Commentary

# Helios—controller of Treg stability and function

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Helios is a member of the Ikaros family (e.g., Ikaros, Eos, Pegasus, and Ailos) of zinc-finger transcription factors and was initially cloned by two independent groups (1,2). Initial studies implicated Helios in cancer due to its as aberrant expression in certain types of leukemia and lymphoma (3-7). However, more recent work has described Helios as being expressed in lymphocytes, particularly a unique subset of CD4+ T cells, termed regulatory T cells (Treg) (8,9).

CD4+ Treg play an essential role in the maintenance of self-tolerance through elaboration of mechanisms that suppress autoreactive lymphocytes. CD4+ Treg can develop naturally in the thymus (nTreg) or can be induced in the periphery (pTreg) (10). Initial work from the Shevach group showed that Helios expression could differentiate nTreg from pTreg (9), although this work is controversial—as others have not observed clear distinction of Treg subsets on the basis of Helios expression (8,11-13). Beyond a marker, functional assessment of Helios initially relied on overexpression of dominant negative versions of Helios or Ikaros, which were difficult to interpret because of the effects of dn forms on the function of other Ikaros family members (14,15). However, a null allele of Helios was generated in mice on a C57BL/6 genetic background, where homozygous loss of Helios drove neonatal lethality (16). Strikingly, on a mixed 129/BL/6 background, mice were viable, healthy and fertile. Further, T cell development and immune homeostasis appeared normal in the absence of Helios (16). The genetic reasons for the neonatal lethal phenotype of Helios-deficient mice on a BL/6 background are completely unclear. Nonetheless, these data suggested that Helios either played little role in T cell development/function or it was redundant with other Ikaros family members.

More recently, however, another group generated mice with a conditional allele of Helios that allowed for tissue-specific deletion of Helios (17). Loss of Helios in most T cells (driven by CD4Cre-driven loss of Helios), did not affect T cell development, function, or immune homeostasis—similar to Helios-deficient mice on a mixed genetic background (16,17). In recent work by the Cantor lab, FoxP3-Cre-driven deletion of Helios did not initially affect the numbers or frequency of Treg. Expectedly, given the phenotype of CD4-Cre and global Helios-deficient mice, deletion of Helios in Treg also did not result in rapid onset autoimmunity, unlike FoxP3-deficient mice (16,17). Interestingly, the authors aged the FoxP3Cre-Helios$^{f/f}$ mice and found that, after 4–5 months of age, FoxP3Cre-Helios$^{f/f}$ mice spontaneously developed a delayed onset autoimmune-like condition with multi-organ inflammation, systemic lymphocyte activation, anti-nuclear and dsDNA antibodies, and kidney pathology (16,17). Further, the authors showed that Helios deficiency did not alter thymic development of Treg, showing that Helios is not required for Treg development. In addition, Helios deficiency did not alter negative selection of conventional T cells, suggesting the defect in autoimmunity was not due to the inability to cull autoreactive conventional T cells in the thymus. Instead, they showed that, unlike Helios-sufficient Treg, Helios-deficient Treg were unable to prevent the development of colitis in Rag2$^{-/-}$ mice driven by the transfer of colitogenic CD4$^{+}$FoxP3$^{-}$ cells. Also, reconstitution of scurfy mice with Helios-sufficient, but not Helios-deficient, bone marrow was able to prevent the spontaneous autoimmune disease that occurs in scurfy mice. Thus, it appears that Treg-specific loss of Helios results in a delayed onset autoimmune

disease that is associated with defective Treg function.

In an attempt to characterize the mechanism(s) underlying the defects in Treg that drive the development of autoimmunity in FoxP3Cre-Helios$^{f/f}$ mice, the authors performed a ChIP-seq experiment to identify genes that associated with Helios. They showed that Helios associated largely with the promoter regions of genes, particularly genes involved in apoptosis/survival. In particular, the authors found that Helios deficient Treg had lower levels of FoxP3, increased active caspase-3, defective expression of CD25, and reduced IL-2-driven STAT5 activation relative to Helios-sufficient Treg. Interestingly, the authors did not demonstrate a substantial loss of Treg in aged FoxP3Cre-Helios$^{f/f}$ mice, suggesting that the effects of Helios on cell survival genes was not sufficient to drive a substantial loss of Treg *in vivo*. Alternatively, there was a loss, but it was offset by a substantial increase in Treg production or proliferation. Regarding the effects of Helios on Treg function, the authors showed that following immunization, Helios-deficient Treg were able to produce cytokines reminiscent of effector cells—such as IFN-γ, IL-17 and TNF-α. Overexpression of a constitutively active version of STAT5 restored levels of FoxP3 and prevented aberrant expression of IFN-γ. Thus, it appears that Helios is critical for maintaining Treg identity, repressing their ability to express effector cytokines.

Since this initial work, two other papers have been published that have largely confirmed and extended the findings of the Cantor group. Work by the Shevach group demonstrated a milder phenotype of FoxP3Cre-Helios$^{f/f}$ mice in terms of autoimmunity, but a strikingly similar ability of Helios-deficient Treg to aberrantly produce inappropriate cytokines (18). While the Shevach group was unable to reproduce the Treg-specific requirement of Helios to prevent colitis in the adoptive transfer model, it is important to note that the controls in the Shevach experiments lacked induction of weight loss and the Tregs that were transferred, were purified on the basis of CD25 rather than by FoxP3-YFP as was done in the experiments by the Cantor group. Thus, their lack of confirmation of the failure of Helios-deficient Treg to control colitis needs to be interpreted with this caveat. However, they were able to demonstrate that Helios-deficient Treg were unable to restrain follicular T cell responses, which likely contributes to the autoantibody production in the FoxP3Cre-Helios$^{f/f}$ mice. Thus, despite the minor differences in these two papers, they both arrive at the conclusion that Helios is critical for the maintenance of Treg stability—at least in terms of cytokine production.

In terms of cancer, a recent paper has capitalized on these initial observations and extended them to suggest that manipulation of Helios may be a useful target to improve anti-tumor responses. In recent work by Kim and colleagues, Helios-deficient Treg were able to substantially infiltrate mouse tumors, however, they were unable to maintain their suppressive phenotype (19). Helios-deficient Treg isolated from B16 or M38 tumors displayed a significantly increased expression of IFN-γ and TNF-α compared to Helios expressing Treg. This dysregulation of Treg stability was associated with the ability of FoxP3Cre-Helios$^{f/f}$ mice to significantly restrain tumor growth. It is important to note that such loss of Treg stability was mainly in tumors, and not in splenic Treg. Helios may thus be a legitimate "checkpoint" target in which one could restrain Treg stability and unleash effector anti-tumor responses. An advantage is that the limited Helios expression in other cell types would suggest minimal off-target effects, should specific suppression of Helios become possible.

Of note, we recently reported in the non-human primate model the existence of Helios$^{lo}$ "inflammatory" Treg, that expressed not only FOXP3, but also RORc and T-bet, and could express a whole array of effector cytokines such as IL-17, IFN-g, IL-8 and IL-2 upon *ex vivo* restimulation (20). Importantly, this subset was more frequent in inflammatory conditions (20), suggesting that Helios could be a major factor active across species to control Treg phenotype during inflammatory responses.

The role of Helios in maintaining Treg stability raises more intriguing questions. First, the factors that maintain Helios expression in Treg are unclear. Further elucidation of the factors that control Helios expression would help identify potential therapeutic targets to manipulate Treg stability. For example, enhancement of Helios expression may promote resistance to autoimmunity. In contrast, methods to reduce the expression of Helios in the context of tumors, may promote Treg to Teff conversion and enhance anti-tumor immunity. In this regard, Kim and colleagues showed that engagement of GITR using an agonistic anti-GITR antibody decreased expression of Helios in Treg and favored their conversion to Teff. When given prophylactically, anti-GITR therapy substantially restricted tumor growth. More importantly, when given therapeutically, anti-GITR significantly retarded tumor growth, although the effects were not nearly as dramatic as when given prior to tumor implantation. Thus, the modest therapeutic effect of anti-GITR-driven in the B16 mouse model suggest that (I) GITR-down modulation of Helios may not be sufficient to unleash effector anti-tumor responses; (II) in addition to its

 

effects on Helios, agonistic anti-GITR may have other pro-tumorigenic properties; (III) downregulation of Helios may be necessary, but not sufficient to allow unrestrained effector response to drive tumor elimination.

In addition to GITR in the context of tumors, the initial data from Cantor and colleagues suggested that IL-2 was potentially important in controlling expression of Helios. Thus, Helios could serve as part of a positive feedback circuit in which IL-2, by promoting expression of Helios, controls the expression of the IL-2R, which reinforces IL-2-driven signaling and maintains expression of Helios. However, we have recently shown, in the context of aging, that despite the significantly lower levels of IL-2 in the serum of aged mice, that aged Treg maintain high levels of Helios expression (21,22). Thus, it is likely that IL-2 is not the only factor that maintains Helios expression in Treg.

Second, by what mechanism(s) does Helios suppress Treg expression of effector cytokines? One simple explanation could be that Helios-driven STAT5 promotes expression of FoxP3 which acts to transcriptionally suppress cytokine expression in Treg. The data showing that caSTAT5 restores FoxP3 levels and reduces effector cytokine expression is consistent with such an explanation. Further, the fact that the ChIP-seq data did not implicate Helios binding to the promoters of cytokine genes also argues against a direct role of Helios in directly controlling cytokine genes. However, it does not exclude the possibility of Helios controlling transcription factors such as t-bet or ROR-γt which are important for elaboration of various Th cytokines. Indeed, Treg from FoxP3Cre-Helios$^{f/f}$ mice have substantially increased expression of t-bet (19). Thus, more work needs to be done to examine the mechanism(s) by which Helios restricts effector cytokine expression by Treg.

Third, does Helios collaborate with other transcription factors known to be involved with control of Treg stability, such as RORγt, AP-1, and FoxP3 itself? Future work examining Helios expression in various conditions of Treg plasticity should determine whether Helios downregulation is associated with situations in which Treg fail to maintain a stable phenotype. For example, given the data in the colitis models, it would be intriguing to determine if Treg-specific reduction in Helios expression is associated with colitis development in models involving a lack of Treg stability. In these contexts, Treg-specific overexpression of Helios may help determine whether reduction of Helios contributes to the loss of Treg stability. However, this approach must be tempered with the acknowledgement that Helios overexpression may lead to non-specific effects and mimicking of other Ikaros factors at high Helios concentrations. Nonetheless, monitoring of Ikaros family members in various Th lineages, ChIP on ChIP, and co-immunoprecipitation experiments should allow for determination of the specific role of Helios in maintenance of Treg stability and the biologic consequences thereof.

Fourth, while Treg clearly acquire some effector function in the absence of Helios, it remains unclear how much of the Treg program is impaired. For example, do Helios-deficient Treg express classic markers associated with Treg function (i.e., CTLA-4, TGF-β, IL-10)? If they retain certain aspects of Treg function, it may explain the delayed and less severe development of systemic autoimmunity in FoxP3Cre-Helios$^{f/f}$ mice. Also, as described above, it will be interesting to determine whether post-thymic development loss of Helios in Treg can result in the loss of peripheral tolerance. Such data would provide further justification for manipulation of Helios as a therapeutic target.

In sum, this data suggests an exciting time in Treg biology. The elucidation of new factors that control Treg stability should lead to the identification of new therapeutics that can control immune tolerance. Future work will hopefully be focused on whether such mechanisms could be exploited to further stabilize Treg and reduce autoimmunity. Conversely, exploitation of factors that reduce Treg stability could be used to enhance anti-tumor and anti-vaccine responses. In this regard, it will be intriguing to determine whether the effects of Helios down modulation are long lasting or transient as one could envision that short-term Treg instability may be sufficient to alter anti-tumor responses, but not result in loss of self-tolerance.

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