/− CD8+ T cells upregulated 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 costimulation 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, 2C). Addition of

**FIGURE 1.** Activation- and cytokine-dependent Ikaros expression in CD8+ T cells. Cells from RAG1−/− OT-I mice were stimulated with soluble CD3 and CD28 Abs (0.5 μg/ml each) for 24 h 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 × 10⁸ 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 (∗1000). Alternatively, stimulated cells were centrifuged onto glass slides and subjected to immunohistochemical staining for Ikaros (B and C). Asterisks indicate intact cells shown magnified in individual panels. Anucleate, apoptotic cells show a low level of background reactivity. Data are representative of three independent experiments. (B and C) Original magnification ∗40. (D) Naive-enriched RAG1−/− OT-I CD8+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 (1.0 μg/ml each) in the presence (right panel) or absence (left panel) of IL-2 (10 ng/ml) for 48 h, and GolgiStop was added for last 4 h of stimulation. Cells were harvested, stained for CD8, CD44, and IFN-γ, and subjected to flow cytometric analysis. Gates were set using an FMO approach, and plots depict IFN-γ expression by activated (CD44hi) cells from duplicate cultures, with numbers indicating percentage IFN-γ+ cells. Data are representative of at least three independent experiments.
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 (29, 30). 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 downregulation of Ikaros by Ikzf1+/− cells (data not shown), indicating that the gain of function exhibited by these cells is entirely dependent on IL-2.

We also assessed expression of the T-box transcription factors T-bet and Eomes (31, 32), as IL-2 induces Eomes expression (8) and Ikaros has been shown to regulate T-bet during CD4+ T cell differentiation (26). TCR and CD28 signals induced T-bet expression by wild-type cells (33), 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 did wild-type cells, and they also exhibited increased T-bet expression in response to IL-2 (Fig. 3E, right panel). Whereas 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 those 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 (31, 32).

Ikaros influences CD8 differentiation via control of autocrine IL-2

The requirement for IL-2 in the differentiation of naive 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+/− CD8+ T cells over 48 h of stimulation. Consistent with previous studies (2), wild-type naive 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 naive cells in this system and measured T cell activation and IL-2 production. Naive, wild-type CD8+ T cells produced very little IL-2, and did so only at supramaximal concentrations of a TCR Ab (5–10 μg/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 a TCR Ab 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 naive 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 whether Ikaros-regulated autocrine IL-2 can also drive the differentiation of neighboring, non–IL-2 producers in a paracrine manner, we used a mixed culture experiment. We mixed naive-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 whether 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, 5F), whereas wild-type cells failed to differentiate under these conditions (Fig. 5A, 5D). 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 whether this was due to the increased autocrine IL-2 production from the CD8$^{+}$ T cells with reduced Ikzf1, cultures were also stimulated in the presence of a neutralizing anti–IL-2 Ab. 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 Ikzf1 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 Ikzf1 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, 34–36). Wild-type CD8$^{+}$ T cells primed through the TCR and CD28 and rechallenged 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, the 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 on 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$^{-/-}$ cocultures, 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$^{-/-}$ cocultures (data not shown), suggesting that the increased tumor cell killing was not due to enhanced Ag recognition by the Ikzf1$^{-/-}$ CTLs. However, we found that tumor cells actively engaged in a conjugate with Ikzf1$^{-/-}$ CTLs were killed more efficiently than tumor cells engaged in a conjugate with wild-type CTLs (Fig. 6D, left panel). Again, the efficiency of wild-type cell-mediated cytotoxicity was increased by exogenous IL-2, reaching levels comparable to those of the Ikzf1$^{-/-}$ CTLs (Fig. 6D, right panel). These data indicate that Ikzf1$^{-/-}$ cells are more efficient at killing tumor cells in an Ag-specific manner once the cells have already engaged their targets. Taken together, these results demonstrate that the autocrine IL-2 that is normally repressed by Ikzf1 is sufficient to drive naive 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 such as IL-2 or IL-12.

**Ikaros regulates antibacterial and antitumor 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 used an IFN-γ–dependent bacterial clearance model using LM-OVA in the context of the OVA-specific OT-I TCR and an IFN-γ–dependent tumor rejection model using the B16 melanoma in the context of a transgenic, melanocyte/melanoma Ag-specific TCR (PMEL). CD8$^{+}$ T cells are crucial for the control of *Listeria* infection, and they 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 (37, 38). We primed naive OT-I or Ikzf1$^{-/-}$ OT-I cells through the TCR and CD28 for 48 h 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 postinfection, as compared with control animals that received no OT-I cells (Fig. 7A). However, LM-OVA replication was reduced by >10-fold by day 3 in animals that received the same number of OT-I cells that lacked one copy of the Ikzf1 gene (Fig. 7A). The enhanced capacity of Ikzf1$^{-/-}$ 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 Ag-specific IFN-γ– and TNF-α–producing cells in the LNs (Fig. 7C). These cytokines have both been linked to bacterial clearance in this model (39–41). These data demonstrate that Ikzf1 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 used the poorly immunogenic B16 melanoma model, together with TCR-transgenic PMEL-1 T cells that recognize the melanocyte/melanoma Ag gp100 (42). Effective cellular immunotherapy of established B16 tumors in this stringent model normally requires the administration of exogenous IL-2 (43) or IL-12 (44, 45) and requires IFN-γ (46). Because our Ikzf1$^{-/-}$ CD8$^{+}$ T cells produce more IFN-γ upon stimulation with IL-12, and IL-12 also improves IL-2 signaling (47, 48), we primed PMEL or Ikzf1$^{-/-}$ 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 h later. Because 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$^{+}$ Th cells. Transfer of PMEL cells that were primed through the TCR and CD28 alone (Fig. 7D, dark gray line) resulted in a modest delay of tumor growth as compared with 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, 7F, black lines). Mice that received the TCR/CD28-primed Ikzf1$^{-/-}$ PMEL CD8$^{+}$ T cells (Fig. 7A, black line) demonstrated a trend toward decreased tumor growth in comparison with 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 with Ikzf1-deficient PMEL cells (Fig. 7E, black line). This is consistent with the fact that Ikzf1$^{-/-}$ CD8$^{+}$ T cells produce more...
IFN-γ in response to IL-12 than do 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 (49). The combination of IL-2 and IL-12 resulted in the most significant tumor delay in recipients of Ikaros+/- PMEL cells compared with tumors growing in recipients of wild-type PMEL cells.
FIGURE 6. Loss of Ikaros function leads to enhanced cytolytic capacity by CD8+ T cells. (A) Naive-enriched RAG1−/− OT-I (dark gray) or Ikzf1−/− OT-I (light gray) CD8+ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 for 48 h in the presence or absence of IL-2 or anti–IL-2. PMA (30 ng/ml) and ionomycin (1 μM) were added for the last 4 h of culture. Expression of granzyme B was assessed by flow cytometry. Data are representative of two independent experiments. Filled black histograms indicate granzyme B FMO negative control. OT-I cells (dark gray) or Ikzf1−/− OT-I cells (light gray) were stimulated for 48 h 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 h. 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 three independent experiments. Statistical significance was determined by a Student t test for triplicate values/group in (B). *p < 0.05, **p < 0.001, ***p < 0.0001. Values plotted in (D) represent means ± SEM of duplicate cultures.

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Discussion

In this study we demonstrate a novel role for Ikaros in the regulation of naive 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 CTLs. Naive 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 on environmental cues such as IL-12 from activated dendritic cells, or IL-2 from activated CD4+ T cells, to license their differentiation into effector CTLs. Ikaros is also a potent repressor of IL-2 and differentiation in CD4+ Th cells (19, 26). Importantly, we find that endogenous CD44hi 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 do naive phenotype cells (S. O’Brien, R.M. Thomas, and A.D. Wells, unpublished observations), a finding predicted by our model in which Ikaros appears to integrate TCR and costimulatory and cytokine signals and mans a global checkpoint for T cell differentiation.

Although some aspects of the hematopoietic lineage-restricted control of Ikaros expression are understood, to our knowledge the type of dynamic “tuning” of Ikaros levels by TCR versus 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 Ikaros 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 CD25lo memory. IL-2 drives CD8 effector differentiation, in part by inducing the T-box transcription factor Eomes (8), which cooperates with its family member T-bet to transactivate IFN-γ (55), and drives feed-forward expression of the high-affinity IL-2 receptor chain, CD25 (30). 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 lymphocytic choriomeningitis virus infection (9), and high concentrations of exogenous IL-2 during CD8+ T cell activation in vitro results in CTLs 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 CD25lo memory precursor cells that arise during lymphocytic choriomeningitis virus infection (9). Although 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, 56–58). 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 they exhibited poor cytotoxicity upon rechallenge with Ag. 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 (59) or converted IL-2Rα−/− CD8+ T cells into competent memory CD8+ T cells (4). Adoptive transfer studies with naive 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 (60–62), 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 when other signal 3 cytokines are present, but are defective in homeostasis, and they exhibit markedly reduced proliferation, cytolytic capacity, and cytokine production when challenged with Ag during the memory phase (4, 5, 56). Consistent with this, the I22 and Ilf4 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 corepressor complexes (63–65), and it is a potent regulator of chromatin structure and DNA methylation at its target genes (26). 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 I22 promoter in naive 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 (26) 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 (26), and we show in the present study that IL-2 (Figs. 1, 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 (49). 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 naive CD8+ T cells and is highly induced upon TCR/CD28 costimulation. Our previous studies indicate that targets such as I22, T-box genes, Gzmb, and Ilf4 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, when a naive 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 Ilf4, 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 naive 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 Bhi CTLs 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 CD25hi 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 antibacterial efficacy of pathogen-specific CD8+ T cells when primed in the absence of inflammatory stimuli. Similarly, barriers to antitumor immunity include immunologic tolerance to self/tumor Ags and the immunosuppressive tumor microenvironment (66). 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 Ag, leading to effective control of tumor growth. Alternatively, 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, type 1 diabetes, and Crohn’s/inflammatory bowel disease in genome-wide association studies (67–69), 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 autoimmunity disease or organ transplant rejection. Taken 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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Disclosures

The authors have no financial conflicts of interest.

References

IKAROS REGULATES CD8+ T CELL DIFFERENTIATION


