Research Article

Nrf2 expression driven by Foxp3 specific deletion of Keap1 results in loss of immune tolerance in mice

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The transcription factor Nrf2 regulates oxidative stress responses. However, the specific function of Nrf2 in Tregs, the central regulators of immune homeostasis, is unclear. Here, we report an unexpected but important role of Nrf2 in Tregs. Nrf2 expression driven by Foxp3 specific deletion of Keap1 resulted in an autoinflammatory phenotype with enhanced effector T cell activation and immune cell infiltrates in the lung. While early postnatal death of mice with Foxp3 specific deletion of Keap1 was most probably due to ectopic Foxp3cre expression and subsequent Keap1 deletion in epithelial cells, bone marrow chimeras suggest that Nrf2 activation intrinsically in Tregs contributes to a loss of Treg cells and diminished peripheral tolerance. Moreover, Nrf2 activation was associated with a loss of Foxp3 expression, but an enhanced glucose uptake and mTOR activity in Tregs, thus mimicking a metabolic phenotype that is associated with impaired lineage stability and cell functioning.

Keywords: immune tolerance · Nrf2 · regulatory T cells · redox metabolism

Introduction

All cells are equipped with an intrinsic mechanism that neutralizes excess ROS and protects against oxidative injury. The transcription factor NF (erythroid-derived 2)-like 2 (Nrf2) acts as master regulator of the so-called oxidative stress response [1]. Under quiescent conditions, cytoplasmic Nrf2 is retained and degraded in the proteasome by Kelch like ECH-associated protein 1 (Keap1) in concert with the E3 ubiquitin ligase Cullin 3 (Cul3) [2]. Cellular stimuli such as oxidative stress induce conformational changes in Keap1, which are followed by the release of Nrf2 from Keap1. Subsequently, Nrf2 translocates to the nucleus and transactivates the expression of genes containing an antioxidant response element (ARE) in their promoter regions. Nrf2 thereby upregulates phase II detoxifying enzymes and antioxidant proteins and plays a vital role in maintaining cellular homeostasis, especially upon exposure of cells to chemical or oxidative stress [3].

Anti-inflammatory effects of Nrf2 activation and the pro-inflammatory effects of Nrf2 deletion have been demonstrated in a large number of studies (reviewed in ref. [4]). The finding that old Nrf2−/− female mice develop an autoimmune syndrome closely resembling human systemic lupus erythematosus underlines the key role played by Nrf2 in autoimmune regulation [5]. However, it is not clear whether Nrf2 contributes to alleviation of
systemic inflammation by enhancing cell survival and cellular antioxidant capacity and thereby reducing autoimmunity responses or by directly suppressing inflammatory responses, as described in macrophages [6]. Especially the role of Nrf2 signaling in Tregs remains less clear, although Tregs serve as central mediators in the prevention of autoimmune disease and ROS and are central to T-cell function. Conflicting results exist regarding the capacity of Tregs to respond to oxidative stress. While Tregs have been reported to be more resistant to oxidative stress-induced cell death than conventional T cells [7], others have found higher vulnerability of Tregs to free oxygen species, which was attributed to their weak Nrf2-associated antioxidant system [8]. T cell specific overexpression of Nrf2 has been shown to lead to increased T regulatory cell development in vivo in a model of acute kidney injury [9]; however, absence of Nrf2 in donor cells also enhanced persistence of Tregs and reduced systemic inflammation in a graft-versus-host disease model in mice [10]. So far, no direct analysis of Nrf2/Keap1 signaling in Tregs has been carried out. We herein describe for the first time unexpected and deleterious consequences of a Foxp3 specific activation of Nrf2 in vivo.

Results

Nrf2 expression driven by Foxp3 specific deletion of Keap1 induces postnatal lethality

TCR stimulation induces nuclear Nrf2 translocation and protein expression [10]. To further address the relevance of Nrf2 activity for T cell differentiation, we analyzed Nrf2 activation in isolated T cells during subsequent differentiation into distinct T cell subsets. To this end, we used ARE-luc mice, which express the LUC enzyme under the control of an ARE-bearing promoter as an indicator of Nrf2 activity. With respect to different T cell subsets, differentiation toward Foxp3+ Treg cells revealed the highest Nrf2 activity (Fig. 1A). We wondered, whether high Nrf2 activation in Treg cells has functional relevance and therefore crossed mice carrying loxp-flanked Keap1 alleles with Foxp3-Cre mice (Foxp3CreKeapfl/fl mice). Very few homozygous Foxp3CreKeapfl/fl mice reached 4 weeks of age (Fig. 1B), most homozygous offspring died at very early time points (Fig. 1C). It is known that Keap1-deficient mice die postnatally due to hyperkeratosis in the esophagus and forestomach [11, 12]. Taking into account that the Foxp3 locus is not exclusively transcribed in Tregs but also outside the lymphoid system, e.g. in epithelial cells of several organs [13], we analyzed esophagus and stomach of neonatal Foxp3CreKeapfl/fl mice on postnatal day 9. Histological analysis revealed abnormal keratinization in esophagus and stomach similar to Keap−/− mice, which confirms that Foxp3CreKeapfl/fl mice die postnatally most probably due to Keap1 deletion in epithelia (Fig. 1D and Supporting Information Fig. 1A) and thus starvation. To ensure that we have a specific overexpression on Nrf2 in Treg cells, we sorted CD4+ Nrp1+ CD25+ Treg cells, and CD4+ Nrp1+ CD25− cells from spleen, isolated BM-derived cells from femur and collected ear tissue and tested for recombinase mRNA expression. Although we found recombinase expression in all cells and in the tissue of Foxp3CreKeapfl/fl mice, there was a significant upregulation within Treg cells (Supporting Information Fig. 1B). Keap1 mRNA was furthermore downregulated in Treg cells but not in non-Treg CD4+ T cells (Fig. 1E) and Nqo1, which is a Nrf2 target gene, was specifically upregulated in Treg cells as well (Fig. 1F). From this, we concluded that despite discrete activity of the CRE recombinase in non-Treg cells in our model, which most probably accounts for postnatal death, there is still a dominant CRE activity and deletion of Keap1 in Treg cells, resulting in activation of Nrf2 in Treg cells as measured by enhanced Nqo1 activity.

Nrf2 activation reduces regulatory T cells in vivo and in vitro

Analysis of surviving Foxp3CreKeapfl/fl and heterozygous Foxp3CreKeapfl/+ mice showed reduced splenic numbers of Tregs in Foxp3CreKeapfl/fl and a trend toward such a reduction in Foxp3CreKeapfl/+ mice (Fig. 2A and B). In addition, Foxp3 expression per cell was significantly reduced in Foxp3CreKeapfl/fl and Foxp3CreKeapfl/+ compared to WT mice (Fig. 2C and D). To exclude secondary effects due to Keap1 deletion in epithelial cells, we generated bone-marrow chimeras, by reconstituting irradiated RAG2−/− mice with BM from either CD45.1 WT or CD45.2 Foxp3CreKeapfl/fl mice, or a mixture of both. Reduced numbers of Tregs and reduced Foxp3 expression occurred in mixed BM chimeras in the CD45.2+ Foxp3CreKeapfl/fl cell populations as well as in mice that were reconstituted with Foxp3CreKeapfl/fl BM compared to WT BM (Figs. 2E and F). The percentages of CD4+Foxp3− cells were enhanced in mixed BM chimeras in the CD45.2+ Foxp3CreKeapfl/fl cell populations as well as in mice that were reconstituted with Foxp3CreKeapfl/fl BM compared to WT BM (Supporting Information Fig. 2). This confirms that side-effects from postnatal starvation or epithelial Keap1 deletion are not the reason for the phenotype present in here. As only a few homozygous Foxp3CreKeapfl/fl mice reached maturity, we performed a subsequent analysis with heterozygous Foxp3CreKeapfl/+ and with Nrf2−/− mice. The Nrf2/Keap1 pathway critically governs cell survival by regulating the cellular antioxidant system [8, 14]. However, heterozygous Foxp3CreKeapfl/+ Tregs did not include altered percentages of apoptotic cells compared to WT Tregs (Supporting Information Fig. 3A). However, in vitro Treg differentiation of Foxp3CreKeapfl/+ CD4+ T cells was largely diminished (Fig. 2G and H). To further confirm these data with CD4+ T cells from non-inflammatory mice, we also performed in vitro Treg differentiation of VAVcreKeapfl/+ CD4+ T mice and could also observe a reduced differentiation towards Foxp3+ cells (Supporting Information Fig. 3B). Vice versa, Nrf2 deficient CD4+ T cells revealed enhanced differentiation toward Foxp3− cells (Fig. 2I). As expected, redox-metabolism was altered, appearing as enhanced ROS levels in Nrf2 deficient Tregs and reduced levels in Foxp3CreKeapfl/fl Treg cells (Fig. 2J and K; Supporting Information Fig. 4A and B). Performing metabolic analysis, Tregs from heterozygous Foxp3CreKeapfl/+ mice showed enhanced
glucose uptake (Fig. 2L; Supporting Information Fig. 4C) but no alterations with regard to mitochondrial masses Supporting Information Fig. 2C), while Tregs from Nrf2−/− mice showed reduced glucose uptake (Fig. 2M; Supporting Information Fig. 4C) but increased expression of phosphorylated mTOR (Fig. 2O; Supporting Information Fig. 4F and H). Interestingly, enhanced glucose uptake and enhanced expression of phosphorylated mTOR were also observed in Foxp3Cre/Keapfl/fl Tregs from mixed BM chimeras, which further suggests that Nrf2 induces cell-intrinsic changes of metabolism in Tregs (Fig. 2R and S; Supporting Information Fig. 4I). As enhanced mTOR activity and a glycolytic phenotype in Tregs is thought to be detrimental to their long-term survival and lineage stability, we suppose that Nrf2 activation enhances mTOR activation in Tregs and that this in turn results in a metabolic phenotype that is associated with reduced expression of Foxp3 and decreased immune suppressive function [19].

Activation of Nrf2 in Foxp3+ cells is associated with a fatal lung and liver inflammation

Surviving Foxp3Cre/Keapfl/fl and, at later time points, Foxp3Cre/Keapfl/fl mice revealed increased CD4+ T cell activation. Frequencies of central (CD44highCD62Lhigh) and effector

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memory T cells (CD4<sup>4<sup>high</sup></sup>/<sup>CD62I<sup>low</sup></sup>) within the CD4<sup>+</sup> population were strongly enhanced in Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> (Fig. 3A) and heterozygous Foxp3<sup>−/+</sup>Keap1<sup>−/−</sup> (Fig. 3A) compared to WT mice, while naive T cells were reduced. CD4<sup>+</sup> T cells of Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> and Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> mice produced significantly higher amounts of IFN-γ (Fig. 3B and C). Moreover, Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> and Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> mice spontaneously developed extensive inflammatory infiltrates in the lungs (Fig. 3D–F). In order to exclude the possibility that inflammation results from epithelial expression of the Foxp3 locus and Keap1 deletion in nonlymphoid cells, we analyzed the BM chimeras that were transferred with Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> cells in irradiated RAG2<sup>−/−</sup> mice for signs of inflammation. Mice that were reconstituted with Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> BM started to lose weight after 3 weeks (Fig. 4A), they furthermore showed enhanced splenic weight (Fig. 4B) and enhanced percentages of IFN-γ producing T cells (Fig. 4C), liver and lung inflammation (Fig. 4D and E) that reflects the inflammatory phenotype of Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> mice. The absolute numbers of CD4<sup>+</sup> cells within the spleen were not altered between mice that were reconstituted with Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> BM and those that received WT BM (Supporting Information Fig. 5A), which shows that the reconstitution with Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> BM worked. These results suggest that constitutive Nrf2 activation leads to a loss of Treg mediated immune tolerance. While the presence of WT Tregs is sufficient to overcome these effects in the mixed BM chimeras, the activation of Nrf2 in all Tregs induces dramatic changes resulting in effector T cell activation and lung inflammation. To finally establish whether the dysfunctions of Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> Tregs are due to metabolic changes in Tregs, we generated Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> BM and treated them with PEG/Tween/NaCl (vehicle) or PEG/Tween/Rapamycin beginning at day 10 after reconstitution. Rapamycin prevented loss of body weight and furthermore increased numbers of Tregs (Fig. 4F and G). This suggests that enhanced mTOR signaling in Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> Tregs might constitute a mechanism by which Nrf2 negatively regulates Tregs cells.

**Discussion**

In conclusion, our study indicates that Nrf2 is a negative regulator of Treg function and that Foxp3 specific activation of Nrf2 results in a loss of immune tolerance and spontaneous accumulation of IFN-γ producing effector T cells and inflammation. This is quite surprising, given that Nrf2 is generally assumed to exert anti-inflammatory effects. Noel et al. genetically deleted Keap1 from CD4<sup>+</sup> T cells, which resulted in higher percentages of Tregs and was protective in ischemia reperfusion-induced acute kidney injury [9]. This suggests that Nrf2 activation improves Treg-mediated suppression of inflammation, which conflicts with our

Figure 2. Nrf2 activation in T<sub>reg</sub> cells reduces Foxp3 expression and enhances mTOR activation. (A) Representative density plot showing CD25<sup>+</sup>Foxp3<sup>+</sup> cells gated on CD4<sup>+</sup> cells in spleens of WT (left) and Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> mice (right) using flow cytometry. The gating strategies are shown in Supporting Information Fig. 3A. (B) Statistical analysis of splenic Treg percentages from WT (n = 17), combined from seven independently performed experiments, Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> (n = 6, combined from two independently performed experiments) and Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> (n = 10, combined from five independently performed experiments) mice. (C) Representative histogram showing overlay of Foxp3 expression in CD4<sup>+</sup>CD25<sup>+</sup> cells from WT (black) and Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> (red) spleens. (D) Statistical analysis of MFI of Foxp3 in 14 WT (combined from nine independently performed experiments), six Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> (combined from five independently performed experiments) and seven Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> (combined from three independently performed experiments) CD4<sup>+</sup>CD25<sup>+</sup> T cells. (E and F) BM chimeric mice were analyzed 6 weeks after transfer of either CD45.1 WT or CD45.2 Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> BM cells alone or a mix of CD45.1 WT and CD45.2 Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> BM cells into lethally irradiated RAG2<sup>−/−</sup> recipient mice (data combined from two independently performed experiments with two to three per experiment, overall five WT BM recipients, five Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> recipients and six mixed BM chimeras). (G) Statistical analysis of Foxp3 MFI in splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells from five WT BM recipients, five Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> recipients and within CD45.2<sup>+</sup> and CD45.1<sup>+</sup> cell populations of six mixed BM chimeras (data combined from two independently performed experiments with two to three mice per experiment). (H) Percentages of splenic CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells of CD45.1 and CD45.2 origin in mixed BM chimeras and BM chimeras, (data combined from two independently performed experiments with two to three mice per experiment). (I) CD4<sup>+</sup>CD25<sup>+</sup> T cells stimulated with anti-CD3/CD28 antibodies in the presence/absence of 5 ng/mL TGF-β for 5 days. Percentages of Foxp3<sup>+</sup> cells combined from four independently performed experiments were determined, each with spleen from one mouse. (J) Representative density plot of (G) in the presence of TGF-β. (I) CD4<sup>+</sup>CD25<sup>+</sup> T cells stimulated with anti-CD3/CD28 antibodies in the presence/absence of 5 ng/mL TGF-β for 5 days. Percentages of Foxp3<sup>+</sup> cells combined from six independently performed experiments were determined, each with spleen from one mouse, using a two-tailed paired t-test. (J) Statistical analysis of ROS<sup>+</sup> cells in splenic pregled CD4<sup>+</sup>CD25<sup>+</sup> T cells from WT (n = 5) and Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> mice (n = 5), combined from two independently performed experiments. (K) Statistical analysis of ROS<sup>+</sup> cells in pregled splenic CD4<sup>+</sup>CD25<sup>+</sup> T cells from WT (n = 3) and Nrf2<sup>−/−</sup> mice (n = 3) from one experiment. (L) 2NBD-glucose incorporation was analyzed in CD4<sup>+</sup>CD25<sup>+</sup> Tregs from WT (n = 4) and Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> (n = 5) mice at different time points, combined from two independently performed experiments. (M) 2NBD-glucose incorporation was analyzed in CD4<sup>+</sup>CD25<sup>+</sup> Tregs from WT (n = 3) and Nrf2<sup>−/−</sup> (n = 3) mice at different time points, combined from two independently performed experiments. (N) Statistical analysis of pmTOR expression in splenic CD4<sup>+</sup>Foxp3<sup>+</sup> cells from WT (n = 8) and Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> mice (n = 7), combined from two independently performed experiments. (O) Statistical analysis of pS6 expression in CD4<sup>+</sup>Foxp3<sup>+</sup> cells from WT (n = 9) and Foxp3<sup>−/−</sup>Keap1<sup>−/−</sup> mice (n = 8), combined from three independently performed experiments. (P) Statistical analysis of pmTOR expression in CD4<sup>+</sup>Foxp3<sup>+</sup> cells from WT (n = 4) and Nrf2<sup>−/−</sup> mice (n = 4), combined from two independently performed experiments. (Q) Statistical analysis of pS6 expression in CD4<sup>+</sup>Foxp3<sup>+</sup> from WT (n = 4) and Nrf2<sup>−/−</sup> mice (n = 4), combined from two independently performed experiments. (R) 2NBD-glucose incorporation was analyzed in mixed BM chimeras CD4<sup>+</sup>CD25<sup>+</sup> Tregs from CD45.1 (n = 3) and CD45.2 (n = 3). Data are from a single experiment. For B, E, F, G, J, K, P, Q, R, and S, two-tailed, unpaired t-tests were used to test significance. For D, N, and O, a two-tailed Mann–Whitney test was used. All results are expressed as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. All data were measured using flow cytometry, the gating strategies are shown in Supporting Information Fig. 3.
A

![Graph showing CD4^+CD82^+ in CD4^+](image)

B

![Flow cytometry plots showing CD4^+ cells with IFN-γ expression](image)

C

![Graph showing IFN-γ^+ in CD4^+](image)

D

![Immunostaining images of WT and Foxp3^creKeap^fl/fl mice](image)

E

![Histological images of tissue sections from 6-11 weeks and 23-40 weeks](image)

F

![Graph showing immune infiltrate area](image)
findings. However, Suzuki et al. showed that Nrf2 inhibits effector T cell activities independently of Tregs and that Nrf2-mediated inflammatory effects in the context of autoimmunity required the integrity of Nrf2 mediated functional modulations of multiple cell lineages [20]. More recently, it was reported that absence of Nrf2 in donor T cells enhanced persistence of Tregs and reduced systemic inflammation in a graft-versus-host disease in mice [10].

Our study is the first to analyze Nrf2 activation specifically in Tregs by using Foxp3<sup>Cre</sup>Keap1<sup>fl/fl</sup> mice and Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> cell reconstituted BM chimeras. We hypothesize that high Nrf2 activity in Tregs, which might occur during oxidative stress prone conditions such as infection, is a feedback mechanism serving to limit Treg expansion and function in order to allow time for a sufficient immune response.

One limitation of our study is that we found CRE expression in non-Treg cells and in tissue of Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> mice. Although we found the most prominent CRE expression and Keap1 downregulation in Tregs, there are also side effects from Keap1 deletion in non-Treg cells and tissue such as hyperkeratosis in the esophagus and forestomach, which might influence function of Treg cells as well. Due to the fact that deletion of Keap1 is not specific for Treg cells in our Foxp3<sup>Cre</sup> model and in-depth mechanistic analyses in BM chimeras are difficult to perform, the use of more specific and inducible cre-flux mouse models will be needed to analyze Nrf2 mediated Treg-intrinsic mechanisms in vivo. Further studies will also attempt to elucidate the exact mechanism by which Nrf2 dysregulates Treg cells. It furthermore remains to be elucidated why the inflammation in our mouse models is mostly restricted to the lungs and liver. Mechanistically, our data suggest that Foxp3 specific activation of Nrf2 results in enhanced mTOR signaling with detrimental effects on cell lineage function and stability. The role of mTOR signaling in immune tolerance and the possibility of targeting metabolic pathways in order to restore immune tolerance are matters of intensive current research. Chap- man et al. showed that mTOR signaling is necessary for Treg cell activation and tissue Treg homeostasis by means of a genetic Treg-specific deletion of mTOR [21]. In contrast, Battaglia et al. showed that rapamycin selectively expands Tregs [22] and Delgoffe et al. showed that mTOR deficient T cells differentiate toward Treg cells [19]. Recent reports link hyperactivation of mTOR to dysregulated T cell responses in autoimmunity [23, 24]. As the roles of mTOR signaling are so complex, it has been suggested that local environmental signals might fine-tune levels of mTOR activity in Tregs [21]. mTOR-dependent metabolic programming might play context-dependent roles in different Treg subsets or under distinct (patho-) physiological conditions [25]. We hypothesize that Nrf2 activation enhances mTOR signaling in Tregs and rapamycin treatment inhibited exaggerated mTOR signaling in our BM chimeras. This re-established normal numbers of Treg cells and prevented weight loss. However, rapamycin treatment also inhibits mTOR signaling in other cells, such as effector T cells and might thereby contribute to a lowering of effector T cell proliferation and numbers of IFN-γ positive cells independently of the direct effects of rapamycin on Tregs. Even if reduced levels of Treg cells and their metabolic shift do constitute the sole cause of a loss of tolerance and tissue inflammation, this question needs to be investigated further with more specific or inducible cre-flux models. Nevertheless, by showing a Treg specific role of Nrf2 in metabolic regulation of immune homeostasis, our study addresses an important and previously unrecognized issue. Further analyses will elucidate the mechanisms of Nrf2 and mTOR interaction in Tregs and how these might influence immune tolerance.

Our study thus demonstrates an as yet unappreciated immune regulatory function of Nrf2, which does make an important contribution toward a better understanding of Nrf2 mediated immune modulation.

**Figure 3.** Foxp3 specific constitutive Nrf2 activation induces inflammation in the lung. (A) Statistical analysis of CD44 and CD62L expression gated on CD4<sup>+</sup> splenic T cells. The left diagram shows percentages of naive T cells from WT (n = 16, combined from seven independently performed experiments), Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> (n = 10, combined from four independently performed experiments) and Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> mice (n = 10, combined from four independently performed experiments). Effector memory T cells from WT (n = 16, combined from seven independently performed experiments), Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> (n = 10, combined from four independently performed experiments) and Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> mice (n = 7, combined from four independently performed experiments) are shown in the middle diagram, and central memory T cells from WT (n = 16, combined from seven independently performed experiments), Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> (n = 10, combined from four independently performed experiments) and Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> mice (n = 7, combined from four independently performed experiments) on the right. For naive T cells (left diagram) a two-tailed Mann–Whitney test was used, for effector memory T cells and central memory T cells (middle and right diagrams) two-tailed, unpaired t-tests were used to test significance. Results are expressed as the mean ± SEM. (B) Representative contour plots showing IFN-γ expression in splenic CD4<sup>+</sup> T cells from WT and Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> mice. Each symbol indicates an individual mouse. Two-tailed, unpaired t-tests were used to test significance. Results are expressed as the mean ± SEM. (C) Statistical analysis of IFN-γ expression in splenic CD4<sup>+</sup> T cells from WT and Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> mice. Each symbol indicates an individual mouse. Two-tailed, unpaired t-tests were used to test significance. Results are expressed as the mean ± SEM. (D) Representative contour plots showing IFN-γ expression in splenic CD4<sup>+</sup> T cells from WT and Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> mice. Each symbol indicates an individual mouse. Two-tailed, unpaired t-tests were used to test significance. Results are expressed as the mean ± SEM. (E) Representative H&E-stained images from lungs of WT, Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> (n = 10, combined from five independently performed experiments) and Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> mice aged 6–8 weeks in one experiment, seven WT mice aged 9–12 weeks in three experiments. (A–C) All data were measured using flow cytometry. (D) Representative immunofluorescent stainings for DAPI (blue) and CD3 (red) in cryo sections of lung tissue from 6 to 7 wk old WT and Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> mice. Arrow indicates infiltrated immune cells (scale bars are 100 µm, 20× magnification). (E) Representative H&E-stained images from lungs of WT, Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup>, and Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> mice. Arrows indicate infiltrated immune cells (scale bars are 200 µm, 10× magnification). (F) Quantification of infiltrated area in H&E-stained lung tissue of WT (n = 22), Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> (n = 26), and Foxp3<sup>Cre</sup>Keap1<sup>−/−</sup> (n = 9) mice, results were combined from five independently performed experiments. Each symbol indicates an individual mouse. A two-tailed Mann–Whitney test was used. Results are expressed as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

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Figure 4. Foxp3 specific constitutive Nrf2 activation induces inflammation in BM chimeras. (A) Loss of weight of irradiated RAG2−/− mice after reconstruction with either CD45.1 WT (n = 5) and CD45.2 Foxp3creKeapfl/fl (n = 5) BM cells alone or a mix of CD45.1 WT and CD45.2 Foxp3creKeapfl/fl BM cells (n = 6). A two-tailed, unpaired test was used to test significance, (data combined from two independently performed experiments with two to three mice per experiments). (B) Splenic weight of irradiated RAG2−/− mice after reconstruction with either CD45.1 WT (n = 5) and CD45.2 Foxp3creKeapfl/fl (n = 5) BM cells. A two-tailed, unpaired test was used to test significance, (data combined from two independently performed experiments with two to three mice per experiments). (C) Statistical analysis of IFN-γ expression in splenic CD4+ T cells from irradiated RAG2−/− mice after reconstruction with either CD45.1 WT or CD45.2 Foxp3creKeapfl/fl BM cells measured by flow cytometry. Each symbol indicates an individual mouse (both n = 5). A two-tailed, unpaired test was used to test significance, data combined from two independently performed experiments with two to three mice per experiments. (D) Representative H&E-stained images from lungs of irradiated RAG2−/− mice after reconstruction with either CD45.1 WT or CD45.2 Foxp3creKeapfl/fl BM cells (scale bars are 100 μm, 10× magnification). (E) Representative hematoxylin and eosin (H&E)-stained images from liver of irradiated RAG2−/− mice after reconstruction with either CD45.1 WT or CD45.2 Foxp3creKeapfl/fl BM cells (scale bars are 100 μm, 10× magnification). (F) Loss of weight of irradiated RAG2−/− mice after reconstruction with Foxp3creKeapfl/fl BM cells and treatment with vehicle (n = 6, data combined from two independent experiments) or vehicle with Rapamycin (n = 6, data combined from two independent experiments). A two-tailed, unpaired test was used to test significance. (G) Percentages of splenic CD4+CD25+Foxp3+ T cells of BM chimeras treated with vehicle (n = 4, one experiment) or vehicle with Rapamycin (n = 4, one experiment) analyzed by flow cytometry. A two-tailed, unpaired test was used to test significance. For (A), (B), (C), (F), and (G), results are expressed as the mean ± SEM. *p < 0.05; **p < 0.01.
Materials and methods

Mice strains

Experiments were performed with sex- and age-matched 6–10 wk old (unless otherwise indicated) Foxp3CreKeapfl/fl and respective WT mice; CD45.1 congenic mice, ARE-luc, and RAG2−/− mice (all C57BL/6 background). CD45.1 mice were provided by F. Tacke and ARE-luc mice were provided by C. Wruck. Foxp3CreKeapfl/fl mice were generated by crossing Keap1-flx mice ([26], provided by C. Wruck) with C57BL/6 Foxp3-IR1 Cre mice (provided by T. Bopp). All mice were bred in our animal facility and kept under standardized conditions. The study was approved by the regional government authorities and animal procedures performed in accordance with German legislation for animal protection.

Mixed BM chimeras

BM cells were isolated from femurs and tibias of age-matched donor animals (WT CD45.1 and Foxp3CreKeapfl/fl CD45.2). RAG2−/− mice were lethally irradiated (2 × 6.8 Gy) and co-infected with 5 × 10⁶ cells of each genotype a/f or injected with 10 × 10⁶ BM cells from either Foxp3CreKeapfl/fl CD45.2 or WT CD45.1 mice. The mice received antibiotic treatment for 14 days. Rapamycin-treated mice received rapamycin/vehicle beginning at day 10 after irradiation. Rapamycin was first solved in ethanol and then diluted in 5.2% PEG/Tween in NaCl. Mice received either vehicle (5.2% PEG/Tween) or 2 mg/kg of body weight rapamycin by i.p. injection every other day.

Flow cytometry

Surface staining with anti-CD4, anti-CD3, anti-CD8, anti-CD25, anti-CD45.1, and anti-CD45.2 (all eBioscience) was performed as described previously [27]. Intracellular Foxp3, pS6, and p-mTOR staining was performed with a FOXP3 staining buffer set (eBioscience, Germany). Mitochondrial mass measurements were performed with 500 nM MitoTracker (ThermoFisher Scientific). Glucose uptake was determined by means of a glucose uptake cell-based kit (Cayman Chemical) according to manufacturers instructions. Cell viability dye (eBioscience) and fluorochrome conjugated Annexin-V (eBioscience) were used to identify apoptotic cells. Flow cytometry was carried out using the FACS Canto II device (BD Biosciences, Germany). Data analysis was performed using FCS Express software.

For gating strategies applied to the data, please refer to Supporting Information Fig. 3 as indicated in the figure captions. We have adhered to the Guidelines for the use of flow cytometry and cell sorting in immunological studies [28].

Histology

For immunofluorescent stainings, 5 µm thick frozen sections of lungs were fixated in acetone and stained against CD3 (Invitrogen). For histological stainings, three micrometer thick paraffin sections of lung, esophagus, stomach, and liver were fixated in methacarn, deparaffinized and stained with H&E. Two photographs of each lung were taken for quantification of lung infiltrates. The area with cell infiltrations was measured and the ratio of infiltrated tissue to total lung tissue analyzed using the ImageJ software (NIH).

T cell differentiation assays

CD4+CD25− T cells (2 × 10⁶ per milliliter) were incubated with plate-coated anti-CD3 (10 µg/mL, eBioscience) and soluble anti-CD28 (eBioscience). T10 cells were left without exogenous cytokines. 10 ng/mL IFN-γ respectively 30 ng/mL IL-4 were added to induce T₅₁ or Tᵥ₂ priming, 5 ng/mL TGF-β was added to induce Treg differentiation and 30 ng/mL IL-6, 1 ng/mL TGF-β, and, 10 ng/mL IL-23 were added to induce Tᵥ₂,17 cells.

ARE-luc reporter gene measurements

Spleen-derived CD4+CD25− T cells (1 × 10⁶ per milliliter) were isolated from ARE-LUC reporter gene mice and differentiated as described above. Subsequently the cells were washed once with PBS and lysed in 150 µL 1 × passive lysis buffer (PLB; Promega, Mannheim, Germany). Lysates (50 µL, technical duplicates) were transferred to a white 96-well microtiter plate. Luminescence was measured in a Glomax96 luminometer (Promega) with an automated injection of 50 µL LUC assay reagent 1 (Luciferase assay system Cat #: E1500, Promega) and 10 s integration time. CyQuant™ assay (Cat. #: C7026, Thermo Scientific, Darmstadt, Germany) was carried out as recommended by the distributor to quantify total DNA amount and normalize raw luminescence data (results are depicted as ratio: luminescence/Cyquant).

Statistics

Results are expressed as the mean ± SEM. Differences between groups were evaluated using two-tailed unpaired or paired (if indicated), Student’s t-test if data were normally distributed. Otherwise, a nonparametric Mann–Whitney test was performed.

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P. K. and A.R. performed the majority of experiments. A.F. performed experiments with ARE-luc mice and helped with data interpretation. C.W., A.S., and N.W. contributed to the writing of
Conflict of interest: The authors declare that there are no commercial or financial conflicts.

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