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CONTRACTING ORGANIZATION: Dana-Farber Cancer Institute
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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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1. 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 CD4 regulatory T cells (Treg) to cancer immunity and define novel and effective therapeutic approaches using multiple experimental approaches including conditional knock-out mouse models, antibody dependent Treg reprogramming and knockout generation using CRISPR/Cas9. Insights gained from this study may allow new therapeutic approaches to CD4 Treg-based cancer immunotherapy of melanoma.

2. KEYWORDS

tumor microenvironment, inflammation, CD4 regulatory T cells, Helios

3. 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) and ACURO.

Development of FoxP-Cre/STAT5b^{intronΔ} 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^{intronΔ} (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$^{EGFPCre\text{-}ERT2}$.Helios$^{fl/fl}$ mice. The contribution of Helios TF to IL-2 responsiveness of Treg under inflammatory conditions.* Percent completion: 95%.

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.* (Percent completion: 100%).

Aim 3. Definition of Treg pathways 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: Candidate IL-23R Ab in development for potential 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 has been validated. Ab dependent blockade and genetic deletion of IL-23R led to delayed tumor growth that is associated with Treg reprogramming. (Percent completed: 70%)

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.

We successfully established and are currently expanding the colony of FoxP3$^{EGFPCre\text{-}ERT2}$.Helios$^{fl/fl}$ mice to further define the contribution of Helios to Treg proliferation/ survival in the face of chronic inflammatory responses of tumors. Our analysis revealed that Helios expression by Treg under inflammatory conditions is essential to maintain Treg stability by ensuring Treg's responsiveness to IL-2, a critical cytokine for Treg survival. Our analysis has shown that converted Treg alone may be sufficient to induce anti-tumor immunity in the adoptive transfer system. Treg from Helios conditional KO mice (Helios$^{fl/fl}$.FoxP3-Cre) in adoptive hosts produce IFNγ and delay tumor progression. As indicated in the year 1 progress report, we analyzed the involvement of STAT5 expression/ activation in Helios-dependent conversion of intratumoral Treg by analyzing expression of Akt and Foxo-1. These studies suggested that conversion of intratumoral Treg may be associated with antigen recognition and local cytokine signaling that may explain acquisition of unstable Treg phenotype selectively within the tumor microenvironment.

Aim 2. Single cell transcriptome analysis of genes associated with conversion of intratumoral Treg into T effector cells:

In year 1, transcriptome analysis with small numbers of cells revealed the dominant molecular pathways correlated with Treg conversion. Over the past year, our analysis of the transcriptome of Treg in spleen and tumor sites with respect to Helios expression revealed a Helios-dependent Treg program within the tumor-tissue microenvironment that is associated with increased expression of genes that control the T effector cell phenotype. Our transcriptome analysis also revealed that ~50% of genes that are upregulated by Helios deficient intratumoral Treg compared to WT tumor Treg belong to STAT4 target genes. Comparison of multiple cytokine signaling suggested that IL-12 may be one of the major cytokines that can induce STAT4 activation, since chronic inflammatory conditions of tumor can deliver a signal through IL-12R on Treg and sustained activation of STAT4 can lead to induction of unstable Treg phenotype. Molecular insight obtained from gene expression profiling may now be applied to the rational design of immunotherapeutics that selectively induce Treg reprogramming in the TME.

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

The functional efficacy of IL-23R blocking in vitro on Treg conversion has been successfully performed, and showed that engagement of IL-23R leads to Treg conversion evidenced by downregulation of FoxP3 and CD25 and de novo expression of IFNγ. In addition, we tested the efficacy of anti-IL23R Ab treatment to anti-tumor immunity in vivo. Using MC38 colon adenocarcinoma model, we found that Ab treatment significantly delayed tumor progression in mice. Inhibition of tumor growth upon anti-IL23R Ab treatment was associated with expression of IFNγ by intratumoral Treg, which in turn de-represses activation of conventional CD4 and CD8 T cells.

Recent analyses have revealed that mice with selective deletion of IL23R in Treg (IL-23R$^{fl/fl}$.FoxP3-^{Cre}) almost completely suppress tumor development after inoculation with MC38 cells.

Our analysis on the involvement of enhanced IL12 signaling in the TME specific Treg conversion revealed that STAT4 activation is a hallmark of induction of Treg instability that is also characterized by IFNγ expression by Treg. STAT4 activation and IFNγ production by Treg is positively correlated with TCR signaling, which may

suggest the phenotype change of Treg selectively in the TME that represents inflammatory condition. Therefore, we measured STAT4 activation in IL-23R deficient Treg and after anti-IL23R Ab treatment (Fig. 1).

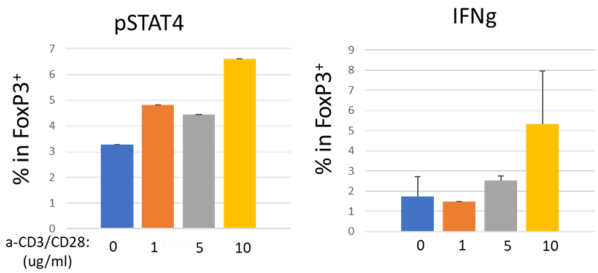

Figure 1: Increased STAT4 activation and expression of IFNg by CD4 Treg in vitro

Preliminary evidence that IL23R-deficient Tregs have increased IL12 sensitivity (**Fig. 2**). To further validate these findings, we will cross B6.IL23R$^{fl/fl}$ mice to IL12-deficient hosts to determine whether the tumor protection phenotype is lost in the absence of IL12.

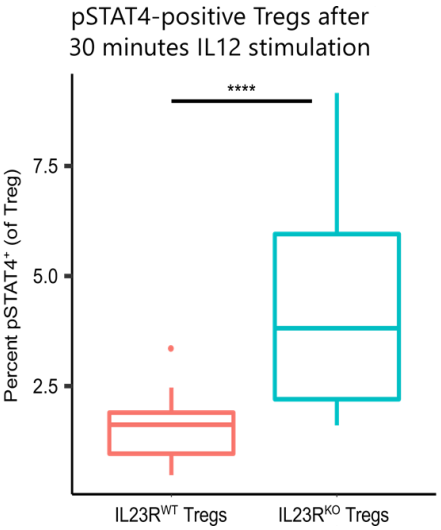

Figure 2: IL23R-deficient Tregs show increased IL12 sensitivity *in vitro.* Tregs with or without IL23R expression were treated with 20 ng/ml IL12 for 30 minutes, after which cells were fixed and stained for phosphorylated STAT4 (pSTAT4). Increased levels of pSTAT4 staining indicates a heightened responsiveness to IL12 stimulation. Results pooled from two independent experiments. Box and whisker plot showing median and quartiles is shown. ****p < 0.0001 by Wilcoxson test.

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 for Aim 1, we will complete expansion of the colony of Helios$^{fl/fl}$.FoxP3$^{EGFPCre\text{-}ERT2}$ mice which harbor an acute deletion of Helios to allow further analysis of the impact on growing tumors. We anticipate that the breeding will be completed in ~3-6 months and experimental studies and analyses in 6-12 months.

Aim 2 is complete.

For Aim 3, we are currently replicating our findings in the B16 melanoma model to assure statistical significance and reproducibility. In addition, based on our observations in the MC38 model, we will test the B16 model and explore the basic molecular mechanisms that underpin this response by performing ATAC-Seq and RNA-Seq. We are also in the process of developing bispecific Abs that allow targeting IL-23R exclusively on intratumoral Treg and may be appropriate for humanization.

4. 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.

5. CHANGES/PROBLEMS

Changes in approach and reasons for change
Nothing to report.

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

6. 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; 140_ 1-20.

Andrew Wight, Jessica M. Sido, Hidetoshi Nakagawa, Lei Wang, 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
June 5, 2019– Poster Presentation, DFCI Cancer Immunology & Virology Retreat, "*Targeting Treg-specific IL23R expression is a potent immunotherapeutic candidate*", Andrew Wight, PhD

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

7. 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:	1 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:	Dr. Nakagawa has been supported by the CRI-Irvington Fellowship which will end 12/31/19; we have therefore included support for 7 months.
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:	2 CM
Contribution to Project:	Dr. Wang works on in vitro and in vivo studies outlined in Aims 3.2 and 3.3.
Funding Support:	Dr. Wang recently received support from the Benacerraf Fellowship in Immunology (1/1/2019) which offsets her salary and allows funding for Dr. Nakagawa (as noted above).

Name:	Andrew Wight, PhD
Project Role:	Research Fellow
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1 CM
Contribution to Project:	Dr. Wight is working on mechanistic studies outlined in Aims 3.2 and 3.3.
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? N/A

8. SPECIAL REPORTING REQUIREMENTS

None

9. APPENDICES –

Abstract for Andrew Wight Poster

POSTER ABSTRACT

Targeting Treg-specific IL23R expression is a potent immunotherapeutic candidate

Andrew Wight, Jessica M. Sido, Lei Wang, Hidetoshi Nakagawa, Hye-Jung Kim, and Harvey Cantor

Cancer Immunology & Virology, DFCI; Immunology, Harvard Medical School

Interleukin 23 (IL23), a member of the interleukin 12 cytokine family, has a poorly understood role in cancer progression. Whole-body knockouts or antibodies targeting the IL23 receptor (IL23R) have had mixed effects on tumor growth. These findings may reflect the contribution of IL23R to expansion of pro-inflammatory Th17 cells and/or modulation of FoxP3- Treg. Here, we show that mice with Treg-specific ablation of the IL23 receptor (Il23raflox × FoxP3cre) mount rapid and protective anti-cancer immune responses accompanied by an early increase of $CD4^+$ memory T-cells. Preliminary results indicate that Treg-specific IL23R targeting may exert its effects through both STAT3-depednent and -independent mechanisms, including promoting intratumoral conversion of targeted Tregs. Moreover, Treg-specific deletion of IL23R did not affect the phenotype of systemic Treg and was not associated with overt signs of systemic autoimmunity. These findings suggest that pharmaceutical agents that mediate Treg-specific interference with IL23 signaling may represent a safe and powerful new cancer immunotherapeutic.

STATEMENT OF WORK

(AUGUST 1, 2018- AUGUST, 15, 2019)

	Timeline (months)	
Specific 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 model 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 • *Findings*: Our analysis revealed that Helios expression by Treg under inflammatory conditions is essential to maintain Treg stability by ensuring Treg's responsiveness to IL-2, a critical cytokine for Treg survival.	100% complete	Drs. Kim, Nakagawa
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. • *Findings*: Our analysis has shown that converted Treg alone may be sufficient to induce anti-tumor immunity in the adoptive transfer system. Treg from Helios conditional KO mice (Helios$^{fl/fl}$.FoxP3-Cre) in adoptive hosts produce IFNγ and delay tumor progression. • *NCE*: We are currently expanding the colony of Helios$^{fl/fl}$.FoxP3$^{EGFPCre\text{-}ERT2}$ mice which will harbor acute deletion of Helios to allow analysis of the impact on growing tumors. We anticipate that the breeding will be completed in ~3-6 months and experimental studies and analyses in 6-12 months.	80% completed **NCE**: 25-36 mos	Drs. Kim, Nakagawa
Development of FoxP$^{3\text{-}Cre}$/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	100% complete	Dr. Kim

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. • *Findings*: As indicated in the progress report, we have analyzed the involvement of STAT5 expression/ activation in Helios-dependent conversion of intratumoral Treg by analyzing Akt and Foxo-1. Our analysis suggested that conversion of intratumoral Treg may be associated with antigen recognition and local cytokine signaling that may explain acquisition of unstable Treg phenotype selectively within the tumor microenvironment.		
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}/STAT5b$^{intron\Delta}$ 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. • *Findings*: As indicated above, analysis of the contribution of STAT5 expression/ activation in Helios-dependent conversion of intratumoral Treg suggested that conversion of intratumoral Treg may be associated with antigen recognition and local cytokine signaling that may explain acquisition of an unstable Treg phenotype selectively within the tumor microenvironment.	100% complete	Drs. Kim, Nakagawa
Milestone(s) Achieved: *Definition of the contribution of Helios to Treg proliferation/ survival in the face of a chronic inflammatory response of tumors* *Determination of the relative contribution of Helios-dependent*		

STAT5 gene expression to the maintenance of Treg population size and stability under chronic inflammation of tumors.		

Specific Aim 2 **Single cell transcriptome analysis of genes associated with conversion of intratumoral Treg into T effector cells**.		
Definition of the genetic basis of Treg conversion into T effector-like cells: the tumor microenvironment (TME) as a site of chronic inflammation: *Subtask 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. • *Findings*: Our analysis of the transcriptome of Treg in the spleen and tumor sites with respect to Helios expression revealed a Helios-dependent Treg program within the tumor-tissue microenvironment that is associated with increased expression of genes that control the T effector cell phenotype.	100% complete	Drs. Kim, Nakagawa
Definition of genetic modification(s) that underlie costimulation-induced conversion. Subtask 1: 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. • *Findings*: Our transcriptome analysis revealed that ~50% of genes that are upregulated by Helios deficient intratumoral Treg compared to WT tumor Treg belong to STAT4 target genes. Comparison of multiple cytokine signaling suggested that IL-12 may be one of the major cytokines that can induce STAT4 activation, since chronic inflammatory conditions of tumor can deliver a signal through IL-12R on Treg and sustained activation of STAT4 can lead to induction of unstable Treg phenotype. Molecular insight obtained from gene expression profiling may now be applied to the rational design of immunotherapeutics that selectively induce	100% complete	Drs. Kim, Nakagawa

Treg reprogramming in the TME.		
Milestone(s) Achieved: *Definition of the genetic events that underlie Treg conversion and potential biomarkers of reprogramming of intratumoral Treg.*		
Specific Aim 3: Definition of receptor-linked pathways in Treg that promote Treg→Teffector conversion: targeting by antibodies.		
3.1. In vitro screen for Abs that induce Treg→Teff conversion. *Subtask 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.	100% complete	Drs. Kim, Nakagawa
3.2. Proof of principle and preliminary definition of lead Ab candidates *Subtask 1*: (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. • *Findings:* The functional efficacy of IL-23R blocking in vitro on Treg conversion has been successfully performed, and showed that engagement of IL-23R leads to Treg conversion evidenced by downregulation of FoxP3 and CD25 and de novo expression of IFNγ. In addition, we tested the efficacy of anti-IL23R Ab treatment to anti-tumor immunity in vivo. Using MC38 colon adenocarcinoma model, we found that Ab treatment significantly delayed tumor progression in mice. Inhibition of tumor growth upon anti-IL23R Ab treatment was associated with expression of IFNγ by intratumoral Treg, which in turn de-represses activation of conventional CD4 and CD8 T cells. • *NCE:* We are currently carrying out this subtask in the B16 melanoma model and will replicate these findings to assure statistical significance and reproducibility, as well as define the molecular mechanisms that underpin this effect.	60% complete **NCE**: 25-36 mos	Drs. Kim, Wang, Wight
3.3. Engagement of the IL-23R Ab and Treg reprogramming.		Drs. Kim, Wang, Wight

Subtask 1: 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 • *Findings*: Recent analyses have revealed that mice with selective deletion of IL23R in Treg (IL-23R$^{fl/fl}$.FoxP3-^{Cre}) almost completely suppress tumor development after inoculation with MC38 cells. • *NCE*: Based on the striking observation in MC38, we are currently testing B16 and exploring the basic molecular mechanisms that underpin this response by performing ATAC-Seq and RNA-Seq. We also plan to develop bispecific Abs that allow targeting IL-23R exclusively on intratumoral Treg.	60% complete **NCE**: 25-36 mos	
Milestone(s) Achieved: *Identification of molecular pathways that are targeted by antibodies and small molecules to reprogram tumor-infiltrating Treg into T effector cells.*		