THE ROLE OF IKAROS IN CD8+ T CELL BIOLOGY

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A DISSERTATION

in

Immunology

Presented to the Faculties of the University of Pennsylvania

in

Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy

2013

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DEDICATION

I would like to thank my friends and family for their support during this PhD endeavor and to my great classmates Peter Morawski and Natalia Ramos-Hernandez.

ACKNOWLEDGMENT

I would like to thank Dr. Andrew Wells for guiding me through this process and getting me through thick and thin during my time at Penn. I also would like to thank the Albelda lab for helping to extend our studies into the new exciting area of CAR-based immunotherapy. I also would like to acknowledge my thesis committee members, Bob Vonderheide, Chris Hunter, Hao Shen and Warren Pear for their support and input into my PhD project. Additionally, I would also like to acknowledge the support of the IGG community.

This work was funded by the NIH-AI054643 and AI070807

ABSTRACT

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Naive CD8+ T cells represent one of the most potent cells in immunity, as they can differentiate into cytolytic T lymphocytes (CTLs) that lyses virally infected cells or tumor cells through the production of IFN-γ, TNF-α, Perforin and Granzyme B. Their ability to differentiate into highly potent CTLs needs to be a tightly regulated process in order to prevent autoimmunity and disease. However, activation in the absence of key support such as cytokine signals or CD4+ T cell help, or constant challenge with antigen can result in hyporesponsiveness of CD8+ T cells and their failure to mount a robust immune response. Interest has grown in studying the epigenetics of T cells, as chromatin accessibility of key cytokine and lytic mediator loci can determine the ability of the T cell to respond to antigen. Identifying the transcriptional regulators of naive CD8+ T cell differentiation and activation is key to learning how to modulate the CD8+ T cell response. Ikaros is a chromatin-remodeling factor that has been identified to regulate autocrine IL-2 production by and the differentiation program of CD4+ T cells in response to TCR and CD28 signals. As CD8+ T cells make little IL-2 and are dependent on paracrine IL-2 or inflammatory signals for their differentiation, we hypothesized that Ikaros could regulate naïve CD8+ T cell differentiation through restriction of autocrine IL-2 production. In this thesis, I demonstrate that naïve CD8+ T cells with only one copy of Ikaros can differentiate *in vitro* into cytolytic effectors with enhanced effector function, and this results from increased autocrine IL-2 production. This enhanced effector function also sparked an investigation into pre-clinical models of cellular immunotherapy for cancer, to determine if the Ikzf1+/- CTLs had enhanced anti-tumor function. In conclusion, modulating Ikaros activity represents a new approach to controlling naïve CD8+ T cell differentiation and effector function. Through being able to produce more autocrine

IL-2, an Ikzf1+/- CD8+ T cell may not require paracrine IL-2 from CD4+ T cells and can possibly resist the “exhausted” CD8+ T cell effector state during chronic antigen exposure. Thus, investigating Ikaros’ role in CD8+ T cell biology will help to elucidate how this chromatin remodeling factor can influence appropriate CD8+ T cells responses to self and foreign antigen, and ensure against inappropriate immunopathology caused by activated CD8+ T cells.

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CHAPTER 1

Introduction

INTRODUCTION

The development of a naive T cell from a lymphoid precursor and its subsequent activation in response to antigen involves multiple gene expression checkpoints that are governed by key transcriptional repressors and activators. The first step of T cell generation involves differentiation from the pluripotent hematopoietic stem cells (HSCs) into common lymphoid precursors (CLPs). The CLPs demarcate a key node in T cell development, as they express a distinct genetic program that predisposes them toward the lymphoid fate-a distinct cell fate from the granulocyte/macrophage precursors (GMPs) lineage. These CLPs give rise to T, B and NK cell precursors, which eventually proceed through their subsequent development into mature lymphocytes. T cell maturation occurs in the thymus, and this process involves distinct gene expression to guide thymocytes through positive and negative selection for T cells that have the appropriate level of avidity for foreign antigen. Upon release into the periphery, a naive T cell responds to appropriate antigen presentation and activates a genetic program that leads to their differentiation into effector and memory T cells. A review of the naïve T cell activation program and its corresponding genetic control of the IL-2 locus will demonstrate the importance of these epigenetic checkpoints in ensuring appropriate T cell activation and differentiation.

Naïve T cell activation

For appropriate naïve T cell activation, both naïve CD4+ and CD8+ T cells require engagement of their T-cell receptor complex (TCR) with a peptide/MHC complex and their CD28 co-stimulatory molecule with a B7 ligand on an activated antigen presenting cell (APC). Naïve CD4+ T cells only require these signals to differentiate into IL-2 producers (1), while naïve CD8+ T cells fail to produce much IL-2 following TCR and CD28 stimulation (2). Instead, the highly potent CD8+ T cells require additional “Signal 3” Cytokines to ensure their appropriate differentiation into cytotoxic lymphocytes (3). This creates a situation for naïve CD8+ T cells being dependent on paracrine IL-2 from activated CD4+ T cells (“CD4 help”) for their continued activation and expansion (4).

In the context of naïve CD4+ T cells, engagement of the TCR and CD28 leads to signaling cascades that promote the activation of the cell and epigenetic changes that led to appropriate gene expression, such as IL-2. The promoter region of IL-2 contains multiple binding sites for TCR and CD28 initiated transcription factors that form an enhancesome in the promoter region and promote its production during T cell activation (5). TCR and CD28 signaling result in the generation of the transcription factors NF-AT, AP-1, NF-κB and their presence on the IL-2 promoter along with the Oct family members can promote IL-2 transcription (5). TCR signaling results in the activation of the Ca^{+2} signaling and the mitogen activating kinase (MAPK) pathways (6). The former is responsible for the generation of the NF-AT, which translocates to the nucleus and binds to the ARRE-1, ARRE-2 and NF-IL2B promoter regions of IL-2 locus (7, 8). The MAPK pathway results in the generation of the c-Fos protein and upon appropriate CD28-mediated activation of the Phosphoinositide-3 kinase (PI3K)/AKT/JNK pathway that results in c-Jun production, can dimerize with c-Jun to form AP-1 (9). Besides driving c-Jun production, CD28 co-stimulation can also activate the PKC-ϕ pathway (6), which will result in the activation of the NK-κB family members. AP-1 is viewed as a primary initiator of IL-2 gene transcription and binds cooperatively with the other transcription factors at the ARRE-1, ARRE-2, NF-IL2B and the CD28RR sites of the IL-2 promoter (7).

Additionally, as CD4+ T cells are a major source of IL-2 (10, 11), their release of IL-2 has to be tightly regulated. This is observed when naïve CD4+ T cells are stimulated with self-antigen, as production of IL-2 by self-reactive CD4+T cells could contribute to autoimmunity and disease (12). As antigen presenting cells that present self-antigen are poorly activated and express few B7 ligands, self-reactive CD4+ T cells that bind the TCR-MHC complex do so in the absence of CD28-B7 ligation. Stimulation in the absence of CD28, results in a state of CD4+ T cell anergy as the CD4+ T cells fail to produce IL-2 upon re-stimulation (13). This can be traced to the lack of AP-1 and NK-κB, which are derived from CD28 co-stimulation. The anergized CD4+ T cells demonstrate increased transcriptional activity of NF-AT to drive anergy-associated genes (14, 15) (16). These cells that are re-stimulated with peptide-MHC complexes in the presence of B7 ligands, fail to produce IL-2, do not undergo clonal expansion and fail to provide paracrine IL-2 for

CD8+ T cell activation. This regulation of IL-2 availability prevents the aberrant activation of CD4+T cells towards self-antigen presentation, and ensures that any self-reactive CD8+ T cells do not receive paracrine IL-2 in the periphery.

The IL-2 locus is also regulated by epigenetic mechanisms, to ensure its appropriate activation. In quiescent CD4+ T cells, the IL-2 promoter is an inactive, but permissive chromatin state (7). Upon CD4+ T cell activation, the IL-2 promoter becomes hyperacetylated (17-19) and becomes demethylated at CpGs (17). In contrast, naïve CD8+ T cells fail to hyperacetylate the IL-2 promoter following TCR and CD28 stimulation (20), again indicating a difference in their ability to produce IL-2. Thus, epigenetic mechanisms are in place to ensure appropriate transcription of the IL-2 gene by naïve CD4+ and CD8+ T cells.

IL-2 activity in a primary immune response

Upon appropriate TCR and CD28 co-stimulation in response to a foreign antigen, naive T cells can produce IL-2 to support their survival, promote cell cycle progression and T cell expansion. Naïve CD4+ T cells produce high amounts of autocrine IL-2 in comparison to naïve activated CD8+ T cells (2, 21) and play a critical role in sustaining an immune response. In a tri-cellular model of activation, activated CD4+ T cells license dendritic cells via CD40L-CD40 engagement (22), and the activated dendritic cell up-regulates more B7 ligands and peptide-MHC complexes. Engagement with a naïve CD8+ T cell forms the tri-cellular model, and the naïve CD8+ T cell becomes exposed to the paracrine IL-2 produced by the activated CD4+ T cell (10, 11, 23). A different version of T cell activation holds that after licensing of a dendritic cell and disengagement from the CD4+ T cell, that a DC up-regulates CD70 and ligates with CD27 (24) on a naïve CD8+ T cell. This engagement results in increased autocrine IL-2 production that ultimately acts during the priming phase to promote memory CD8+ T cell formation (25). While the sources of IL-2 differ in these two models, it is still apparent that IL-2 is a critical factor during a primary immune response. During the initial T cell expansion phase, IL-2 acts in a positive feed forward loop with the high affinity IL-2Rα (CD25) (26, 27), that is expressed on activated CD4+ and Cd8+ T cells following antigen stimulation. This high affinity IL-2Rα complexes with the IL-

2Rβ (CD122) and the common γ chain receptor (CD132) to form the IL-2 receptor on activated T cells (28). The paracrine IL-2 during the primary immune response promotes increased CD25 expression on T cells, as depletion of CD4+ T cells and their paracrine IL-2 in a primary immune response results in a failure of CD8+ T cells to up-regulate much CD25 (29). IL-2 leads to signaling through the associated PI3K/Akt pathway (30) or the JAK/STAT5 pathway (31). PI3K/AKT signaling can activate the mTOR pathway, which has been demonstrated to regulate the balance of effector and memory CD8+ T cells through inducing the transcription factor T-Bet (32). Prolonged STAT5 signaling can also bias the CD8+ T cells towards a short-lived effector phenotype(33). IL-2 signaling is also connected to cell cycle progression (16), IFN-γ production (34, 35) or up-regulation of key transcription factors such as Bcl-2 (1) or Eomes in T cells (36). IL-2 can also regulate its production through the induction of the transcription factor Blimp-1 in T cells, as Blimp-1 has been associated with reducing IL-2 production (37) and driving terminal effector CD8+ T cell differentiation (38, 39).

The level of IL-2 during the primary immune response has to be finely tuned as too much IL-2 can result in activation induced cell death (AICD) (1) and results in ill-timed T cell death during the critical expansion phase (4). Additionally, the presence of IL-2 during the priming phase of the immune response has been demonstrated to influence both effector CD8+ T cell (33, 36, 40, 41) and memory Cd8+ T cell formation (42-44). The provision of IL-2 during the contraction phase of the immune response can also result in increased T cell numbers due to increased cell proliferation and increased apoptosis resistance (4, 45). Thus, IL-2 availability needs to be tightly regulated during an immune response to determine the appropriate amount of antigen experienced T cells that persist.

Overall the availability of IL-2 during an immune response can influence the numbers of activated CD4+ and CD8+ T cells and also control their duration and differentiation into effector cells. As IL-2 transcription is influenced by multiple transcription factors from TCR and CD28-mediated signaling cascades, it is important to identify transcriptional repressors that can integrate TCR and CD28 signaling for the appropriate production of IL-2. These transcriptional repressors can regulate IL-2 availability and prevent immunopathology, through ensuring that this

critical cytokine for T cell activation and differentiation is released after receiving appropriate antigen and B7-costimulatory signals.

Ikaros

One such factor is Ikaros (Ikzf1), a C2H2-motif zinc finger transcription factor that has 4 N-term and two C-term zinc fingers (Fig. 1). There are 8 isoforms of Ikaros that are generated due to alternative splicing, and Ik1 and Ik2 are the predominant isoforms in T cells (46, 47). Ikaros is part of the Ikaros family, which includes Helios (Ikzf2), Aiolos (Ikzf3), Eos (Ikzf4), and Pegasus (Ikzf5) (48). Ikaros expression is found in all hematopoietic cells, lymphoid lineages and some myeloid cell subsets (49). Ikaros is implicated as a lymphocyte-specific lineage factor as mice that are homzygous for the deletion of exon 3-4 (Dominant Negative) (50) or in exon 7 (Ikaros Null system) (51) in both alleles fail to develop T, B, and NK cells and lack peripheral lymph nodes. Additionally, Ikaros acts as a tumor suppressor gene as T cells that express the truncated isoforms or engineered to be heterozygous for the exon 3-4 deletion (DN+/-) give rise to lymphomas and leukemias (52, 53). Loss of complete Ikaros expression can bias cells towards a transformed state (54). Similarly, reduced Ikaros or Ikaros exon deletions have been characterized in human malignancies such as acute lymphoblastic leukemia (ALL) (55), acute myeloid leukemia (AML) (56) and chronic myelogenous leukemia (CML) (54). Thus, Ikaros is a key factor for T cell lineage regulation.

Ikaros acts through the formation of higher order chromatin binding complexes. DNA-binding to the GGGAA consensus binding site binding site (50) (46) (57) is achieved through binding of at least 3 zinc fingers, especially by the exon 3-4 zinc fingers (50). Mice heterozygous for this deletion results in enhanced ability of the truncated Ikaros isoforms to inhibit Ikaros and the other family members (50, 52) and develop leukemias and lymphomas (52) (Fig 1A and B**)**. Ikaros forms cluster of dimers that associate with HDAC-containing Nucleosome Deacetylase Complexes (NURDs) and repress chromatin through associating with Sin3a (58), Mi-2B (59) or in an HDAC-independent manner with CtBP (60). Conversely, Ikaros can also act as an activator of gene expression, through association with the SWI/SNF complex (61). Ikaros forms toroid

structures that localizes with the pericentromeric heterochromatin (53), an area associated with gene silencing and resulting in Ikaros being mainly identified as a transcriptional repressor.

Another area of active research is on upstream regulators of Ikaros' activity, as Ikaros regulates many different target genes (Fig. 2) and thus requires accurate precision to be inhibited or activated. Phosphorylation of Ikaros is one regulatory mechanism, as Casein Kinase II activity on Ikaros results in its decreased binding activity (62) and can result in increased expression of cell cycle genes such as c-Myc (63) and promote the G1-S transition phase (64). Other kinases such as spleen tyrosine kinase (SYK) and Bruton's tyrosine kinase (BTK) have also been demonstrated to phosphorylate Ikaros in a similar fashion (65, 66). Hyper-phosphorylated Ikaros is targeted for degradation (67) and complete loss of this tumor suppressor gene can bias towards the development of leukemia and lymphomas (64, 67). Phosphorylation activity is opposed by the activity of protein phosphatase 1 (PP1), that promotes Ikaros' DNA binding activity (67), recruitment to pericentromeric heterochromatin (62) and resist leukemic transformation. Ikaros' chromatin remodeling activity at the DNA levels is also regulated, as SUMOlyation at K58 and K240 residues diminishes Ikaros repressive function through inhibiting its ability to interact with HDACs (68). Thus, a combination of phosphorylation and SUMOlyation activity help to regulate Ikaros activity, although the direct associations with T cell function and proliferation remain to be worked out.

Ikaros in lymphocyte development

In the context of HSCs, Ikaros is expressed in early hematopoietic development and observed in fetal thymus, liver and spleen (46, 69). In the adult, Ikaros expression is limited to the lymphoid organs and blood leukocytes (57). Deficiencies in Ikaros during these developmental stages can influence cell type expression. Mice that express a dominant negative form of Ikaros typically have increases in GMPs (50) and MEPs (70) along with severe anemia, resulting from decreases in erythroid precursors (50). Thus, alterations in Ikaros activity can impact hematopoeisis.

With developing thymocytes, Ikaros activity is instrumental in regulating the development of

a productive TCR, through regulation of RAG1 (70) and Tdt (71) genes, which assist in VDJ recombination of TCR related genes. Similarly, Ikaros also regulates productive pre-BCR formation through genetic control of the Igll1 locus (72) (73) in developing B cells. Ikaros is highly expressed in thymocytes (69) and restricts thymocyte transitions through enforcing appropriate pre-TCR signals in order to proceed (74). Following productive pre-TCR generation, Ikaros also regulates the CD4+CD8+ double positive to single positive CD4+ or CD8+ thymocyte transition as it has been linked to regulating both CD4 (75) and CD8 co-receptor expression (76, 77). In the Ikaros DN system, there is an increase in CD4+ expressing thymocytes (75, 78) and both DP and DN thymocytes give rise to transformed cells (52), indicating that Ikaros plays a role in the thymocyte to naive T cell transition.

Ikaros in mature lymphocytes

Mature lymphocytes are split into B and T cells and form the humoral and cell mediated immunity branches (CMI) of adaptive immunity. The humoral immune response involves the activation of B cells, which secrete antibodies that are important for neutralization and antibody dependent cell cytoxicity (ADCC) mechanisms to clear pathogens. The activation of B cells is dependent on the CD4+ T cell arm of the CMI branch, as CD4+ T cells produce key cytokines such as IL-4(79) to promote B cells immune responses. Ikaros has been implicated in the regulation of different B cell targets such as transcription factors, cell cycle factors and chromatin modifying agents(73). Aiolos also plays a role in B cell function as Aiolos-/- mice have increased immature B cells, increase in autoantibody production, and development of B cell lymphomas (80). Thus, Ikaros and its family member Aiolos play a role in regulating B cell development and activation.

With mature T lymphocytes, Ikaros is known to have an extensive role in CD4+ T cells. First, Ikaros plays a key role in CD4+ T cell anergy. This state of T cell unresponsiveness occurs when self-reactive CD4+ T cells escape negative deletion mechanisms in the thymus and circulate into the periphery. To prevent aberrant activation of autoreactive CD4+ T cells, naive CD4+ T cells requires both TCR and CD28 co-stimulation. In the absence of CD28 co-stimulation, specifically

by non-activated APCs in non-inflamed environments, these self-reactive CD4+ T cells fail to become activated and re-engagement with TCR and CD28 signals in the periphery results in their inability to be activated or produce IL-2(13). This anergic state is correlated with increasing levels of Ikaros(14) and Ikaros is responsible for the increased de-acetylation of the IL-2 locus in anergized CD4+ T cells (81). The direct regulation of the IL-2 locus by Ikaros in CD4+ T cells(19, 81) can also reverse this state of anergy, as in vitro anergized Ikaros-deficient CD4+ T cells produce more IL-2 and avoid anergy (19). Additionally, IL-2 signaling can reduce Ikaros levels in a T cell (82) and it is plausible that Ikaros and IL-2 act in a negative feedback loop. Thus, Ikaros has an appreciable role for regulating CD4+ T cell responses to self-antigen through direct regulation of the IL-2 locus. This regulation of IL-2 by Ikaros is also being extended to the other Ikaros family members, such as Helios in Tregs(83) and Aiolos in Th17 CD4+ T cells(84).

In the context of CD4+ T cell differentiation, Ikaros represses Th1 differentiation under Th2-polarizing conditions through the regulation of the T-box transcription factor T-Bet(85) and IFN-γ gene expression(86). Under Th2-polarizing conditions, Ikaros-deficient CD4+ T cells up-regulate T-Bet and produce more IFN-γ than wild-type polarized Th2 cells(86), indicating that Ikaros regulates the plasticity of the Th1 and Th2 lineages. Additionally, Th2-polarized Ikaros-deficient cells also demonstrate less repressive chromatin marks at the IFN-γ locus than wild-type Th2-polarized cells, indicating that Ikaros has an epigenetic role on this locus. This regulation of IFN-γ, helps to ensure appropriate immune responses as Ikaros-deficient Th2 polarized CD4+ T cells respond to an *in vivo* Th2 polarizing stimulus in a Th1-responsive manner through aberrant production of IFN-γ (86). In summary, Ikaros plays a critical role in both regulating CD4+ T cell activation in response to appropriate stimulation and their differentiation.

Thesis Focus

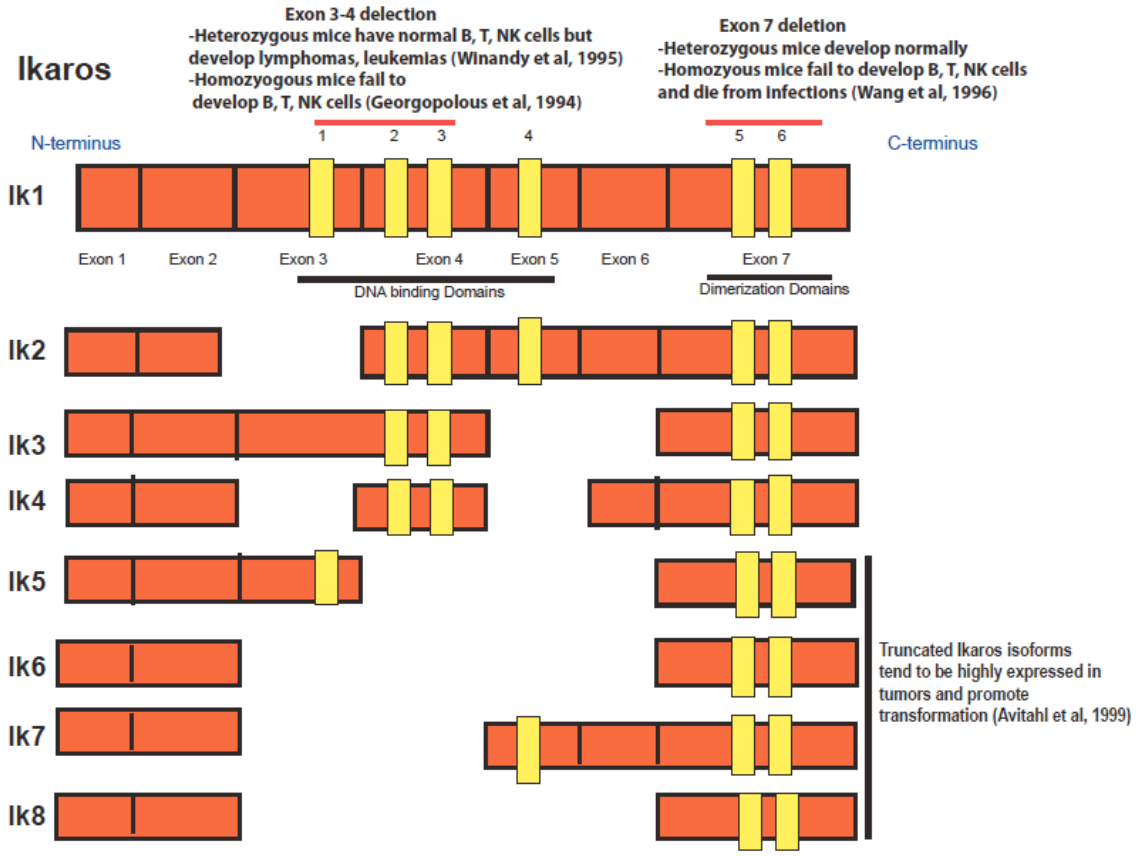
As Ikaros has been characterized to restrict autocrine IL-2 production in CD4+ T cells via transcriptional repression(19, 81) and regulates CD4+ T cell differentiation(86), I hypothesize that Ikaros restricts autocrine IL-2 production in naive CD8+ T cells as means to regulate naïve CD8+ T cell differentiation. Thus, I have sought to determine how Ikaros influences naive CD8+ T cell

differentiation through regulation of autocrine IL-2 production. In Chapter 2, I examine how Ikaros' regulation of autocrine IL-2 production in naive CD8+ T cells influences their differentiation into effector CTLs.

However, Ikaros activity is not restricted to just IL-2 regulation in CD8+ T cells and has been linked to multiple genes (Fig. 2), and influences effector factors such as IFN-γ (86) and Granzyme B (87, 88) in T cells. Thus, I have sought to also examine how Ikaros influences the effector program, and in Chapter 3 I examine how CTLs with reduced Ikaros expression can improve the efficacy of chimeric antigen receptor (CAR) engineered T cells. Finally, in the concluding chapter I examine some preliminary data that indicates a role for Ikaros in memory CD8+ T cells. Overall, Ikaros plays an important role in regulating the naive, effector and memory states of CD8+ T cell biology.

Figures

1A.


Ikaros
Exon 3-4 delection
-Heterozygous mice have normal B, T, NK cells but develop lymphomas, leukemias (Winandy et al, 1995)
-Homozygous mice fail to develop B, T, NK cells (Georgopolous et al, 1994)
Exon 7 deletion
-Heterozygous mice develop normally
-Homozyous mice fail to develop B, T, NK cells and die from infections (Wang et al, 1996)
N-terminus
C-terminus
1
2
3
4
5
6
Ik1
Exon 1
Exon 2
Exon 3
Exon 4
Exon 5
Exon 6
Exon 7
DNA binding Domains
Dimerization Domains
Ik2
Ik3
Ik4
Ik5
Ik6
Ik7
Ik8
Truncated Ikaros isoforms tend to be highly expressed in tumors and promote transformation (Avitahl et al, 1999)

1B

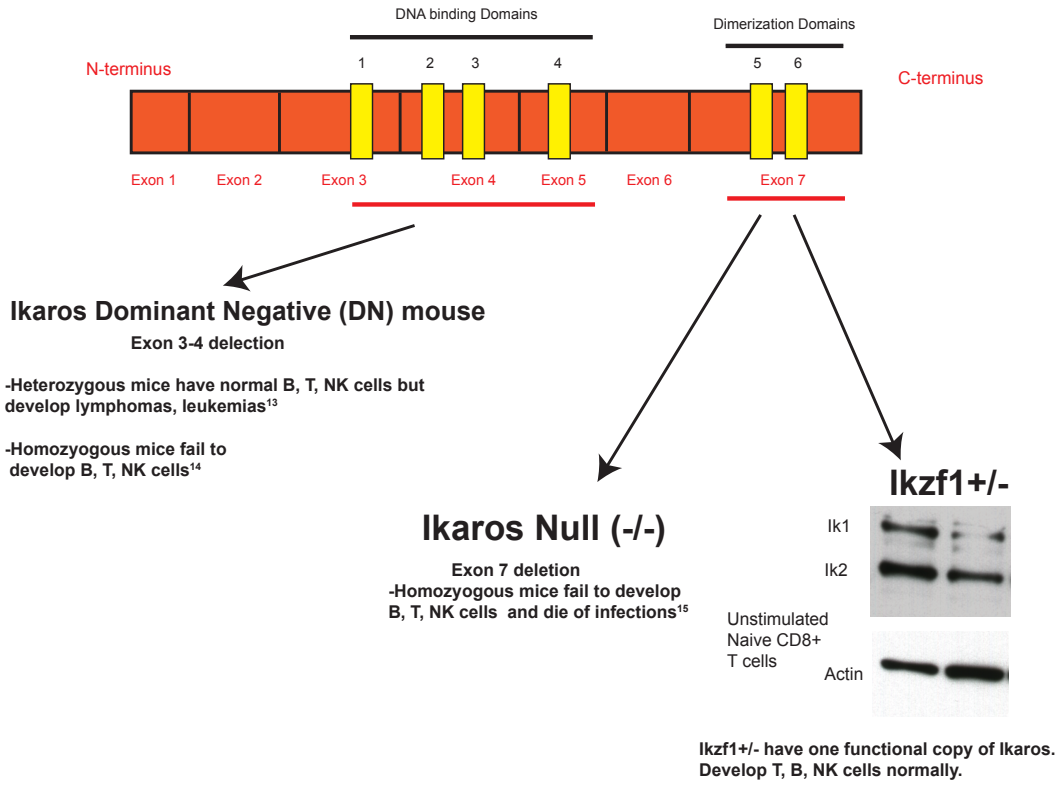


Figure 1: The 8 known isoforms of Ikaros and mouse models

Ikaros has 7 exons, with 4 N-terminal DNA binding C2H2 zinc fingers and 2 C-terminal dimerization zinc fingers (Yellow). Ik1 and Ik2 isoforms are the predominant isoforms in T cells (46, 57, 69). Deletion of Exon 3-4 regions results in the loss of DNA-binding activity and the resulting truncated protein can act to inhibit full length Ikaros and the other family members via the remaining dimerization domains. This results in a dominant negative activity in mice that are heterozygous (52) for this deletion. Deletion in Exon 7 results in a non-productive transcript and mice heterozygous for this deletion has 50% reduction in Ikaros (53), while homozygous mice lack T, B, NK cells (51)

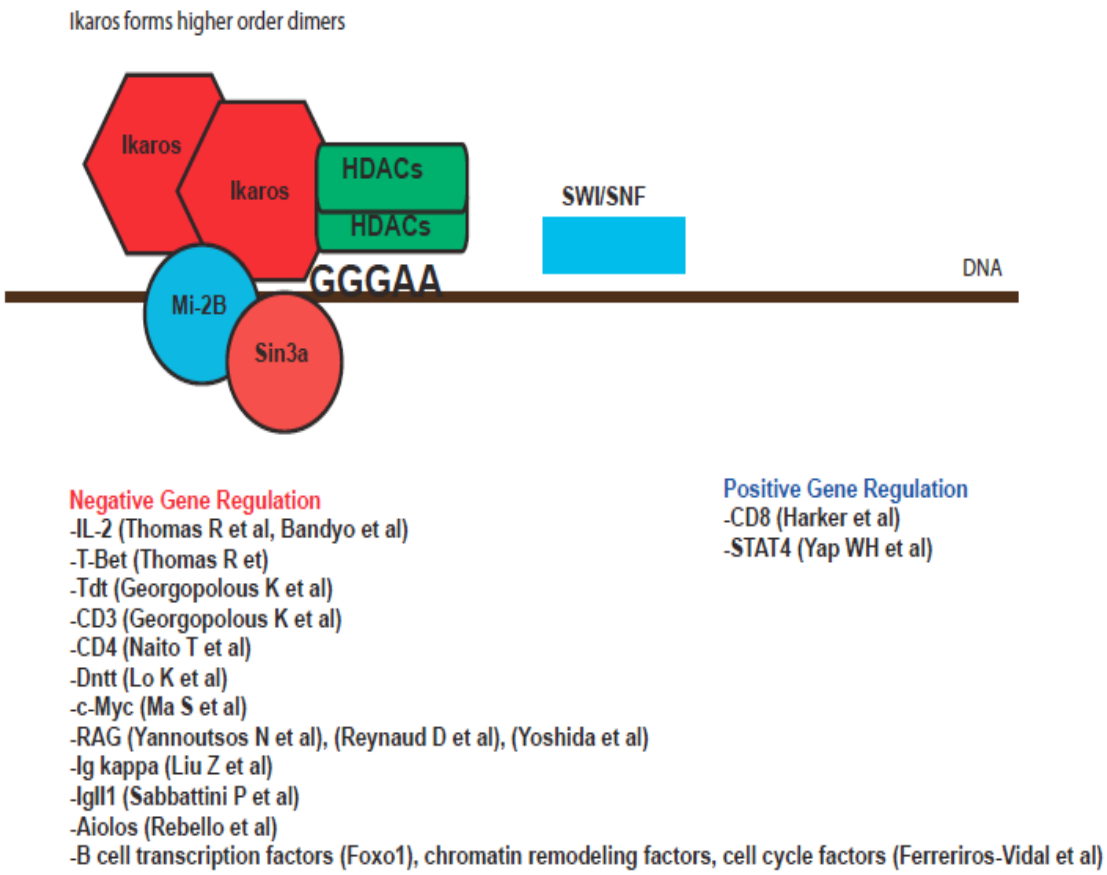


Figure 2: Ikaros can act in a repressive or positive action on gene targets.

Through the formation of higher order chromatin remodeling complexes and associations with NURD and Mi-2B, Sin3a, CtB or SWI/SNF complexes, Ikaros can regulate the gene targets listed in a positive or negative fashion.

CHAPTER 2[1]

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

[1] Figures modified from the submitted manuscript to *Journal of Immunology*, by O'Brien S et al., and titled "Ikaros imposes a barrier to CD8+ T cell differentiation by restricting autocrine IL-2 production"

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 naïve 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. Using a reductionist in vitro system, we find that CD8+ T cell activation 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 Ikzf1 failed to upregulate Ikaros, produced autocrine IL-2, and differentiated into IFN-γ-producing CTL in response to TCR/CD28 stimulation alone, and IL-2 was necessary for this gain of effector function. Furthermore, Ikzf1-haploinsufficient CD8+ T cells were able to help neighboring, non-IL-2-producing cells to differentiate into IFN-γ-producing effectors. Therefore, by repressing autocrine IL-2 production, Ikaros ensures that naïve 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 immunopathology, especially as inappropriate CTL activation has been linked to autoimmune disease such as type I diabetes, vitiligo, and multiple sclerosis.

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, costimulation of naïve CD8+ T cells through the TCR and CD28 does not result in efficient autocrine IL-2 production, and is not sufficient for differentiation into cytolytic effectors. In addition to TCR and CD28, CD8+ T cells require pro-inflammatory cytokines for their differentiation. For instance, IL-12, type I IFN 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 is required during the priming phase for effective T cell memory formation, as “unhelped” CD8+ T cells fail to generate memory(20, 42, 44). However, high levels of IL-2 can promote terminal effector CD8+ T cell generation at the expense of memory formation (33, 36, 40). Thus, strict regulation of IL-2 production during initial phases of an immune response ensures 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 (10, 11, 23). However, in some systems helper T cells can license CD8 cells to produce their own IL-2 (24), which is required during initial priming in order to generate robust memory recall responses (25).

Little is known about how autocrine IL-2 is restricted in naïve CD8+ T cells. Recently, it was shown that Ikaros, a transcriptional repressor required for lymphocyte development, restricts autocrine IL-2 production in mature CD4+ T cells (19, 81). 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, RAG1$^{-/-}$ and OT-1 mice were purchase from JAX. *Ikzf1+/-* mice were a kind gift of Dr. Katia Georgepolous, 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.

Cell Sorting - Single cell suspensions from spleen and LN of polyclonal mice were enriched for CD8+ T cells through use of depleting antibodies against CD4 (GK1.4), MHC II (M5/114), anti-FcR (2.4G2), anti-B220 (RA3-6B2) antibodies and Qiagen magnetic goat anti-rat IgG beads (#310107), and 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, and MHC II-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), 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 Invitrogen 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, 0.66ug/ml) for 4-6 hours. 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, and then re-suspended in FACS Buffer. For transcription factor staining, intracellular staining was achieved with the eBioscience FoxP3 kit and standard protocols were followed.

Cell Culture - Naïve sorted CD8+ T cells were stimulated in 96-well or 24-well plates, which were coated with anti-CD3/CD28 antibodies in PBS. 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 400ug/ml G418 (Invitrogen).

Immunoblot analysis - Immunoblotting was performed for determining the Ikaros isoform expression. For immunoblot, $0.5x10^6$ cells were lysed with Laemmli buffer, boiled, and subjected to SDS-PAGE using Criterion 10% precast Tris-HCl gels (Bio-Rad). Electrophoresed proteins were transferred to nitrocellulose membrane using a Trans-Blot system (Bio-Rad). The membrane was washed 3X with 0.1% Tween-20 in 1X PBS, and stained with a C-terminus reactive goat anti-mouse antibody to Ikaros (SC-9861, Santa Cruz). Blots were washed and incubated with secondary HRP-conjugated anti-goat antibody (1:10,000) for 1 hour at room temperature. Membranes were then washed and developed using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific). The image was developed on a Kodak X-ray

film. Membranes were then stripped with Restore Plus Western Stripping Buffer (Thermo Scientific) for 10 minutes, washed 3X, blocked, and stained with anti-goat antibody to β-actin staining (Santa Cruz) to normalize loading.

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 deparaffinize slides. Stained slides were analyzed on a Leica DM 2500 microscope with a 40x HCX PL Fluotar objective (∞/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 (1.0ug/ml) for 48 hours and in presence or absence of IL-2 (10ng/ml). Cells were then harvested, counted and re-suspended at 0.5e6 cells/ml in complete RPMI and rested overnight at 37C. After overnight rest, effectors were mixed at 10:1 ratio with CFSE-labeled EL4 or EL4.OVA cells and incubated for 3 hours, 37C. After 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 (5ug/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. Cytotoxicity = (% Cytotoxicity of CD8+ T cells - % Cytotoxicity of Control Tumor cells without CD8+ T cells/ %Maximum cytolysis-%minimum cytolysis).

In vivo tumor assay: Naïve purified wild-type and Ikzf1+/- PMEL CD8+ T cells were stimulated for 3 days with plate bound anti-CD3/CD28 (1.0ug/ml) for 3 days and in the presence or absence of IL-12 (20ng/ml) or IL-12+IL-2 (10U/ml). After activation, cells were harvested and 1e6 cells were transferred into B6 mice. After 24 hours, mice were challenged with 1e5 B16 melanoma subcutaneously and tumor growth was followed. Tumors were measured with a ruler and tumor volume was calculated as (LxW^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 Ik1 and Ik2 DNA binding isoforms of Ikaros, with no evidence for expression of the smaller isoforms that lack the DNA binding domain (Fig. 3A). Ikaros exhibited a punctate nuclear pattern by immunohistochemical staining (Fig. 3B), consistent with previous studies (53). When costimulated through the TCR and CD28 in the absence of exogenous cytokines, naïve cells showed marked upregulation of Ik1 and Ik2 (Fig. 3A and B) and failed to differentiate into IFN-γ producing effector cells (Fig. 3D). The addition of exogenous IL-2 reversed the accumulation of Ik1 and Ik2 proteins (Fig. 3A and C), and promoted differentiation of naïve CD8+ T cells into IFN-γ producing effectors (Fig. 3D). These results suggest that Ikaros, a known repressor of CD4+ T cell differentiation (19, 86) 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 (51), and over mice expressing a dominant-negative mutant of Ikaros (50), which develop fatal lymphomas early in life (52, 53)in that Ikzf1+/- mice show normal hematopoietic development and do not develop tumors (53). Naïve, quiescent CD8+ T cells with only one functional allele of Ikzf1 failed to upregulate Ik1 and Ik2 in response to TCR/CD28 stimulation (Fig 4A and B). This failure was not due to under-stimulation of the cells by our *in vitro* priming system as the wild-type and Ikzf1+/- CD8+ T cells up-regulated CD69, CD25 and CD44 following TCR and CD28 stimulation (Supp. Fig. 1). Consistent with our hypothesis that Ikaros

negatively regulates CD8 differentiation, Ikzf1+/- CD8+ T cells were able to differentiate in response to TCR/CD28 costimulation alone (Fig. 4C), giving rise to frequencies of IFN-γ producers comparable to that observed in wild-type cultures given exogenous IL-2 (Fig 4C). The addition of exogenous IL-2 blunted the induction of Ikaros in wild-type cells (Fig. 4A and B), but led to nearly complete loss of Ikaros in the Ikzf1+/- cells (Fig. 4A and B). 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 (26, 27). 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 signal 3-independent CD25 and IFN-γ expression (Fig. 4C) and prevented down-regulation of Ikaros by Ikzf1+/- cells (Fig. 4A) 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 (89, 90), as IL-2 induces Eomes expression(36), and Ikaros has been shown to regulate T-bet during CD4+ T cell differentiation (86). The strong TCR and CD28 signals in this system led to similar induction of T-bet under all conditions (91) in both wild-type and Ikzf1+/- CD8+ T cells (Fig 4D), but induction of Eomes by wild-type CD8+ T cells required exogenous IL-2. However, 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 4D). These data indicate that Ikaros also regulates the expression of Eomes, a key factor for CD8+ T cell effector differentiation (89).

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, wild-type naïve CD8 cells produced very little autocrine IL-2 in response to TCR/CD28 costimulation (Fig. 5A). 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. 5A). This gain of IL-2 production could also be observed at the single-cell level immediately after TCR and CD28 stimulation (Fig. 5B).

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 1.25ug/ml concentrations of αTCR antibody (Fig. 5C). Similarly, increasing the strength of CD28 costimulation at a fixed, high concentration of αTCR 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. 5C). 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. 4C). While this was clearly dependent upon IL-2 secretion by Ikzf1+/- CD8 cultures (Fig. 4C), our ICS data indicate that this IL-2 is produced from relatively few cells at any one time (Fig 5B). 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 mutant 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 CD25hi, IFN-γ producing cells, while wild-type cells failed to differentiate under these conditions (Fig 6A). However, wild-type CD8+ T cells in the presence of Ikzf1+/- CD8+ T cells became CD25hi, and a significant frequency was able to produce IFN-γ as measure by intracellular staining (Fig 6A) and ELISA (Fig. 6B). 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, indicating that the IL-2 from the Ikaros-mutant CD8+ T cells was necessary for both the autocrine and paracrine effects on differentiation (Fig 6A). 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 indirectly 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 (33, 36, 92, 93). 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. 7A), and required the addition of exogenous IL-2 for high-level expression (Fig. 7A). However, Ikzf1+/- effector cells were able to induce Granzyme B to high levels in the absence of exogenous cytokines (Fig 7A), and this gain of Granzyme B expression was completely dependent upon IL-2 (Fig 7A). To assess cytolytic activity, wild-type and Ikzf1+/- OT-I cells were challenged *in vitro* with EL4 thymoma targets engineered to express OVA (EL4.OVA). Wild-type OT-I cells primed through the TCR and CD28 exhibited relatively poor cytotoxicity against EL4.OVA target cells as measured by 7-AAD exclusion (Fig 7B) unless IL-2 was provided during the priming phase (Fig. 7B). However, consistent with their increased IL-2 and Granzyme B expression, TCR/CD28 primed Ikzf1+/- OT-I cells exhibited a cytotoxic capacity equal to that observed in wild-type cells stimulated in the presence of IL-2 (Fig 7B). Together, our data indicate 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 can regulate anti-tumor function *in vivo*

To test if the results of the *in vitro* cytotoxicity assay could translate *in vivo*, we utilized the well-characterized B16 melanoma model. Transgenic PMEL-1 T cells recognize the tumor antigen gp100(94), which is overexpressed by this poorly immunogenic tumor. Effective anti-tumor immunotherapy of established B16 tumors in mice usually requires the administration of exogenous IL-2(95) or IL-12(96, 97) with adoptive transferred PMEL effectors and peptide vaccination. Effective anti-tumor strategies also involves increased MHC I expression on B16 melanoma cells, and IFN-γ has been implicated as key cytokine for this system (98). Since our Ikzf1+/- CD8+ T cells produce more IFN-γ upon stimulation with IL-12 (Fig 4C**)**, and IL-12 also improves IL-2 signaling (99, 100), we elected to prime our wild-type and Ikzf1+/- PMEL cells with TCR, CD28 and in the presence or absence of IL-12 or IL-12+IL-2 signals. To test the fitness of these effectors, the cultured effector CD8+ T cells were adoptively transferred into B6 mice and 24 hours later, injected with the highly aggressive and poorly immunogenic B16 melanoma. As the transferred cells are the only tumor specific population in the mice, this tumor challenge assesses the anti-tumor immunity of these *in vitro* cultured effectors and responsiveness in absence of antigen specific CD4+ helper T cells.

Over the course of the experiment, the wild-type effectors primed in the presence of TCR and CD28 alone promoted minor tumor delay by day 22 (Fig. 8A). Culturing these wild-type effectors in the presence of IL-12 or IL-2+IL-12 promoted some tumor delay by day 19 in comparison to the TCR and CD28 primed wild-type effectors, but by day 22 these stimulated effectors failed to control tumor. Mice that received the TCR and CD28 primed Ikzf1+/- effectors demonstrated a small, although not statistically significant, trend in tumor delay in comparison to the wild-type effectors. Culturing these effectors in the presence of IL-12 did result in more pronounced tumor delay in comparison to wild-type (Fig. 8B**)**, indicating that these Ikzf1+/- effectors have increased sensitivity to IL-12(101), produce more IFN-γ (Fig 4C), and promotes better effector function in comparison to wild-type. More significant tumor delay was observed for the Ikzf1+/- effectors that were stimulated with IL-2 and IL-12 (Fig. 8C). The increased tumor delay exhibited by the Ikzf1+/- effectors in both the IL-12 and the IL-2/IL-12 stimulated groups demonstrates that the IL-12 alone

group is similar to the IL-2+IL-12 group and enhancing IL-2 signaling. This increase in autocrine IL-2 in combination with the inflammatory cytokine IL-12 results in Ikzf1+/- CD8+ effector generation that has enhanced cytolytic function towards a poorly immunogenic tumor. Thus, activating CD8+ T cells with reduced levels of Ikaros in the presence of an inflammatory cytokines can promote an immune response to a self-antigen and Ikaros possibly influences CD8+ T cell tolerance.

DISCUSSION

In this chapter, we demonstrate a novel role for Ikaros in the regulation of naïve CD8+ T cell differentiation through the control of autocrine IL-2. TCR signals in the absence of cytokines results 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 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 cells (19, 81, 86), where a similar role for IL-2 in the repression of Ikaros expression has been shown at the mRNA level (82). Thus, Ikaros appears to integrate TCR, costimulatory and cytokine signals, where it mans a global checkpoint for T cell differentiation.

IL-2 has important roles in CD8+ T cell effector differentiation and memory. IL-2 drives CD8 effector differentiation, in part by inducing the T-box transcription factor Eomes (36, 40), which cooperates with its family member T-bet to transactivate IFN-γ, perforin, and Granzyme B gene expression (89, 90). STAT5, a transcription factor activated by IL-2R signaling, has been shown to promote accessibility of the Ifnγ promoter to binding by T-bet (102), and drives feed-forward expression of the high affinity IL-2 receptor chain, CD25 (27). 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 (33), and high concentrations of exogenous IL-2 during CD8+ T cell activation *in vitro* results in CTL with limited survival and homeostatic capacity (36). In contrast, low levels of IL-2 promote the generation of effectors with reduced cytotoxicity and increased memory potential (36), resembling the CD25lo memory precursor cells that arise during LCMV infection (33). 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 (42, 103, 104). 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, mimicking IL-2 activity during primary immune responses also resulted in increased recall responses (105) or converted IL-2Rα-/- CD8+ T cells into competent memory CD8+ T cells (42). Adoptive transfer studies with naïve OT-I IL-2-/- into B6 mice and subsequent LM-OVA infection also demonstrated that these cells fail to generate memory recall response (25). These studies all demonstrate that naïve CD8+ T cells inability to respond to paracrine IL-2 or produce autocrine IL-2 during a primary immune response will affect their requisite memory recall responses.

The main cellular source of IL-2 is CD4+ T cells, and along with CD40-mediated licensing of dendritic cell maturation (22, 106, 107), IL-2 represents a major paracrine mechanism for CD4 help for CD8+ T cell responses (10, 11, 23). 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 (42, 44, 103). Consistent with this, the il2 and ifnγ loci are epigenetically silenced in unhelped CD8+ T cells, at the level of both DNA methylation and chromatin structure (20, 43). 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 (24, 25), 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 is a zinc finger DNA binding protein that interacts with the NURD, Sin3a and CtBP transcriptional co-repressor complexes (58-60), and is a potent regulator of chromatin structure and DNA methylation at its target genes (86). 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 (86), and are resistant to anergy induction (19, 81). 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 (86), and we show in this current study that IL-2 (Fig. 3 and 4) and IL-12 (Fig. 4A) 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 (108).

These studies suggest 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, Tbox genes, Gzmb, and Ifnγ 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 Ifnγ, 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 not only able to produce IL-2 and drive their own differentiation, they are able to help neighboring, non-IL-2 producing CD8 cells in a paracrine manner to differentiate into $CD25^{hi}$ IFNγ+CTL. By producing more autocrine IL-2 following TCR and CD28 stimulation, these Ikzf1+/- CTLs also

have increased cytolytic function *in vitro* due to increased Granzyme B function. Their ability to produce more IL-2, IFN-γ and Granzyme B, makes them ideal effectors for anti-tumor immunity. Our *in vivo* tumor challenge model with a poorly immunogenic B16 melanoma demonstrated that the Ikzf1+/- effectors that were primed with IL-12 or IL-2+IL-12 had enhanced anti-tumor responsiveness to a self-antigen. While the wild-type cytokine primed effectors did not control the tumor as well, this could be due to the use of the poorly immunogenic B16 as the more highly immunogenic EL4.OVA system is typically utilized in the tumor prevention model(32). Differences in CTL curative ability to EL4.OVA and B16.OVA established tumors following vaccination with OVA has also been noted in the literature(109). Thus, we observe that the increased sensitivity of Ikzf1+/- CD8+ T cells to IL-12(101) in combination with their increased autocrine IL-2, results in robust CTL responses to a highly aggressive, poorly immunogenic tumor.

This may have implications for autoimmunity as promoting Ikaros function could lead to novel treatments for autoimmune disease or organ transplant rejection. Conversely, inhibition of Ikaros activity could improve vaccination or anti-tumor immunity, where the lack of IL-2 derived from CD4+ T cells in the tumor microenvironment poses a challenge for anti-tumor CD8+ T cells (110). Importantly, we find that 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 (Fig. 15), a finding predicted by our model of Ikaros function. 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.

Figures

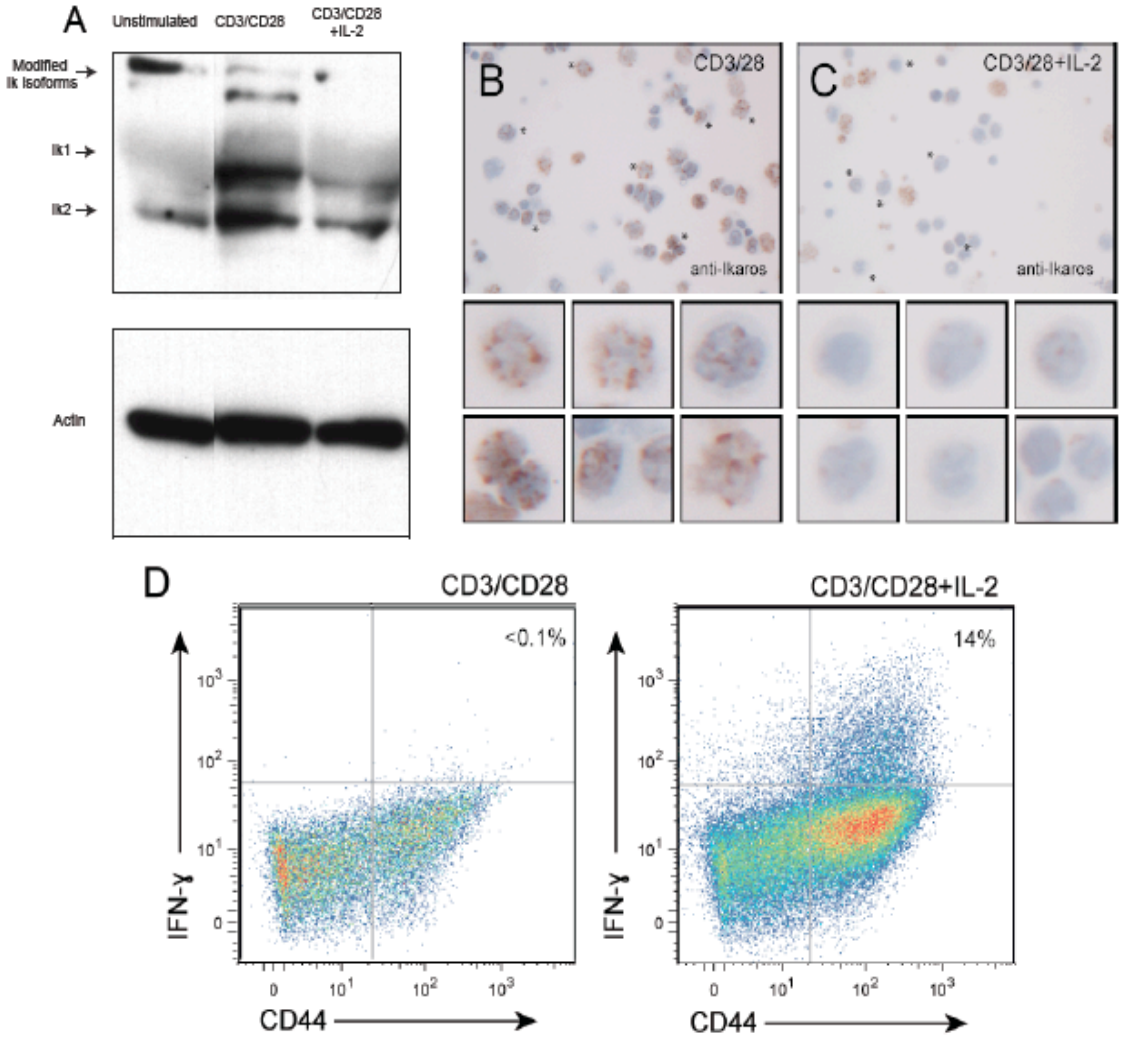


Figure 3: Activation- and cytokine-dependent Ikaros expression in CD8+ T cells.

Cells from RAG1-/- OT-1 mice were stimulated with plate bound anti-CD3 and anti-CD28 antibodies (1.0 µg/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, 0.33x106 cell equivalents), and probed with antisera against Ikaros (top panels) or actin (bottom panels). The predominant Ik1 and Ik2 isoforms are indicated.

Alternatively, stimulated cells were centrifuged onto glass slides and subjected to immunohistochemical staining for Ikaros (B and C). Cells marked with an asterisk are highlighted in individual panels at higher magnification. Anucleate, apoptotic cells show a low level of background reactivity. Data are representative of 3 independent experiments. D. Naïve-enriched RAG1-/- OT-I CD8+ T cells were stimulated with plate bound anti-CD3 and anti-CD28 (1.0, 0.5 μg/ml) 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 CD8+ (CD44hi) T cells, with numbers indicating % IFN-γ+ cells. Data are representative of 3 independent experiments.

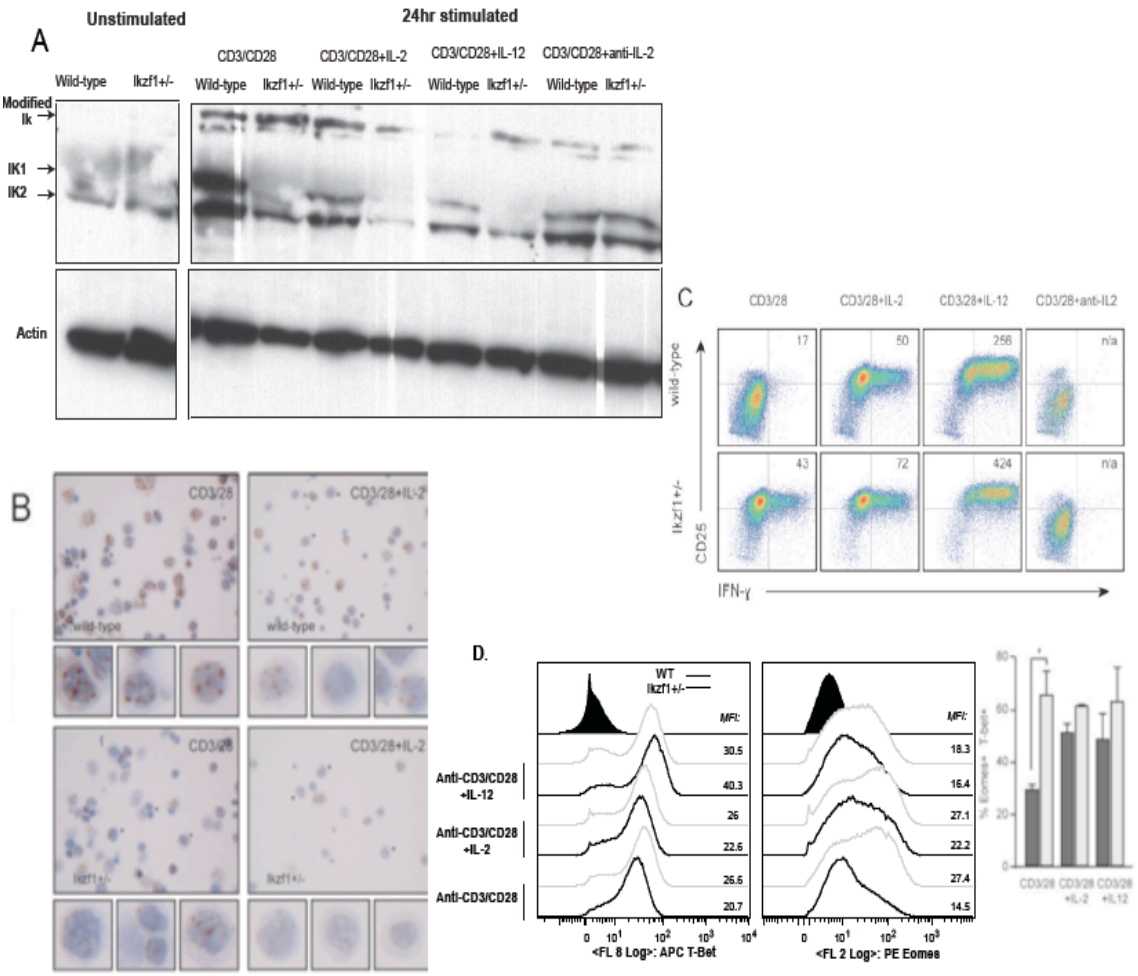


Figure 4: CD8+ T cells hemizygous for the Ikzf1 gene express less Ikaros and show reduced differentiation requirements compared to homozygous cells.

Cells from RAG1-/- OT-1 Ikzf1+/+ (wild-type) or RAG1-/- OT-1 Ikzf1+/- mice were stimulated and subjected to immunoblot (A) and immunohistochemical (B) analysis as in Fig. 3. Cells marked with an asterisk are highlighted in individual panels at higher magnification. Data are representative of 2 independent experiments. CD8+ T cells from Ikzf1+/+ or Ikzf1+/- OT-I mice were sorted by naïve phenotype, stimulated with plate bound anti-CD3/CD28 (1.0, 0.5 µg/ml) plus IL-2, IL-12 (10 ng/ml) or anti-IL-2 (10 µg/ml) for 48 hours, and analyzed for CD44, IFN-γ, and

CD25 expression in (C) by flow cytometry as in Fig. 3D. Data are representative of 2 independent experiments. Naïve sorted polyclonal CD8+ T cells (CD62Lhi CD44lo) from wild-type (black line) and Ikzf1+/- (grey line) mice were stimulated in Fig. 2C although without the anti-IL-2 condition, and assessed for CD44, T-Bet and Eomes expression by flow cytometry. Solid black histograms depict FMO control. Data are representative of 2 independent experiments. For the plots in D, * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$ by Student's T-test.

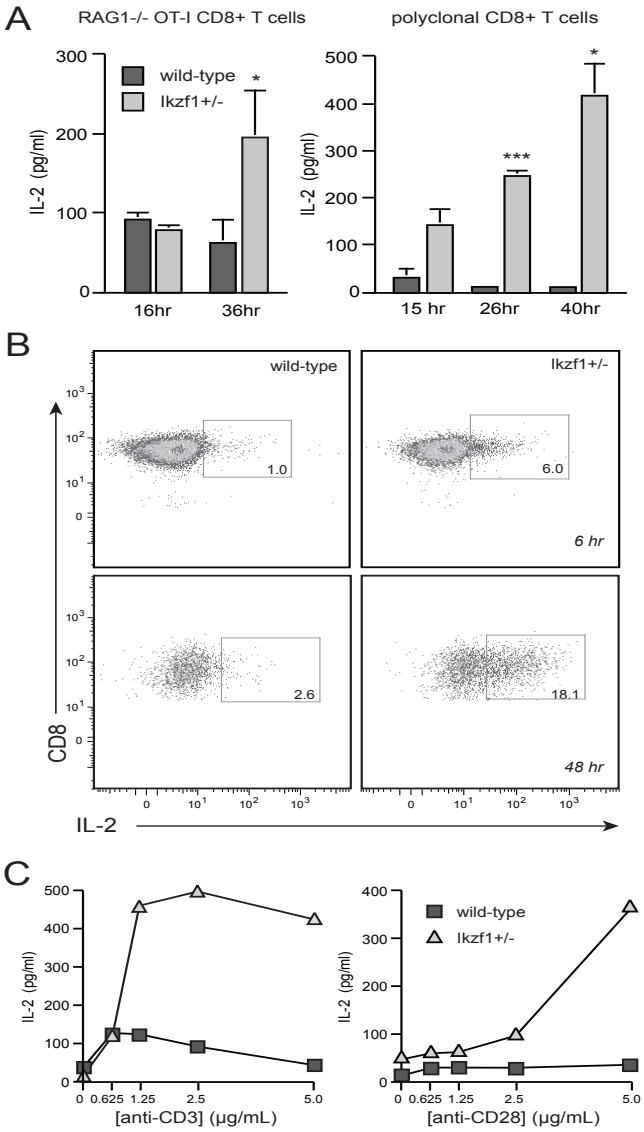


Figure 5:Ikzf1+/- CD8+ T cells produce more autocrine IL-2 than wild-type CD8+ T cells.

Naïve-enriched CD8+ T cells from Ikzf1+/+ or Ikzf1+/- B6 or OT-I mice were stimulated as in Fig. 3, and culture supernatants were assayed for IL-2 production by ELISA at the indicated time points (A) or by intracellular staining at 6 (top panels) and 48 (bottom panels) hours post-

stimulation (B). Plots in B are gated on activated cells. In C, naïve-enriched Ikzf1+/+ or Ikzf1+/- 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). Data are representative of 2 independent experiments. * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$ by Student's T-test.

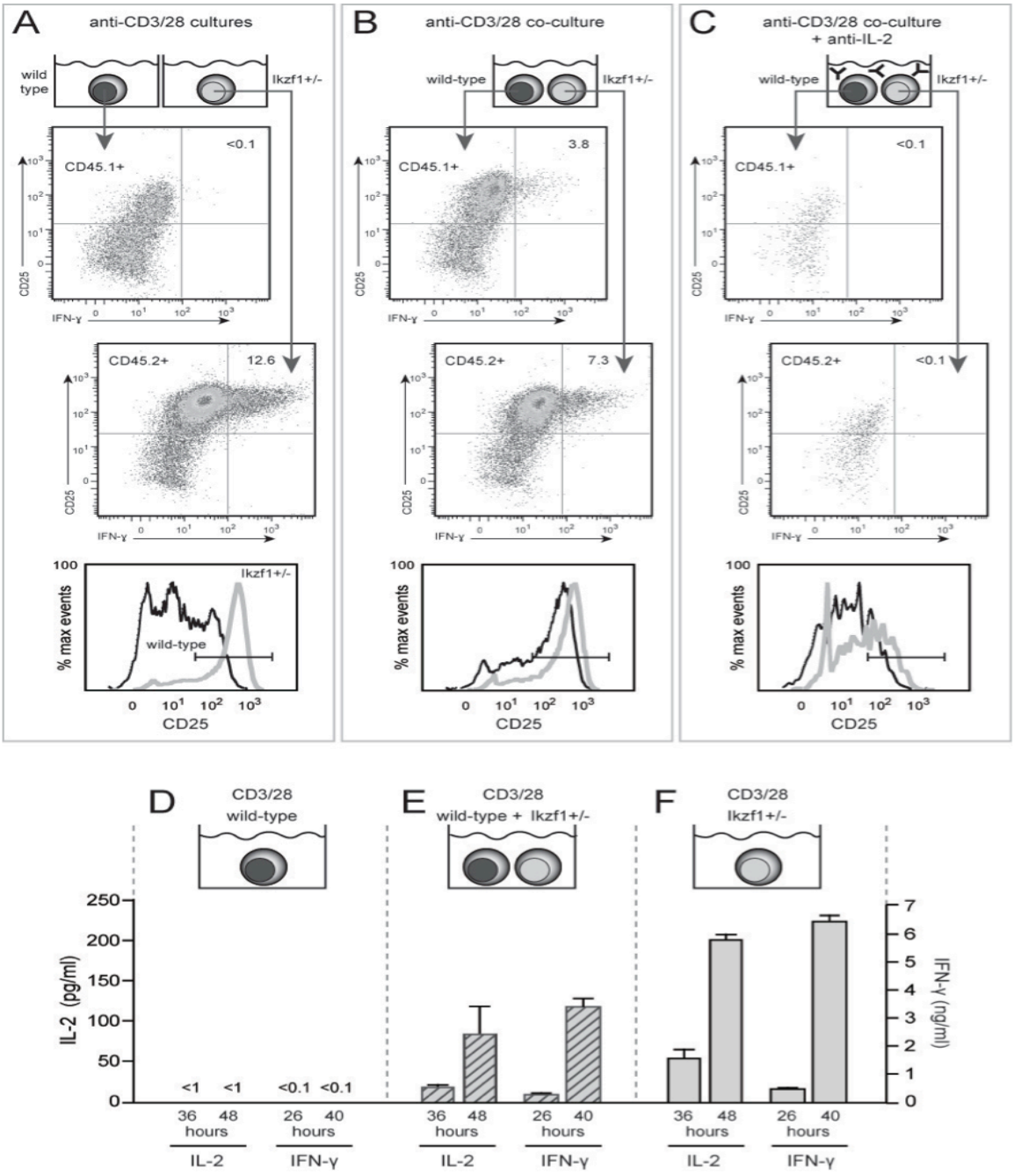


Figure 6: IL-2 produced by Ikzf1+/- CD8+ T cells can act in a paracrine fashion to induce differentiation of wild-type CD8+ T cells.

Naïve-enriched CD8+ T cells from Ikzf1+/+ (CD45.1+) or Ikzf1+/- (CD45.2+) mice were stimulated with plate bound anti-CD3 and anti-CD28 (1.0, 0.5µg/ml, respectively) in individual cultures 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 (10μg/ml). 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. * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$ by Student's T-test.

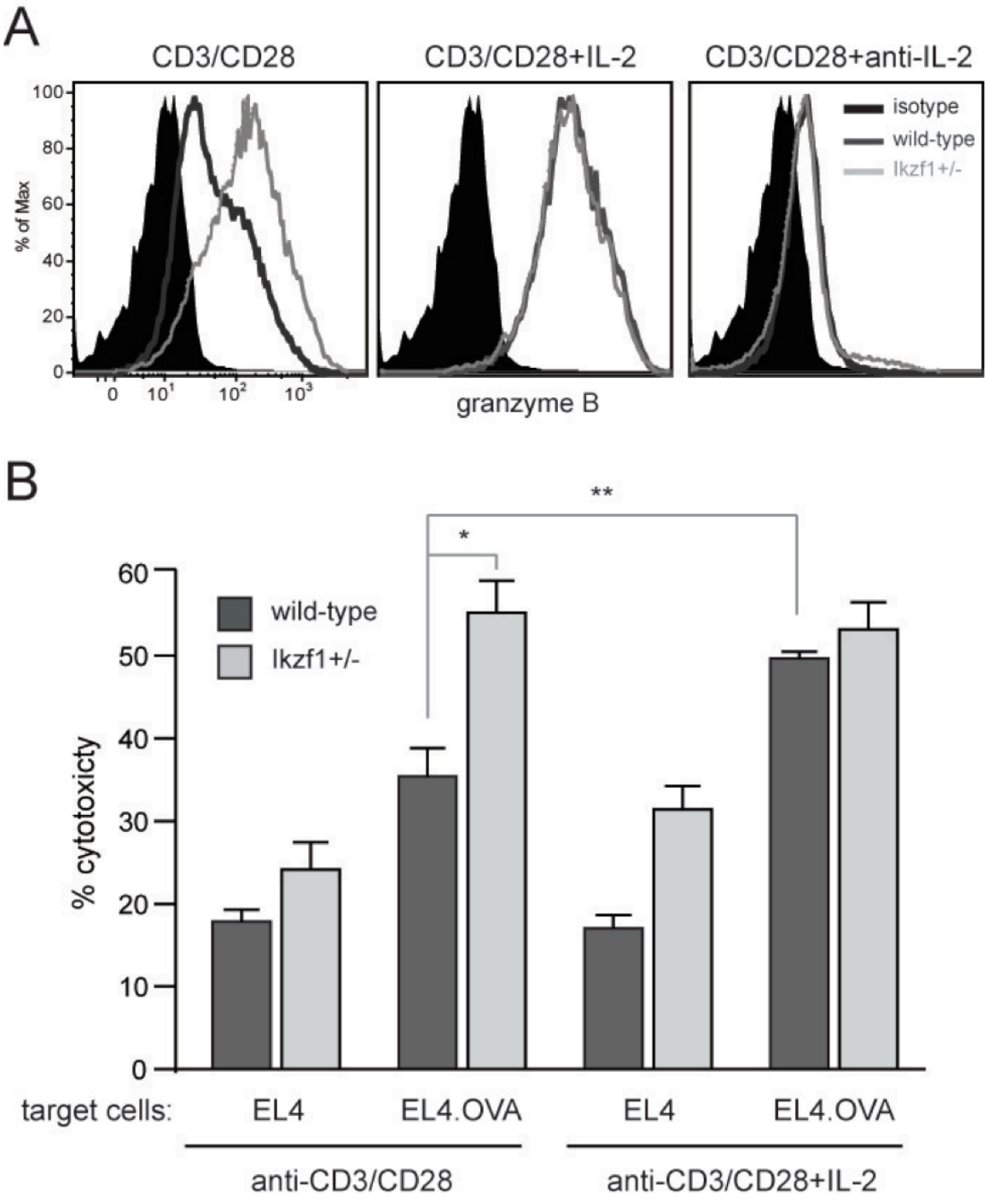


Figure 7: Loss of Ikaros function leads to enhanced cytolytic capacity by CD8+ T cells.

A. Naïve-enriched CD8+ T cells from Ikzf1+/+ (dark gray) or Ikzf1+/- (light gray) OT-I mice were stimulated with plate-bound anti-CD3 and anti-CD28 (1.0 μg/ml) for 48 hours in the presence or absence of IL-2 or anti-IL-2. PMA (30ng/ml) and ionomycin (1uM) 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. B. Ikzf1+/+ (dark gray) or Ikzf1+/- (light gray) OT-I cells were stimulated for 48 hours as in A, rested in medium overnight, then mixed at a 10:1 ratio with CFSE-labeled EL4 or EL4.OVA targets for 3 hours. Cytotoxicity against the EL4 targets was assessed by flow cytometry as described in the methods section. Data are representative of 2 independent experiments. * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$ by Student's T-test.

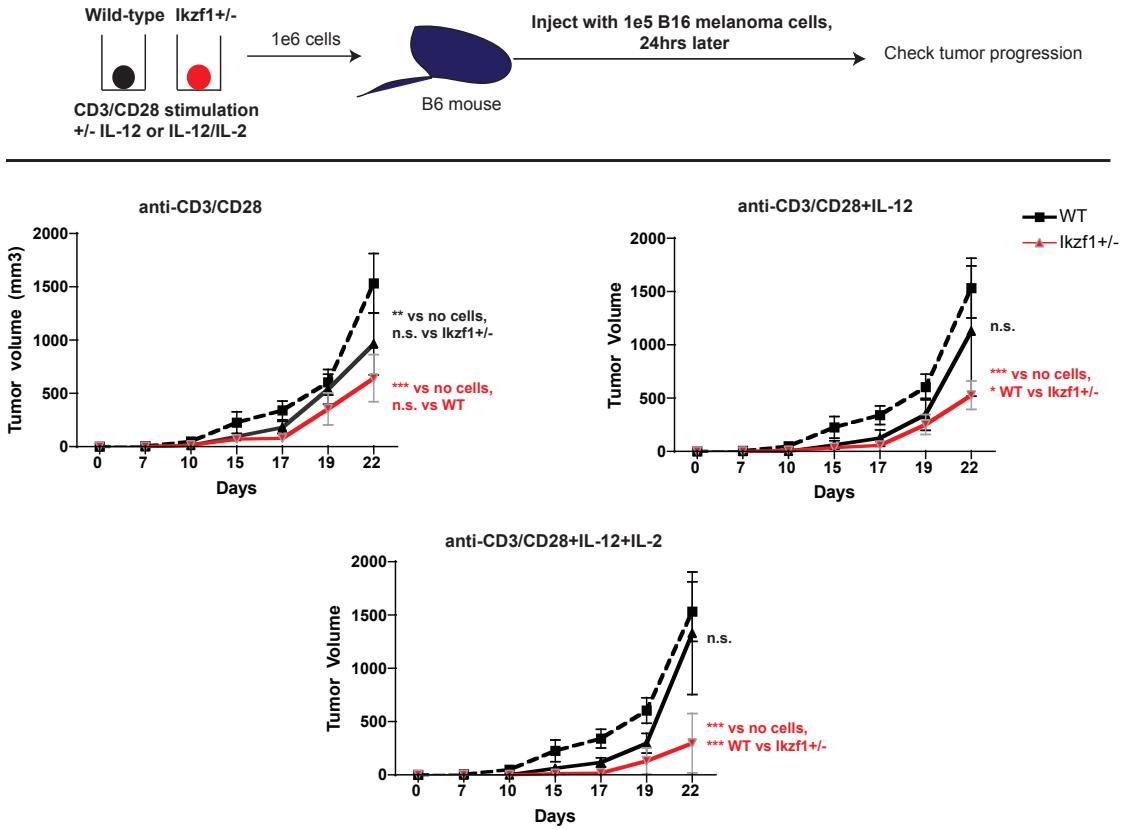
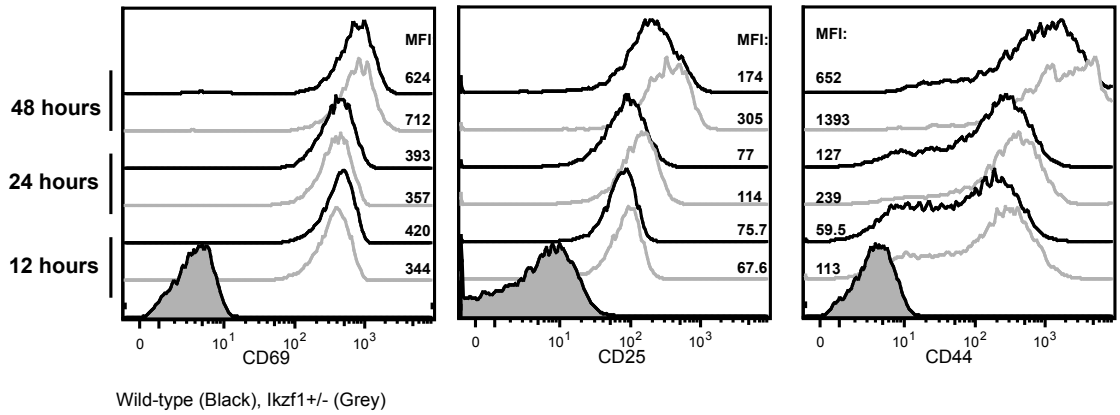


Figure 8. Self-reactive naive CD8+ T cells with reduced Ikaros are more sensitive to IL-2 and IL-12 signals and differentiate into cytolytic effectors with enhanced anti-tumor immunity.

Wild-type (Black) and Ikzf1+/- (Grey) PMEL naïve CD8+ T cells were cultured in the presence or absence of IL-12 or IL-12+IL-12 along with TCR and CD28 stimulation and adoptively transferred into B6 mice, and 24 hours later challenged with B16 melanoma. Each group had 3 mice and each mouse was injected into two sites with 1e5 B16 melanoma. Tumors were evaluated up to 22 days before mice were sacrificed. * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$ by 2-way ANOVA.

Supplemental Figures



Supplemental Figure 1: CD69, CD25 and CD44 activation are similar for Wild-type and Ikzf1+/- naïve CD8+ T cells following initial activation with TCR and CD28

Naïve wild-type and Ikzf1+/- CD8+ T cells were stimulated for 12, 24 and 48 hours with plate bound CD3/CD28 (1.0ug/ml) and then harvested for surface staining of CD69, CD25 and CD44. Representative of two experiments.

CHAPTER 3

Inhibition of Ikaros activity augments the tumoricidal capacity of CD8+ T cells expressing chimeric antigen receptors[2]

[2] Wang and O'Brien in preparation

Abstract

Adoptive transfer of T cells engineered with chimeric antigen receptors (CARs) has showed significant promise in the treatment of cancer. These engineered T cells exhibit improved efficacy in comparison to endogenous anti-tumor CTL due to improved tumor recognition and antigen binding. This is achieved through the combination of an antibody extracellular single chain fragment variable region (scFv) against a tumor antigen and an intracellular TCR signaling domain. However, issues with CAR therapy remain, such as their ability to overcome the immunosuppressive tumor microenvironment and negative T cell signaling regulators that prevent aberrant T cell immune responses. Ikaros is one such negative regulator of T cell activation, as Ikzf1+/- CD8+ T cells demonstrate enhanced *in vitro* cytolytic function due to their increased IL-2, IFN-γ and Granzyme B production. We hypothesized that the enhanced effector function of Ikzf1+/- CD8+ T cells could be utilized to improve the efficacy of CAR transduced T cells towards both tumor cells and tumor stromal fibroblasts. We demonstrate that anti-mesothelin CAR CTLs with reduced levels of Ikaros consistently lysed tumors and stromal fibroblasts, and mediated *in vivo* tumor regression better than wild-type cells transduced with the same CAR. This increased anti-tumor activity was accompanied by increased levels of IFN-γ, Granzyme B and TNF-α, which characterize the activity of fully differentiated anti-tumor CTLs. Thus, inhibition of this negative regulator of T cell activity has resulted in increased efficacy against tumor cells and tumor stroma, and may provide a new approach for improving the efficacy of adoptively transferred CAR CTLs to treat cancer.

INTRODUCTION

In order to have an effective anti-tumor immune response, a CD8+ T cell has to overcome many different obstacles. As many cancers are recognized as "self" through their expression of tumor associated antigens (TAA) (111), most T cells fail to recognize the cancerous cell. Additionally, through negative selection mechanisms in the thymus, most high avidity self-reactive T cells are deleted in order to prevent autoimmunity (112), and any self-reactive T cells that do escape typically have low avidity TCRs (113) (114). Thus, a pool of self-reactive CD8+ T cells can exist in the periphery, but due to central tolerance mechanisms, these cells have poor reactivity to the tumor.

One new approach to overcoming these hurdles is through the advent of chimeric antigen receptors (CARs). This new technology fuses together an extracellular tumor antigen directed antibody-derived single chain fragment variable (scFv) region with an transmembrane CD8α hinge region and intracellular TCR and costimulatory signaling domains such as 4-1BB (115). This construct bypasses the issues of low avidity TCRs, removes MHC Class I restriction and allows for targeting of a wider array of cancer-associated antigens. CAR therapy has advanced into the clinic and led to some recent clinic successes with B cell lymphomas (116) (117).

While advances in CAR therapy have brought improved anti-tumor efficacy, there are still hurdles to overcome. CAR-engineered T cells need to overcome both intrinsic negative regulators of T cell signaling such as diacylgylcerol kinases (118), and negative co-stimulatory proteins(such as PD-1(119, 120), CTLA-4(121), TIM-3(120) and BTLA(122)) as both contribute to the dampening of the T cell immune response. Tumor infiltrating lymphocytes typically express some combination of these negative co-stimulatory receptors and engagement with their respective ligand results in the inactivation of TILs. These TILs express less IL-2, TNF-α and IFN-γ(120), and as a result are less efficient at tumor control(121). This decrease in cytokine production could be linked to epigenetic changes at the cytokine gene locus, although presently there are few reports demonstrating that TILs have increased repressive marks at cytokine genes(123). However, TILs that are activated in the absence of CD4+ T cells in the tumor microenvironment

(110), are also similar to “unhelped” effector cells that also produce less IFN-γ than helped counterparts, due to expressing more repressive chromatin markers at the IFN-γ locus (20). Thus, by targeting transcription factors that epigenetically modify the IL-2 and IFN-γ loci, it could be possible to reverse the loss of cytokine gene expression in CAR-engineered tumor infiltrating lymphocytes, bypass the function of negative regulators, and thus improve tumor immunotherapy.

One such negative regulator is Ikaros, which is a zinc finger DNA binding protein that negatively regulates gene expression through the recruitment of chromatin remodeling complexes, such as Sin3A(59), CtBP(60) and HDACs(58). Ikaros has been characterized as a negative regulator of IL-2 production in both CD4+(19) and CD8+ T cells (Chapter 2). T cells that express dominant negative isoforms of Ikaros (IkDN+/-)(52)have less repressive chromatin marks on the IL-2 and IFN-γ loci(86) and produce more of these cytokines upon stimulation(19, 86). Similarly, activated naive Ikzf1+/- CD8+ T cells produce more autocrine IL-2 and can differentiate into effectors with enhanced cytolytic function *in vitro* due to increased IFN-γ and Granzyme B production (Chapter 2). This enhanced cytolytic function in Ikzf1+/- CD8+ T cells makes Ikaros an attractive target to inhibit for tumor immunotherapy. Thus, I hypothesized that introducing a CAR construct into T cells with reduced Ikaros expression would improve their ability to differentiate into CTLs with enhanced lytic function.

In this chapter, I demonstrate that the ability of CAR-transduced T cells to respond to antigens expressed by tumors and the tumor stroma is enhanced in T cells with reduced Ikaros (IkDN+/- and Ikzf1+/-), and that these CAR-engineered Ikaros-deficient T cells have improved CTL function *in vitro* and *in vivo*. Thus, targeted inhibition of this negative transcription regulator, Ikaros, in CD8+ T cells may provide a new avenue for improving the efficacy of CAR therapy.

MATERIALS AND METHODS

Mice, Antibodies - Wild-type CD45.2, were purchased from JAX. Ikaros DN+/- and Ikzf1+/- mice were a kind gift of Dr. Katia Georgepolous, and were backcrossed on a B6 background for greater than 12 generations. All procedures were approved by The Children's Hospital of Philadelphia Research Institute animal use and care committee.

T cell transductions- cDNAs for a mesothelin CAR or a FAP CAR, which consists of an antibody-binding region for human mesothelin or mouse FAP fused to a CD8 transmembrane domain, CD3z, and 4-1BB costimulatory domains, were subcloned into a MIGR retrovirus. This vector expresses GFP. Transfections were conducted with Lipofectamine 2000 (Invitrogen) and infectious particles were collected from transfected 293T with the MIGR retrovirus and helper plasmids. T cells were purified from single cell suspensions of wild-type, Ikzf1+/- and IkDN spleens as suggested by the manufacturer (Miltenyi Biotec). Cells were stimulated with plate bound anti-CD3 (1 mg/ml) and anti-CD28 (2 mg/ml) in 100U/ml IL-2 at 4e6 cells/well in 24 well plates. After 48 hours, 1e6 cells/well were mixed with retrovirus (1ml crude viral supernatant) in 24 well plate coated with Retronectin (50 mg/mL; Clontech) and centrifuged without braking at room temperature for 30 minutes at 1,200 g. After overnight incubation, cells were expanded with 100 U/mL of IL-2 for 7 days. After 7 days, transduction efficiency was checked for %GFP+ expression by flow cytometry on a Beckman Coulter CyanADP.

Bead Preparation- BSA or human mesothelin (1 or 5ug, RayBiotech, #230-00043) were chemically cross-linked to tosylactivated 4.5mm Dynabeads (Invitrogen #140-13) as previously described(124)

In vitro Cytotoxicity and IFN-γ ELISA Assays- T cells were co-cultured with luciferase-expressing mouse mesothelioma cell line AE17meso or the parent cell line, AE17, at different effector to target ratios for 18 hours, in triplicate in 96 well round bottom plates. Cell lysis was determined by lucerifase release as previously detailed(124). Supernatants were also characterized for IFN-γ by

ELISA kit (Biolegend). This assay was also conducted with NIH 3T3 cell lines that were engineered to express mouse FAP.

In vivo tumor models- For the Winn's Assay, T cells were mixed at the effector:target ratio of 0.5:1 with the AE17meso or AE17 and injected into the right flank of B6 mice. Tumor volume was assessed by calipers and over a 16-day period until mice were euthanized. For established tumor model, B6 matched mice were injected with 2e6 AE17meso cells. 7 days later, mice were injected after tumors grew to 100mm3. Mice received via tail vein injections 10e6 CAR-engineered wild-type or Ikzf1+/- T cells. Tumors were measured by calipers and assessed for additional 10 days. Tumor volume was calculated by the formula 0.52(a^2)(b), with a representing the minor axis and b representing the major axis.

Flow cytometric assays- Fluorochrome conjugated antibodies against anti-mouse IFN-γ (XMG1), anti-mouse CD25 (PC61), anti-mouse IL-2 (JES6-1A12), anti-mouse CD8 (53-6.7), 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 antibody to anti-mouse Granzyme B (NGZB) was purchased from eBioscience. Fluorochrome antibodies to anti-mouse TNF-α (MP6-XT22) and anti-mouse CD69 (H1.2F3) were purchased from BD Biosciences. For intracellular cytokine staining, cells were treated with Golgi Stop (BD Biosciences, 0.66ug/ml) for 4-6 hours. 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, and then re-suspended in FACS Buffer.

RESULTS

To study how reduced Ikaros expression in a CAR-engineered T cell could enhance their anti-tumor effect, we utilized the Ikzf1+/- mouse model. Ikzf1+/- T cells express approximately 50% less Ikaros protein than wild-type TCR and CD28 stimulated T cells (Chapter 2) (53) and also develop an intact hematopoietic systems in comparison to nullizygous mice (51) and avoid lymphoma development like the Ikaros dominant-negative system(52).

As Ikaros is a negative regulator of CD8+ T cell function (Chapter 2), we sought to adapt them to our CAR transduction system to test if they could improve the efficacy of CAR therapy. We initially tested them with an anti-human mesothelin CAR, which has a antibody derived soluble chain Fragment variable (scFv) region to mesothelin, which is typically overexpressed in human mesotheliomas, pancreatic and ovarian cancers(125). This extracellular domain is coupled to a CD8α transmembrane domain and to intracellular 4-1BB and CD3ζ signaling domains.

As CTLs produce IFN-γ and lytic mediators such as Granzyme B and TNF-α, we assessed for any differences in production of these factors by the wild-type and Ikzf1+/- CD8+ T cells. Additionally, we also assessed their ability to respond to low and high amounts of antigen availability by mixing the T cells with anti-mesothelin coated beads with 1 or 5ug of mesothelin. As a positive control, cells were also stimulated with PMA/I. With the wild-type T cells, they expressed similar frequencies of IFN-γ regardless of the amount of mesothelin antigen present (Fig 9A). The wild-type cells also expressed similar levels of Granzyme B and TNF-α regardless of the amount of antigen they were presented with (Fig 9A). In contrast, the Ikzf1+/- T cells produced more IFN-γ, Granzyme B and TNF-α at low dose of mesothelin coated beads in comparison to the wild-type T cells. At the higher dose of mesothelin-coated beads, the Ikzf1+/- continued to produce more of these factors in comparison to wild-type. Thus, the Ikzf1+/- T cells produce more lytic mediators at low dose of antigen in comparison to wild-type T cells.

To test if the increased IFN-γ, Granzyme B and TNF-α of Ikzf1+/- T cells could contribute to enhanced lysis, cells were mixed at different ratios with the parental cell line, AE17 or the mesothelin expressing cell line, AE17meso. These cell lines also express luciferase, which was

utilized as readout of cytolytic function. With the parental cell line, both the wild-type and Ikzf1+/- T cells failed to produce IFN-γ or lyse cells in response to AE17 (Fig 10A and B). In the presence of AE17meso, the level of IFN-γ production and lysis by the wild-type T cells declined as the number of wild-type cells to target cells were titered down (Fig 10A and B). In contrast, at high effector to target ratios the Ikzf1+/- T cells produced more IFN-γ and had increased lysis than wild-type and continued to do so at even very low effector to target ratios (Fig 10A and B). Thus, the observed increases in IFN-γ, Granzyme B and TNF-α contributed to increased cytolytic function *in vitro* by the Ikzf1+/- T cells.

To further demonstrate that reducing Ikaros levels in effector CTLs could result in enhanced effector function, we also utilized the Ikaros DN+/- system. These mice are heterozygous for a deletion in exon 3-4 of Ikaros, and result in truncated Ikaros isoforms that act in a dominant negative fashion on Ikaros(52) and reduce Ikaros at levels greater than the Ikzf1+/- T cells. In utilizing the same *in vitro* cytotoxicity assay with anti-human mesothelin CAR engineered wild-type and Ikaros DN T cells, the Ikaros DN+/- T cells also demonstrated greater levels of IFN-γ production and increased lysis in comparison to their wild-type counterparts (Fig 11A and B). Thus, greater reductions of Ikaros activity continued to result in enhanced production of IFN-γ and cytolytic function.

To verify that these in vitro results could be applied *in vivo*, we utilized two *in vivo* models. In the Winn's Assay, the wild-type T cells failed to provide much delay in AE17meso tumor growth and the tumors grew appreciably (Fig 12A). In contrast, the Ikzf1+/- T cells were able to provide more durable tumor delay. To further follow up on these results, these set of T cells were also adoptively transferred into mice with established AE17meso tumor. Following T cell transfer, the wild-type cells provide minimal tumor delay and tumor volume began to increase 10 days post-transfer (Fig 12B). In contrast, the adoptively transferred Ikzf1+/- T cells were more effective at delaying tumor growth, as the tumor volume remained stable and did not increase appreciably as with the wild-type cells post-transfer (Fig 12B). Thus, in two *in vivo* models of cancer, the Ikzf1+/- transduced T cells displayed better anti-tumor immunity than their wild-type counterparts.

The tumor microenvironment is composed of multiple cell types that produce pro-angiogenic factors (126) and thus represent a logical target to inhibit tumor growth. One such cell type of interest is tumor stromal fibroblasts, which express the fibroblast activation protein (FAP), and is typically expressed in most tumors. Thus, we also decided to test if an anti-FAP CAR in Ikzf1+/- T cells could have an additive anti-tumor effect, just as we demonstrated with the anti-mesothelin CAR transduced T cells.

In *in vitro* assays with a FAP-expressing 3T3 cell line, the wild-type T cells produced minimal levels of IFN-γ production (Fig 13A). Additionally the wild-type cells were only efficient at lysis with high effector to target cell ratios (Fig 13B). In contrast, Ikaros deficiency resulted in these transduced T cells producing more IFN-γ and increased cytolytic function even at lower effector to target ratios (Fig 13A and B). Thus, reduced Ikaros in T cells expressing a FAP CAR results in their enhanced effector function *in vitro*. To determine if targeting FAP-expressing cells *in vivo* could result in diminished tumor growth by AE17meso tumors, mice with established AE17meso were injected either with wild-type or Ikzf1+/- transduced anti-human FAP CAR T cells. Mice receiving wild-type transduced cells provided minimal tumor delay and the AE17meso tumors continued to grow. In contrast, the Ikzf1+/- transduced T cells were able to delay tumor growth (Fig 13C), and demonstrate that targeting the tumor stroma could prevent their pro-angiogenic properties that are necessary for tumor growth.

DISCUSSION

CAR therapy represents a new therapeutic avenue in the field of cancer immunotherapy. It represents a major step forward through improving the binding affinity of a T cell to a non-MHC I restricted tumor antigen and also removing the requirement for co-stimulatory molecules through utilizing intracelluar co-stimulatory signaling domains in the CAR construct. This study demonstrates for the first time that targeting of a negative transcriptional regulator, Ikaros, in CAR transduced T cells results in enhanced anti-tumor activity *in vivo*. Thus, Ikaros inhibition could represent a new therapeutic target for CAR therapy.

Revitalizing TILs in the tumor microenvironment represents a major goal of cancer immunotherapy and has been targeted in many different ways. As TILs up-regulate negative ligands such as CTLA-4(127), PD-1(119, 120), TIM-3(120), or BTLA(122), these cells typically develop a hypofunctional state and lose their ability to produce key effector cytokines and lytic products. Strategies to restore TIL function have involved the use of antibodies to these inhibitors or their respective ligands and have resulted in the restoration of effector function to this population of cells. However, while blockade of these inhibitors results in better anti-tumor immunity, there have also been instances in which these T cells cause autoimmunity (127-129). Another approach to revitalize TILs has been through the targeting of intrinsic T cell signaling regulators. Improved CAR efficacy has been demonstrated upon inhibition of diacylglycerol kinases(124), as T cells lacking DGK-α/ζ activity no longer inhibit the Ras signaling pathway.

In this chapter, I report on a new approach to improving T-cell mediated immunotherapy, through targeting of Ikaros, a transcriptional repressor involved in T cell differentiation. In CD4+ T cells, Ikaros regulates the Th1 and Th2 differentiation pathway through tight regulation of T-Bet expression in Th2 polarized cells. In Th2 polarized Ikaros-deficient CD4+ T cells, the cells take on a Th1 phenotype through being able to produce IFN-γ and express the Th1-transcription factor T-Bet(86). Likewise in Ikzf1+/- naive CD8+ T cells, these cells only require TCR and CD28 co-stimulation as their increased autocrine IL-2 production promotes their ability to differentiate into CD25hi, Eomes+, IFN-γ, Granzyme B+ effector cells (Chapter 2) with enhanced effector function. Additionally, this negative repressor has been associated with the direct repression of IL-2 in T

cells(19), and T cells with reduced Ikaros also exhibit more permissive chromatin marks at the IFN-γ locus(86). Finally, Ikaros is an ideal candidate to target for restoration of T cell effector function, as CD4+ T cells deficient in Ikaros are resistant to anergy (19) and CD8+ T cells no longer require Signal 3 cytokines (3) for CD8+ T cell effector differentiation (Chapter 2). Thus, T cells with reduced Ikaros expression are able to differentiate into T cells with enhanced effector function and bypass the typical requirements for appropriate differentiation.

In the context of priming T cells in the tumor microenvironment, utilizing CD8+ T cells with reduced Ikaros presents added advantages. The tumor microenvironment is devoid of inflammatory cytokines to mediate CD8+ T cell differentiation and may not have appropriate CD4+ T cell help present for CD8+ T cell differentiation, however Ikzf1+/- CD8+ T cells can overcome these issues as they can be activated in the absence of these factors (Chapter 2). Due to chronic antigen being present in the tumor microenvironment, the TILs upregulate negative co-stimulatory ligands and upon their engagement, results in decreased IL-2, TNF-α and IFN-γ, and possibly due to increased repressive marks(123). By targeting Ikaros activity in CD8+ T cells, this hyporesponsive state can possible be reversed as Ikaros deficient T-cells have permissive chromatin at cytokine genes(86) and typically produce more cytokines(86)(Chapter 2). Thus, an Ikzf1+/- TIL may thrive in the immunosuppressive microenvironment due to their enhanced functionality.

As demonstrated in our *in vitro* systems, the Ikzf1+/- T cells can thrive with low levels of antigen present and produce high amounts of IFN-γ, TNF-α and Granzyme B. This is an added advantage in a tumor environment with low antigen availability, as the Ikzf1+/- T cells can produce high amounts of lytic mediators. Additionally, the high amount of IFN-γ would be expected to mediate additional anti-tumor activity, as IFN-γ has been denoted to up-regulate Class I MHC expression on the tumor(130) and thus improving its immunogenicity, recruiting macrophages to the tumor for phagocytic activity(131), anti-angiogenic activity(132) and driving STAT1 mediated function of Th1 cells(133). The increase in the lytic mediators of TNF-α and Granzyme B in our Ikzf1+/- CD8+ T cells results in increased killing of tumor targets *in vitro* and *in*

vivo, again demonstrating that a CD8+ T cell with reduced Ikaros in the tumor microenvironment will have enhanced effector function (Chapter 2).

While CAR therapy has been initially developed to target antigens expressed on cancer cells, such as CD19(116) and mesothelin (124, 134, 135), it is now expanding to include other cell types that contribute to tumor growth. CARs are now being developed towards VEGFR-1(136), and FAP-α (137). Our results with the anti-FAP CAR-engineered Ikzf1+/- T cells demonstrate that targeting the tumor stroma is a viable option for controlling tumor growth. (137). Thus, inhibition of a negative transcriptional regulator can improve the efficacy of both CAR-engineered T cells directed toward tumor antigens and non-tumor antigens.

In targeting both tumor cells and the tumor stroma, CAR-engineered T cells on an Ikzf1+/- background demonstrate enhanced anti-tumor effects *in vitro* and *in vivo.* As mesothelin is a self-antigen, and expressed in mesothelial cells that line the lung, heart, spleen, kidney and testis, (138) it is plausible that anti-mesothelin CAR-T cells could have some off-target effects. While our *in vivo* experiments have not demonstrated any toxicity, there have been reports of CAR-engineered T cells causing toxicity(139, 140). Thus, with our Ikzf1+/- CAR-engineered T cells requiring little antigen in order to promote their activation (Fig. 9), we will need to determine if Ikaros deficiency may promote any off target effects.

In conclusion, our report demonstrates that Ikaros could be a valid target for inhibition, as means to improve the efficacy of CAR expressing TILs. Inhibiting Ikaros expression to 50% levels as in the Ikzf1+/- T cells (53) or further reduced in the Ikaros DN+/- T cells demonstrates how CTLs with reduced Ikaros have enhanced effector function. One could envision studies in which CAR transduced T cells are treated with an inhibitor of Ikaros, to promote the differentiation of the CD8+ T cell into a CTL with enhanced anti-tumor activity. This may help anti-tumor CD8+ T cells to overcome the hurdles of the immunosuppressive tumor microenvironment.

FIGURES

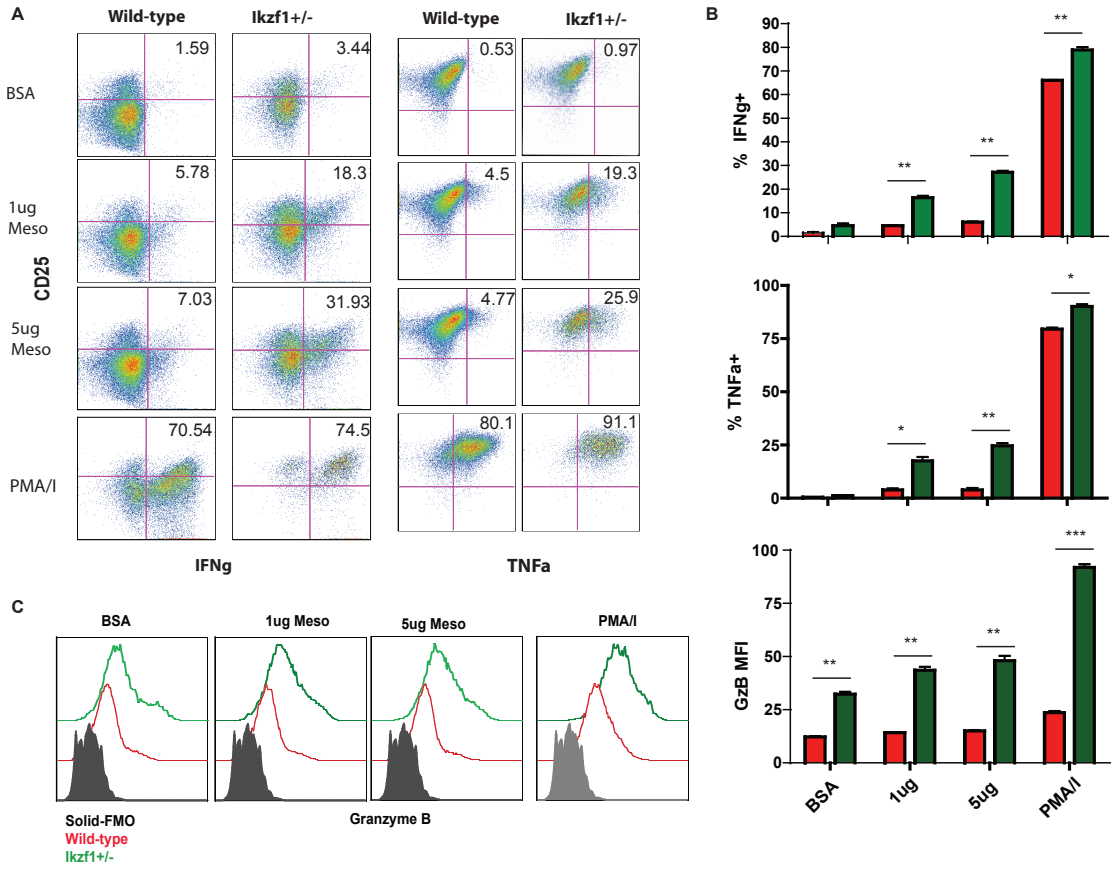


Figure 9: Ikzf1+/- T cells produce more IFN-γ, TNF-α and Granzyme B in comparison to wild-type stimulated T cells with low or high dose of antigen-coated mesothelin beads.

Wild-type and Ikzf1+/- CAR-engineered T cells were mixed with BSA, mesothelin (1ug, 5ug) coated beads or PMA/I (30ng/ml, 1nM) for 6 hours and assessed by ICS. T cells were assessed for IFN-γ (A), TNF-α (B) and Granzyme B (C) by ICS. * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$ by Student's T-test.

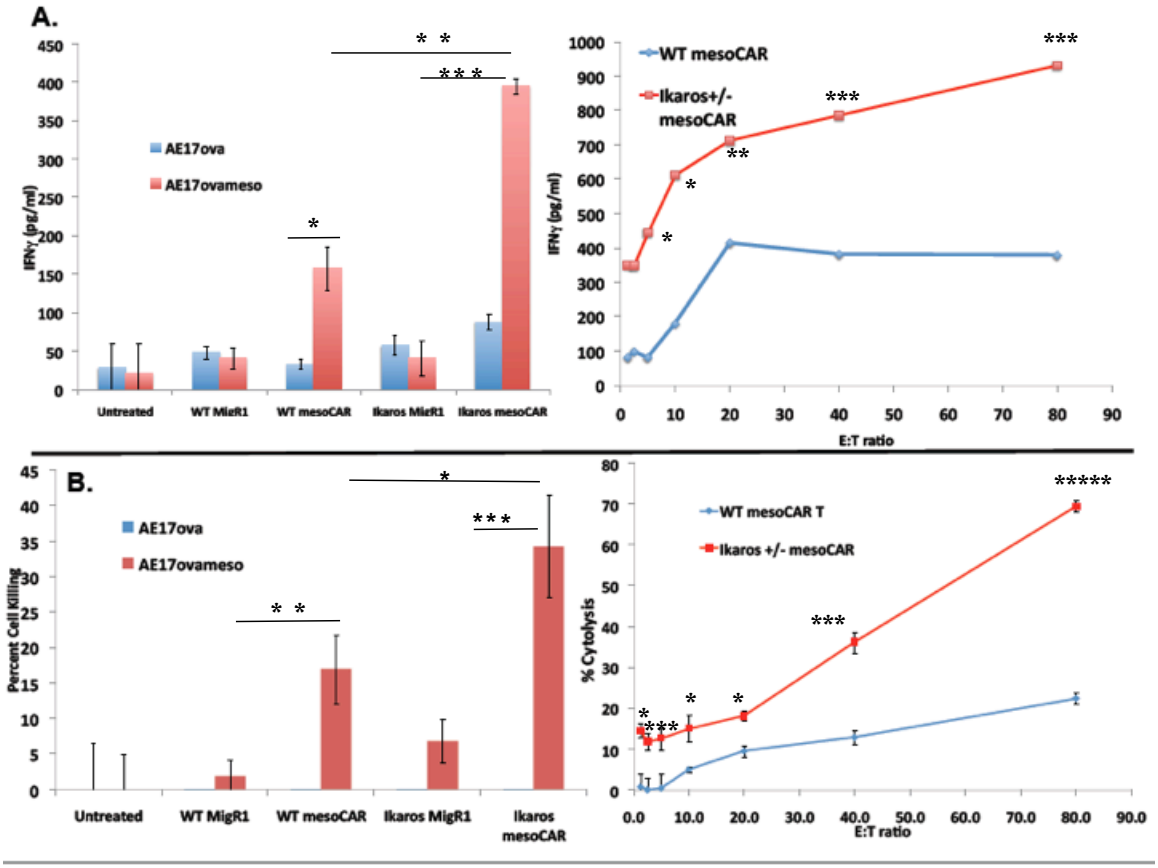


Figure 10: Ikzf1+/- T cells transduced with anti-human mesothelin CAR produce more IFN-γ and Granzyme B, and have increased cytolytic function in vitro upon challenge with antigen expressing tumor cell line.

Wild-type and Ikzf1+/- transduced T cells with anti-human mesothelin CAR were challenged with the luciferase-expressing tumor cell line AE17 or AE17meso at different effector to target rations for 18 hours and supernatants were collected for IFN-γ ELISA (A). Release of luciferase was used as a read-out for in vitro cytotoxicity (B). * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$ by Student's T-test.

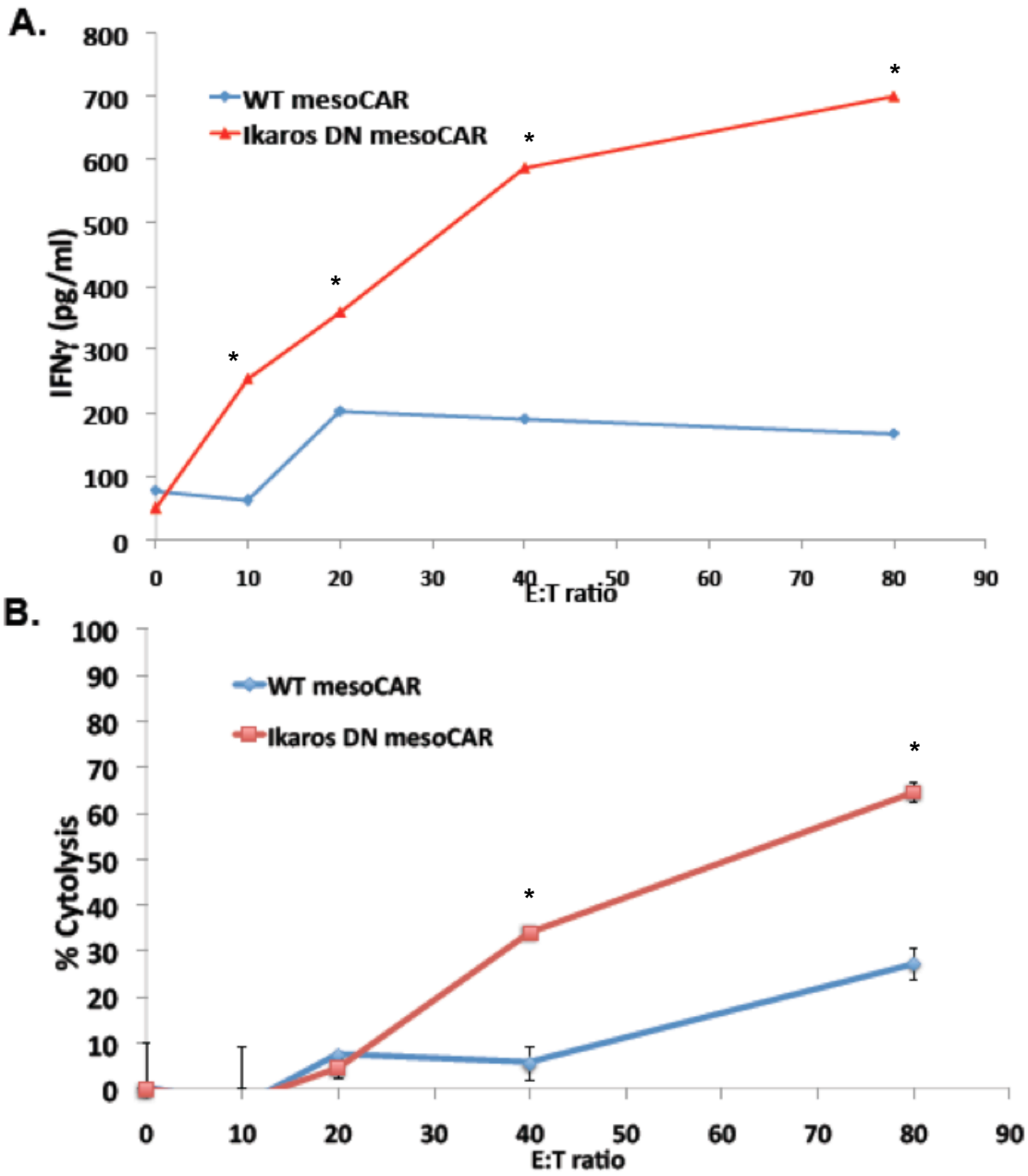


Figure 11: Anti-mesothelin CAR-engineered Ikaros DN+/- T cells have enhance cytolytic function in vitro and produce more IFN-γ than wild-type T cells

Wild-type and IkDN+/- transduced T cells with anti-human mesothelin CAR were challenged with the luciferase-expressing tumor cell line AE17 or AE17meso at different effector to target ratios for 18 hours and supernatants were collected for IFN-γ ELISA (A). Release of luciferase was used as a read-out for in vitro cytotoxicity (B). 5-10 mice per group were used. * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$ by Student's T-Test.

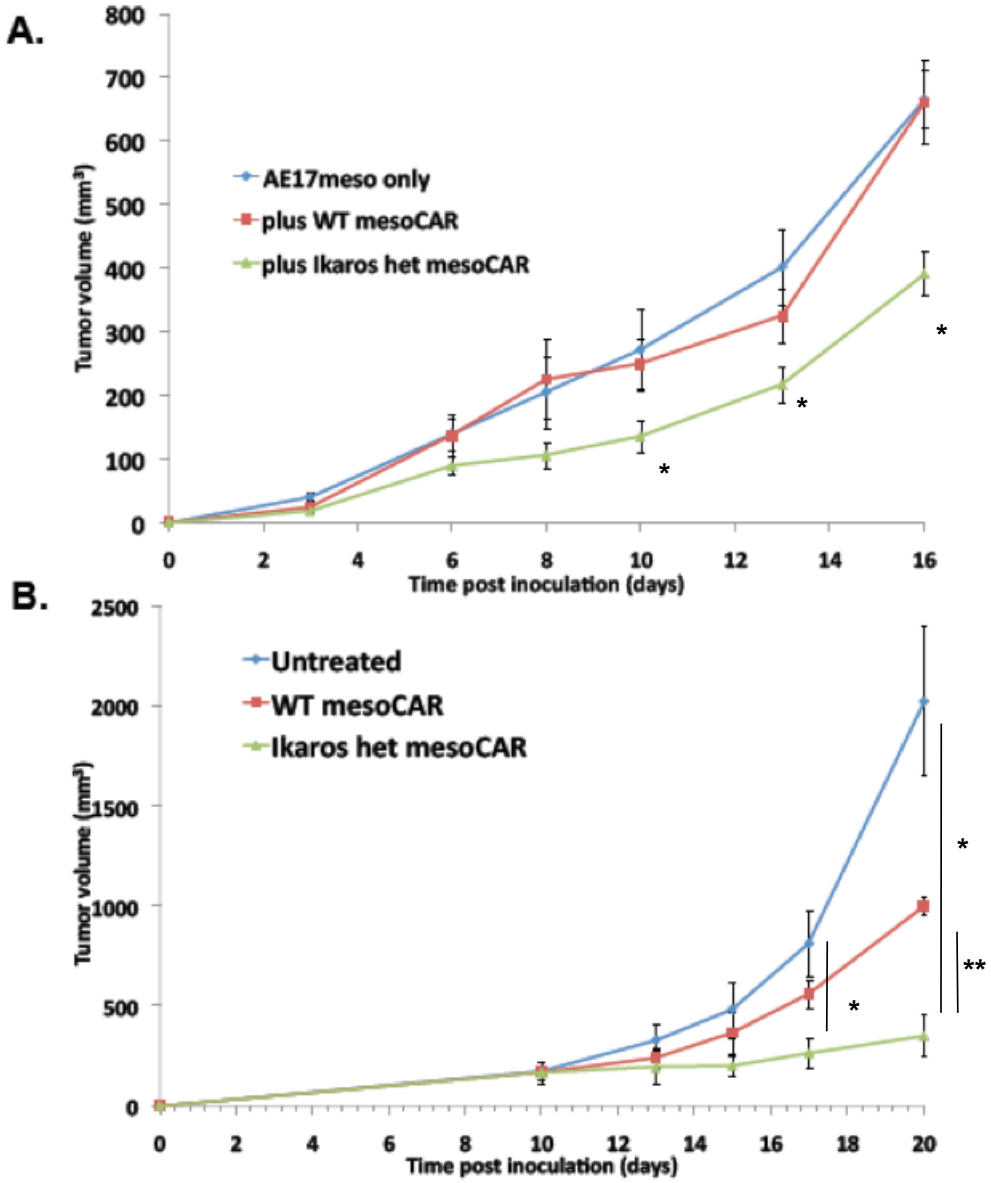


Figure 12: Ikzf1+/- T cells transduced with anti-human mesothelin CAR have enhanced anti-tumor in vivo properties in comparison to wild-type transduced T cells.

In 12A, a Winn's assay was performed by having wild-type (square) and Ikzf1+/- (diamond) transduced T cells mixed with the mesothelin-expressing tumor cell line AE17 (AE17meso) at a ratio of 0.5:1 (T cell: Target cell) and then subcutaneously injected into B6 mice. Tumor volume was measured over a 16-day period. In 10B, B6 mice were injected with 2e6 AE17meso cells and grew to 100mm3. 10 days post-injection, tumor bearing mice were separated into untreated

(diamond) or received 10e6 wild-type (square) or Ikzf1+/- (triangle) transduced anti-human mesothelin CAR T cells and tumor volume was measured following the injections. 5-10 mice per group were utilized. * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$ by Student's T-test.

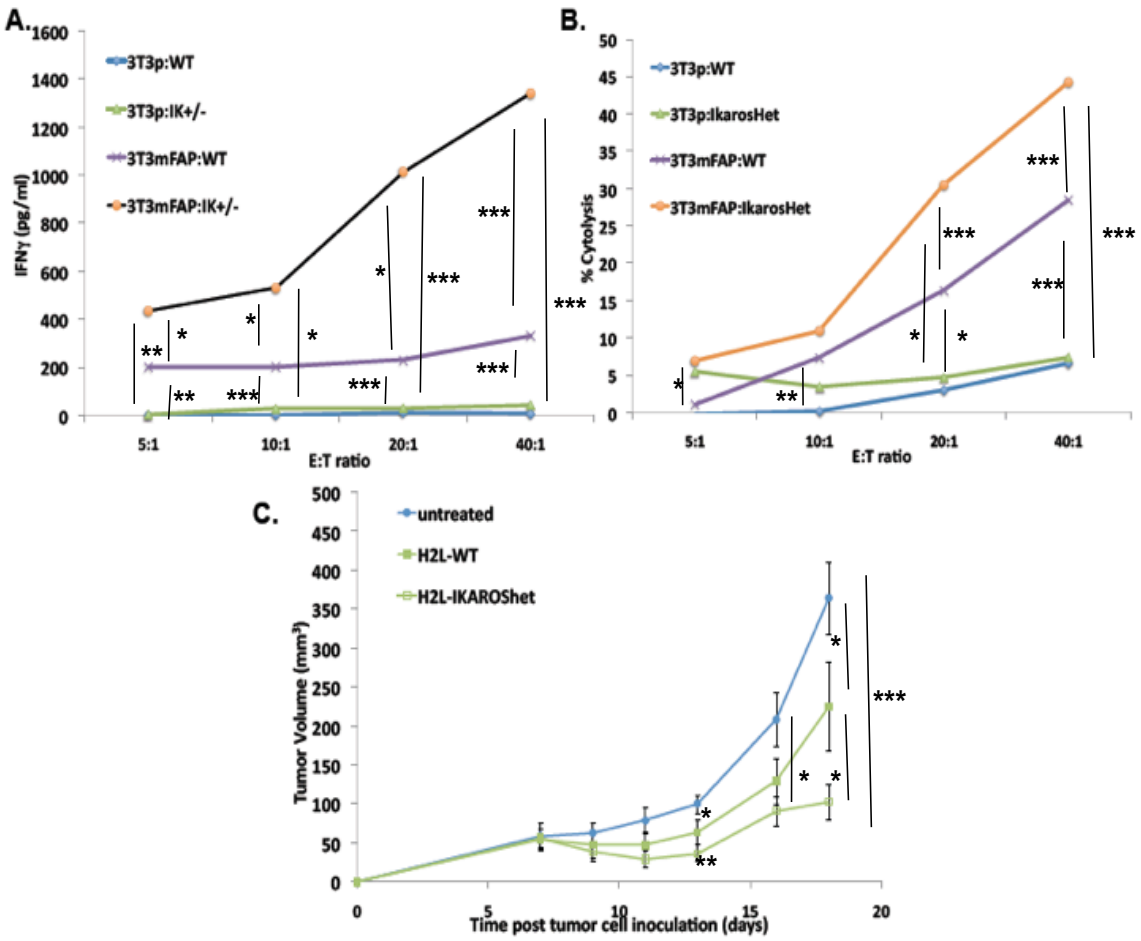


Figure 13: Ikzf1+/- transduced T cells with anti-FAP CAR have enhanced IFN-γ and cytolytic functions in vitro in comparison to wild-type transduced cells and promote tumor delay of AE17meso implanted tumors.

As in Figure 8, wild-type and Ikzf1+/- transduced T cells were assessed for their ability to produce IFN-γ and kill a 3T3 cell line expressing FAP by ELISA (A) and through a luciferase release assay (B). In C, B6 mice were injected with 2e6 AE17meso cells and after tumors grew to be 100mm3,

mice were either untreated (circle) or injected with 10e6 wild-type (square) or Ikzf1+/- transduced anti-human FAP CAR T cells. Tumors volume was measured following initial tumor dose inoculation and after T cell transfer. For tumor model, 5-10 mice were used per group. * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$ by Student's T-test.

Chapter 5

Future Directions

In this chapter, I highlight some preliminary data that has interesting future implications and reveals new roles for Ikaros in the context of CD8+ T cell biology. These preliminary data outlines how naïve Ikzf1+/- CD8+ T cells can differentiate *in vitro* in response to TCR signals alone. This result is similar to how memory CD8+ T cells become activated in the absence of inflammatory signals and CD28 signals, and regulate Ikaros expression. Finally, I conclude with implications for how targeted inhibition of Ikaros could bypass the requirement for CD4+ T cell help and lead to the revitalization of TILs and exhausted CD8+ T cells.

Ikaros enforces the CD28 costimulatory requirement for naïve CD8+ T cell activation

As outlined in Chapter 2, naïve Ikzf1+/- can differentiate with TCR and CD28 signals in the absence of inflammatory cytokines and CD4 help due to their increased autocrine IL-2. As IL-2 signals can replace the CD28 costimulation requirement(141) and Ikaros enforces the CD28 costimulatory requirement for IL-2 activation in CD4+ T cells(19), I investigated if our naïve Ikzf1+/- CD8+ T cells could differentiate in our *in vitro* priming system in the presence of TCR signals alone or with anti-IL-2.

In using the same *in vitro* priming approach in Chapter 1, naive sorted polyclonal or OT-1 cells from wild-type and Ikzf1+/- mice were stimulated only in the presence of plate bound anti-CD3. In the absence of CD28 signals and exogenous cytokines, the activated wild-type cells failed to become IFN-γ producing CTLs (Fig. 14A and C). In contrast, the stimulated Ikzf1+/- CD8+ T cells could differentiate into IFN-γ producing CTLs and this differentiation program was again observed due to increased autocrine IL-2 production (Fig. 14B). These activated Ikzf1+/- CD8+ T cells from the polyclonal or OT-1 system could differentiate into IFN-γ producing CTLs with only TCR signals. This differentiation program was abrogated in the presence of neutralizing anti-IL-2.

Ikaros expression was also characterized, and in the absence of both CD28 and cytokine signals, wild-type naive CD8+ T cells up-regulated Ikaros more than wild-type CD8+ T cells stimulated with TCR, CD28 and cytokine signals (Fig 14D). However, the Ikzf1+/- naive CD8+ T

cells failed to up-regulate as much Ikaros protein expression as the wild-type cells (Fig. 14D). Thus, like in CD4+ T cells(19), Ikaros may also act to enforce CD28 co-stimulatory signals and cytokine signals for appropriate naive CD8+ T cell differentiation. These results will have to be further characterized, especially due to the effects of the increase autocrine IL-2 on Ikaros regulation and cell cycle(63) in these naïve Ikzf1+/- CD8+ T cells. Thus, appropriate Ikaros expression is essential for ensuring that naive CD8+ T cells differentiate into highly potent cytotoxic lymphocytes once appropriate TCR, CD28 and cytokine signals are received.

Ikaros is not up-regulated in memory CD8+ T cells upon re-stimulation

This finding that Ikzf1+/- naive CD8+ T cells can differentiate in the absence of CD28 and cytokines signals, is similar to memory CD8+ T cell activation. Memory CD8+ T cells quickly respond to antigen presentation in the periphery, and do not require co-stimulation or inflammatory cytokines to rapidly induce IFN-γ, Granzyme B and Perforin(142-144). The ability of memory cells to quickly up-regulate cytolytic factors implies that the chromatin around these genes is more accessible in comparison to naive CD8+ T cells, which fail to produce these factors quickly(142). Interestingly, memory CD8+ T cells have been characterized to have increased histone acetylation(20) and partial methylation of CpGs(142) of the IFN-γ locus in comparison to the hypoacetylated and fully methylated CpGs in naive CD8+ T cells, and quickly lose the remaining CpG methylation upon antigen re-stimulation(142). Chromatin marks around IFN-γ, Perforin and Granzyme B have also been characterized to be more accessible in comparison to naive CD8+ T cells(145, 146), and this results in increased mRNA transcripts for re-stimulated memory CD8+ T cells in comparison to stimulated naive CD8+ T cells(145). Thus, memory Cd8+ T cells have distinct epigenetic profiles from naive CD8+ T cells, as they need to produce lytic factors in a rapid fashion.

Due to the difference in epigenetic states of naive and memory CD8+ T cells, this hints at a possible role for Ikaros' function in memory CD8+ T cells. Ikaros has been characterized to regulate the IFN-γ(86) and IL-2 loci (19, 81), and T cells with reduced Ikaros demonstrate

increased cytokine function and more accessible chromatin around these cytokine genes (86)-similar to memory T cell function.

Additionally, our experimental data with naive Ikzf1+/- CD8+ T cells has implications for Ikaros function in memory CD8+ T cell generation. Activated naive Ikzf1+/- up-regulate Eomes upon activation, and Eomes has been implicated in both driving the effector program(90) and linked to memory CD8+ T cell formation (90, 147). The increased expression of CD25 by Ikzf1+/- CD8+ T cells *in vitro* could also influence memory formation, as CD25hi CD8+ T cells could bias toward a terminally differentiated effector phenotype at the expense of memory precursors (33, 36). Finally, the ability of naive Ikzf1+/- CD8+ T cells to differentiate in response only to TCR or TCR/CD28 signals and produce IFN-γ and Granzyme B in a rapid fashion is very similar to memory CD8+ T cell activation.

Finally, we have preliminary data about Ikaros expression in memory CD8+ T cells that may indicate its role in memory function. Sorted naive (CD62L+CD44-) and memory populations (CD44+CD62L+) from uninfected wild-type and Ikzf1+/- mice were characterized for their Ikaros expression in unstimulated or TCR/CD28 stimulated cells (Fig. 15A). As expected, in the absence of inflammatory cytokines, Ikaros was up-regulated in the activated naive population. However, the activated wild-type memory cells demonstrated less Ikaros up-regulation in comparison to their wild-type effectors (Figure 15A). Interestingly, the activated wild-type memory CD8+ T cell population also expressed similar Ikaros levels as the activated Ikzf1+/- memory CD8+ T cells. This may demonstrate that wild-type memory CD8+ T cells induce lower Ikaros levels in order to quickly activate their effector program. In re-stimulation of wild-type and Ikzf1+/- memory cells, it is readily apparent that the wild-type cells produce more IFN-γ and IL-2 in comparison to their activated effectors (Fig 15B). Additionally, the Ikzf1+/- memory cells express more IFN-γ and IL-2 than the wild-type memory cells and further demonstrate that decreased Ikaros levels in memory cells results in increased cytokine production. These findings needs to be further characterized, but it is tantalizing to speculate that wild-type memory CD8+ T cells have lower Ikaros expression in order to reduce the negative regulation that Ikaros has on the effector genes programs. Essentially, due to the need for rapid effector gene activation in memory CD8+ T cells, it is

possible to hypothesize that lower Ikaros expression would result in less epigenetic repression. As Ikzf1+/- naive CD8+ T cells can differentiate into CTLs with enhanced effector function and in similar fashion to memory CD8+ T cell re-activation, then it is plausible that there could be link to reduced Ikaros expression and the rapid re-activation of the effector program in memory CD8+ T cells.

Ikaros enforces the requirement for CD4+ T cell help

This preliminary data in conjunction with Chapter 2 and Chapter 3, demonstrate that Ikaros enforces CD4+ T cell help to gain appropriate effector differentiation. The naïve *Ikzf1+/-* CD8+ T cells have an increased ability in comparison to wild-type CD8+ T cells to differentiate *in vitro* in the absence of CD4+ T cells and the IL-2 they provide (Fig. 3, 4). Due to their increased autocrine IL-2 and their ability to only respond to TCR and CD28 signals, these Ikzf1+/- CD8+ T cells can act like IL-2 producing CD4+ T helper cells. Their increased IL-2 production can act in a paracrine fashion on wild-type naïve CD8+ T cells, and through IL-2 negative regulation of Ikaros(82) promote the differentiation of the wild-type CD8+ T cells into IFN-γ producing CTLs (Fig. 6). This again demonstrates the need for Ikaros to tightly restrict autocrine IL-2 production, to ensure that naïve CD8+ T cells produce IL-2 under appropriate circumstances and prevent paracrine IL-2 driven CD8+ T cell differentiation in a non-specific manner.

With Ikzf1+/- CD8+ T cells acting in a similar fashion as CD4+ Th cells, it is plausible that they can bypass the need for CD4+ T cell licensing of DCs(22) or CD4+ T cell derived IL-2 (10, 11, 23) and become activated in the absence of CD4+ T cell help. The increased tumor killing by Ikaros-modified CAR T cells in Chapter 3 may demonstrate this. In this model, no lymphodepletion is required to create “space” for these transferred cells, which are composed of greater than 90% CD8+ T cells. The lack of transferred CAR-specific CD4+ T cells tests the fitness of the transferred CTLs to become activated in the absence of CD4+ T cell help. Previous tumor models have demonstrated that paracrine IL-2 from CD4+ Th assists activated tumor specific CTLs (95, 110) and that lymphodepletion assists adoptive T cell transfer immunotherapy (148). Our *in vivo* tumor data demonstrate that the transferred Ikaros modified CAR T cells have

enhanced *in vivo* killing in the absence of CD4+ help and the lack of lymphodepletion. Thus, it will be interesting to determine if the enhanced killing by our Ikaros modified CAR-transferred CTLs in our tumor model have increased IL-2 production and thus bypass the requirement for CD4+ T cell help in the tumor microenvironment.

Revitalizing tumor infiltrating lymphocytes and exhausted CD8+ T cells through Ikaros modulation

Our data from the *in vivo* tumor models in Chapter 2 and Chapter 3 indicate that it is plausible that controlling Ikaros' activity represent a possible avenue for the rejuvenation of CD8+ T cell function. In the context of the tumor microenvironment, a tumor infiltrating CD8+ T cells has to overcome its low avidity TCR (113, 114), the lack of CD4+ T cell help(95, 110), having to overcome engagement with negative co-stimulatory ligands (120) (119) (122) (121), immunosuppressive cytokines and regulatory T cells (126). Additionally, a TIL activated in the absence of CD4+ help might resemble an "unhelped" CD8+ T cells (42, 44, 103) and thus have increased repressive marks at cytokine gene loci (20, 123). TILs isolated from tumors demonstrate decreased responsiveness to antigen and are rejuvenated *in vitro* through culture in cytokines (148) in order to utilize for adoptive cell transfer therapy.

As detailed in Chapter 3, Ikaros represents a viable target to restore CD8+ T cell effector function. Through utilizing anti-mesothelin CARs to overcome low TCR avidity issues(115), and expressing them in T cells that express 50-90% less Ikaros expression, these transduced T cells are able to demonstrate enhanced anti-tumor function *in vivo*. Additionally, these results are not due to differences in autocrine IL-2 production, as both wild-type and Ikzf1+/- or IkDN T cells were initially cultured in high amounts of IL-2. This indicates that Ikaros is also regulating other components of the CTL effector pathway, as the T cells with reduced Ikaros express more IFN-γ, TNF-α and Granzyme B (Fig. 9). These factors are critical for anti-tumor activity and demonstrate that by inhibition of a transcriptional repressor in CTLs, it is possible to overcome some of the challenges associated with tumor immunotherapy.

The possibility of restoring CD8+ effector function via Ikaros modulation could also have implications for CD8+ T cell exhaustion and chronic infections. The inability to clear virally

infected cells by CTLs results in the persistence of antigen and chronic infections. The persistence of antigen results in the up-regulation of multiple inhibitory receptors such as PD-1, TIM-3, 2B4, LAG-3(144) on CD8+ T cells and results in their transition to an “exhausted” state as they lose the ability to produce effector cytokines such as IL-2, IFN-γ, and TNF-α(144, 149). Thus, being able to restore these antigen-specific exhausted CD8+ T cells is especially important in the context of chronic infections like HIV. The use of anti-PD-1 antibodies has represented one method for the restoration of effector function(150, 151), however groups have also reported on the provision of CD4+ help(152) or the administration of exogenous IL-2 (4, 153) as possible methods to restore the function of these exhausted CD8+ T cells. As our Ikzf1+/- CD8+ T cells demonstrate an increased ability to produce IL-2 in comparison to wild-type CD8+ T cells following 48 hours of chronic stimulation with plate bound anti-CD3/CD28 antibodies (Fig. 5B), it is plausible that Ikzf1+/- CD8+ T cells could be resistant to the induction of “exhaustion”. Introducing a P14 transgenic TCR that recognizes the LCMV-antigen gp33 onto Ikzf1+/- CD8+ T cells (P14-Ikzf1+/-), transferring into a B6 host and then challenging with the Clone 13 strain of LCMV to induce chronic infection, would be a viable way to test this. Thus, this increased ability to produce autocrine IL-2 by Ikzf1+/- CD8+ T cells may present a possible pathway to preventing exhaustion in CD8+ T cells.

Overall, these preliminary data indicate that reduced Ikaros expression can alter the differentiation program of naïve CD8+ T cells to one that is similar to memory CD8+ T cell activation. Additionally, the requirement to quickly respond to antigen could require inhibition of Ikaros induction in memory CD8+ T cells and thus modulating Ikaros may represent a therapeutic strategy to change the effector state of the CD8+ T cell.

FIGURES

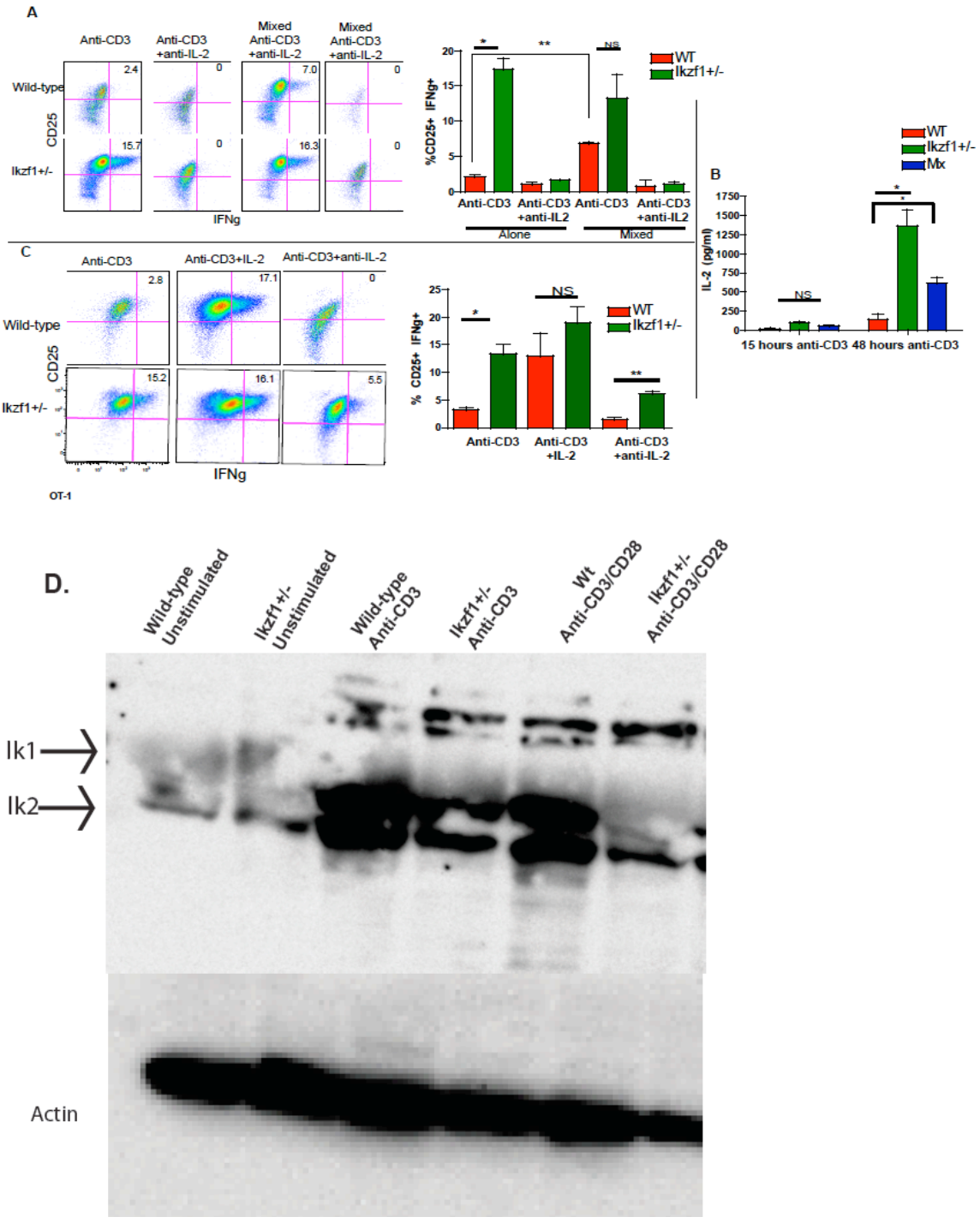


Figure 14: Naive Ikzf1+/- CD8+ T cells can differentiate into IFN-γ producing CTLs in response to only TCR signals.

Naive sorted wild-type (CD45.1) or *Ikzf1+/-*(CD45.2) CD8+ T cells were stimulated in with plate-bound anti-CD3 (0.5ug/ml) alone or mixed 1:1 and in the presence or absence of anti-IL-2 (10ug/ml) for 48 hours and assessed by ICS (A) or for IL-2 production by ELISA (B). Naive purified CD8+ T cells from wild-type and *Ikzf1+/-* RAG OT-1 mice were stimulated in the same fashion as in 13A, and also assessed by ICS for IFN-γ production (C). Ikaros expression in naive sorted wild-type or *Ikzf1+/-* CD8+ was assessed in both unstimulated or stimulations with anti-CD3 (0.5ug/ml) or anti-CD3/CD28 (0.5ug/ml) for 24 hours by Western Blot. * is $p<0.05$, ** is $p<0.01$, and *** is $p<0.001$ by Student's T-test.

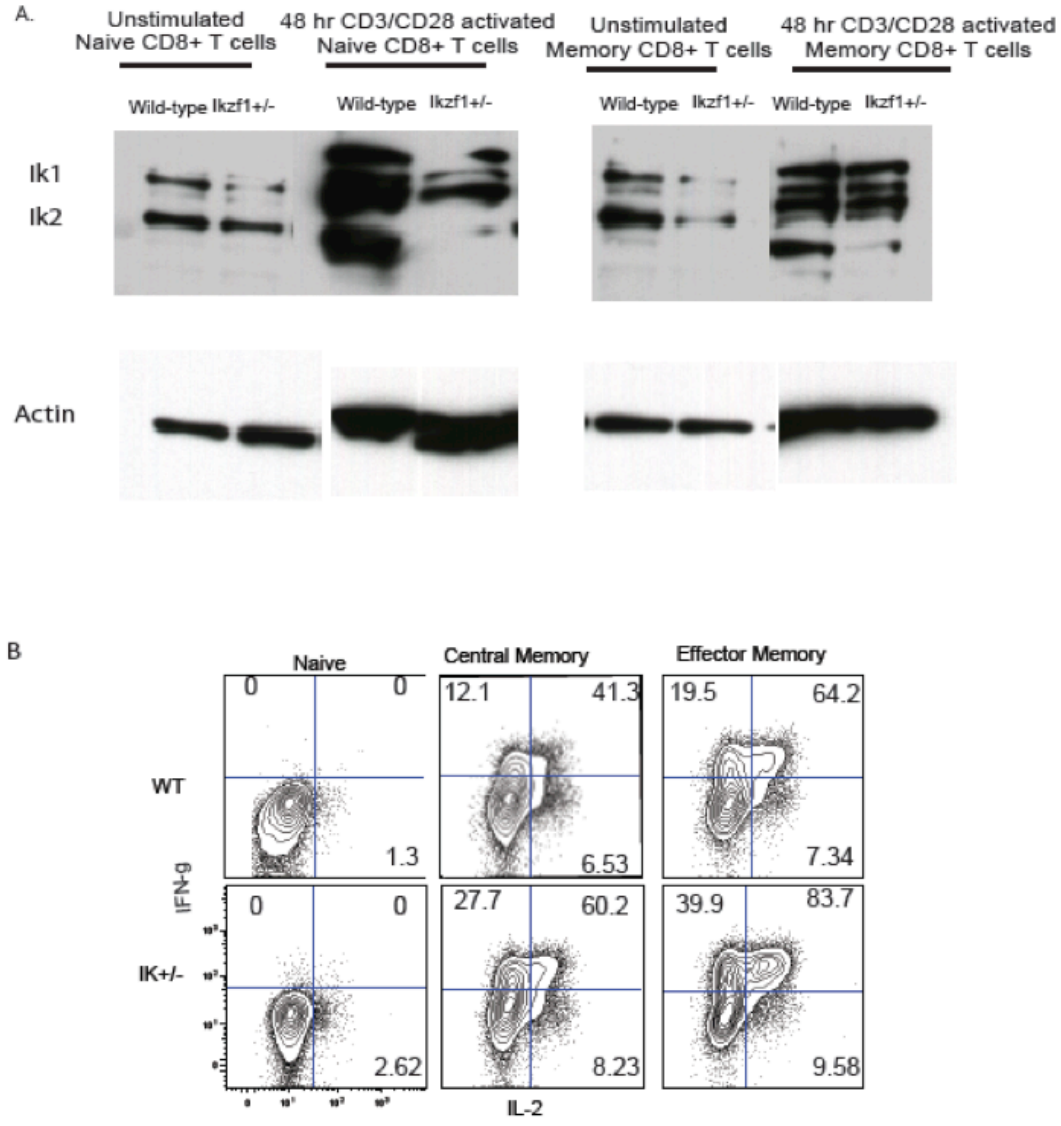


Figure 15: Activated wild-type memory CD8+ T cells express less Ikaros in comparison to TCR/CD28 stimulated wild-type effectors and produce more IFN-γ+ and IL-2 upon re-stimulation

Naive sorted cells (CD62L+CD44-) and memory CD8+ T cells (CD62L+CD44hi) cells were isolated from wild-type and *Ikzf1+/-* mice and were left unstimulated or stimulated with plate

bound anti-CD3 and anti-CD28 (1.0, 0.5ug/ml) for 48 hours. Ikaros expression was characterized by Western Blot (A) from 0.5e6 cell equivalents. Similarly, naive, effector and central memory CD8+ T cells were re-stimulated with PMA/I (30ng/ml, 1uM) and analyzed by ICS (B).

Chapter 6

Discussion: Ikaros and CD8+ T cell biology

In the context of naïve CD8+ T cells, they require TCR, CD28 and Signal 3 Cytokines (154) for their appropriate differentiation into effector cells. In our *in vitro* priming system in Chapter 2, I demonstrated how Ikaros protein levels are regulated by TCR, CD28 and cytokine signals. Naïve wild-type CD8+ T cells primed with TCR, CD28 and cytokines such as IL-2 or IL-12 (Fig. 3 and 4) failed to induce much Ikaros expression and the cells were able to differentiate into IFN-γ producing effector cells (Fig. 4). Conversely, in the absence of IL-2 and IL-12, TCR and CD28 signals resulted in high Ikaros protein induction and the wild-type cells failed to differentiate into CTLs. Thus, TCR and CD28 signals alone induce high amounts of Ikaros protein levels; possibly through TCR and CD28 mediated signals that drive Ikaros transcription (Fig. 16A).

This induction of Ikaros hints at a role for Ikaros enforcing cytokine signaling to drive appropriate naïve CD8+ T cell differentiation. With IL-2, Ikaros enforces CD8+ dependency on CD4 help from the periphery for their differentiation. In the absence of paracrine IL-2 from activated CD4+ T cells (11, 155), or appropriately licensed DCs that promote CD8+ production of IL-2 via CD27-CD70 engagement(24), these wild-type CD8+ T cells fail to receive CD4+ T cell mediated help and fail to differentiate. Additionally, our data has demonstrated for the first time that Ikaros is regulated by IL-12, which is produced by activated DCs and again showcases a possible link between the inflammatory environment and Ikaros regulation of the naïve CD8+ T cell differentiation program. Reduced Ikaros expression results in increased sensitivity of the naïve CD8+ T cells to cytokines signals(101) and helps to drive their effector function, especially when IL-2+IL-12 combination is administered (Fig. 8). With IL-12 acting to enhance IL-2 signaling through mediating enhanced CD25 expression (99) (100), it is possible to envision both CD4+ T cells and inflammatory cytokines from activated dendritic cells during an anti-viral immune response contributing to drive repression of Ikaros expression and enhanced CTL differentiation. As Type I IFN and IL-21 have been characterized as inflammatory cytokines (40) (3), it will be interesting to also determine if they have a similar regulatory role with Ikaros. Thus, Ikaros enforces a dependency on cytokine signals derived from CD4+ T cells (CD4 help) or activated DCs to ensure appropriate differentiation during an inflammatory immune response.

In the presence of cytokine signals, it is possible that IL-2 or IL-12 signaling could act to block the TCR/CD28 mediated transcription of Ikaros (Fig 16B) and result in decreased Ikaros protein (Fig 3 and 4). Additionally, as Ikaros is regulated by phoshorylation (65-67), it is possible that cytokine signaling could act via an unknown kinase to promote hyperphosphorylation of Ikaros, and targeting it to the ubiquination pathway(67) (Fig. 16B). Ikaros degradation results in less repression of target genes, and cytokine signaling could then promote gene expression of factors that promote CD8+ T cell differentiation (Eomes (36) and T-bet(156)) and cell cycle progression. Finally, cytokines signaling could induce factors that compete with Ikaros for binding at effector gene promoters and thus inhibit Ikaros' ability to bind to its target genes and prevent its repressive ability. Thus, while my data indicates a role for IL-2 and IL-12 signaling in regulating Ikaros activity in activated CD8+ T cells, there still remain many avenues to investigate in how these cytokine signals regulates Ikaros.

This high induction of Ikaros in TCR and CD28 stimulated naïve CD8+ T cells could also serve to prevent CD8+ T cell activation against self-antigen. When presented with foreign antigen in an inflammatory environment (Fig. 17), the naïve CD8+ T cell has appropriate stimulus, fails to induce much Ikaros, and can differentiate into a CTL to clear out the pathogen. However, presentation of self-antigen in the periphery is typically done in the absence of inflammatory signals and hence these wild-type naïve CD8+ T cells up-regulate more Ikaros and fail to differentiate.

This ability to differentiate between self and foreign pathogen environments is especially important for these cytotoxic CD8+ T cells, as aberrant activation can result in immunopathology. This is apparent in cases of molecular mimicry, as viral specific CD8+ T cells that have been previously activated by an inflammatory environment can target self-antigen that has similar epitopes as the viral pathogen. In the RIP-LCMV model, LCMV specific CD8+ T cells can cause murine diabetes or liver damage through targeting these organs that express gp33(157). While the etiology is unclear, effector CTLs have been implicated in causing immunopathology associated with type 1 diabetes (158), multiple sclerosis (159), rheumatoid arthritis (160), and liver hepatitis(161). Thus, appropriate licensing of naïve CD8+ T cells is important to restricting

CD8+ T cell mediated immunopathology and Ikaros up-regulation appears to play a clear role through autocrine IL-2 restriction.

This restriction of autocrine IL-2 is especially important, as self-reactive naïve CD8+ T cells activated in the presence of IL-2 can mediate both tumor rejection and immunopathology. In the same transgenic system as utilized in Figure 8, the Restifo lab has demonstrated that activated PMEL cells supplied with IL-2 can target self-antigen and cause effective anti-tumor immunity(95) and also induce vitiligo (95, 162) and ocular autoimmunity(163). As our work has demonstrated that IL-2 up-regulates Granzyme B in activated CD8+ T cells (Fig 7), this has implications for immunopathology as Granzyme B+ CD8+ T cells have been associated with systemic lupus erythematosus(164). While the Ikzf1+/- mice fail to demonstrate any signs of autoimmunity, it will be interesting to determine if their increased autocrine IL-2 production *in vivo* in response to self-antigen or viral antigen could result in pathology. As exogenously supplied during the CD8+ T cell contraction phase of an infection response results in increased viral specific CD8+ T cells(4), it could be possible that viral specific Ikzf1+/- CD8+ T could promote inflammation through delayed contraction. Thus, induction of Ikaros in absence of inflammatory signals represses autocrine IL-2 production, and plays a critical role in preventing CD8+ T cell mediated immunopathology.

A negative of this prevention of CD8+ T cell mediated immunopathology is the challenge of activating a self-reactive CD8+ T cells against a tumor antigen. As tumor cells express self-antigen and the tumor microenvironment contains immunosuppressive cytokines and lack CD4+ T cells(126, 165), it is possible that tumor-reactive CD8+ T cell would up-regulate Ikaros and prevent their response to the tumor. As demonstrated in Chapter 3, using T cells with reduced Ikaros via the Ikzf1+/- and IkDN models demonstrates that repressing Ikaros activity could represent a possible pathway to revitalizing tumor-reactive CD8+ T cells, and possibly bypass required CD4+ T cell help in the tumor microenvironment (95, 110). Thus, it will be interesting to determine if poorly responsive TILs up-regulate Ikaros in response to self-antigen, and if the use of Ikaros shRNA(19) could remove the block on differentiation.

After naïve CD8+ T cells receive appropriate differentiate signals, they can give rise to both effectors and memory precursors(166). These memory CD8+ T cells are now antigen

experienced and licensed to have a rapid recall response to pathogen(143). It now appears that Ikaros also plays a role in memory CD8+ T cell biology. As outlined in Chapter 5 (Fig.16), it appears that while quiescent wild-type memory CD8+ T cells express moderate levels of Ikaros, they fail to induce Ikaros to the same levels as naïve CD8+ T cells following TCR and CD28 signals. While this result is preliminary, it is possibly due to increased autocrine IL-2 by memory CD8+ T cells that IL-2 acts in a negative feedback loop on Ikaros(167). Thus, memory CD8+ T cells may not induce as much Ikaros in order to promote their rapid differentiation into effector cells (Fig. 17).

In summary, Ikaros is a transcription repressor that has multiple roles in CD8+ T cell biology and its expression can influence the ability of a CD8+ T cell to respond appropriately to antigen. While its down-regulation in response to IL-2 and IL-12 promotes appropriate CTL differentiation and response to inflammatory stimuli, the lack of CD4 help and inflammatory signals in the tumor microenvironment could induce its up-regulation and promote poor anti-tumor immune responses. Ikaros acts as a sensor to ensure that the tightly regulated cytotoxic program of naïve CD8+ T cells is appropriately released during infection and prevents aberrant immunopathology. Thus, being able to modulate Ikaros expression could have implications for tumor immunotherapy or the rescue of exhausted CTLs, through promoting increased autocrine IL-2 production to drive increased CTL differentiation and bypass CD4+ T cell help.

Figures

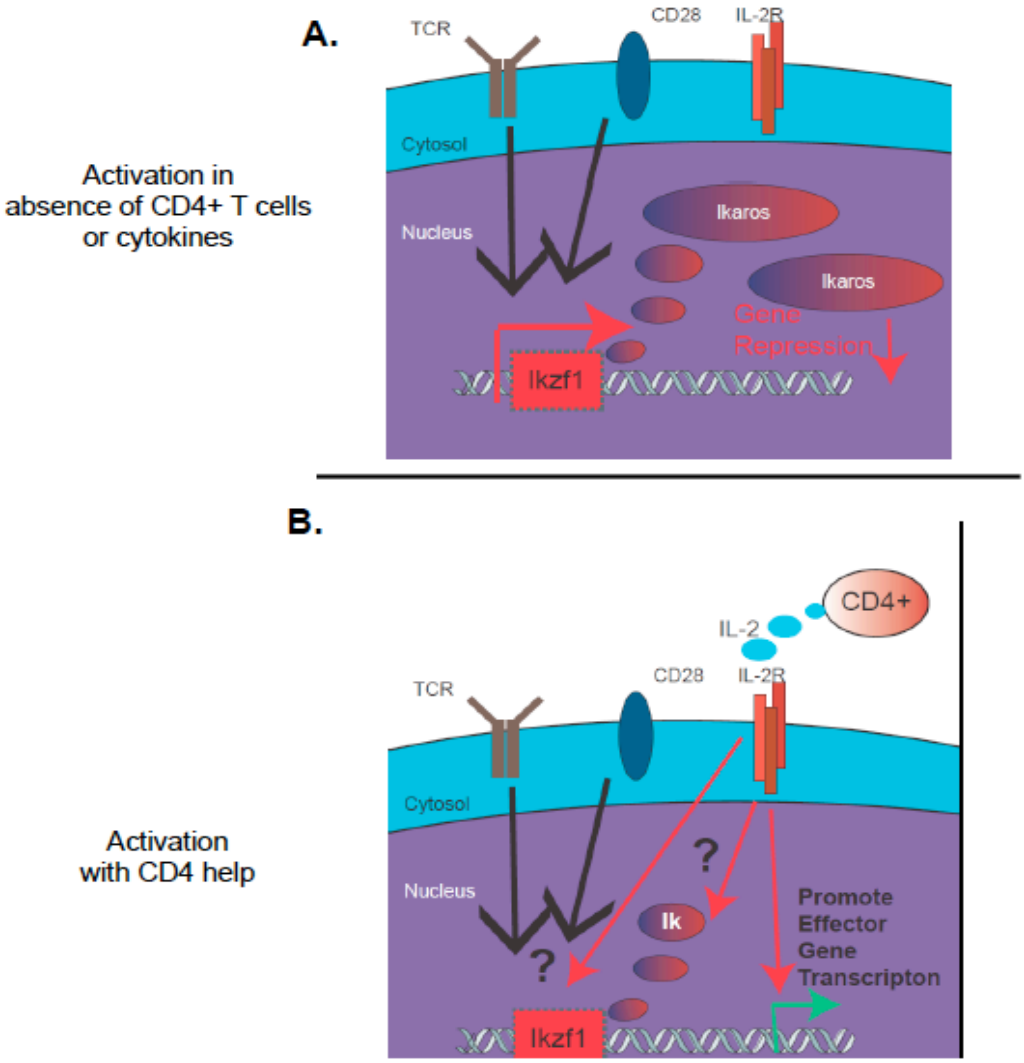


Figure 16- Possible regulatory mechanism of Ikaros

In A, TCR and CD28 signals alone act to induce Ikaros through possibly initiating Ikaros transcription, resulting in increased Ikaros protein expression. This results in gene repression. In the presence of CD4+ T cells and paracrine IL-2 (B), IL-2 signaling could inhibit Ikaros transcription, promote Ikaros protein degradation and also promote factors that compete with Ikaros at effector genes.

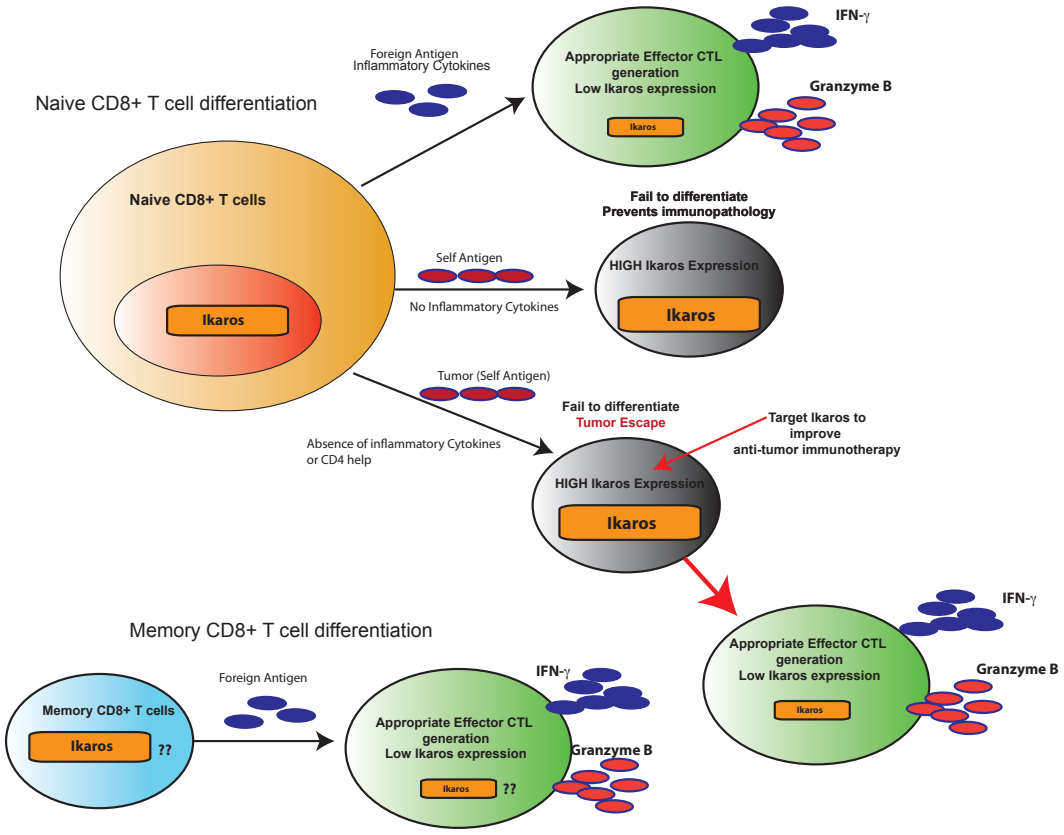


Figure 17-Ikaros in CD8+ T cell biology

In naïve CD8+ T cells, there is little active Ikaros present. Upon activation with TCR, CD28 and inflammatory cytokines, Ikaros production is inhibited and the naïve CD8+ T cell can differentiate into a CTL to eliminate the foreign pathogen.

Activation in the absence of inflammatory cytokines, results in induction of Ikaros and a failure of the naïve CD8+ T cell to differentiate into an effector CTL and prevents immunopathology. This poses a challenge in the tumor microenvironment, as CD8+ T cells fail to respond to self-antigen. Modulating Ikaros, as in Chapter 3, results in the restoration of effector function and ability to respond to self-antigen.

A memory Cd8+ T cell does not induce as much Ikaros as naïve CD8+ T cell and can be activated in the absence of inflammatory signals as it only requires being activated by a recall

antigen. The activated memory cell then can quickly differentiate and respond to the antigen of interest. Thus, Ikaros plays a role in regulating the different activation stages of CD8+ T cell differentiation.

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