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Helios defines T cells being driven to tolerance in the periphery and thymus

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Abbreviations:

H/Kα	H^+/K^+ ATPase α-subunit
H/Kβ	H^+/K^+ ATPase β-subunit
ILN	Inguinal lymph node
PgLN	Paragastric lymph node
pTreg cell	Peripherally-derived regulatory T cell
SP	Single positive

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Summary

The expression of the Ikaros transcription factor family member, Helios, has been shown to be associated with T-cell tolerance in both the thymus and the periphery. To better understand the importance of Helios in tolerance pathways, we have examined the expression of Helios in TCR-transgenic T cells specific for the gastric H^+/K^+ ATPase, the autoantigen target in autoimmune gastritis. Analysis of H^+/K^+ ATPase-specific T cells in mice with different patterns of H^+/K^+ ATPase expression revealed that, in addition to the expression of Helios in $CD4^+Foxp3^+$ regulatory T (Treg) cells , Helios is expressed by a large proportion of $CD4^+Foxp3^-$ T cells in both the thymus and the paragastric lymph node, (PgLN), which drains the stomach. In the thymus, Helios was expressed by H^+/K^+ ATPase-specific thymocytes that were undergoing negative selection. In the periphery, Helios was expressed in H^+/K^+ ATPase-specific $CD4^+$ T cells following H^+/K^+ ATPase presentation and was more highly expressed when T-cell activation occurred in the absence of inflammation. Analysis of purified H^+/K^+ ATPase-specific $CD4^+Foxp3^-Helios^+$ T cells demonstrated that they were functionally anergic. These results demonstrate that Helios is expressed by thymic and peripheral T cells that are being driven to tolerance in response to a genuine autoantigen.

Introduction

Helios, an Ikaros transcription factor family member, is expressed during the early stages of haematopoietic development in the embryo. However in the adult, expression is largely T cell-restricted, and is found within developing thymocytes and also mature T cells, where it is preferentially expressed by $CD4^+Foxp3^+$ regulatory T (Treg) cells [1-6]. Helios consists of two distinct zinc-finger domains: one at the N-terminus, which mediates binding to specific DNA sequences, and the second at the C-terminus, which functions to mediate dimerization of Helios with other Ikaros transcription factor family members [2, 4].

A number of studies have focused on identifying a role for Helios in T cells. Helios was initially postulated to act as a marker capable of discriminating between thymus-derived Treg cells and peripherally-derived Treg (pTreg) cells, however accumulating data appears to refute this hypothesis [1, 6-10]. Other work has demonstrated that $Helios^-$ and $Helios^+$ Treg cells exhibit distinct properties, with $Helios^+$ Treg cells displaying increased suppressive function and stability [1, 10-12]. However, additional roles for Helios have also been suggested, including that of a mediator of T-cell homeostasis, a marker of T-cell activation within both Treg cells and $Foxp3^-$ conventional T cells, or a marker associated with Th2-cell differentiation [7, 13-17]. Two recent papers have demonstrated a potential for Helios to be expressed in association with tolerogenic T-cell responses [18, 19]. Helios has been postulated to act as a suppressor of *Il2* gene transcription by binding to the *Il2* promoter and inducing epigenetic modifications [18]. Within the thymus, thymocytes with TCRs that strongly bind self-peptide–MHC complexes express Helios, and thereby Helios appears to be the first marker capable of distinguishing between positive and negative selection [19].

Given these findings, we were interested to characterise the expression of Helios within a model of autoimmune disease. Autoimmune gastritis represents an excellent disease to investigate this, as work using a variety of murine systems has allowed a thorough characterisation of the events that lead to tolerance to the antigen target of the disease, the gastric H^+/K^+ ATPase, a bona fide autoantigen. The gastric H^+/K^+-ATPase is composed of two subunits: the catalytic α-subunit (H/Kα) and the highly glycosylated β-subunit (H/Kβ) [20-22]. Thymic events contribute little to the tolerance of H^+/K^+-ATPase–specific T cells as

neither subunit is presented in the thymus [23-26]. As a result, tolerance to the H^+/K^+-ATPase appears to be established in the periphery: the H^+/K^+-ATPase is presented exclusively by $CD11c^+$ DCs of the PgLN, and this leads to tolerance of H/Kα-specific T cells, which is established by clonal deletion and regulation by Treg cells [27, 28].

In the present study, we analysed the expression of Helios in the thymus and periphery of A23 TCR-transgenic mice, a severe and spontaneous model of autoimmune gastritis in which all T cells are directed toward an epitope derived from the H/Kα [29]. Expression of Helios was analysed in a range of A23 TCR-transgenic mice on different backgrounds of H/Kα expression. From these analyses, we have shown that Helios is expressed in self-reactive H/Kα-specific T cells being driven to undergo negative selection in the thymus and also coincides with the development of functional anergy in peripheral H/Kα-specific T cells following antigen-driven activation in local draining lymph nodes.

Results

Helios is expressed in thymic and peripheral A23 TCR-transgenic T cells upon H/Kα presentation

Helios is expressed in thymocytes undergoing negative selection and also in peripheral T cells where it acts to suppress IL-2 gene transcription [18, 19], and thus Helios expression is associated with tolerogenic T-cell responses. In order to investigate the influence of antigen presentation on the expression of Helios in A23 TCR-transgenic T cells, the expression of Helios by $CD4^+Foxp3^-$ conventional T cells and $CD4^+Foxp3^+$ Treg cells was analysed in thymi and periphery lymphoid organs of A23 TCR-transgenic mice with different patterns of H/Kα expression. Three different strains of A23 TCR-transgenic mice were analysed: A23.H/K$\alpha^{-/-}$ mice, which lack presentation of H/Kα in all organs [30], A23 mice on a WT background of H/Kα-expression, wherein the H/Kα is not presented in the thymus but is presented in the periphery [24], and A23.IE-H/Kβ mice, which express H/Kβ in the thymus under the control of the MHC class II I-Eα promoter [31], facilitating presentation of H/Kα and thus representing a strain in which negative selection of H/Kα-specific T cells occurs [24].

In thymi of A23.H/K$\alpha^{-/-}$ and A23 mice, approximately 80% of the CD4 single positive (SP) thymocytes that were $Foxp3^+$ also expressed Helios (Fig. 1A and B), in agreement with previous studies [1, 6]. A significantly higher proportion and number of Treg cells in thymi of A23.IE-H/Kβ mice were $Helios^+$ when compared with the Treg cells present in A23.H/K$\alpha^{-/-}$ and A23 mice (Figure 1A, B and C). Helios expression was also analysed within $Foxp3^-$ thymocytes. In thymi of A23.IE-H/Kβ mice, the proportion and number of CD4 SP $Foxp3^-$ thymocytes that expressed Helios was far greater than observed in A23 and A23.H/K$\alpha^{-/-}$ mice (Fig. 1A- C). These $Helios^+$ CD4 SP cells most likely represent thymocytes whose TCRs bind strongly to the H/Kα/MHC-II and are therefore being driven to undergo negative selection [19].

In the periphery, approximately 80 – 90% of the Treg cells in the PgLNs, inguinal lymph nodes (ILNs) and spleens of the three strains of A23 TCR-transgenic mice expressed Helios (Figure 2A and B). The proportion of Helios$^+$ Treg cells within the PgLNs of A23.IE-H/Kβ mice was significantly higher than observed in A23 and A23.H/K$\alpha^{-/-}$ mice, although the difference was quite small (Figure 2A – C).

In the PgLNs of A23 mice, approximately 50% of the CD4$^+$Foxp3$^-$ cells expressed Helios (Figure 2A – C). This was only observed in PgLNs, and not in ILNs or spleens where the H/Kα is not presented (Figure 2A). This population of cells was not observed in the PgLNs of A23.HK$\alpha^{-/-}$ mice, which lack presentation of H/Kα in all tissues and organs, or in the PgLNs of A23.IE-H/Kβ mice, in which the H/Kα is presented in the periphery (Figure 2A). In thymi of A23.IE-H/Kβ mice, presentation of the H/Kα leads to negative selection and the efficient deletion of H/Kα-specific thymocytes at the CD4 SP stage. A minority of T cells fully mature in thymi of A23.IE-H/Kβ mice and leave that organ, however the levels of CD4 and TCR expression on these cells is lower when compared with A23 mice, which presumably allows such cells to escape negative selection [24]. Therefore, a likely explanation is that mature CD4$^+$Foxp3$^-$ cells that seed the periphery in A23.IE-H/Kβ mice are unable to bind to H/Kα with high enough affinity to induce Helios expression in PgLNs.

Collectively, these results demonstrate that the majority of H/Kα-specific Treg cells are Helios$^+$ in the thymus and the periphery. In addition, H/Kα-specific CD4$^+$Foxp3$^-$ T cells can express substantial levels of Helios, as evident within H/Kα-specific thymocytes undergoing negative selection, an observation that is in agreement with a recent publication [19], and also in the periphery in response to H/Kα presentation.

Helios is induced on A23 TCR-transgenic T cells following antigen exposure in vivo

It was particularly striking that Helios expression was observed in approximately 50% of CD4$^+$Foxp3$^-$ cells in the PgLNs of A23 mice (Figure 2). Within this strain of mice, there is no presentation of H/Kα in the thymus, rather, the PgLN is the site at which A23 TCR-transgenic T cells are first presented with H/Kα epitopes. These results indicate that Helios

may be expressed in $CD4^+Foxp3^-$ cells in response to H/Kα presentation. To investigate this, $CD4^+$ T cells from peripheral LNs of A23.H/K$\alpha^{-/-}$ mice were transferred to WT and H/K$\alpha^{-/-}$ recipients. Prior to transfer, the donor A23.H/K$\alpha^{-/-}$ cells were analysed for Foxp3 and Helios expression (Figure 3A, C and D). Approximately 2% of $CD4^+Foxp3^-$ cells expressed Helios and approximately 80% of Treg cells expressed Helios.

Four days after transfer, the number of transferred A23.H/K$\alpha^{-/-}$ cells was significantly lower in the PgLNs of the H/K$\alpha^{-/-}$ recipients in comparison with the WT recipients (Figure 3B, identification of transferred cells using CD90 congenic marker is shown in Supporting Information Fig. 1). This indicates that the A23.H/K$\alpha^{-/-}$ cells are unable to proliferate in the absence of the H/Kα, inhibiting their ability to persist in these mice. Analysis of the PgLNs of the recipient mice revealed that the proportion of $Foxp3^-Helios^+$ A23.H/K$\alpha^{-/-}$ cells was significantly higher following transfer to the WT recipients in comparison with the proportion observed prior to transfer, whereas the proportion of $Foxp3^-Helios^+$ cells in the H/K$\alpha^{-/-}$ recipients following transfer was unchanged (Figure 3C). This indicates that presentation of H/Kα drives the expression of Helios in the transferred A23.H/K$\alpha^{-/-}$ cells. On the other hand, the proportion of the transferred $CD4^+$ cells that were $Foxp3^+Helios^+$ was significantly lower following transfer to both the WT and the H/K$\alpha^{-/-}$ recipients in comparison with the proportion prior to transfer (Figure 3D).

Taken together, the transfer of $CD4^+$ A23.H/K$\alpha^{-/-}$ T cells to WT and H/K$\alpha^{-/-}$ recipients supports the potential role of Helios as a marker of antigen exposure in peripheral $CD4^+Foxp3^-$ cells. However, A23.H/K$\alpha^{-/-}$ Treg cells appear to slightly decrease expression of Helios following transfer to WT and H/K$\alpha^{-/-}$ recipients.

Expression of Helios by $CD4^+Foxp3^-$ A23 TCR-transgenic T cells is inhibited during inflammation

The results thus far suggest that Helios is expressed by peripheral $CD4^+Foxp3^-$ A23 TCR-transgenic T cells in an H/Kα-dependent manner. It is unclear whether the expression of Helios in H/Kα-specific T cells is indicative of a particular T-cell fate or function. Helios

cannot be used as a marker to identify H/Kα-specific Treg cells that are extrathymically derived, as H/Kα-specific pTreg cells comprise a population of both Helios$^-$ and Helios$^+$ cells (Supporting Information Fig. 2), a result consistent with previous publications [7, 9, 10, 13]. Helios has been demonstrated to play a role as a suppressor of IL-2 gene transcription [18], therefore we reasoned that Helios may be expressed in peripheral CD4$^+$Foxp3$^-$ cells in which anergy is being induced. To test this idea we compared Helios expression in T cells in a tolerogenic environment compared with an inflammatory situation. PC-GMCSF mice express GM-CSF within the stomach leading to spontaneous development of autoimmune gastritis [32]. In these mice H$^+$/K$^+$ ATPase-specific T cells are activated and develop into pathogenic effector T cells, contrasting to the situation in WT mice where H$^+$/K$^+$ ATPase-specific T cells recognise antigen but are tolerised and do not develop into effector T cells (unpublished data). Therefore, CD4$^+$ T cells from A23.H/Kα$^{-/-}$ mice were transferred to WT and PC-GMCSF recipients and analysed for Helios expression.

Six days later, the A23.H/Kα$^{-/-}$ cells had expanded and accumulated to a higher degree (Figure 4A, identification of transferred cells using CD90 congenic marker is shown in Supporting Information Fig. 3) and had divided to a greater extent (Figure 4B and C) in the inflammatory environment within the PgLNs of the PC-GMCSF mice in comparison with WT mice. The proportion of A23.H/Kα$^{-/-}$ cells that were Foxp3$^-$Helios$^+$ was significantly lower in the PC-GMCSF recipients compared with the proportion in the WT recipients (Figure 4C and D), and in addition the level of Helios expression by the Foxp3$^-$Helios$^+$ cells in PC-GMCSF recipients was lower when compared with WT recipients (Figure 4C and E). On the other hand, the proportion of A23.H/Kα$^{-/-}$ cells that were Foxp3$^+$Helios$^+$ was not significantly different between the WT and PC-GMCSF mice, although the level of Helios expression by the Foxp3$^+$Helios$^+$ cells was higher in the WT recipients (Figure 4C, F and G).

Collectively, these results demonstrate that inflammatory conditions favouring the development of effector T cells are less conducive for the expression of Helios by CD4$^+$Foxp3$^-$ A23.H/Kα$^{-/-}$ T cells when compared with the steady state.

Helios$^+$ cells display anergic properties following peptide stimulation in vitro

To assess if Helios$^+$ cells in the PgLNs of A23 mice may be anergic in function, we stimulated naive A23.H/K$\alpha^{-/-}$ and antigen-experienced A23 PgLN cells with A23 peptide *in vitro* (Supporting Information Fig. 4). Only approximately 2% of CD4$^+$Foxp3$^-$ cells in the naïve A23.H/K$\alpha^{-/-}$ cells are Helios$^+$, and stimulation of these cells lead to production of IL-2 and TNFα (Supporting Information Fig. 4A and B). On the other hand, approximately 50% of CD4$^+$Foxp3$^-$ cells are Helios$^+$ in antigen-experienced A23 mice and cytokine production was not detected in these cells. Additionally, A23 cells retained Helios expression following stimulation (Supporting Information Fig. 4C and D).

The above *in vitro* experiments suggests that Helios expression may be associated with T-cell anergy, but the data are complicated by the relative impurity of the T cells. In order to definitively demonstrate a relationship between Helios expression and anergy, we devised a strategy for the purification of Treg-free Helios$^+$ and Helios$^-$ CD4$^+$ T cells from PgLNs of A23 mice. Since expression of Helios is associated with T-cell activation [7, 13] and in an autoimmune arthritis model, autoantigen reactive CD4$^+$ T cells become anergic and upregulate the expression of the cell surface markers folate receptor 4 (FR4) and CD73 [33], we decided to examine if CD62L, CD44, FR4 and CD73 could be used to isolate Helios-expressing CD4$^+$Foxp3$^-$ A23 PgLN cells.

It was found that the majority of CD4$^+$Foxp3$^-$Helios$^+$ cells displayed an activated phenotype as demonstrated by expression of CD62L and CD44. A higher proportion of CD4$^+$Foxp3$^-$ Helios$^+$ cells were activated CD62L^{lo}CD44^{hi} cells than CD4$^+$Foxp3$^-$Helios$^-$ cells (Figure 5A and B). On the other hand, CD62L and CD44 expression was similar on A23 Treg cells regardless of whether they were Helios$^-$ or Helios$^+$. These results suggest that Helios expression in CD4$^+$Foxp3$^-$ A23 TCR-transgenic T cells is associated with T-cell activation, consistent with the H/Kα-dependent expression of Helios in A23 TCR-transgenic T cells (Figure 3).

Approximately 75% of the CD4$^+$Foxp3$^-$Helios$^+$ cells were CD73$^-$FR4$^+$, which was far higher than the proportion of CD4$^+$Foxp3$^-$Helios$^-$ cells that were CD73$^-$FR4$^+$ (Figure 5C and D).

Few $CD4^+Foxp3^-Helios^+$ A23 PgLN cells were $CD73^+FR4^+$. On the other hand, CD73 and FR4 expression was similar on A23 Treg cells regardless of whether they were $Helios^-$ or $Helios^+$.

Based on this analysis, a strategy was devised to enrich $CD4^+Foxp3^-Helios^+$ and $CD4^+Foxp3^-Helios^-$ T cells from PgLNs of A23 mice in order to analyse their function (Figure 6). Firstly, to exclude Treg cells, $CD4^+$ live cells were gated on $CD25^-CD73^-$ cells, as the majority of Treg cells are $CD25^+CD73^+FR4^+$. To obtain a population of $Foxp3^-Helios^+$ cells, the $CD4^+CD73^-CD25^-$ population was gated on $CD62L^-FR4^+$ cells, and to obtain the $Foxp3^-Helios^-$ population, the $CD4^+CD73^-CD25^-$ population was gated on $CD62L^-FR4^-$ cells (Figure 6A). Using this strategy, $Foxp3^-Helios^+$ cells could be reproducibly enriched by flow cytometric sorting to a purity of >80% of $CD4^+$ live T cells and $Foxp3^-Helios^-$ cells to a purity of >70% (Figure 6B, C and Supporting Information Fig. 5). Importantly, very few of the purified cells are convincingly $Foxp3^+$ (Figure 6B).

To address whether $CD4^+Foxp3^-Helios^+$ cells in PgLNs of A23 mice are anergic, $Foxp3^-Helios^+$ and $Foxp3^-Helios^-$ cells were purified using the strategy described above, labelled with CFSE and stimulated *in vitro* with A23 peptide and irradiated splenocytes for four days.

The number and proportion of $Foxp3^-Helios^+$ cells that were live after four days of culture was significantly lower than $Foxp3^-Helios^-$ cells (Figure 7A, B and C). In the remaining live cells, CFSE dilution analysis indicated that $Foxp3^-Helios^+$ cells proliferated less than $Foxp3^-Helios^-$ cells, at both peptide concentrations (Figure 7A, D and E). Furthermore, $Foxp3^-Helios^-$ cells produced high levels of IL-2 whereas very little IL-2 was detected in the cultures of $Foxp3^-Helios^+$ cells (Figure 7F). Taken together, these results demonstrate that antigen-experienced $CD4^+Foxp3^-Helios^+$ T cells from PgLNs of A23 mice are anergic, as they proliferate less than $CD4^+Foxp3^-Helios^-$ cells, do not produce IL-2, and have poor survival in culture.

Discussion

Helios, an Ikaros transcription factor family member, is expressed by thymocytes undergoing negative selection and has also been shown to function as an inhibitor of IL-2 expression in peripheral T cells [18, 19]. These publications suggest a role for Helios in tolerogenic responses, therefore here we have characterised the expression of Helios in the thymus and the periphery of A23 TCR-transgenic mice, a well-characterised model of an organ-specific autoimmune disease directed towards a genuine autoantigen [24, 27-29, 34].

Helios has been suggested to be a marker of activation in both Foxp3$^-$ and Foxp3$^+$ T cells [13, 18]. Consistent with these reports, Helios was expressed in peripheral CD4$^+$Foxp3$^-$ A23 TCR-transgenic T cells in response to H/Kα presentation. In PgLNs of A23 mice, a population of CD4$^+$Foxp3$^-$ T cells expressed Helios$^+$, and this population was not apparent in other peripheral lymphoid sites where H/Kα epitopes were not presented, such as the ILN and spleen. Following transfer to H/Kα-sufficient recipients, there was a larger population of Foxp3$^-$Helios$^+$ T cells within A23.H/Kα$^{-/-}$ cells in comparison with that observed prior to transfer, indicating either an expansion of this population or the *de novo* expression of Helios within the transferred T cells following H/Kα presentation. This was not observed when the A23.H/Kα$^{-/-}$ T cells were transferred to H/Kα$^{-/-}$ recipients, indicating that Helios expression was dependent on H/Kα expression. Furthermore, analysis of CD44 and CD62L expression by Foxp3$^-$Helios$^+$ T cells in PgLNs of A23 mice revealed that they had a more activated phenotype than Foxp3$^-$Helios$^-$ T cells.

Helios has been shown to act in T cells to repress transcription of the *Il2* gene, therefore we reasoned that expression of Helios in peripheral antigen-experienced A23 TCR-transgenic T cells could be indicative of T-cell anergy [18]. Indeed, H/Kα exposure under tolerogenic conditions resulted in a larger population of H/Kα-specific Foxp3$^-$Helios$^+$ T cells than that observed during inflammation, as evidenced by the transfer of A23.H/Kα$^{-/-}$ T cells to WT or PC-GMCSF mice. To test the function of H/Kα-specific Foxp3$^-$Helios$^+$ T cells, we designed a sorting strategy employing cell surface markers to enrich Foxp3$^-$Helios$^+$ or Foxp3$^-$Helios$^-$ T cells. PgLN cells from A23 mice were analysed for expression of CD73 and FR4, given that CD73 and FR4 have been identified as novel anergy markers in self-reactive glucose-6-

phosphate isomerase-specific $CD4^+Foxp3^-$ T cells in a mouse model of autoimmune arthritis [33]. $Foxp3^-Helios^+$ cells expressed more FR4 than $Foxp3^-Helios^-$ cells, however, only a moderate proportion of $Foxp3^-Helios^+$ cells expressed CD73. This contrasts to the afore mentioned study in which anergic cells expressed high levels of CD73 in addition to FR4 [33]. A sorting strategy employing CD73, CD25, FR4 and CD62L allowed enrichment of $Foxp3^-Helios^+$ and $Foxp3^-Helios^-$ A23 PgLN cells for functional analysis *in vitro*. $Foxp3^-Helios^+$ cells produced minimal IL-2, proliferated less than $Foxp3^-Helios^-$ cells and survived poorly in culture, demonstrating that Helios expression in peripheral A23 TCR-transgenic T cells is indicative of antigen-experienced cells that are anergic in function.

Within thymi of A23.IE-H/Kβ mice, where overexpression of the H/Kβ facilitates presentation of the H/Kα and negative selection [23-26], a distinct population of CD4 SP $Foxp3^-$ T cells expressed Helios, which was not evident in A23 or A23.H/$K\alpha^{-/-}$ mice, indicating that expression of Helios in CD4 SP non-Treg cells in the thymus is driven by H/Kα presentation. Daley et al. demonstrated that Helios expression is induced during negative selection, which is consistent with our finding that Helios is expressed by $Foxp3^-$ CD4 SP cells in A23.IE-H/Kβ mice during negative selection [19]. In line with this, there was an absence of a distinct population of Helios-expressing $CD4^+Foxp3^-$ T cells in the PgLNs of A23.IE-H/Kβ mice, in contrast to A23 mice. Our data suggests that these T cells are unable to bind with a high enough affinity to H/Kα epitopes in the PgLN in order to induce Helios expression. Therefore, our analyses indicate that Helios distinguishes the negative selection of T cells that are specific for a genuine autoantigen.

Helios has been postulated to act as a marker that discriminates between thymus-derived Treg cells and pTreg cells [6], however, Helios was unable to be used as a marker for H/Kα-specific pTreg cells, a result in agreement with others [7, 9, 10, 13]. In thymi of A23.IE-H/Kβ mice, a greater amount of Treg cells expressed Helios in comparison with Treg cells in thymi of A23 and A23.H/$K\alpha^{-/-}$ mice. Thus, antigen presentation in thymi of these mice appears to drive the expression of Helios in $Foxp3^+$ Treg cells. The expression of Helios was high in $Foxp3^+$ Treg cells within the periphery of the three strains of A23 TCR-transgenic mice, in agreement with previous observations [1, 6]. Helios has been suggested to be a marker of activation in both $Foxp3^-$ and $Foxp3^+$ T cells [13, 18]. Whether H/Kα expression was

required to induce or maintain the expression of Helios in H/Kα-specific Foxp3$^+$ Treg cells in the periphery was unclear. Studies have demonstrated an important role for Helios in Treg cells, with Helios being required for optimal suppressive function, stability and cytokine regulation [1, 10-12, 18]. These observations warrant further investigation into Helios$^-$ and Helios$^+$ Treg cells during the course of autoimmune gastritis.

In conclusion, using a model of autoimmune gastritis, we have demonstrated that Helios expression can be used as a marker of tolerogenic CD4$^+$ T-cell responses directed towards a genuine autoantigen. In addition to being expressed by H/Kα-specific Foxp3$^+$ Treg cells in the thymus and the periphery, Helios is also expressed by Foxp3$^-$ H/Kα-specific T cells undergoing negative selection in the thymus and, in addition, is expressed by anergic Foxp3$^-$ H/Kα-specific T cells in the periphery. Thus Helios may be a useful marker to define T cells that are being driven to tolerance in the periphery as well as the thymus.

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Materials and Methods

Mice

WT BALB/cCrSlc [35] congenic CD90.1 mice, RAG-1$^{-/-}$ [36], A23 TCR-transgenic mice [29], H/Kα-deficient mice [30] and IE-H/Kβ-transgenic mice [31] were housed under specific pathogen-free conditions at the Bio21 Molecular Science and Biotechnology Institute, the University of Melbourne. PC-GMCSF mice [32] were housed under conventional conditions at the Department of Zoology, University of Melbourne. All strains of mice were backcrossed at least ten times to BALB/cCrSlc mice. A23.H/Kα-deficient mice were generated by intercrossing A23 TCR-transgenic mice and H/Kα-deficient mice; A23.IE-H/Kβ-transgenic mice were generated by intercrossing A23 TCR-transgenic mice and IE-H/Kβ-transgenic mice; and A23.RAG-1$^{-/-}$ mice were generated by intercrossing A23 TCR-transgenic mice with BALB/c.RAG-1$^{-/-}$ mice. All mice and experiments were approved by the University of Melbourne Animal Experimentation Ethics Committee.

Antibodies and flow cytometry

All antibodies were purchased from eBioscience unless otherwise stated. Anti-CD44–biotin (IM7), anti-CD4–eFluor 450 (RM4-5), anti-CD8α–PE-Cy7 (53-6.7), anti-CD62L–FITC (MEL-14), anti-CD90.2–PerCP (30-H12), anti-CD25–PerCP-Cy5.5 (PC61.5), anti-V alpha 2–biotin (B20.1), anti-FR4–PE-Cy7 (eBio12A5), anti-CD73–biotin (TY/11.8), anti-Foxp3–APC (FJK-16s) and Fixable Viability Dye eFluor 780. Streptavidin–V500, anti-CD90.2–biotin (53-2.1) and anti-CD90.1–PerCP (OX-7) (BD Biosciences). Anti-Helios–PE (22F6) (Biolegend). Intracellular staining of Foxp3 and Helios was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience). Flow cytometry acquisition was performed on a LSR Fortessa^{TM} flow cytometer (BD, California, USA), and analysis was performed using FlowJo software (Tree Star, Inc., USA).

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Preparation and transfer of CD4$^+$ A23.H/K$\alpha^{-/-}$ T lymphocytes

CD4$^+$ cells were isolated from peripheral LN cells of A23.H/K$\alpha^{-/-}$ mice, which expressed both CD90.1 and CD90.2. Briefly, single cell suspensions were resuspended in F4/80 and B220 hybridoma supernatants in addition to an antibody directed to CD8α (53.6.72, BioX-Cell), and the antibody-bound cells were removed using anti-rat IgG-coated magnetic beads (QIAGEN). The CD4$^+$ T cells were then labelled with CFSE as previously described [37]. CD4$^+$ CFSE-labelled A23.H/K$\alpha^{-/-}$ T cells (2×10^6) were then transferred to WT or H/K$\alpha^{-/-}$ CD90.1 congenic mice or to WT or PC-GMCSF CD90.2 congenic mice by i.v. injection. A23.H/K$\alpha^{-/-}$ T cells were identified in the recipient mice by CD4 and CD90.2 or CD90.1 expression.

In vitro T-cell proliferation assay

Single cell suspensions were prepared from the PgLNs of A23 and A23.H/K$\alpha^{-/-}$ mice and CFSE labelled as previously described [37]. LN cells ($1x10^5$) were stimulated with 0, 5 or 10 μg/mL of H/K$\alpha_{630\text{-}641}$ peptide (PIT AKA IAA ASV G) for two or four days in RPMI-1640 media supplemented with 10% FCS, 2mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and 50 μM 2-mercaptoethanol. For sorting of Foxp3$^-$Helios$^+$ and Foxp3$^-$Helios$^-$ cells, single cell suspensions were prepared from the PgLNs of A23 mice, sorted as described in Figure 6 and CFSE labelled. Sorted Foxp3$^-$Helios$^+$ and Foxp3$^-$Helios$^-$ cells (5×10^4) were stimulated with 5×10^4 irradiated splenocytes as antigen presenting cells, and peptide as described above. After culture, cells were harvested and stained for analysis by flow cytometry. Supernatants were collected and analysed for the presence of T-cell cytokines using the BD Cytometric Bead Array Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences). Cytometric Bead Array data was analysed using FCAP Array™ software (BD Biosciences) to determine cytokine concentration.

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Statistical analysis

Statistical analysis was performed using GraphPad Prism 5. $p > 0.05$ was considered not significant.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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Figure 1. Helios expression in thymi of A23 TCR-transgenic mice on different backgrounds of H/Kα expression.
Thymi of 6–8 week old A23.H/K$\alpha^{-/-}$, A23 and A23.IE-H/Kβ mice were collected and analysed for expression of Foxp3 and Helios by flow cytometry. (A) Representative flow cytometric plots are gated on lymphocytes (left panel) and show CD4 and CD8 expression. Flow cytometric plots in right panel are gated on $CD4^+CD8^-$ single positive (SP) cells and show Foxp3 and Helios expression. The (B) proportion and (C) number of CD4 SP $Foxp3^+$ and $Foxp3^-$ cells that were $Helios^+$ were quantified by enumerating cells in the appropriate flow cytometric gates as shown in (A). Data are shown as mean + SEM of $n = 6$ (A23.H/K$\alpha^{-/-}$), $n = 4$ (A23), $n = 8$ (A23.IE-H/Kβ) and are pooled from four individual experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ (Mann Whitney test).

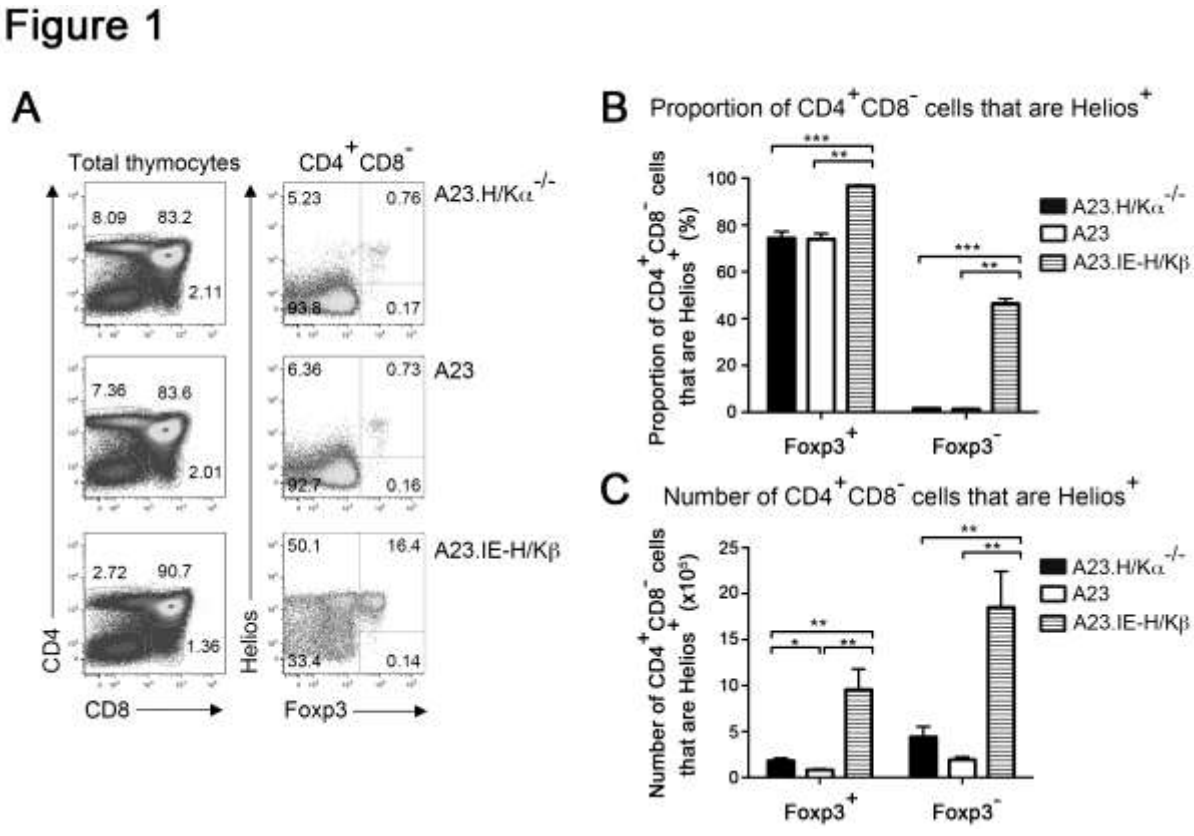

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Figure 2. Helios expression in the periphery of A23 TCR-transgenic mice on different backgrounds of H/Kα expression.

Paragastric lymph nodes (PgLNs), Inguinal lymph nodes (ILNs) and spleens of 6–8 week old A23.H/K$\alpha^{-/-}$, A23 and A23.IE-H/Kβ mice were collected and analysed for expression of Foxp3 and Helios by flow cytometry. (A) Representative flow cytometric plots are gated on $CD4^+$ cells and show Foxp3 and Helios expression. The (B) proportion and (C) number of $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells that were $Helios^+$ within the PgLNs was quantified enumerating cells in the appropriate flow cytometric gates as shown in (A). Data are shown as mean + SEM of $n = 6$ (A23.H/K$\alpha^{-/-}$), $n = 4$ (A23), $n = 8$ (A23.IE-H/Kβ) and are pooled from four individual experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ (Mann Whitney test).

Figure 2

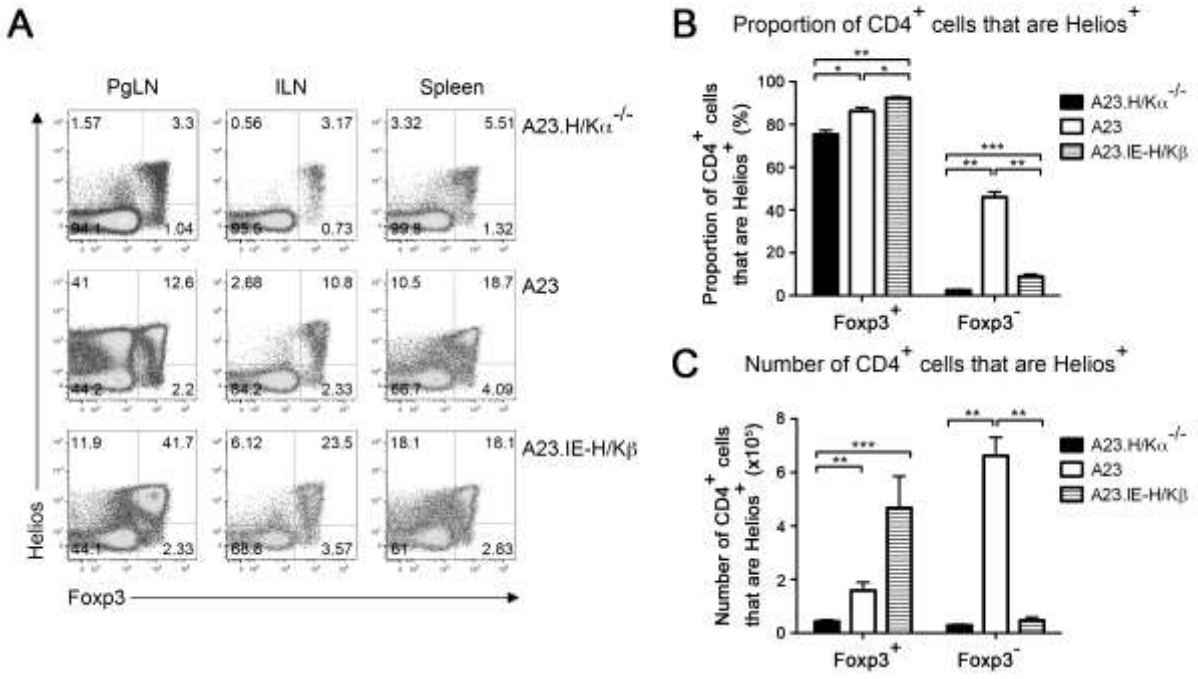

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Figure 3. Conventional CD4$^+$ A23.H/Kα$^{-/-}$ T cells express Helios following transfer to H/Kα-replete but not H/Kα-deficient recipients.

A23.H/Kα$^{-/-}$ CD4$^+$CD90.1$^+$CD90.2$^+$ cells were transferred into CD90.1 WT or H/Kα$^{-/-}$ recipients. Four days later, PgLNs were collected for analysis of marker expression in the transferred cells by flow cytometry. A) A23.H/Kα$^{-/-}$ peripheral LN cells were analysed for expression of Foxp3 and Helios prior to transfer and four days following transfer. Representative flow cytometric plots are gated on CD4$^+$ cells (left panel) or CD4$^+$CD90.2$^+$ cells (middle and right panels) and show Foxp3 and Helios expression. (B) The number of transferred CD4$^+$CD90.2$^+$ cells was quantified by flow cytometry in the PgLNs. The proportions of (C) CD90.2$^+$CD4$^+$Foxp3$^-$ and (D) CD90.2$^+$CD4$^+$Foxp3$^+$ cells that were Helios$^+$ were quantified in the PgLNs by flow cytometry. (B–D) Each symbol represents an individual mouse, except for prior to transfer data, which represents pooled A23.H/Kα$^{-/-}$ CD4$^+$CD90.1$^+$CD90.2$^+$ cells from several mice. Means are shown with horizontal bars. Data are pooled from three individual experiments(n = 6 (WT), n = 5 (H/Kα$^{-/-}$)). *p < 0.05; **p < 0.01; ***p < 0.001 (Mann Whitney test).

Figure 3

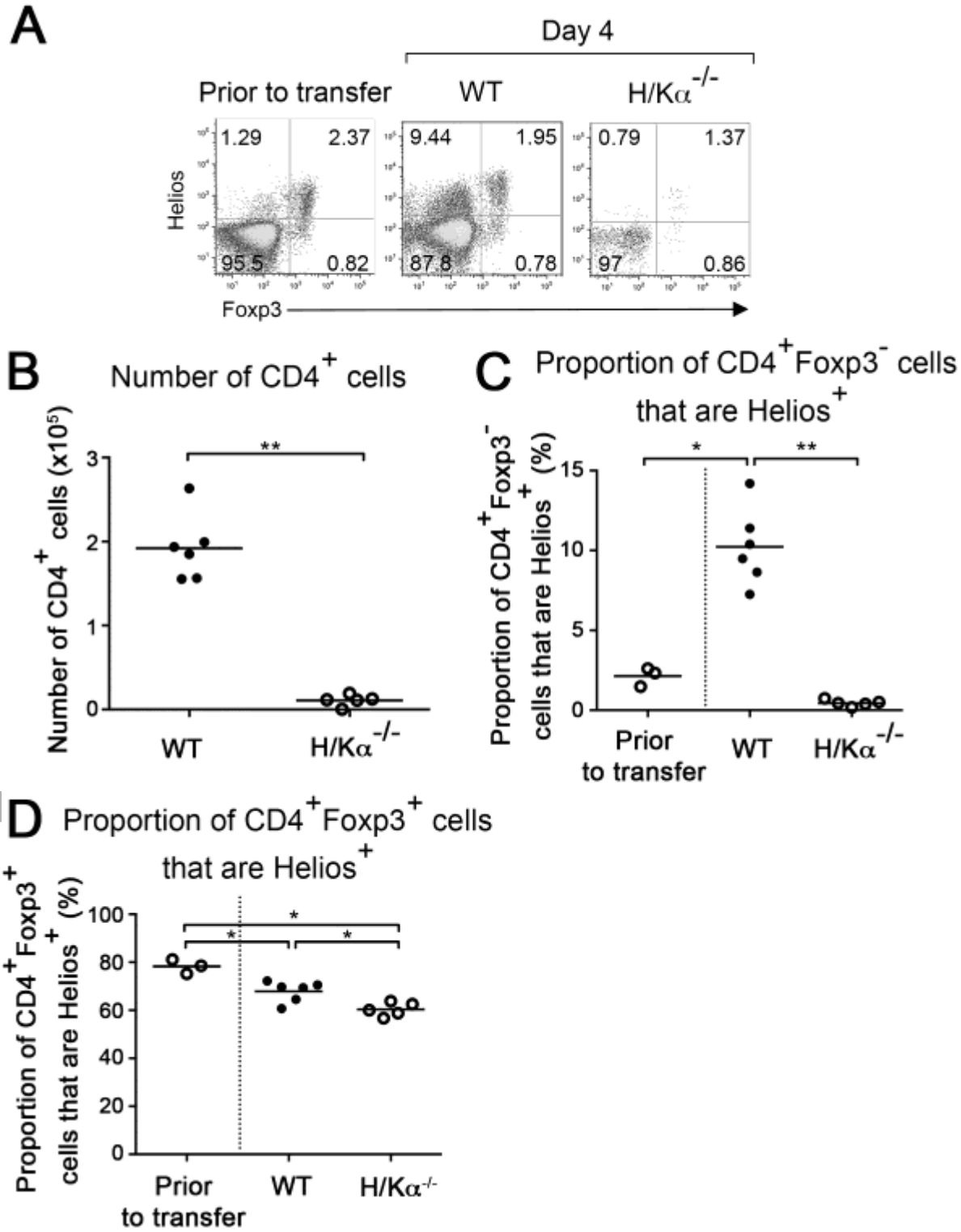

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Figure 4. Expression of Helios by CD4$^+$Foxp3$^-$ A23 TCR-transgenic T cells is inhibited under inflammatory conditions.

CFSE-labelled A23.H/K$\alpha^{-/-}$ CD4$^+$CD90.1$^+$CD90.2$^+$ cells were transferred into CD90.2 WT or PC-GMCSF recipients. Six days later, the PgLNs were collected for analysis of marker expression in the transferred cells by flow cytometry. (A) The number of transferred CD4$^+$CD90.1$^+$ cells was quantified in the PgLNs by flow cytometry. (B) Division of the A23.H/K$\alpha^{-/-}$ cells was analysed by quantifying the proportion of CD4$^+$CD90.1$^+$ cells in each CFSE division. Each symbol represents mean ± SD of six (WT) or eight (PC-GCSF) samples pooled from two independent experiments. (C) Representative flow cytometric plots are gated on CD4$^+$CD90.1$^+$Foxp3$^-$ cells (left two panels) or CD4$^+$CD90.1$^+$Foxp3$^+$ cells (right two panels) and show CFSE and Helios expression. The proportions of (D) CD90.1$^+$CD4$^+$Foxp3$^-$ cells or (F) CD90.1$^+$CD4$^+$Foxp3$^+$ cells that were Helios$^+$ were quantified in the PgLNs. The MFIs of Helios on (E) CD90.1$^+$CD4$^+$Foxp3$^-$ cells or (G) CD90.1$^+$CD4$^+$Foxp3$^+$ cells that were Helios$^+$ were quantified. (A, D–G) Each symbol represents an individual mouse and means are shown with horizontal bars. Data are pooled from two individual experiments(n = 6 (WT), n = 8 (PC-GMCSF)). *p < 0.05; **p < 0.01; ***p < 0.001 (Mann Whitney test).

Figure 4

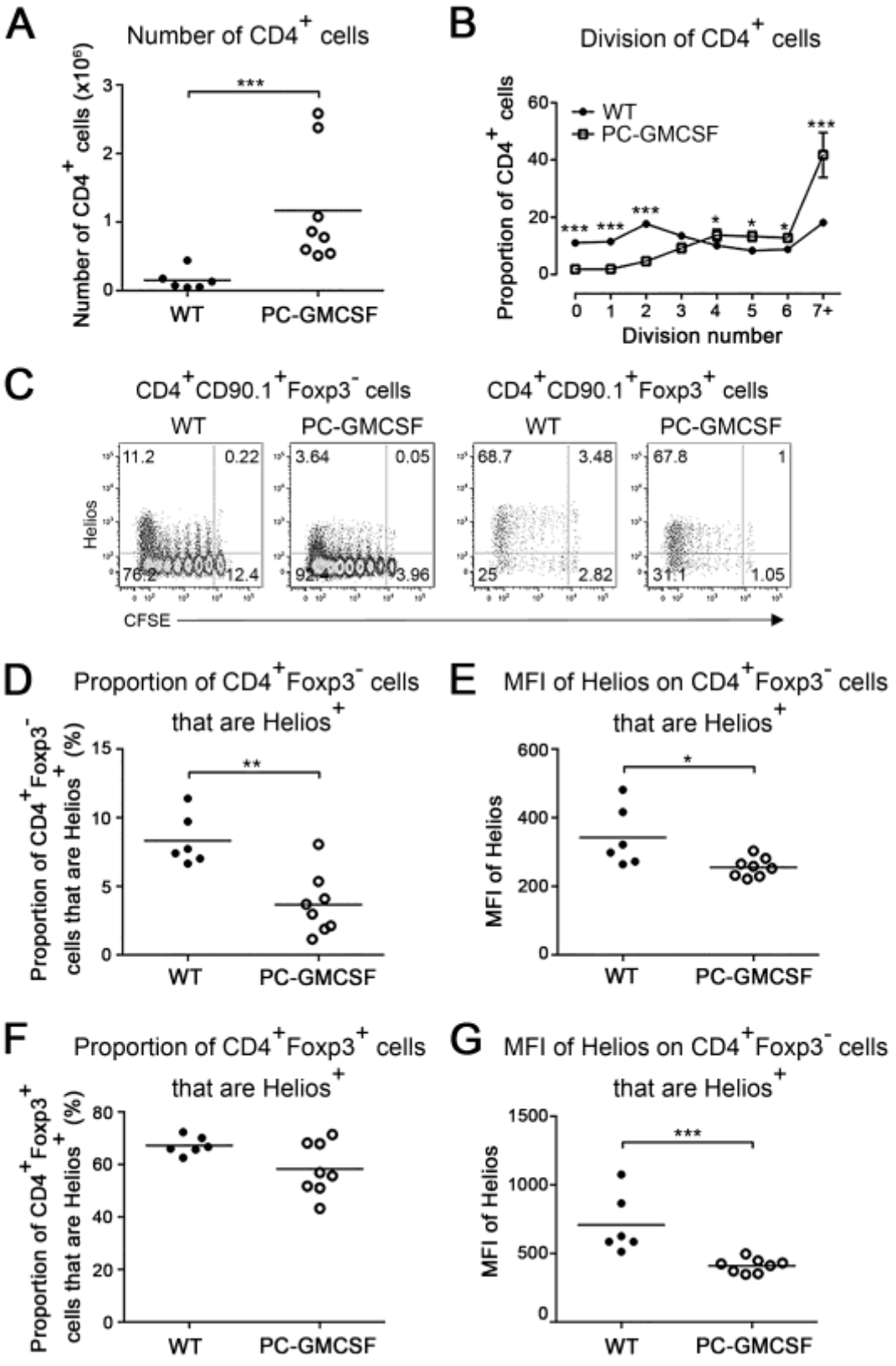

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Figure 5. Characterisation of surface markers expressed by Foxp3$^-$Helios$^+$ cells. PgLNs of 6–8 week old A23 mice were collected and analysed for expression of CD4, Foxp3, Helios, CD62L, CD44, CD73 and folate receptor 4 (FR4) by flow cytometry. (A) Representative flow cytometric plots are gated on CD4$^+$Foxp3$^-$ cells (top panel) and CD4$^+$Foxp3$^+$ cells (bottom panel) that are either Helios$^+$ (left panel) or Helios$^-$ (right panel). Plots show CD62L and CD44 expression. (B) The proportion of CD4$^+$Foxp3$^-$ and CD4$^+$Foxp3$^+$ cells that were Helios$^-$ or Helios$^+$ and also CD62L^{lo}CD44^{hi} was quantified by flow cytometry. Data are pooled from two individual experiments; $n = 7$. (C) Representative flow cytometric plots are gated on CD4$^+$Foxp3$^-$ cells (top panel) and CD4$^+$Foxp3$^+$ cells (bottom panel) that are either Helios$^+$ (left panel) or Helios$^-$ (right panel). Plots show CD73 and FR4 expression. (D) The proportions of CD4$^+$Foxp3$^-$ and CD4$^+$Foxp3$^+$ cells that were Helios$^-$ or Helios$^+$ and also CD73$^-$FR4$^+$ were quantified by flow cytometry. In (B) and (D) each symbol represents an individual mouse and means are shown with horizontal bars. Data are pooled from two individual experiments ($n = 8$). $^{**}p < 0.01$; $^{***}p < 0.001$ (Mann Whitney test).

Figure 5

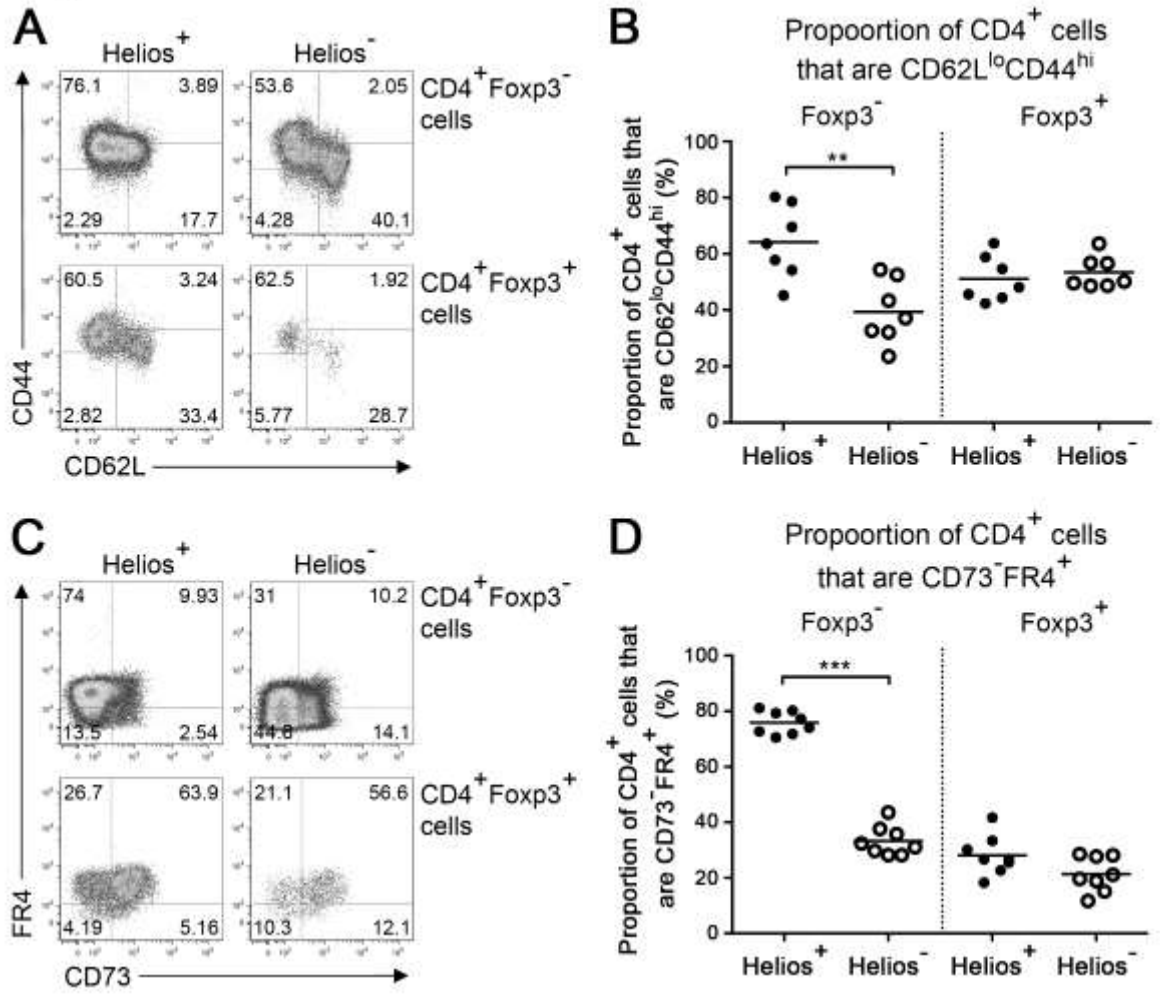

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Figure 6. Purification of Foxp3$^-$Helios$^+$ and Foxp3$^-$Helios$^-$ cells from PgLNs of A23 mice. (A) Strategy to allow purification of Foxp3$^-$Helios$^+$ and Foxp3$^-$Helios$^-$ cells. PgLNs of 6–8 week old A23 mice were collected and analysed for expression of CD4, Fixable Viability Dye, CD25, CD73, CD62L, FR4, Foxp3 and Helios by flow cytometry. Representative plots from one of two independent experiments show gating on the indicated population. (B) Purification of Foxp3$^-$Helios$^+$ and Foxp3$^-$Helios$^-$ cells by flow cytometric sorting. PgLNs of 6–8 week old A23 mice were collected and stained for CD4, Fixable Viability Dye, CD25, CD73, CD62L and FR4. CD62L$^-$FR4$^+$ or CD62L$^-$FR4$^-$ populations were sorted as illustrated in (A). Aliquots of PgLN cells were collected and fixed and stained for Foxp3 and Helios. Representative flow cytometric plots from one of five individual experiments are gated on CD4$^+$ live cells and show Foxp3 and Helios expression. (C) The proportion of CD4$^+$ live cells that were Foxp3$^-$Helios$^+$ or Foxp3$^-$Helios$^+$ was quantified by flow cytometry. Each symbol represents an individual mouse and means are shown with horizontal bars. Data are pooled from five individual experiments(n = 5).

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Figure 6

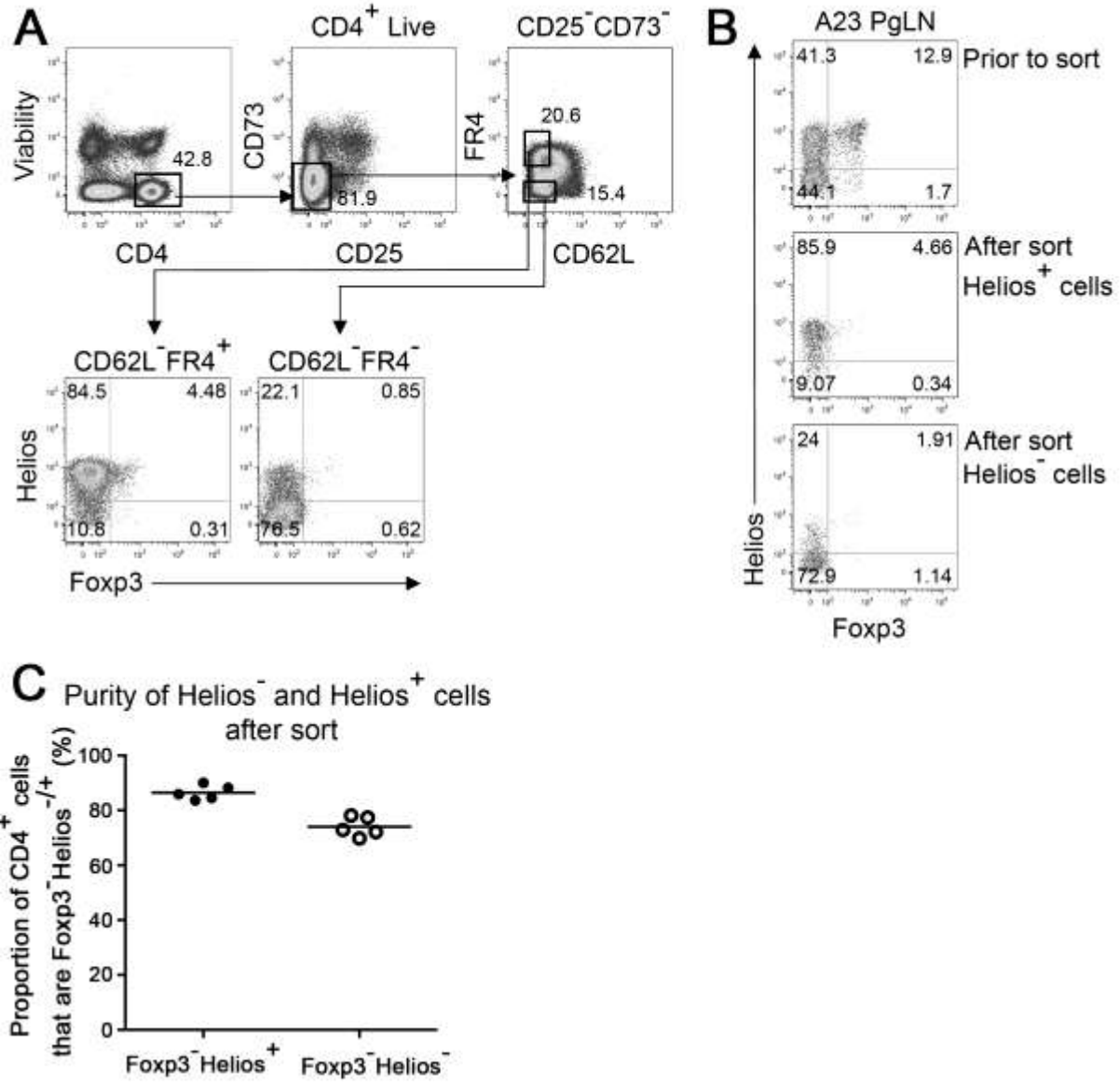

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Figure 7. Helios$^+$ cells display anergic properties following peptide stimulation in vitro. Foxp3$^-$Helios$^+$ and Foxp3$^-$Helios$^-$ cells were purified by flow cytometric sorting from PgLNs of A23 mice, labelled with CFSE and stimulated in vitro with A23 peptide at 0, 5 or 10 μg/mL. After four days, supernatants were collected and cells harvested. Cells were analysed by flow cytometry for expression of CD4, Fixable Viability Dye and dilution of CFSE. A) Representative histograms show Foxp3$^-$Helios$^+$ and Foxp3$^-$Helios$^+$ cells stimulated with 5 μg/mL (top panel) and 10 μg/mL (bottom panel) of A23 peptide. Histograms are gated on CD4$^+$ cells and show Fixable Viability Dye expression (left panel) (Live cells are negative for Fixable Viability Dye) or dilution of CFSE (right panel). The (B) number and (C) proportion of CD4$^+$ live cells were quantified by flow cytometry. Division of the purified Helios$^+$ and Helios$^-$ cells stimulated with (D) 5 μg/mL or (E) 10 μg/mL was analysed by quantifying the proportion of CD4$^+$ live cells in each CFSE division. (F) Culture supernatants were analysed for IL-2 by cytometric bead array. Dashed line indicates limit of detection. All data show mean + SEM or ± SEM (n = 5) and are representative of five individual experiments

performed in duplicate or triplicate wells. *$p < 0.05$; **$p < 0.01$ (Mann Whitney test).

Figure 7