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14. ABSTRACT

Accumulation of activated and suppressive regulatory T cells (Treg) within the tumor microenvironment (TME) is a major obstacle to the development of efficient anti-tumor immunity. Although Treg depletion can enhance anti-tumor immune responses, autoimmune sequelae can complicate this approach. To analyze the impact of transcription factor Helios on $FoxP3^{+}$ CD4 Treg in lymphoid tissues, we determined that Helios activates the IL2R–STAT5 pathway to enhance FoxP3 expression and maintain Treg suppressive activity. The observation that Helios-deficient Treg enhancement of anti-tumor immunity may reflect conversion of unstable $Helios^{+}$ Treg into T effector cells (Teff) within tumors was tested by inducing Treg lineage instability to promote anti-tumor immunity. During the first year of funding, we performed transcriptome analysis of intratumoral Treg, which revealed that Helios deficient intratumoral Treg adopt a genetic program that is typical of effector Th1 and Th2 cells. We also tested the feasibility of enhancement of anti-tumor immune responses by Treg conversion by targeting IL-23R using antibodies or genetic mouse models. Hypothesis driven analysis of the mechanism of Treg reprogramming upon blockade of IL-23 signaling is currently underway. These findings are consistent with our hypothesis that antibody-based approaches to reprogram tumor-infiltrating Treg into T effector cells represent a potential immunotherapeutic approach to the treatment of melanoma.

15. SUBJECT TERMS

tumor microenvironment, inflammation, CD4 regulatory T cells, Helios

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Introduction

While immunotherapeutic approaches to melanoma have gained traction in the clinic, regulatory T cells remain an understudied area of potential clinical importance. Here we delineate the contribution of antigen-specific CD8 regulatory T cells (Treg) to cancer immunity and define novel and effective therapeutic approaches using multiple experimental approaches including single cell TCR analysis, conditional knock-out mouse models and TCR knockin generation using CRISPR/Cas9. Insights gained from this study may allow new therapeutic approaches to CD8 Treg-based cancer immunotherapy of melanoma.

Keywords

tumor microenvironment, inflammation, CD4 regulatory T cells, Helios

Accomplishments

What were the major goals of the project?

Aim 1. Definition of the contribution of the Helios TF to proliferation, survival and stable FoxP3 expression by CD4 Treg within the microenvironment of murine melanoma.

IL-2 responsiveness of Helios-deficient Treg: reduced STAT5b activation.

Subtask 1: To examine IL-2 responsiveness and STAT5 activation, FoxP3^{+} CD4 Treg will be harvested from (n=2) Helios$^{+/+}$, Helios$^{fl/fl}$/CD4-Cre and Helios$^{fl/fl}$/FoxP3$^{YFP\text{-}Cre}$ mice followed by FACS analysis

Experimental analysis of Helios-deficient Treg responses during the progression of melanoma: analysis of FoxP3$^{EGFPCre\text{-}ERT2}$.Helios$^{fl/fl}$ mice in which Helios deletion is acutely induced upon tamoxifen administration.

Subtask: Melanoma will be induced by injection of B16/F10 cells into FoxP3$^{EGFPCre\text{-}ERT2}$ (n=5) and FoxP3$^{EGFPCre\text{-}ERT2}$.Helios$^{fl/fl}$ mice (n=5) followed by tamoxifen administration and monitoring of tumor growth. At day 7, 14 and 21, mice will be sacrificed and analyzed for proliferation, apoptosis and expression of survival markers by FACS analysis.

N.B. For all in vivo approaches, experiments are performed with groups of 5 mice and repeated a minimum of three times and maximum of 5 times for a total of 25 mice per experimental approach. These approaches have been approved by the DFCI IACUC full review (protocol 03-036).

Development of FoxP-Cre/STAT5b$^{intron\Delta}$ mice by CRISPR/Cas 9

Subtask 1: In collaboration with S. Dougan, we will generate the above mouse strain by microinjection of (n=5) C57Bl/6 zygotes. 2 months later, founder strains will be confirmed and backcrossed to FoxP3-Cre mice.

Subtask 2: To analyze survival, proliferation and anergic phenotype of CD4 Treg, Treg from FoxP-Cre/STAT5b$^{intron\Delta}$ (n=2) and FoxP-Cre (WT) (n=2) mice will be adoptively transferred into TCR$\alpha^{-/-}$ hosts (n=5) along with OT-II CD4 cells (n=2) and OT-II/CFA peptide, before analysis for proliferation, apoptosis and surface phenotype.

Determine whether mutation of Helios binding sites within the STAT5 gene locus recapitulates the Helios-deficient phenotype of CD4 Treg during melanoma development.

Subtask 1: Following induction of melanoma in FoxP3-Cre (WT) and FoxP-Cre/STAT5bintronΔ mice (n=5), tumor growth will be monitored for 3 weeks, followed by sacrifice and ex vivo analysis of proliferation, apoptosis and anergic phenotype of CD4 Treg in spleen and tumor in each group by FACS.

Subtask 2: To determine whether expression of STAT5b-CA can rescue the functional phenotype of FoxP3^{+} CD4 Treg in Helios$^{fl/fl}$.FoxP3-Cre mice, we will transduce CD4 Treg from Helios$^{fl/fl}$.FoxP3-Cre mice with control retrovirus or retrovirus expressing STAT5b-CA and measure suppressive activity of Treg after transfer into Rag2^{-}

$^{/-}$ hosts (n=5) followed by melanoma induction. Three weeks later, mice will be sacrificed for ex vivo analysis of forced expression of STAT5-CA by FACS to distinguish STAT5-dependent vs. STAT5-independent components of the Helios-deficient Treg phenotype.

Milestone(s) Achieved: Definition of the contribution of Helios to Treg proliferation/ survival in the face of chronic inflammatory responses of tumors: establishment of a colony of FoxP3$^{EGFPCre-ERT2}$.Helios$^{fl/fl}$ mice is underway. The contribution of Helios TF to IL-2 responsiveness of Treg under inflammatory conditions has been partially defined. Percent completion: 40%.

Aim 2. In vivo single cell transcriptome analysis of the genes responsible for conversion of intratumoral Treg into T effector cells.

2.1. Definition of the genetic basis of Treg conversion into T effector-like cells: the tumor microenvironment (TME) as a site of chronic inflammation:

Subtask 2.1: Melanoma will be induced using B16/F10 cells injected s.c. into Helios WT (n=5) and KO (n=5) mice followed by monitoring of tumor growth. Two weeks later, mice will be sacrificed for ex vivo analysis of tumor-infiltrating T cells based on FACS analysis of intracellular YFP signals (YFP^{hi}, YFP^{med}, YFP^{lo}). Transcriptome analyses will be performed with RNAs extracted from sorted YFP^{hi}, YFP^{med}, YFP^{lo} cells and significantly enriched molecular networks and signaling pathways will be assessed.

2.2. Definition of genetic modification(s) that underlie costimulation-induced conversion.

Subtask 2.2: Melanoma will be induced by injection of B16/F10 cells into FoxP3^{YFP}-Cre.Helios^{venus} mice (n=5) followed by treatment with isotype control or anti-GITR Ab at days 0, 3, 6 and 9. Three weeks later, mice will be sacrificed and analyzed by FACS for the presence of CD69^{+}YFP^{hi}Venus^{hi}, CD69^{+}YFP^{med}Venus^{med}, CD69^{+} YFP^{lo}Venus^{lo} cells followed by transcriptome analysis and pathway modeling to identify potential conversion modules.

Milestone(s) Achieved: Definition of the genetic events that underlie Treg conversion and potential biomarkers of reprogramming of intratumoral Treg: transcriptome analysis with small numbers of cells was performed and the dominant molecular pathways correlated with Treg conversion identified (Percent completion: 60%).
Data obtained from this analysis will be used to select potential Treg surface molecules that will be targeted to induce Treg conversion in the TME (Percent completion: 60%)

Aim 3. Definition of Treg pathways that that inhibit Helios expression and allow Treg → Teffector conversion of intratumoral but not systemic Treg.

3.1. In vitro screen for Abs that induce Treg→Teff conversion.

Subtask 3.1: To detect the converted Treg phenotype we used FACS analysis of RFP for FoxP3 and YFP for IFNγ. Isolated CD4 Treg (RFP^{+}YFP^{-}) stimulated with anti-CD3/CD28 Ab in the presence of IL-2 and IL-4 to mimic an inflammatory environment.

3.2. Proof of principle and preliminary definition of lead Ab candidates

Subtask 3.2: (in vitro studies) To analyze the effect of engagement of IL-23R by blocking Ab on Treg phenotype, we will culture isolated CD4 Treg with isotype or anti-IL23R Abs in the presence of inflammatory cytokine IL-4. The outcome of this signaling on Treg will be analyzed by FACS analysis of expression of FoxP3, CD25 and IFNγ.

3.3. Engagement of the IL-23R Ab and Treg reprogramming.

Subtask 3.3: To validate the functional efficacy of this Ab candidate for reprogramming of Treg, we will induce melanoma in B6 mice followed by antibody treatment (n=5) or no treatment (n=5). Three weeks later, mice will be sacrificed for ex vivo analysis of the cellular and molecular mechanisms that enhance antitumor immunity according to FACS analysis of the numbers and phenotype of intratumoral and splenic Treg and CD8 T cells compared between Ab treated and non-treated groups.

Deliverable: should have potential Ab candidate ready for humanization process

Milestone(s) Achieved:
Identification of molecular pathways that are targeted by antibodies and small molecules to reprogram tumor-infiltrating Treg into T effector cells: the contribution of IL-23R signaling to Treg stability is identified. Ab dependent blockade and genetic deletion of IL-23R led to delayed tumor growth that is associated with Treg reprogramming
(Percent completed: 80%)

What was accomplished under these goals?

Aim 1. Definition of the contribution of the Helios TF to proliferation, survival and stable FoxP3 expression by CD4 Treg within the microenvironment of a murine melanoma.

Analysis of the contribution of IL-2 signaling in Helios dependent stabilization of Treg under inflammatory conditions revealed that Helios-deficient Treg display reduced responsiveness to IL-2 according to measurement of levels of activated STAT5. Helios-deficient Treg obtained from $Helios^{fl/fl}$.CD4-Cre, and $Helios^{fl/fl}$.FoxP3-Cre mice showed diminished percentages of p-STAT5 compared to Helios WT Treg. Based on the current paradigm of signaling pathway upon TCR and IL2R engagement that is associated with Treg survival during the immune response, we measured the activation of the PI3K-Akt pathway leading to activation of Foxo1. Our in vitro and in vivo experiments revealed that Helios deficient CD4 Treg enhance the downstream signaling of IL2R (AKT-Foxo axis) that leads to aberrant expression of genes associated with an unstable Treg phenotype (**Fig. 1**). One Foxo1 target gene is IFNγ, and transmigration of Foxo-1 upon phosphorylation often results in derepression of IFNγ expression. Therefore, dysregulated AKT-Foxo pathway in Helios deficient Treg may represent an important component of the molecular basis for acquisition of an unstable phenotype under inflammatory conditions. Further dissection of the upstream signaling events that lead to enhanced AKT activation in the absence of Helios is currently underway.

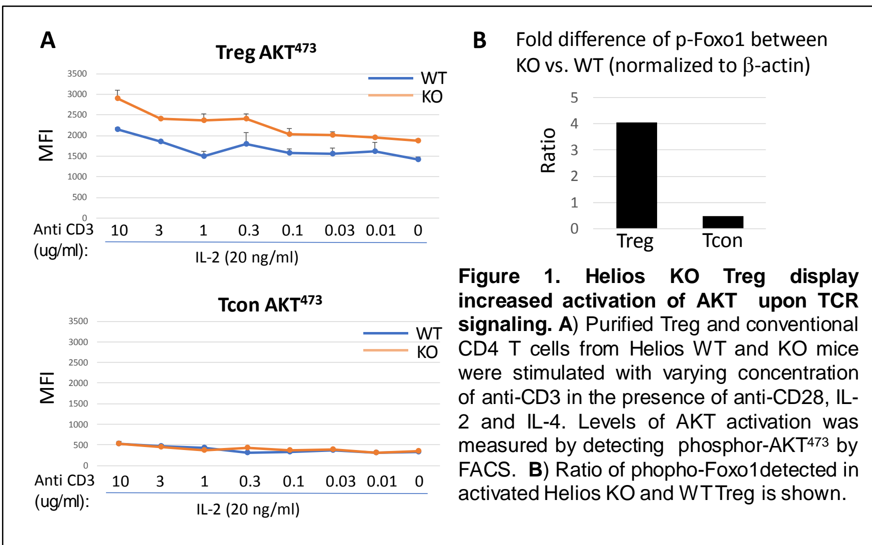

Figure 1. Helios KO Treg display increased activation of AKT upon TCR signaling. A) Purified Treg and conventional CD4 T cells from Helios WT and KO mice were stimulated with varying concentration of anti-CD3 in the presence of anti-CD28, IL-2 and IL-4. Levels of AKT activation was measured by detecting phosphor-AKT^{473} by FACS. **B**) Ratio of phopho-Foxo1detected in activated Helios KO and WTTreg is shown.

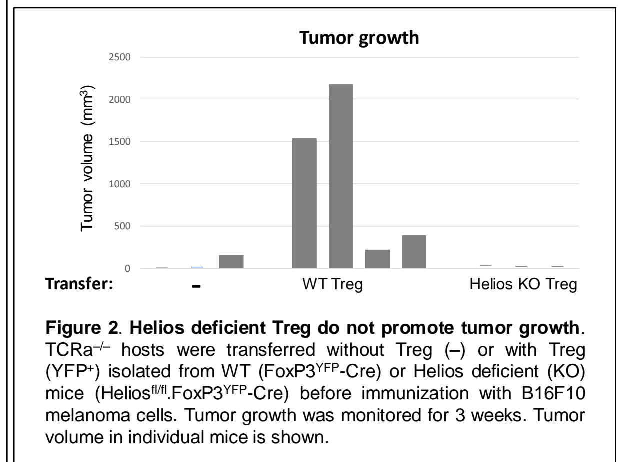

Figure 2. **Helios deficient Treg do not promote tumor growth**. $TCRa^{-/-}$ hosts were transferred without Treg (–) or with Treg (YFP^{+}) isolated from WT ($FoxP3^{YFP}$-Cre) or Helios deficient (KO) mice ($Helios^{fl/fl}$.$FoxP3^{YFP}$-Cre) before immunization with B16F10 melanoma cells. Tumor growth was monitored for 3 weeks. Tumor volume in individual mice is shown.

We have analyzed the individual effects of Helios deficient Treg on tumor growth by transferring Helios WT and KO Treg into $TCRa^{-/-}$ hosts, which lack the entire pool of T cells. While $TCRa^{-/-}$ hosts transferred with WT Treg showed rapid growth of melanoma, those that received Helios deficient Treg did not display tumor growth after three weeks, the maximum monitoring period allowed by our protocol (**Fig. 2)**. We believe that this observation of delayed growth of melanoma in $TCRa^{-/-}$ hosts harboring Helios deficient Treg is due to their unstable Treg phenotype and genetic reprogramming under the chronic inflammatory condition of tumors as shown below (see data in Aim 2).

Aim 2. Single cell transcriptome analysis of genes associated with conversion of intratumoral Treg into T effector cells: In our analysis of single cells at different stages of conversion, identified according to levels of YFP expression (FoxP3^{YFP}-Cre), we performed gene expression profiling of small numbers (~400 cells) of intrasplenic and intratumoral Treg from WT and Helios KO mice (**Fig. 3**). These analyses revealed that effector cell conversion of Helios-deficient Tregs within the tumor-tissue microenvironment was associated with increased expression of genes that control T effector cell phenotype, including the master transcription factors T-bet and Gata-3 for Th1 and Th2 effector cells, along with prototype cytokines for each Th subset. This analysis also provided a list of genes that are differentially expressed only by intratumoral Treg between WT and Helios KO mice while intrasplenic Treg in both genotypes showed similar levels of expression. These gene sets will be used for further identification of target genes for selective reprogramming of intratumoral Treg as proposed in Aim 3.

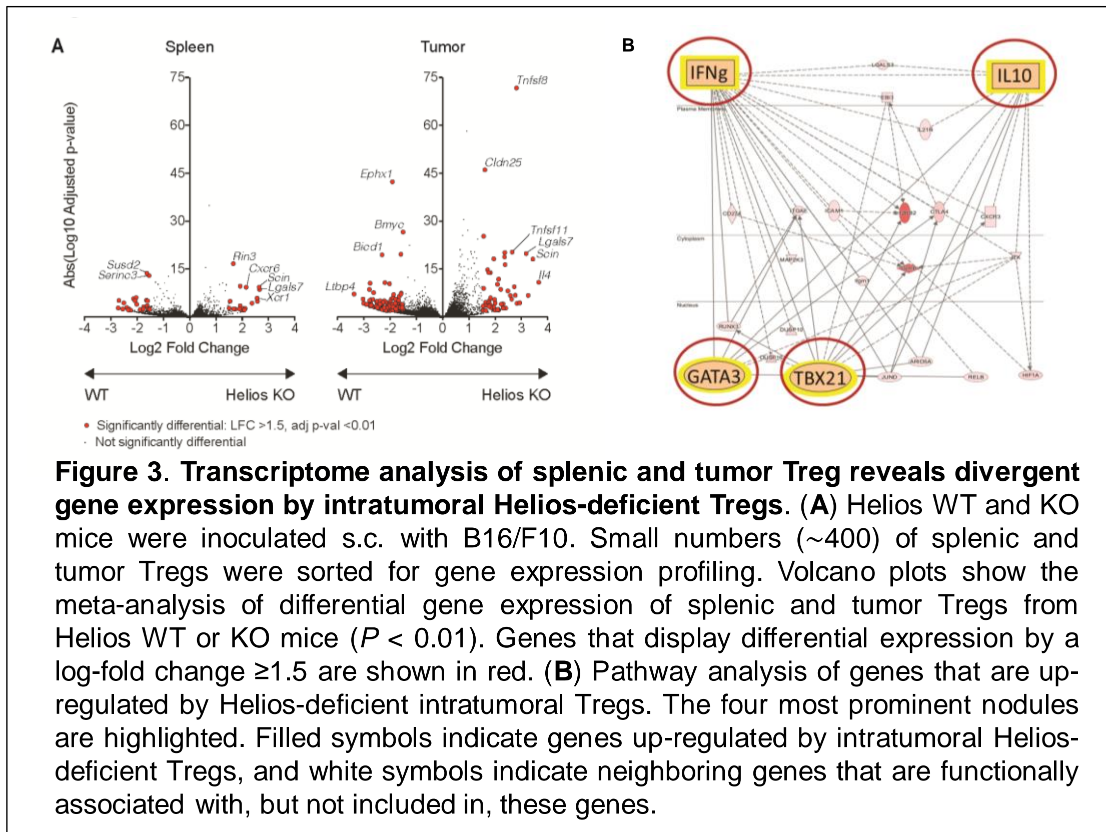

Figure 3. **Transcriptome analysis of splenic and tumor Treg reveals divergent gene expression by intratumoral Helios-deficient Tregs**. (**A**) Helios WT and KO mice were inoculated s.c. with B16/F10. Small numbers (~400) of splenic and tumor Tregs were sorted for gene expression profiling. Volcano plots show the meta-analysis of differential gene expression of splenic and tumor Tregs from Helios WT or KO mice ($P < 0.01$). Genes that display differential expression by a log-fold change ≥1.5 are shown in red. (**B**) Pathway analysis of genes that are up-regulated by Helios-deficient intratumoral Tregs. The four most prominent nodules are highlighted. Filled symbols indicate genes up-regulated by intratumoral Helios-deficient Tregs, and white symbols indicate neighboring genes that are functionally associated with, but not included in, these genes.

Aim 3. Definition of receptor-linked pathways that promote Treg →T effector conversion: targeting by antibodies:

Our in vitro screen and in vivo validation of Treg surface markers for identification of molecular targets for Treg conversion revealed that Ab-mediated engagement of IL-23R can lead to Treg conversion under inflammatory conditions (**Fig. 4**).

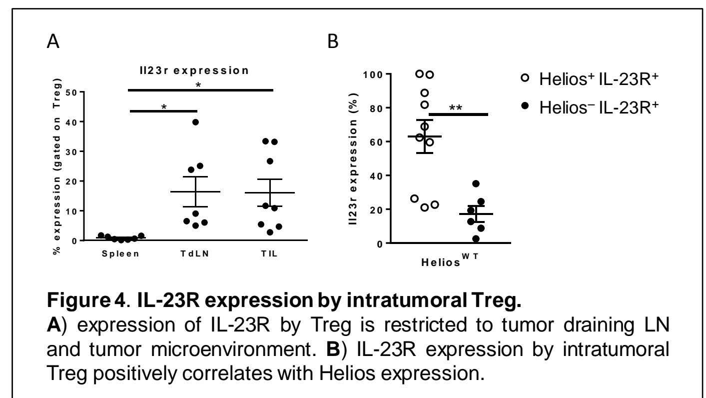

Figure 4. **IL-23R expression by intratumoral Treg.**
A) expression of IL-23R by Treg is restricted to tumor draining LN and tumor microenvironment. **B**) IL-23R expression by intratumoral Treg positively correlates with Helios expression.

In sum, during the first year of funding we obtained data from several experimental approaches proposed in our original Aims:

1) Profiling of IL-23R expression by Treg: IL-23R is upregulated by Treg in tumor-draining LNs and intratumoral Treg. IL-23R expression also shows a correlation with Helios expressed by intratumoral Treg and converted intratumoral Treg express reduced levels of IL-23R.

2) Impact of IL-23R expression on Treg stability using genetic models: Mice with Treg specific deletion of IL23R showed delayed tumor growth and IL23R-deficient Treg displayed decreased stability as evidenced by reduced Foxp3 and IFNγ expression (**Fig. 5**).

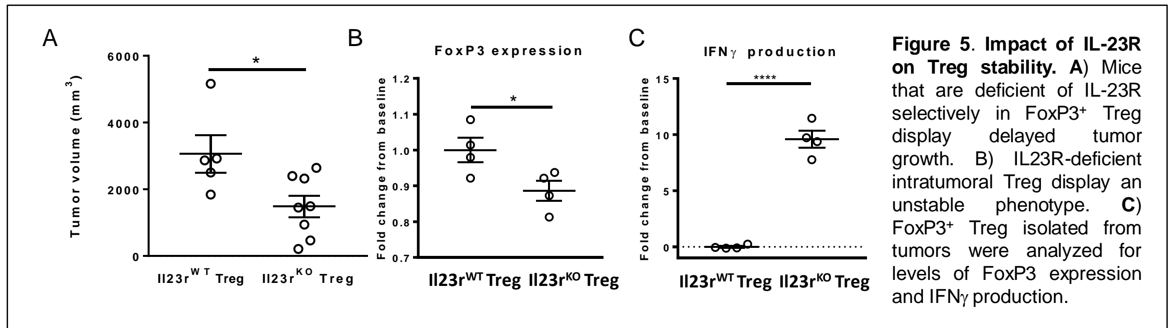

Figure 5. Impact of IL-23R on Treg stability. A) Mice that are deficient of IL-23R selectively in FoxP3$^+$ Treg display delayed tumor growth. B) IL23R-deficient intratumoral Treg display an unstable phenotype. **C)** FoxP3$^+$ Treg isolated from tumors were analyzed for levels of FoxP3 expression and IFNγ production.

3) Therapeutic application in a preclinical tumor model: Administration of IL-23R Ab into tumor-bearing mice delayed tumor growth and increased antitumor immune responses, which were associated with phenotypic changes in Treg. To test therapeutic applicability of anti-IL-23R Ab, we injected anti-IL23R Abs <u>after</u> tumor induction at a time point when palpable tumors were observed (**Fig. 6**). Blockade of IL-23R led to significantly delayed tumor growth and characterization of immune cells in the spleen and tumor revealed that intratumoral Treg display reduced numbers and an unstable phenotype including expression of the IFNγ effector cytokine.

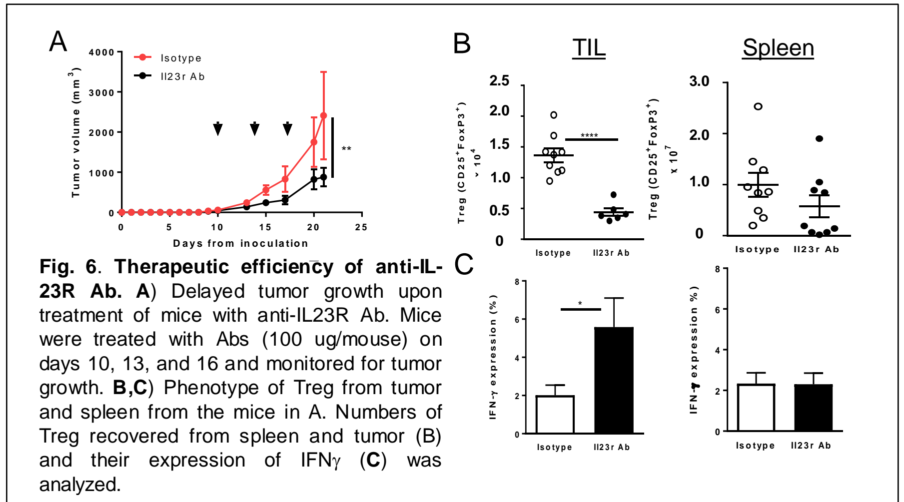

Fig. 6. Therapeutic efficiency of anti-IL-23R Ab. A) Delayed tumor growth upon treatment of mice with anti-IL23R Ab. Mice were treated with Abs (100 ug/mouse) on days 10, 13, and 16 and monitored for tumor growth. **B,C)** Phenotype of Treg from tumor and spleen from the mice in A. Numbers of Treg recovered from spleen and tumor (B) and their expression of IFNγ (**C**) was analyzed.

What opportunities for training and professional development has the project provided?

Nothing to report

How were the results disseminated to communities of interest?

Nothing to report

What do you plan to do during the next reporting period to accomplish the goals?

In the next reporting period, i) we will identify molecular mechanisms by which engagement of IL-23R leads to reprogramming of intratumoral Treg by testing the following hypotheses.

a) Based on the shared usage of the IL12Rβ1 subunit by IL-23R and IL-12R, we will test the hypothesis that Ab mediated blockade of IL-23R may lead to increased availability of IL-12R by Treg, which may enhance IL-12R dependent signaling.

b) IL-23 signaling has been shown to upregulate expression of the androgen receptor (AR). Binding of AR leads to epigenetic changes within the FoxP3 locus that stabilizes Treg differentiation. Using genome editing and biochemical analysis, we will test the impact of IL-23 signaling on AR expression leading to FoxP3 upregulation in conjunction with tumor growth and phenotype stability of intratumoral Treg. In this experiment, our well-established colony of IL-23R$^{fl/fl}$.FoxP3-Cre mice will serve as the basic genetic tool to address this mechanistic question. ii) To select the best of class target for Treg reprogramming in the TME, we will test a set of Treg surface molecules identified from our transcriptome analysis (Figure 2, above). We have established CRISPR mediated knockout, collection of agonistic and antagonistic Abs and genetic mouse models that are deficient in these candidate molecules for analysis of their impact on Treg conversion in the context of melanoma.

Impact

What was the impact on the development of the principal discipline(s) of the project?
Nothing to report.

What was the impact on other disciplines?
Nothing to report.

What was the impact on technology transfer?
Nothing to report.

What was the impact on society beyond science and technology?
Nothing to report.

Changes/Problems

Changes in approach and reasons for change
We will not produce STATΔ mice, since the involvement of Helios in IL-2 responsiveness leading to Treg survival in the TME has been tested (see Figure 1). The involvement of STAT5 expression/activation in Helios-dependent stability of Treg will be further tested using alternative approaches, including FACS analysis of activation of Akt and Foxo-1 and CRISPR-mediated deletion of intermediary and downstream molecules using primary cells.

Actual or anticipated problems or delays and actions or plans to resolve them
Nothing to report

Changes that had a significant impact on expenditures
Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents
Nothing to report

Products

Publications, conference papers, and presentations

Journal publications

Hidetoshi Nakagawa, Lei Wang, Harvey Cantor, Hye-Jung Kim. New insights into the biology of CD8 regulatory T cells. *Advances in Immunology* 2018:83; *in press*.

Jessica M. Sido, Hidetoshi Nakagawa, Hye-Jung Kim, Harvey Cantor. Requirement for IL23R to maintain Treg stability in the tumor microenvironment. *Manuscript in preparation*.

Books or other non-periodical, one-time publications
Nothing to report

Other publications, conference papers, and presentations
2017– Presentation: Trainee Talk, DFCI Cancer Immunology & Virology Retreat, "*Role of Il23/Il23r in Treg stability*"

Website(s) or other Internet site(s)
Nothing to report

Technologies or techniques
Nothing to report

Inventions, patent applications, and/or licenses
Nothing to report

Other Products
Nothing to report

Participants and Other Collaborating Organizations

What individuals have worked on the project?

Name:	Harvey Cantor, M.D.
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-3313-2478
Nearest person month worked:	1 CM
Contribution to Project:	No change
Funding Support:	N/A

Name:	Hye-Jung, Ph.D.
Project Role:	Lecturer
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2 CM
Contribution to Project:	No change
Funding Support:	N/A

Name:	Hidetoshi Nakagawa, M.D., Ph.D.
Project Role:	Postdoctoral Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	2 CM
Contribution to Project:	No change
Funding Support:	N/A

Name:	Lei Wang, Ph.D.
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 CM
Contribution to Project:	Dr. Wang recently began work on the molecular mechanisms of IL-23 signaling and Treg stability in the tumor microenvironment.
Funding Support:	N/A

Name:	Anne Gonzalez
Project Role:	Research Technician
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 CM
Contribution to Project:	No change
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Organization Name: N/A

Special Reporting Requirements

None