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NR4A family members regulate T cell tolerance to preserve immune homeostasis and suppress autoimmunity

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ABSTRACT

The NR4A family of orphan nuclear receptors (Nr4a1-3) plays redundant roles to establish and maintain Treg identity; deletion of multiple family members in the thymus results in Treg deficiency and a severe inflammatory disease. Consequently, it has been challenging to unmask redundant functions of the NR4A family in other immune cells. Here we use a competitive bone marrow chimera strategy, coupled with conditional genetic tools, to rescue Treg homeostasis and unmask such functions. Unexpectedly, chimeras harboring Nr4a1−/− Nr4a3−/− (DKO) bone marrow develop autoantibodies and a systemic inflammatory disease despite a replete Treg compartment of largely wild-type origin. This disease differs qualitatively from that seen with Treg-deficiency and is B cell-extrinsic. Negative selection of DKO thymocytes is profoundly impaired in a cell-intrinsic manner. Consistent with escape of self-reactive T cells into the periphery, DKO T cells with functional, phenotypic, and transcriptional features of anergy accumulate in chimeric mice. Nevertheless, we observe upregulation of genes encoding inflammatory mediators in anergic DKO T cells, and DKO T cells exhibit enhanced capacity for IL-2 production. These studies reveal cell-intrinsic roles for the NR4A family in both central and peripheral T cell tolerance, and demonstrate that each is essential to preserve immune homeostasis.

INTRODUCTION

Since the initial discovery of regulatory T cells (Treg) and their recognition as a distinct T cell lineage dependent upon the transcription factor FOXP3, it has been shown that they are absolutely essential for immune homeostasis and tolerance to self
Indeed, Foxp3-deficient mice and mice with a loss-of-function mutation in Foxp3 (Scurfy) rapidly develop an autoimmune disease characterized by cytokine storm, immune cell proliferation and infiltration, autoantibody production, and death typically by 4 weeks of age (1-4). Conversely, re-introducing Treg is sufficient to prevent this disease (2). However, extensive cell-intrinsic mechanisms that operate in other immune cell lineages are also essential to maintain tolerance to self, including processes such as deletion and hypo-responsiveness of self-reactive lymphocytes (termed anergy) (5).

Prior work has implicated a small family of orphan nuclear hormone receptors (encoded by Nr4a1-3) in several of these processes. Most notably, NR4A family members play redundant roles upstream of Foxp3 to maintain Treg identity and function; deletion of multiple family members in the thymus results in profound Treg deficiency and a severe “Scurfy-like” disease that phenocopies Foxp3-deficient mice (6, 7). As a result, it has been difficult to isolate redundant functions of this family in other immune cell populations. Yet this remains an important area to explore since the NR4A family are widely expressed and thought to be druggable targets that may facilitate manipulation of immune cell function in the context of autoimmune disease, tumor immunotherapy, and hematologic malignancies (8-11).

Nr4a1-3 (encoding NUR77, NURR1, and NOR-1, respectively) are rapidly upregulated in response to mitogenic stimuli, including antigen receptor ligation, and are thought to function as constitutively active transcription factors without a confirmed endogenous ligand (12). As a result, not only are these family members upregulated in T and B cells after acute antigen encounter, but also in Treg in the steady-state, in thymocytes undergoing negative selection, and in self-reactive, anergic, or exhausted
lymphocytes in response to chronic antigen stimulation (6, 9, 13-19). Indeed, the NR4A family has been argued to play a tolerogenic role in all these contexts. The NR4A family selectively restrains the survival and expansion of B cells that encounter antigen (signal 1) in the absence of co-stimulation (signal 2) (15, 20). Similarly, overexpression of either Nr4a1 or Nr4a3 mediates antigen-induced cell death in the thymus, while a dominant-negative transgenic (Tg) construct had the opposite effect (13, 21, 22). However, Nr4a1-/- mice exhibit extremely subtle defects in thymic negative selection (23, 24), suggesting possible redundancy among the family members. Nr4a1 and Nr4a3 also play non-redundant roles in peripheral conventional T cells (Tconv): most notable among these are roles for Nr4a1 in CD4+ T cell anergy (17) and an additive role for all 3 family members in CD8+ T cell exhaustion (9). Finally, it has been argued that Nr4a1 and Nr4a3 redundantly maintain myeloid homeostasis since, in their absence, a myeloproliferative disease is observed (25). However, unmasking redundancy between NR4A family members in many of these contexts has been hampered by profound immune dysregulation that develops in the absence of functional Treg.

We sought to bypass this obstacle by generating competitive bone marrow (BM) chimeras harboring both wild-type (WT) cells (that could reconstitute a functional Treg compartment) and DKO cells (lacking both Nr4a1 and Nr4a3) to isolate cell-intrinsic immune functions for the NR4A family. Unexpectedly, mixed chimeras harboring both WT and DKO BM rapidly developed anti-nuclear autoantibodies (ANA) and a systemic inflammatory disease, despite a replete Treg compartment of largely WT origin. The disease that developed in BM chimeras was B cell-extrinsic and qualitatively different from that in germline DKO mice. We found that negative selection of DKO thymocytes in
competitive chimeras was profoundly impaired in a cell-autonomous manner. DKO Tconv cells with phenotypic, functional, and transcriptional features of antigen experience and anergy accumulate in these chimeras, suggesting escape of self-reactive T cells into the periphery. However, self-reactive DKO CD4+ Tconv cells nevertheless exhibit expression of inflammatory mediators and exaggerated capacity for IL-2 production, suggesting that anergy is defective. Our findings unmask essential, redundant roles for the NR4A family in both central and peripheral T cell tolerance to maintain immune homeostasis.

RESULTS

Systemic immune dysregulation in mice with germline deficiency of Nr4a1 and Nr4a3

Nr4a1, 2, and 3 are all expressed in thymocytes, Treg, and peripheral T cells, but the expression of Nr4a2 is minimal under steady-state conditions (Supplementary Figure 1A; immgen.org). To unmask redundant functions of the NR4A family, we generated mice lacking germline expression of both Nr4a1 and Nr4a3. We used Nr4a1fl/fl mice to generate Nr4a1-deficient mice with germline excision of the loxP-flanked locus and bred this with a CRISPR-generated Nr4a3−/− line that we recently described (20). An independently-generated line of Nr4a1−/− mice in widespread use has been reported to express a truncated NUR77 protein encoded by exon 2 of Nr4a1 (23, 26). Our newly generated Nr4a1−/− Nr4a3−/− mice (germline DKO, denoted as gDKO here-in) do not express exon 2 of Nr4a1 consistent with the prior analysis of Nr4a1fl/fl mice (26).
gDKO mice were born at Mendelian ratios but exhibited severe runting (Figure 1A) and invariably died before 4 weeks of age, consistent with observed mortality in an independent gDKO line generated with distinct Nr4a1 and Nr4a3 null alleles (25). As previously reported for CD4-cre $Nr4a1^{+/+}$ $Nr4a3^{-/-}$ mice (6), our gDKO mice exhibit near-complete loss of FOXP3$^+$ Treg in both thymus and periphery (Figures 1B-D). gDKO mice also exhibit severe thymic atrophy, but loss of peripheral Treg is disproportionate relative to a more modest reduction of total splenocytes (Supplementary Figures 1B, C). Concurrently, we observed expansion of a unique population of CD4$^+$CD25$^+$ FOXP3$^-$ T cells in the thymus and also in the periphery that may represent cells that have either lost or failed to upregulate expression of FOXP3, described elsewhere (Figures 1B, Supplementary Figures 1D-G) (6, 27, 28). Importantly, and consistent with prior reports, neither $Nr4a1^{-/-}$ nor $Nr4a3^{-/-}$ single knockout (SKO) mice exhibit Treg loss, expansion of this unique cell population, or frank disease (Figures 1B-D, Supplementary Figures 1B-G) (6).

**Cell intrinsic Treg defect in the absence of Nr4a1 and Nr4a3**

Systemic inflammatory disease and associated thymic atrophy preclude the study of thymic development and mature Tconv cells in gDKO mice (Supplementary Figure 1B). Similar mortality observed in both germline (25) and CD4-cre conditional mouse lines (6) suggested to us that disease in gDKO animals might be due to Treg deficiency in both settings. We reasoned that restoring functional Treg could unmask cell-intrinsic functions of NR4A family in other immune cell populations. To do so, we generated competitive chimeras to allow WT donor BM to reconstitute a functional Treg
compartment. Equal proportions of congenically marked donor BM from CD45.2 gDKO and CD45.1/2 WT mice were transplanted into lethally irradiated CD45.1 BoyJ recipients (Figure 1F). In parallel, we generated control chimeras in which CD45.1 hosts were reconstituted with a mixture of CD45.2 WT and CD45.1/2 WT BM (Figure 1E). In addition, we also generated mixed chimeras with a low proportion of gDKO donor BM (1:5 ratio) to further ensure development of a WT Treg compartment (Figure 1G). We assessed reconstitution and immune phenotypes of chimeras at sequential time points between 6-14 weeks post-transplant.

Consistent with studies of CD4-cre chimeras, we observed a profound cell-intrinsic disadvantage for DKO Treg in the thymus and spleen when compared to CD4SP thymocytes (Figures 1H-O) (6). Similar results were reproduced with DKO:WT 1:5 chimera (Figure 1P, Q, Supplementary Figures 1H-K). FOXP3 and CD25 expression in DKO Treg was reduced (Supplementary Figures 1L-O), consistent with a role for the NR4A family in ‘maintenance’ of Treg identity (18). Most importantly, the Treg compartment was restored and Treg number was comparable between DKO:WT chimera and WT:WT chimera (Figures 1J, M, Supplementary Figures 1J, K). We also confirmed Treg were largely reconstituted from WT donor in DKO:WT 1:5 chimera (Figures 1P, Q). This allowed us to explore the cell-intrinsic roles of the NR4A family in other immune cell types.

Thymic atrophy is partially rescued in competitive chimeras

gDKO mice exhibit severe thymic atrophy with marked reduction of all thymocyte subsets and disproportionate loss of DP thymocytes (Figures 2A, B). We postulated
that this might be an indirect consequence of Treg deficiency and systemic inflammation in gDKO mice, since DP thymocytes are sensitive to glucocorticoid-induced apoptosis (29). Indeed, profound thymic atrophy is partially rescued in DKO:WT 1:1 chimeras and fully rescued in DKO:WT 1:5 chimeras within the first 6 weeks of reconstitution (Supplementary Figure 2A). However, progressive thymic atrophy was observed over time in DKO:WT chimeras (relative to WT:WT control chimera). This led us to focus on early time points to isolate cell-intrinsic roles for the NR4A family during thymic development (6 weeks post-transplant).

NR4A expression is dispensable for thymic β-selection

We previously showed, using a fluorescent reporter of Nr4a1 transcription (NUR77-eGFP), that GFP is upregulated at the β-selection checkpoint during thymic development, raising the possibility that Nr4a1 and family members might play a functional role here (14). Immature double negative (DN) thymocytes (lacking both CD4 and CD8 expression) recombine the TCRβ chain, which pairs with pre-TCRα to signal in an antigen-independent manner at the ‘β-selection’ checkpoint (30). This occurs during the DN3 stage of development; pre-selection DN3a thymocytes are CD25^{hi}CD44^{lo} and forward scatter (FSC)-low, while DN3b thymocytes that have traversed this checkpoint successfully express the same surface markers but are larger (FSC-high) (31) (Supplementary Figure 2B). We probed β-selection in both 1:1 and 1:5 DKO:WT competitive chimeras, but identified no advantage for either DKO or WT CD45.2 cells relative to competitor CD45.1/2 WT cells (Figure 2C).
DKO thymocytes have a profound cell-intrinsic defect in negative selection

Studies of two independent NUR77-eGFP reporter lines as well as transcriptional analysis have shown that Nr4a genes are upregulated at the positive selection checkpoint and are especially enriched among thymocytes undergoing negative selection (14, 18, 32). Overexpression of full-length and truncated dominant-negative constructs suggested that NUR77 and NOR-1 redundantly mediate thymic negative selection (13, 22, 33, 34), yet Nr4a1−/− mice exhibit only subtle defects (23, 24). We reasoned that DKO:WT competitive chimeras could unmask cell-intrinsic, redundant functions of the NR4A family during thymic selection. Indeed, we observe a striking advantage for DKO cells in CD4SP and CD8SP subsets relative to DP in both 1:1 and 1:5 chimeras but not at an earlier stage (Figures 2D, E), suggesting either enhanced positive selection or impaired negative selection. However, we do not see an advantage for DKO cells in post-selection DP thymocytes relative to pre-selection DP thymocytes, arguing against a role during positive selection (Supplementary Figures 2C, D).

To test the hypothesis that DKO thymocytes escape negative selection, we assessed antigen-induced apoptosis by detection of activated Caspase 3 (aCasp3) in thymocytes from chimeras. We observed reduced aCasp3+ DKO relative to WT thymocytes after in vitro TCR-stimulation (Figures 2F, G, I, J). By contrast, we saw no difference between donors in control WT:WT chimeras (Figures 2H, K). Notably, we also saw no significant difference in aCasp3 expression in SKO thymocytes relative to co-cultured WT (Supplementary Figures 2E, F). Moreover, mixed chimeras generated with either Nr4a1−/− or Nr4a3−/− SKO mice revealed only a small competitive advantage for CD8SP cells, suggesting a largely redundant role for these family members during
negative selection that is only unmasked when both family members are lost (35) (Supplementary Figure 2G). We conclude that DKO thymocytes escape negative selection, and show for the first time that this is a profound effect in a physiological context, independent of either a TCR Tg or NR4A misexpression.

**Myeloproliferative disorder in DKO mice is a non-cell-autonomous effect of NR4A-deficiency**

Previous studies report that a severe myeloproliferative disorder develops in the first weeks of life in independently generated gDKO mice (25). This was not seen in SKO animals lacking only one Nr4a family member, although mice lacking three out of four Nr4a alleles (i.e. Nr4a1+/−Nr4a3−/− or Nr4a1−/−Nr4a3+/−) did eventually succumb to a similar disease at much later time points (36). Consistent with this, we observed profound expansion of side-scatter (SSC)-high cells infiltrating all hematopoietic tissues and lymphoid organs in gDKO mice; this included not only BM and spleen (Supplementary Figure 3), but was especially pronounced in lymph nodes and thymus (Figures 3A-D). These SSChi cells are CD11b+ but largely Gr1−. Since Treg-deficient animal models like Scurfy and Foxp3-deficient mice similarly exhibit myeloid expansion (2, 3, 37), we hypothesized that the myeloproliferative disorder observed in gDKO animals was due to Treg deficiency. Consistent with this possibility, myeloid expansion is observed in CD4-cre Nr4a1+/− Nr4a3+/− mice, but not in mixed chimeras generated with WT donor BM (6). Resolving this question with gDKO cells has important implications since it has been argued that the NR4A family may represent important drug targets in myeloid leukemic diseases (8, 25, 36). Indeed, in our DKO:WT chimeras,
myeloid expansion is suppressed (even after 12 weeks of reconstitution) and DKO cells exhibit no competitive advantage in these compartments (*Figures 3A-I, Supplementary Figure 3A, B, D, E*). We did observe a minor infiltration of SSC\(^{hi}\) CD11b\(^+\) cells into the thymus of 1:1 DKO:WT but not WT:WT chimeras, and here as well the effect of *Nr4a*-deficiency was cell-extrinsic (*Figures 3D, H, I*). Taken together, these data support our hypothesis that the myeloproliferative disorder observed in gDKO animals is due to a non-cell-autonomous impact of *Nr4a* deletion.

Abnormal B cell homeostasis in DKO mice is a non-cell-autonomous effect of NR4A-deficiency

Like other Treg deficient models, gDKO mice exhibit spontaneous polyclonal B cell activation and differentiation under steady-state conditions (*Figures 4A, B, Supplementary Figures 4A-F*). We recently identified a cell-intrinsic role for the NR4A family in restraining Ag-induced B cell expansion in the absence of co-stimulation, including in the context of B cell tolerance (15, 20). We therefore sought to determine to what extent spontaneous B cell activation and differentiation in gDKO mice (under homeostatic conditions) were attributable to a B cell-intrinsic role for the NR4A family. Chimeras did not reveal a competitive advantage or disadvantage for DKO cells during splenic B cell development apart from a subtle disadvantage in the MZ compartment (*Supplementary Figure 4G*) (20). B cells in 1:1 DKO:WT chimeras expressed higher levels of activation markers than B cells in WT:WT chimera (*Figure 4C, Supplementary Figures 4H, I*). However, this did not differ between donor genotypes within individual chimeras, suggesting a B cell-extrinsic effect of NR4A-
deficiency. We observed expansion of GC B cells and CD138\(^+\) cells in DKO:WT
chimeras relative to WT:WT control chimeras, but this was similarly B cell-extrinsic
(Figures 4D-I). Indeed, no expansion of the GC or CD138\(^+\) compartment was evident
under steady-state conditions in mice lacking Nr4a1/3 exclusively in the B cell
compartment (mb1-cre \(Nr4a1^{fl/fl}\) \(Nr4a3^{-/-}\)), even when aged to 40 weeks (Figures 4J-
M). Nor could we detect an advantage for mb1-cre DKO B cells in a competitive setting
(Supplementary Figures 4J-L). We conclude that there is evidence of a spontaneous
polyclonal B cell activation and differentiation in gDKO chimeras, but it is a B cell-non-
autonomous effect of NR4A-deficiency.

Reconstitution of WT Treg in competitive chimeras does not rescue DKO CD8\(^+\) T
cell homeostasis
Progressive thymic atrophy (Supplementary Figure 2A) and spontaneous B
activation and differentiation in DKO:WT chimeras (Figure 4) suggested the
development of a systemic autoimmune and inflammatory state despite replete and
largely WT Treg compartment. We next probed the mature T cell compartment to
understand the source of this immune dysregulation. gDKO mice exhibit expanded
effector-memory compartment and nearly complete loss of naive CD8\(^+\) T cells (Figures
5A, B, Supplementary Figures 5A, B). However, despite reconstitution of a replete
Treg compartment of WT origin (Figure 1M), DKO:WT chimeras nevertheless exhibit
marked accumulation of CD44\(^{hi}\)CD8\(^+\) T cells relative to WT:WT control chimeras
(Figures 5C, D), and moreover, DKO T cells accumulate in this compartment (Figure
5E). In addition, these CD44\(^{hi}\)CD8\(^+\) DKO T cells upregulate PD-1 expression,
suggesting an exhausted state (Supplementary Figures 5C, D). These observations reveal a T cell-intrinsic role for the NR4A family in CD8+ T cell homeostasis.

Abnormal DKO CD8+ T cell homeostasis in competitive chimeras is due to a cell-intrinsic role for Nr4a1 and Nr4a3 during thymic development

To test whether abnormal DKO CD8+ T cell homeostasis reflects a requirement for the NR4A family during thymic selection or exclusively in the periphery, we took advantage of a CD8-cre construct driven by the E8I enhancer that expresses specifically in mature CD8SP and peripheral CD8+ T cells in order to generate CD8-cre Nr4a1fl/fl Nr4a3−/− mice (CD8-cre cDKO) (38) (Figures 5F, G). We can confirm that this cre is not active until after thymic DP stage and positive selection checkpoint are traversed, because NUR77 expression in the mature CD4 lineage of CD8-cre cDKO mice is intact (Figures 5F, G). Since accumulation of CD44hiCD8+ T cells was not observed in CD8-cre cDKO mice (Figure 5H, Supplementary Figure 5E), we conclude that this phenotype must be attributable to a role for the NR4A family earlier in development and likely reflects escape of self-reactive CD8+ T cells into the periphery due to impaired negative selection.

Cell-intrinsic accumulation of CD4+ DKO T cells with anergic phenotype in competitive chimeras
gDKO mice exhibit an expanded CD4+ T cell effector-memory compartment that is not evident in DKO:WT chimeras (Figures 6A-C). However, CD4+ T cell homeostasis is not restored in these chimeras; rather DKO CD4+ T cells accumulate in the CD44hi...
(memory) compartment and upregulate well-established markers of anergy (CD73 and FR4) in a cell-intrinsic manner (Figures 6D-G) (39). Similarly, expansion of anergic CD4+ T cells was exaggerated in 1:1 DKO:WT chimeras relative to both control chimeras and SKO mice (Supplementary Figures 6A-H). Moreover, even phenotypically 'naive' DKO CD44loCD62Lhi CD4+ T cells in mixed chimeras upregulated CD73 and FR4, suggestive of antigen encounter (Figure 6D, E, Supplementary Figures 6A, C, F) (19). Taken together, these data are consistent with escape of self-reactive DKO CD4+ T cells from negative selection in the thymus (Figure 2) and acquisition of an anergic phenotype in the periphery.

Impaired TCR signaling in anergic DKO T cells from competitive chimeras

Canonical functional features of anergic T cells include defective proximal TCR signal transduction and impaired IL-2 production (40, 41). Therefore, we next assessed TCR-induced Erk phosphorylation in T cells from DKO chimeras via a well-established flow-based assay. Since anergic surface markers were largely preserved after TCR stimulation and methanol permeabilization, we were able to gate cells according to CD73 and FR4 expression, and on this basis defined cells as non-anergic, intermediate anergic, or anergic (Supplementary Figure 7A). Consistent with this surface phenotype, we observed progressively impaired TCR-induced Erk phosphorylation of WT memory CD4+ T cells across these populations (Figures 7A, B). Within each gate, DKO CD4+ T cells were even more refractory to TCR stimulation than WT cells from the same chimera. Strikingly, unlike cells of WT origin, naive anergic DKO T cells were as refractory as memory anergic T cells. These data suggest that DKO CD4+ T cells not
only upregulate markers of anergy (Figure 6), but acquired functional features of anergy and did so to an even greater extent than WT.

Since we observed the accumulation of DKO CD44^{hi}CD8^{+} T cells with increased PD-1 expression in DKO:WT chimeras (Figure 5, Supplementary Figures 5C, D), we utilized the same approach to assess functional characteristics of DKO CD8^{+} T cells. We found that DKO CD44^{hi}CD8^{+} T cells exhibit impaired Erk phosphorylation relative to WT cells within the same chimera (Supplementary Figures 7B-D).

Of note, Erk phosphorylation downstream of PMA stimulation was intact in both genotypes across all gated populations, suggesting a proximal rather than distal defect in TCR signaling among ‘tolerant’ T cells (Figure 7A, Supplementary Figure 7C). Importantly, TCR-induced Erk phosphorylation was robust in naive / non-anergic DKO T cells (Figures 7A, B, Supplementary Figures 7C, D), implying that defective signal transduction was an acquired feature of tolerant T cells. Collectively, these data suggest that self-reactive DKO T cells escape negative selection and acquire both phenotypic and functional features of antigen experience.

**Upregulation of anergy-associated genes in DKO CD4^{+} T cells**

To define the transcriptome of anergic DKO CD4^{+} T cells using an unbiased approach, we undertook RNA-sequencing of DKO and WT cells sorted from 1:5 chimeras. We gated on CD4^{+}CD25^- cells to exclude a large fraction of FOXP3^{+} Treg and sorted CD44^{hi}CD62L^{lo}CD73^{hi}FR4^{hi} “anergic” cells (Supplementary Figure 7E). In parallel, we also sorted naive CD4^{+} T cells from each genotype for analysis ex vivo and following 3h in vitro TCR stimulation. Since we observed the expansion of anergic cells
in the naive CD4+ T cell compartment, we gated on CD73loFR4lo within the CD44loCD62Lhi gate to collect the least self-reactive naive cells. Indeed, sorted “naive” DKO cells do not express anergy-associated genes ex vivo (Supplementary Figure 7F, Figure 7C). By contrast, “anergic” DKO cells exhibited pronounced upregulation of a subset of anergy-related genes relative to “anergic” WT cells (Figure 7C). This may reflect a high degree of self-reactivity in this compartment due to escape from negative selection. Importantly, although gene expression diverges between DKO and WT anergic cells, much of their transcriptome is shared (Supplementary Figures 7F, G). These include negative regulators such as Lag3, Pdcd1/PD-1, Rnf128/GRAIL and Spry1 that serve to suppress proximal TCR signaling and may account, at least in part, for the defect in ERK phosphorylation we observed in DKO cells (Figure 7C). Indeed, we confirmed upregulation of PD-1 and LAG3 expression in DKO anergic cells by flow staining (Supplementary Figure 7H, I). By contrast, Treg-associated genes such as Foxp3, Ikzf4/Eos and Lrrc32/GARP are not expressed in anergic DKO cells (Figure 7D), consistent with previous studies revealing an essential role for the NR4A family in induction and maintenance of Treg fate (6, 7, 28, 42). Further validating our data set, gene set enrichment analysis (GSEA) revealed that genes repressed by overexpression of Nr4a1 (17) are enriched in DKO anergic cells (Figure 7E), and conversely, genes upregulated by Nr4a1 overexpression (17) are enriched in WT anergic cells (Figure 7F) (9, 17). Strikingly, a subset of genes encoding pro-inflammatory cytokines and mediators is upregulated in anergic DKO cells (Figure 7G), including Th1-related genes (Tbx21 and Ifng) and Th2-related genes (Gata3 and Il4). This is consistent with prior reports identifying a role for the NR4A family in the repression of Th1 and Th2 cell
differentiation (7, 42) and may contribute to immune dysregulation observed in DKO:WT chimeras.

Cell-intrinsic defect in peripheral CD4+ DKO T cell tolerance

In order to define how such dysregulated gene expression may arise and also to control for self-reactivity of the DKO T cell repertoire, we next sought to identify the immediate targets of the NR4A family in sorted CD73−FR4− “naive” CD4+ T cells (Supplementary Figures 7E). We compared the expression of primary response genes (PRGs) in sorted naive WT and DKO cells following TCR stimulation (Supplementary Figure 8A). We selected an early 3 hour time point in order to capture peak NR4A protein induction and to enrich for direct transcriptional targets (18, 43). Principle component analysis (PCA) segregated DKO from WT cells following acute TCR stimulation, though less robustly than for DKO and WT anergic cells (Supplementary Figures 8B, 7G). We focused our attention on PRGs that were differentially expressed between WT and DKO cells (Figure 8A). Among those under-induced in DKO cells, we identified Bcl2l11/BIM, and negative regulators of TCR signaling including Cblb, Dusp4, and Tnfaip3/A20. Conversely, we observed over-induction of inflammatory mediators such as Ccl4, Il2 and Tnf in DKO cells. We found that genes downregulated by Nr4a1 over-expression are highly enriched in acutely TCR-stimulated naive DKO cells, while the opposite is true for genes upregulated by Nr4a1 over-expression (Figures 8B, C).

Impaired IL-2 production is among the most characteristic features of anergic T cells, while exogenous IL-2 can override anergy in some settings (40, 41), suggesting its dysregulation in DKO T cells may contribute to disruption of T cell tolerance in our
Indeed, *Il2* has been previously implicated as a target of the NR4A family in the context of anergy and exhaustion (9, 17). We first assessed secreted IL-2 in culture supernatants of *Nr4a1*−/− or *Nr4a3*−/− SKO CD4+ T cells across a broad titration of TCR stimulation (**Figure 9A**). Both SKO genotypes secrete higher amounts of IL-2 compared to WT, suggesting an additive role for the NR4A family in IL-2 regulation. We next sought to assess IL-2 responses by DKO T cells. To do so, we cultured T cells from DKO:WT chimeras with anti-CD3 and then assessed the capacity for IL-2 production following maximal restimulation with PMA/ionomycin. We observed that, after TCR stimulation, DKO CD4+ T cells acquire a much higher capacity for IL-2 production relative to WT, and this is cell-intrinsic (**Figures 9B, C**). This result is not due to Treg deficiency in DKO compartment (**Supplementary Figure 8C**). *Nr4a1*−/− or *Nr4a3*−/− SKO CD4+ T cells each exhibit a less robust but independent increase in capacity for IL-2 production relative to WT (**Supplementary Figure 8D**). Moreover, *Il2* transcript is upregulated in anergic DKO cells relative to anergic WT cells directly ex vivo (**Figure 9D**). These data suggest that the role of the NR4A family in restraining IL-2 production is not completely redundant, but rather additive, and impacts naive as well as anergic CD4+ T cells.

DKO CD8+ T cells also exhibited a much higher capacity for IL-2 production than WT cells from the same mixed chimera (**Supplementary Figures 8E, F**). Furthermore, CD8+ T cells from CD8-cre cDKO mice exhibit a nearly identical phenotype that is more robust than in SKO CD8+ T cells from *Nr4a3*−/− or CD8-cre *Nr4a1*fl/fl mice (**Supplementary Figure 8G**). These data suggest that the NR4A family negatively
regulate the IL-2 locus in peripheral CD8\(^+\) T cells in a manner that is additive and cell-
intrinsic, and independent of self-reactivity.

**Restoring WT Treg compartment in competitive chimeras alters autoantibody repertoire but does not suppress autoimmunity**

gDKO mice exhibit spontaneous, early-onset development of autoantibodies (Figures 10A, B). Indirect immunofluorescence assay (IFA) for autoantibodies revealed both nuclear and cytosolic staining suggesting a widespread loss of B cell tolerance that occurs with complete penetrance before 4 weeks of age, recapitulating observations in Treg-deficient mice (4). It is possible that this is attributable in part to loss of T follicular regulatory (Tfr) cells in gDKO as seen in other Treg-deficient mice (44). Indeed, we identify a profound cell-intrinsic defect for DKO cells in Tfr (but not Tfh) compartments in chimeras (Supplementary Figures 9A, B) (7, 45). Although older Nr4a3\(^{-/-}\) (but not Nr4a1\(^{-/-}\)) mice exhibit very low titer autoantibodies with a similar pattern (Supplementary Figures 9C, D), B cell tolerance is largely preserved in SKO mice. To our surprise, despite reconstitution of the Treg (and Tfr) compartment in DKO:WT chimeras with cells of WT origin, we nevertheless observed the development of high titer autoantibodies even at early time points after reconstitution (Figures 10C, D). Cytosolic staining by autoantibodies was largely eliminated, but anti-nuclear autoantibodies persisted in both 1:1 and even 1:5 DKO:WT chimeras (Figures 10E, F, Supplementary Figures 9E, F). This suggests that the development of autoimmunity in DKO chimeras was not attributable to a residual or partial Treg defect. The titer of ANA in DKO:WT chimeras increased with age (Figure 10G, Supplementary Figure 9E).
This correlated with progressive accumulation of anergic CD4+ T cells, thymic atrophy, and development of polyclonal B cell activation and spontaneous GC expansion, suggestive of evolving immune dysregulation in these chimeras. By contrast, mice in which B cells conditionally lack both Nr4a1 and Nr4a3 (mb1-cre cDKO) did not develop ANA even after 40 weeks (Supplementary Figures 9G, H). These data imply that ANA in DKO:WT chimeras are not attributable to a B cell-intrinsic role for the NR4A family. Rather, we propose that - although reconstituting a WT Treg compartment suppresses lethal immune dysregulation in DKO chimeras - tolerance is not fully restored, and this may be due to a profound defect in both negative selection and peripheral T cell tolerance.

DISCUSSION

A vital and redundant role for NR4A factors in the Treg compartment has made it challenging to isolate and dissect other functions for this family in immune tolerance and homeostasis (6, 7). Unfortunately, conditional genetic strategies alone cannot disentangle requirements for the NR4A family during thymic selection from their obligate function in Treg. Here we used competitive BM chimeras to reconstitute a functional Treg compartment of WT origin, and this enabled us to unmask additional essential roles for the NR4A family in the preservation of both central and peripheral T cell tolerance under homeostatic conditions.

We confirmed a cell-intrinsic requirement for Nr4a1 and Nr4a3 in the Treg compartment as previously reported with CD4-cre conditional DKO and TKO mice (6, 7, 28, 42). Concurrently, we also observed expansion of DKO CD25+ FOXP3− cells both in
gDKO mice and DKO chimeras. Sekiya and colleagues propose that this compartment contains highly self-reactive T cells that failed to assume Treg fate and yet escaped censorship by negative selection (28). Formal fate-mapping studies will be important to test this hypothesis. This population of cells may contribute to immune dysregulation in DKO chimeras, but importantly, cells diverted from the Treg fate cannot account numerically for excess DKO SP thymocytes that escape negative selection in mixed chimeras, especially since equal or greater advantage for DKO cells relative to WT is observed in CD8SP thymocytes relative to CD4SP.

By contrast, we showed that myeloid cell expansion in gDKO mice is not cell-intrinsic because it is almost entirely suppressed in DKO chimeras, and is instead likely attributable to loss of Treg as observed in other Treg-deficient mouse models (2, 3, 37). In support of this hypothesis, CD4-cre TKO mice develop a similar myeloproliferative disorder that is also rescued in competitive chimeras generated with mixtures of CD4-cre TKO and WT donor BM (6). This result emphasizes the need to critically re-assess the therapeutic potential of the NR4A family as drug targets in myeloproliferative disorders.

One of the earliest functions identified for the NR4A family is an essential role in antigen-induced cell death and during thymic negative selection, but these studies relied on mis-expression of full length and truncated NR4A Tg constructs under the control of the proximal Lck promoter (which is active early during the DN stage of thymic development) (13, 21, 22, 33, 34). By contrast, studies of Nr4a1−/− mice have revealed subtle phenotypes, consistent with redundancy among family members (23, 24, 46). Here we were able to unmask the redundancy between Nr4a1 and Nr4a3 during thymic
negative selection in a physiological setting for the first time. It is estimated that six
times more thymocytes are negatively than positively selected in a given time frame
(47). Since both 1:1 and 1:5 DKO chimeras harbor 4- to 6-fold more CD4SP and
CD8SP DKO cells relative to WT cells when normalized to the pre-selection DP
compartment (**Figure 2E**), we propose that NR4A-dependent deletion may account for
most or all negative selection. Prior studies implicate the NR4A family in negative
selection by both ubiquitous and tissue-restricted antigens (TRA) (22, 24, 48-50).
Though our data does not directly distinguish between the two, the striking amplitude of
rescue seen in DKO chimeras suggests escape from negative selection by ubiquitous
self-antigens (proposed to account for 75% of all deletion) and possibly TRA as well, but
this remains to be determined (47, 51).

Caspase-3 is activated in thymocytes upon TCR stimulation and in the process of
negative selection (52). Reduced aCasp3 expression of in vitro stimulated DKO
thymocytes suggests caspase-dependent TCR-induced apoptosis is mediated, at least
in part, by the NR4A family. BIM/Bcl2l11, a member of Bcl-2 family that can promote
Caspase-3 activation, is also essential for thymic negative selection and may represent
a transcriptional target for *Nr4a1* (24, 53). Although we find that *Nr4a1* and *Nr4a3*
collectively promote *Bcl2l11* transcription in naive CD4+ T cells (**Figure 8A**), it has also
been shown that NUR77 can promote apoptosis by directly binding BCL-2 in the
cytosol, inducing a conformational change that exposes its BH3 pro-apoptotic domain in
a manner that is independent of transcriptional activity of NUR77 (49, 54). It will be
important to define which effectors downstream of NR4As mediate negative selection in
vivo. It remains to be determined how additional instructional signals modulate NR4A.
function to either promote Treg differentiation or, alternatively, drive deletion of self-reactive thymocytes.

We observe the accumulation of DKO CD44\textsuperscript{hi}CD8\textsuperscript{+} T cells in DKO competitive chimeras, and this is eliminated in CD8-cre cDKO mice in which cre-mediated deletion occurs only after thymic selection is complete (Figure 5). We also observed a marked accumulation of CD4\textsuperscript{+} DKO T cells with transcriptional and functional features of anergy (Figure 6, 7). We propose that these phenotypes reflect escape of self-reactive T cells into the periphery due to a defect in thymic negative selection.

Recent work suggests that \textit{Nr4a1} is required for induction and/or maintenance of CD4\textsuperscript{+} T cell anergy (17); over-expression of \textit{Nr4a1} drives upregulation of a subset of anergy-related genes, whereas deletion of \textit{Nr4a1} prevents generation of functionally tolerant T cells. Similarly, \textit{Nr4a} TKO CAR T cells evade exhaustion and eliminate tumors (9). Although DKO T cells acquire features of tolerance in chimeras, we nevertheless observed the development of systemic immune dysregulation and ANA in DKO chimeras despite reconstitution of a functional Treg compartment, suggesting a residual defect in functional anergy. It remains to be determined if DKO T cells are also resistant to Treg-mediated suppression. We identified upregulation of inflammatory mediators (e.g., \textit{Il2} and \textit{Tnf}) and impaired induction of negative regulators (e.g., \textit{Cblb}, \textit{Tnfaip3} and \textit{Bcl2l11/BIM}) in naive DKO CD4\textsuperscript{+} T cells following acute TCR stimulation (Figure 8). These and other transcriptional targets of the NR4A family may contribute to impaired peripheral tolerance. Indeed, although suppression of IL-2 production is among the most characteristic features of anergic T cells, we report enhanced capacity for IL-2 production in SKO T cells (consistent with prior studies of \textit{Nr4a1}^{-/-} T cells (17)).
and much more so in DKO T cells (Figure 9). We suggest this reflects a role for the NR4A family in epigenetic remodeling of the Il2 locus in response to TCR stimulation.

Indeed, NR4A transcription factors modulate chromatin structure in the setting of chronic antigen engagement (9, 17), and interrogation of a recently published ATACseq data set reveals differentially accessible regions of open chromatin (OCR) near the Il2 locus in Nr4a3−/− CD8+ T cells following 12 h TCR stimulation (GSE143513) (55). We propose that self-reactive DKO T cells that have escaped negative selection, Treg differentiation, and peripheral anergy accumulate in the periphery and drive ANA production in DKO chimeras. It remains to be defined whether defective central or peripheral tolerance (or both) are most relevant for the development of autoimmunity in DKO chimeras, and whether specific Th subsets (such as Tfh) play a role (42).

Nearly complete redundancy between Nr4a1 and Nr4a3 are evident in Treg and during negative selection; deletion of both family members is necessary to unmask these roles. By contrast, regulation of B cell responses (20) and CD8+ T cell exhaustion (9) by the NR4A family appear additive. Based on published work (17) and our observations of the IL-2 module in SKO and DKO T cells, we speculate that regulation of CD4+ T cell anergy is similarly additive, but this remains to be fully addressed.

Although expression of Nr4a2 is low in the T cell lineage under steady-state conditions, we also cannot exclude the possibility that Nr4a2 compensates for and partially masks some immune phenotypes in DKO cells, especially in the context of inflammatory stimuli.

We propose that Nr4a1 and Nr4a3 regulate layered T cell tolerance mechanisms to preserve immune homeostasis under steady-state conditions (see model,
Supplementary Figure 10). In addition, it is likely that NR4A factors also serve to counter-regulate inflammatory stimuli and promote a return to homeostasis. Indeed, negative feedback by NR4A restrain responses to LPS in myeloid cells (56) and to antigen stimulation in B cells (20), and suppresses inflammation in immune-mediated disease models (57). Although it remains unclear whether endogenous ligands regulate NR4A function in vivo, small molecule NUR77 agonist (10) and antagonist (11) compounds have been reported. Agonists might be useful to suppress autoimmunity and maintain transplant tolerance. Antagonizing NUR77 and perhaps other NR4A family members could have applications for cancer immunotherapy (9). Since redundancy among NR4A family members is important in both negative selection and in Treg, selectively targeting individual NR4A family members may allow modulation of antigen-specific T and B cell responses without disrupting global immune homeostasis. Conversely, our studies unmask Treg-independent and redundant roles for Nr4a1 and Nr4a3 in maintaining T cell tolerance under homeostatic conditions, with important implications for drug design.

MATERIALS AND METHODS

Mice. Nr4a1<–/–, Nr4a1fl/fl, and Nr4a3<–/– mice were previously described (6, 20, 23). Nr4a1fl/fl were previously obtained from Catherine Hedrick (La Jolla Institute for Immunology, La Jolla, CA) with permission from Pierre Chambon (University of Strasbourg, Strasbourg, France) (6). Nr4a1<–/– mice were obtained from The Jackson Laboratory and this line is used throughout the manuscript exclusively as single germline knockout comparator (23), Nr4a3<–/– mice were generated in our laboratory as
previously described (20). CD8-cre and mb1-cre were obtained from The Jackson Laboratory (38, 58). C57BL/6 mice were from The Jackson Laboratory and CD45.1+ BoyJ mice were from Charles River Laboratories. To generate germline DKO Nr4a1−/− Nr4a3−/− mice, we bred Nr4a3−/− and Nr4a1fl/fl mice with germline recombination of the loxp-flanked locus, and confirmed loss of exon 2 both by genomic DNA PCR and transcript qPCR. All strains were fully backcrossed to C57BL/6 genetic background for at least 6 generations. Mice of both sexes were used for experiments between the ages of 3 and 10 weeks except for BM chimeras as described below.

**Antibodies and Reagents.**

**Abs for surface markers:** Abs to B220, CD3, CD4, CD8, CD11b, CD11c, CD19, CD21, CD23, CD25, CD44, CD45.1, CD45.2, CD62L, CD69, CD73, CD86, CD93 (AA4.1), CD138, CXCR5, Fas, FR4, γδTCR, GL7, Gr1, IgD, MHC-II, NK1.1, PD-1, and pNK conjugated to fluorophores were used (BioLegend, eBiosciences, BD, or Tonbo). See also Supplementary Material.

**Abs for intra-cellular staining:** FOXP3 Ab conjugated to APC or FITC (clone FJK-16s, Invitrogen). Anti-active Caspase-3 (aCasp3) Ab conjugated to APC (clone C92-605, BD Pharmingen). Anti-NUR77 conjugated to PE (clone 12.14, Invitrogen). Anti-IL-2 Ab conjugated to PE (clone JES6-5H4, Invitrogen). Anti-pERK (Phospho-p44/42 MAPK (T202/Y204) (clone 197G2, Cell Signaling) Rabbit Ab. Goat Anti-Rabbit IgG (H+L) conjugated to APC (Jackson ImmunoResearch).

**Stimulatory Abs:** Anti-CD3 (clone 2c11) and anti-CD28 (clone 37.51) (BioLegend). Goat anti-Armenian Hamster antibody (Jackson ImmunoResearch).
**ELISA reagents**: 96-well, high-binding, flat-bottom, half-area, clear polystyrene Costar Assay Plate (Corning). Mouse anti-dsDNA IgG-specific ELISA Kit (Alpha diagnostic.)

Mouse IL-2 DuoSet ELISA and DuoSet ELISA Ancillary Reagent Kit 2 (R&D Systems).

**Anti-nuclear antibody (ANA)**: NOVA Lite™ HEP-2 ANA Substrate Slide and mounting medium (INOVA Diagnostics, Inc, #708100); FITC Donkey Anti-Mouse IgG (Jackson ImmunoResearch).

**Culture Media**: RPMI-1640 + L-glutamine (Corning-Gibco), Penicillin Streptomycin L-glutamine (Life Technologies), HEPES buffer [10mM] (Life Technologies), B- Mercaptoethanol [55mM] (Gibco), Sodium Pyruvate [1mM] (Life Technologies), Non-essential Amino acids (Life Technologies), 10% heat inactivated FBS (Omega Scientific).

**Flow Cytometry**. Cells were analyzed on a Fortessa and sorted on Aria (Becton Dickson). Data analysis was performed using FlowJo (v9.9.6 or v10.7.1) software (Becton Dickson).

**Intracellular staining to detect active Caspase 3**. Following in vitro stimulation, cells were permeabilized and stained with APC-aCasp3, according to the manufacturer’s protocol (BD Cytofix/Cytoperm kit).

**FOXP3 staining**. FOXP3 staining was performed utilizing a FOXP3/transcription factor buffer set (eBioscience) in conjunction with APC or FITC anti-FOXP3, as per manufacturer’s instructions.
Intracellular staining to detect IL-2. Splenocytes were stimulated with plate-bound anti-CD3 Ab for 20 hours followed by a 4-hour treatment with 20 ng/ml of phorbol myristate acetate (PMA) (Sigma) and 1 μM of ionomycin (Calbiochem) and protein transport inhibitor cocktail (eBioscience) per manufacturer’s protocol. Following in vitro stimulation, cells were permeabilized and stained, according to the manufacturer’s protocol (BD Cytofix/Cytoperm kit).

Intracellular staining to detect NUR77. Following 2-hour in vitro stimulation with 20 ng/ml of PMA and 1 μM of ionomycin, cells were fixed in a final concentration of 4% paraformaldehyde for 10 min, permeabilized at −20 °C with 100% methanol for 30 min and, following washes and rehydration, stained with primary antibody for 60 min at 20 °C (room temperature).

Live/dead staining. LIVE/DEAD Fixable Near-IR Dead Cell Stain kit (Invitrogen). Reagent was reconstituted in DMSO as per manufacturer’s instructions, diluted 1:1000 in PBS, and cells were stained at a concentration of 1 × 10^6 cells /100 μl on ice for 15 minutes.

In vitro T cell culture and stimulation. Flat bottom 96 well plates were coated with varying doses of anti-CD3 with or without 2 mg/ml anti-CD28 at 4°C overnight. Splenocytes, lymphocytes or thymocytes were harvested into single cell suspension. Splenocytes were subjected to red cell lysis using ACK buffer. Cells were plated at a
concentration of $5 \times 10^5$ cells/100 μl complete RPMI media in antibody coated flat bottom 96 well plates for varying time.

**Bone marrow chimeras.** Host mice were irradiated with two doses of 530 rads, 4 hours apart, and injected on the same day IV with a total of $2 \times 10^6$ donor BM cells at varying ratios (1:1 or 1:5 or without mixture, as noted). Chimeras were sacrificed 6-14 weeks after irradiation for downstream analyses.

**CD4$^+$ T cell purification.** CD4$^+$ T cell purification was performed utilizing magnetic-activated cell sorting (MACS) separation, per the manufacturer’s instructions. In brief, pooled spleen and/or lymph nodes were prepared utilizing the CD4$^+$ T Cell Isolation Kit (Miltenyi Biotec) and purified by negative selection through an LS column (Miltenyi Biotec). Purified CD4$^+$ T cells were then subjected to in vitro culture.

**Phospho-flow.** Splenocytes were rested at 37°C in serum-free RPMI for 30 minutes. Cells were then stimulated with 10 μg/ml of anti-CD3 (clone 2c11) for 30 seconds followed by 50 μg/ml of anti-Armenian hamster crosslinking antibody for 2 minutes, or PMA for 2 minutes. Stimulated cells were fixed with 2% paraformaldehyde and permeabilized with methanol at -20°C overnight. Cells were then stained with surface markers and pErk at 20°C.

**RNA-sequencing.** DKO and WT CD25$^-$CD4$^+$ T cells were sorted from competitive chimeras to identify either naive (CD44$^{lo}$CD62L$^{hi}$CD73$^{lo}$FR4$^{lo}$) or anergic (CD44$^{hi}$CD62L$^{lo}$CD73$^{hi}$FR4$^{hi}$) populations. Cell populations were sorted directly into RLT
+ 1% beta-mercaptoethanol (BME) buffer. In parallel, sorted naive CD4\(^+\) T samples were stimulated ex vivo with 8 \(\mu\)g/ml of plate-bound anti-CD3 and 2 \(\mu\)g/ml of anti-CD28 for 3 h and lysed in RLT/BME buffer. Libraries were generated by Emory Integrated Genomics Core (EIGC): RNA was isolated using the Quick-RNA MicroPrep kit (Zymo, 11-328M). 2000 cell equivalent of RNA was used as input to SMART-seq v4 Ultra Low Input cDNA Synthesis kit (Takara, 634888) and 200 pg of cDNA was used to generate sequencing libraries with the NexteraXT kit (Illumina, FC-121-10300). Libraries were pooled at equimolar ratios, and sequenced on the NovaSeq6000 with a PE100 configuration using a NovaSeq 6000 SP Reagent Kit. Fastq files were trimmed for adapters and low quality base pairs using Fastp (59) then aligned to mouse genome assembly mm10 using STAR (60). FeatureCounts (61) was used to obtain read count data and a paired differential expression analysis comparing samples from the same chimeras was performed with edgeR (62). Heatmaps and principal component analysis plots were generated using the ClustVis online tool (https://biit.cs.ut.ee/clustvis/) (63).

Gene set enrichment analysis (GSEA) was performed with GSEA (v4.1.0) software (UC San Diego and Broad institute) (64, 65). Fastq and TPM data are publicly available (GEO accession number: GSE178782) and Supplementary Data 1 contains analyses.

**ELISA for IL-2 detection.** Purified lymph node CD4\(^+\) T cells were cultured on anti-CD3/28 coated plate at 1 \(\times\) 10\(^5\) cells per well. Plates were spun and supernatants were harvested after 24h or 48 h. IL-2 concentrations in supernatants were measured using a commercial ELISA kit, per the manufacturer’s instructions (R&D Biosystems). In brief, 96-well plates were coated with 1 \(\mu\)g/ml of capture anti-IL-2 antibody. Supernatants
were diluted serially, and IL-2 was detected with detection anti-IL-2 antibody. ELISA plates were developed with mixture of tetramethylbenzidine and peroxidase, then stopped with 2 N sulfuric acid. Absorbance was measured at 450 nm using a spectrophotometer (SpectraMax M5; Molecular Devices).

**ELISA for serum anti-dsDNA.** Serum was harvested from blood collected by lateral tail vein sampling or cardiac puncture postmortem. Serum anti-dsDNA titer was measured with a commercial ELISA kit, per the manufacturer’s instructions (Alpha diagnostic). In brief, sera were added to plates coated with dsDNA. Anti-dsDNA titer was detected with anti-IgG-HRP. ELISA plates were developed, and absorbance was measured as described above.

**Anti-nuclear antibody (ANA).** Serum ANA was detected with NOVA Lite™ HEP-2 ANA Substrate Slide as per manufacturer’s instructions except for using FITC-conjugated donkey anti-mouse IgG secondary antibody. Images were captured with a Zeiss Axio Imager M2 widefield fluorescence microscope. Images were processed with Zen Pro (Zeiss). To measure titer, serum was serially diluted 2-fold from 1:40 to 1:1280. HEP-2 ANA slides were stained with diluted serum. Images were read by a rheumatologist in a blinded manner and titer was determined as the detectable lowest dilution of each sample.

**Statistical analysis.** Statistical analysis and graphs were generated using Prism v9 (GraphPad Software, Inc). Graphs show mean ± SEM unless otherwise stated. Student’s unpaired or paired t-test was used to calculate the P values for all
comparisons of two groups, and correction for multiple comparisons across time points or doses was then performed using the Holm–Šídák method. One-way or two-way analysis of variance (ANOVA) with follow-up Tukey’s test or Dunnett’s test were performed when more than two groups were compared with one another. Fisher’s exact test was used to compare the difference in proportions of two groups. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Study approval. All mice were housed in a specific pathogen-free facility at UCSF according to the University and National Institutes of Health guidelines. The protocol for use of mice was reviewed and approved by UCSF Institutional Animal Care Use Committee (San Francisco, CA).

Author contributions
R.H., H.V.N., J.L.M. and J.Z. conceived of and designed the experiments. R.H., H.V.N. and J.L.M performed the experiments. R.H., H.V.N., J.L.M., R.M. and J.Z. analyzed the data. R.H. and J.Z. wrote the manuscript. R.H., H.V.N., J.L.M. and J.Z. edited the manuscript.

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FIGURE LEGENDS

Figure 1. Systemic immune dysregulation and Treg deficiency in mice with germline deficiency of Nr4a1 and Nr4a3

A. Nr4a1<sup>−/−</sup>Nr4a3<sup>−/−</sup> (gDKO) mouse (red arrow) compared to healthy littermate, 4 w; Representative of n = 8.

B. Flow plots show splenic CD4<sup>+</sup> T cells with FOXP3<sup>+</sup>Treg gate in mice of each genotype. Representative of 5 mice/genotype.

C, D. Quantification of thymic (C) and splenic (D) Treg cell number (n ≥ 5, 3 to 4-week-old gDKO and 5 to 6-week-old mice with other genotypes).

E-G. Schematics depict competitive BM chimera design.

H, I. Flow plots show thymic CD4SP (H) or splenic CD4<sup>+</sup> T cell (I) sub-populations in 1:1 DKO:WT chimeras. Representative of 6 (H) or 10 (I) chimeras.

J-O. Quantification of thymic (J) or splenic (M) Treg cell number in 1:1 chimeras. Ratio of CD45.2 to CD45.1/2 for thymic (K, L) or splenic (N, O) Treg, CD25<sup>+</sup>FOXP3<sup>−</sup> and CD25<sup>−</sup>FOXP3<sup>−</sup> cells in 1:1 chimeras, normalized to DP thymocytes (n ≥ 3 (J-L) or 6 (M-O), pooled from 2 sets of independently generated chimeras 6-10 w post-transplant.

P, Q. Flow plots show thymic CD4SP (P) or splenic CD4<sup>+</sup> T cell (Q) sub-populations in 1:5 DKO:WT chimera. Representative of ≥ 3 chimeras from one chimera setup.

Graphs depict mean +/- SEM. Statistical significance was assessed by one-way ANOVA with Tukey’s test (C, D, K, L, N, O) or two-tailed unpaired Student’s t-test (J, M). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. NS, not significant.
**Figure 2. DKO thymocytes have a cell-intrinsic defect in negative selection**

A. Flow plots show thymic subsets in WT, *Nr4a3−/−*, *Nr4a1−/−* and gDKO mice. Representative of n ≥ 4 mice/genotype.

B. Quantification of thymic subset cell number as gated in (A); (n ≥ 4, 3 to 4-week-old gDKO and 5 to 6-week-old mice of other genotypes).

C. Ratio of CD45.2 to CD45.1/2 thymocytes among thymic DN3a and DN3b subsets (as gated in Supp. Fig. 2B), normalized to DN2 subset (n = 3-4 chimeras).

D. Flow plots show thymic subsets in competitive chimeras. Representative of ≥ 3 mice/genotype.

E. Ratio of CD45.2 to CD45.1/2 thymic subsets as gated in (D) normalized to DP subset (n ≥ 3). Data in C-E were from 6-7 w post-transplant chimeras pooled from 3 sets of independently generated chimeras.

F-K. Thymocytes from 1:1 DKO:WT chimeras were cultured with varying doses of plate-bound anti-CD3 and 2 μg/ml of anti-CD28 for 24 h. Cells were stained to detect CD4/CD8 surface markers, followed by permeabilization and detection of active Caspase3 (aCasp3). Representative plots show aCasp3 expression in WT CD45.1/2 and DKO CD45.2 DP (F) and CD4SP (I) thymocytes from 1:1 DKO chimeras cultured with 10 μg/ml anti-CD3. Quantification % aCasp3+ cells among DP (G, H) or CD4SP (J, K) in 1:1 DKO:WT (G, J) or 1:1 WT:WT (H, K) chimeras (n = 3 from one chimera setup). Graphs depict mean +/- SEM. Statistical significance was assessed by one-way (B) or two-way (C, E) ANOVA with Tukey’s test or two-tailed unpaired Student’s t-test with the Holm–Šidák method (G, H, J, K). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. NS, not significant.
Figure 3. Myeloproliferative disorder in DKO mice is a non-cell-autonomous effect of NR4A deficiency

A-D. Lymph nodes cells (A, B) and thymocytes (C, D) from WT and gDKO mice (A, C) or 1:1 DKO:WT chimeras (B, D) were stained to detect CD11b and Gr1 expression. Shown are representative plots of ≥ 5 mice.

E-H. Quantification of CD11b+Gr1− cells in lymph nodes (E, F) and thymocytes (G, H) from WT, Nr4a3−/−, Nr4a1−/− and gDKO mice (E, G) (n ≥ 5, 3 to 4-week-old gDKO and 5 to 6-week-old mice of other genotypes) and from WT:WT = 1:1 and DKO:WT = 1:1 chimeras (F, H) (n ≥ 3 pooled from 2 sets of independently generated chimeras).

I. Ratio of CD45.2 to CD45.1/2 for CD11b+Gr1− cells in lymph nodes, thymus, and spleen from WT:WT = 1:1 and DKO:WT = 1:1 chimera (n ≥ 3 pooled from 2 sets of independently generated chimeras).

Graphs depict mean +/- SEM. Statistical significance was assessed by one-way ANOVA with Tukey’s test (E, G), two-tailed unpaired Student’s t-test with (I) or without (F, H) the Holm–Šidák method. **P < 0.01; ****P < 0.0001. NS, not significant.
Figure 4

A) Splenic B220^+ Nr4a3^−/−, Nr4a1^−/−, and gDKO

B) CD69 (MFI)

C) CD69 (MFI)

D) Splenic B220^+ IgD^−

E) % of GC B cells of total B cells

F) Ratio CD45.2:CD45.1:CD45.1/2 (GC B cells)

G) Splenocytes

H) CD138^+ B220^−

I) Ratio CD45.2:CD45.1:CD45.1/2 (CD138^+ cells)

J) Splenic B220^+ IgD^− mb1-cre, mb1-cre cDKO

K) % of GC B cells of total B cells

L) CD138^+ B220^− mb1-cre, mb1-cre cDKO

M) % of CD138^+ of splenocytes mb1-cre, mb1-cre cDKO
Figure 4. Abnormal B cell homeostasis in DKO mice is a non-cell-autonomous effect of NR4A deficiency

A. Representative flow plots showing CD69 expression on splenic B cells from WT (shaded gray histogram) and overlaid Nr4a3−/−, Nr4a1−/− or gDKO mice.

B. Quantification of CD69 MFI as in (A) (data in A, B represent n ≥ 5, 3 to 4-week-old gDKO and 5 to 6-week-old mice of other genotypes).

C. Quantification of CD69 MFI on splenic B cells of each donor genotype in competitive 1:1 chimeras (n = 3 from one chimera setup).

D, G. Representative flow plots show FAS^hi/GL7^+ GC B cells pre-gated on B220^+IgD^lo splenocytes (D) and CD138^+ splenocytes (G) from competitive chimeras.

E-I. Frequency of GC B cells among total B cells (E), ratio of CD45.2 to CD45.1/2 GC B cells normalized to B220^+IgD^hi naive B cells (F), ratio of CD138^+ to B220^+ splenocytes (H), ratio of CD45.2 to CD45.1/2 CD138^+ cells normalized to B220^+CD138^- cells (I) from competitive chimeras as gated in D, G (data in D-I represent n ≥ 6 pooled from 3 sets of independently generated chimeras).

J-M. Representative flow plots show GC B cells (J) and CD138^+ cells (L) in spleen from host chimeras transplanted with either mb1-cre or mb1-cre Nr4a1^0/0 Nr4a3−/− (cDKO) BM after 40 weeks. Frequency of GC B cells among total B cells (K) and CD138^+ cells among splenocytes (M) (n ≥ 3).

Graphs depict mean +/− SEM. Statistical significance was assessed by one-way ANOVA with Tukey’s test (B) or Dunnett's test (E, F, H, I), or two-tailed unpaired Student’s t-test with (C) or without (K, M) the Holm–Šidák method. *P < 0.05; **P < 0.01; ****P < 0.0001. NS, not significant.
Figure 5. Reconstitution of WT Treg compartment does not restore CD8+ T cell homeostasis in competitive chimeras

A. Splenocytes from WT, Nr4a3+/−, Nr4a1+/− and gDKO mice were stained to detect CD8+ T cell subsets on the basis of CD44 and CD62L expression. Plots are representative of ≥ 5 mice/genotype.

B. Quantification of splenic CD44hiCD8+ T cells as gated in A (n ≥ 5, 3 to 4-week-old gDKO and 5 to 6-week-old mice of other genotypes).

C. Flow plots showing the peripheral CD8+ T cell subsets in competitive chimeras, as described for A above. Representative of ≥ 7 chimeras of each type.

D. Quantification of splenic CD44hiCD8+ T cells from chimeras as gated in C at varied time points post-transplant (n ≥ 3).

E. Ratio of CD45.2 to CD45.1/2 for CD8+CD44hi population as gated in C, normalized to naive CD8+CD44loCD62Lhi gate (n ≥ 3). Data in C-E pooled from 2 sets of independently generated chimeras.

F, G. Thymocytes and splenocytes from CD8-cre and CD8-cre Nr4a1fl/fl Nr4a3−/− (cDKO) mice were stimulated with PMA and ionomycin (PMA/Io) for 2 h. Flow plots show intracellular NUR77 expression following fixation and permeabilization within thymic and splenic T cell subsets (F). Quantification of NUR77 MFI in T cell subsets (G) (n = 3 mice/genotype).

H. Quantification of splenic CD8+CD44hi T cells from CD8-cre, Nr4a3−/−, CD8-cre Nr4a1fl/fl and CD8-cre cDKO mice (n = 3 mice/genotype).
Graphs depict mean +/- SEM. Statistical significance was assessed by one-way ANOVA with Tukey’s test (B, H) or two-way ANOVA with Dunnett's test (D, E). *P < 0.05; ***P < 0.001; ****P < 0.0001. NS, not significant.
**Figure 6. Accumulation of anergic DKO CD4+ T cells in competitive chimeras**

A. Splenocytes from WT, Nr4a3−/−, Nr4a1−/− and gDKO mice were stained to detect CD4+ T cell subsets on the basis of CD44 and CD62L expression. Plots are representative of ≥5 mice/genotype.

B. Quantification of splenic CD4+ CD44hiCD62Llo T cells as gated in A (n ≥ 5, 3 to 4-week-old gDKO and 5 to 6-week-old mice of other genotypes).

C. Quantification of splenic FOXP3−CD4+ CD44hiCD62Llo T cells as gated in Supp. Fig. 6A from competitive chimeras at indicated time points post-transplant (n ≥ 3, pooled from 2 sets of independently generated chimeras).

D. Splenocytes from 12 weeks post-transplant DKO:WT = 1:5 chimera were stained to detect anergic T cell subsets. Flow plots depict CD73hiFR4hi (anergic) T cells within CD44loCD62Lhi (naive), CD44hiCD62Lhi and CD44hiCD62Llo (memory) compartments of CD4+FOXP3− cells of each donor genotype. Representative of 7 chimeras, generated in one set.

E-G. Ratio of CD45.2 to CD45.1/2 within CD73hiFR4hi gate among naive (E), CD44hiCD62Lhi (F) or memory (G) CD4+ T cell compartments, as gated in D. Shown are WT:WT = 1:1 and DKO:WT = 1:5 chimeras at indicated time points post-transplant (n ≥ 3 pooled from 2 sets of independently generated chimeras). Ratios were normalized to naive CD4+ T cells. Graphs depict mean +/- SEM. Statistical significance was assessed by one-way (B) ANOVA with Tukey’s test, two-way ANOVA with Dunnett’s test (C) or two-tailed unpaired Student’s t-test with the Holm–Šidák method (E-G). *P < 0.05; **P < 0.01; ****P < 0.0001. NS, not significant.
Figure 7. Functional and transcriptional characteristics of anergic CD4⁺ T cells in competitive chimeras

A. Splenocytes from DKO:WT = 1:5 chimera were stimulated with anti-CD3 for 30 seconds followed by secondary crosslinking antibody for 2 minutes, or alternatively with PMA for 2 minutes. Cells were fixed, permeabilized, and then stained to detect surface markers, FOXP3, and pErk. Representative histograms showing intra-cellular pErk expression in non-anergic (CD73loFR4lo; NA), int-anergic (CD73intFR4int; IA) or anergic (CD73hiFR4hi; A) among naive (CD44loCD62Lhi) or memory (CD44hiCD62Llo) CD4⁺ T cells gated as Supp Fig. 7A. Dashed line shows the threshold of positive gate. Plots are representative of n = 6 mice.

B. Quantification of %pErk⁺ as in A above (n = 3 biological replicates, representative of n = 2 independent experiments from one chimera setup). Graphs depict mean +/- SEM. Statistical significance was assessed by two-tailed unpaired Student’s t-test with the Holm–Šidák method. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

C, D. Naive or anergic CD4⁺ T cells from CD45.1 (WT) or CD45.2 (DKO) cells gated as in Supp Fig. 7E were sorted directly into RLT buffer for RNA sequencing. ClustVis heatmaps depict expression of selected genes associated with anergy (C) or Treg (D).

E, F. GSEA plots for the genes downregulated (E) or upregulated (F) by Nr4a1 (Nature. 2019;567(7749):525-9.) against differentially expressed genes (DEGs) in DKO and WT anergic cells. DEGs were defined as genes upregulated in DKO compared to WT anergic cells with p < 0.05. NES = normalized enrichment score, FDR = false discovery rate.

G. Heatmap shows expression of selected inflammatory mediators.
Figure 8. Transcriptional targets of NR4A family in acutely stimulated naive CD4$^+$ T cells

A. ClustVis heatmap shows overlap primary response genes (PRGs) and differentially expressed genes (DEGs) in TCR-stimulated naive CD4$^+$ T cells. PRGs defined as genes upregulated in stimulated WT naive CD4$^+$ T cells relative to ex vivo with FDR < 0.05, log CPM > 1, and log2 fold change > 1.5. DEGs were defined as in Fig. 7.

B, C. GSEA plots for the genes downregulated (B) or upregulated (C) by Nr4a1 (Nature. 2019;567(7749):525-9.) against DEGs in DKO and WT stimulated naive CD4$^+$ T cells. NES = normalized enrichment score, FDR = false discovery rate.
**Figure 9.** NR4A family negatively regulates IL-2 production in CD4⁺ T cells

A. CD4⁺ T cells were isolated by negative selection from lymph nodes and cultured in plates coated with indicated dose of anti-CD3 + anti-CD28 for 24 h (left) or 48 h (right). IL-2 concentration in supernatant was measured with ELISA (n = 3 biological replicates).

B. Lymph node cells from 10 weeks post-transplant DKO:WT = 1:1 chimera were cultured in plates coated with indicated doses of anti-CD3 for 20 h. Then cells were re-stimulated with PMA, ionomycin and brefeldin for additional 4 h. Representative histograms of 3 mice showing intracellular IL-2 in CD4⁺ cells of each donor genotype.

C. Quantification of %IL-2⁺ as described for C above (n = 3 biological replicates from one chimera setup).

D. Transcripts per million (TPM) of Il2 detected with RNA-sequencing in WT and DKO cells sorted as described.

Graphs depict mean +/- SEM. Statistical significance was assessed by two-way ANOVA with Tukey’s test (A), two-tailed unpaired Student’s t-test with the Holm–Šidák method (C), or a paired differential expression analysis with EdgeR comparing samples from the same chimeras (D). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. NS, not significant.
Figure 10. Restoring Treg compartment in competitive chimeras alters autoantibody repertoire but does not restore tolerance

A-D. Anti-nuclear antibody (ANA) immunofluorescence images. 1:40 diluted serum of indicated mice were applied to Hep-2 substrate slides, washed, and stained with FITC-anti-mouse IgG. Images are representative of biological replicates as quantified below in E, G.

E. Graphs depict frequency of negative, nuclear, or nuclear+cytoplasmic Hep-2 cell staining patterns in WT:WT = 1:1 chimera, DKO:WT = 1:1 chimera and gDKO (1:40 dilution). Data includes analysis of serum from 2 sets of independently generated chimeras 6-12 w post-transplant. Statistical significance was assessed by Fisher’s exact test. ****P < 0.0001.

F. Quantification of anti-dsDNA antibody from n=9 WT:WT 1:1 chimeras and n=17 DKO:WT 1:1 chimeras determined by ELISA, pooled from 2 sets of individually generated chimeras. Statistical significance was assessed by two-tailed unpaired Student’s t-test. **P < 0.01.

G. ANA titer determined with serial 2-fold dilution of serum from chimeras at indicated time points post-transplant stained as in A-D.
Supplementary Figure 1. Supporting data for Figure 1
A. Expression of Nr4a family mRNA in T cell subsets quantified via RNA-seq. Data are exported from Immgen database (http://rstats.immgen.org/Skyline/skyline.html).
B. C. Quantification of viable thymocyte (B) and splenocyte (C) cell number from WT, Nr4a3–/–, Nr4a1–/– and Nr4a1–/– Nr4a3–/– (gDKO) mice. (Data in C-G include n ≥ 5 biological replicates/genotype, 3 to 4-week-old gDKO and 5 to 6-week-old mice with the other genotypes were analyzed).
D. Representative flow plots showing thymic Treg gate in WT, Nr4a3–/–, Nr4a1–/– and Nr4a1–/– Nr4a3–/– (gDKO) mice, as determined by FOXP3 and CD25 expression. Plots are representative of at least 5 mice/genotype.
E, F. Quantification of cell number (E) and frequency (F) of thymic CD4SP CD25+FOXP3+ cells as gated in (D).
G. Quantification of splenic CD4+CD25+FOXP3+ cell number as gated in Fig. 1B (n ≥ 5 biological replicates, 3 to 4-week-old gDKO and 5 to 6-week-old mice with the other genotypes were analyzed).
H. I. Ratio of CD45.2 to CD45.1/2 for Treg, CD25+FOXP3+ cells and CD25+FOXP3+ cells among thymic CD4SP (H) or splenic CD4+ (I) in DKO:WT = 1:5 chimera (n = 3 biological replicates from one chimera setup). Ratios were normalized to DP thymocytes.
J, K. Quantification of thymic (J) and splenic (K) Treg cell number in WT:WT = 1:1, DKO:WT = 1:1 and 1:5 chimera at 6 weeks post-transplant (n = 3 biological replicates pooled from 2 sets of individually generated chimeras).
L-O. Quantification of FOXP3 (L, M) or CD25 (N, O) expression (MFI) on splenic Treg cells of each donor genotype from WT:WT = 1:1 chimeras (L, N) and DKO:WT = 1:1 (M, O) determined via flow staining. Lines connect donor genotypes within an individual chimera (n = at least 6 biological replicates from one chimera setup). Graphs depict mean +/- SEM. Statistical significance was assessed by one-way ANOVA with Tukey’s test (B, C, E-K) or two-tailed paired Student’s t-test (L-O). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. NS, not significant.
A. Quantification of thymocyte cell number in WT:WT = 1:1, DKO:WT = 1:1 and 1:5 chimera at indicated time points post-transplant (n ≥ 3 biological replicates pooled from 3 sets of individually generated chimeras).

B. Representative flow plots show gating strategy to identify pre- and post- b selection subsets DN3a/b in 1:1 DKO:WT chimera. Dump staining includes CD4, CD8, CD3, CD19, y6CR, NK1.1, pNK, CD11b, Gr1, and CD11c. Plots are representative of 4 chimeras.

C. Representative flow plots show gating strategy to detect pre- and post-positive selection DP subsets in DKO:WT = 1:5 chimera. Plots are representative of 4 chimeras.

D. Ratio of CD45.2 to CD45.1/2 of pre-selection, post-selection 1 and post-selection 2 DP thymocytes (as gated in C) in chimeras, normalized to 3 chimeras.

E. F. Thymocytes from CD45.2 WT, Nr4a3−/− and Nr4a1−/− were mixed with CD45.1 WT thymocytes in 1:1 ratio. Cells were co-cultured, stimulated and stained as described for Figure 2F, I. Quantification of frequency of aCasp3+ cells among DP (E) or CD4SP (F) with indicated dose of anti-CD3 (n = 3 biological replicates). Error bars indicate SEM.

G. Ratio of CD45.2 to CD45.1/2 thymic subsets in WT:WT = 1:1 chimera and Nr4a3−/−:WT = 1:1 chimera, normalized to DP thymocytes (n = 2 biological replicates from one chimera setup). Error bars indicate SEM.

Supplementary Figure 2. Supporting data for Figure 2

Graphs depict mean ± SEM. Statistical significance was assessed by two-way ANOVA with Tukey’s test (A, D) or two-tailed unpaired Student’s t-test with the Holm–Šidák method (E, F). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. NS, not significant.
Supplementary Figure 3. Expansion of myeloid cells in spleen and bone marrow of gDKO mice

A-C. Splenocytes (A) and BM cells (C) from WT and gDKO mice, as well as splenocytes from DKO:WT = 1:1 chimera (B) were stained as described for Supplementary Figure 3. Expansion of myeloid cells in spleen and bone marrow of gDKO mice

**P < 0.01; ***P < 0.001; ****P < 0.0001. NS, not significant.

Graphs depict mean +/- SEM. Statistical significance was assessed by one-way ANOVA with Tukey’s test (D, F) or two-tailed unpaired Student’s t-test (E).

**P < 0.01; ***P < 0.001; ****P < 0.0001. NS, not significant.
Supplementary Figure 4. Supporting data for Figure 4
A. Representative flow plots show FAS^+ GL7^+ GC B cells from WT and gDKO mice as described for Figure 4D. Plots are representative of 6 mice.
B. Quantification of frequency of GC B cells, as gated in (A) above, among total B cells from WT, Nr4a3^−/−, Nr4a1^−/− and gDKO mice (n ≥ 5 biological replicates).
C. Quantification of CD86 MFI on splenic B cells from WT, Nr4a3^−/−, Nr4a1^−/− and gDKO mice (n ≥ 4 biological replicates).
D. Quantification of FAS^+ cells among B220^+ cells in spleen from WT, Nr4a3^−/−, Nr4a1^−/− and gDKO mice (n ≥ 5 biological replicates).
E. Representative flow plots show CD138^+ cells in splenocytes from WT and gDKO mice. Plots are representative of 4 mice.
F. Quantification of ratio of CD138^+ to B220^+ cells, as gated in (E) above, from WT, Nr4a3^−/−, Nr4a1^−/− and gDKO mice (n ≥ 4 biological replicates).
G. Ratio of CD45.2 to CD45.1/2 cells in spleen from WT and gDKO mice (n ≥ 4 biological replicates).

Supplementary Figure 4. Supporting data for Figure 4
A. Representative flow plots show FAS^+ GL7^+ GC B cells from WT and gDKO mice as described for Figure 4D. Plots are representative of 6 mice.
B. Quantification of frequency of GC B cells, as gated in (A) above, among total B cells from WT, Nr4a3^−/−, Nr4a1^−/− and gDKO mice (n ≥ 5 biological replicates).
C. Quantification of CD86 MFI on splenic B cells from WT, Nr4a3^−/−, Nr4a1^−/− and gDKO mice (n ≥ 4 biological replicates).
D. Quantification of FAS^+ cells among B220^+ cells in spleen from WT, Nr4a3^−/−, Nr4a1^−/− and gDKO mice (n ≥ 5 biological replicates).
E. Representative flow plots show CD138^+ cells in splenocytes from WT and gDKO mice. Plots are representative of 4 mice.
F. Quantification of ratio of CD138^+ to B220^+ cells, as gated in (E) above, from WT, Nr4a3^−/−, Nr4a1^−/− and gDKO mice (n ≥ 4 biological replicates).
G. The ratio of CD45.2 to CD45.1/2 for transitional1 (T1), T2/3, follicular (Fo) and marginal zone (MZ) B cell subsets (gated on the basis of B220, CD21, CD23, and CD93) from WT:WT and DKO:WT = 1:1 chimera, normalized to T1 subset (n = 6 biological replicates from one chimera setup).
H, I. Quantification of CD86 (H) and MHC II (I) MFI on splenic B cells of each donor genotype in in WT:WT and DKO:WT = 1:1 chimera (n = 3 biological replicates from one chimera setup).
J. Representative flow plots showing FAS^+ GL7^+ GC B cells in spleen from mb1-cre:WT = 1:1 chimera and mb1-cre Nr4a1^−/− Nr4a3^−/− (cDKO):WT = 1:1 chimera on the left. Right-hand plots depict CD45.2 cDKO donor and CD45.1/2 WT donor populations. Plots are representative of n = 4 biological replicates.
K. Quantification of frequency of GC B cells among total B cells as described for J above.
L. Ratio of CD45.2 to CD45.1/2 GC B cells in spleen from chimeras described in (J) above, normalized to B220^+ IgD^− cells (data in K, L represent n = 4 biological replicates from n = 2 independent experiments with one chimera setup).

Graphs depict mean +/- SEM. Statistical significance was assessed by Brown-Forsythe ANOVA (B, F), one-way ANOVA with Tukey’s test (C, D) or two-tailed unpaired Student’s t-test with (G, H, I) or without (K, L) the Holm–Šidak method. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. NS, not significant.
Supplementary Figure 5. Supporting data for Figure 5
A, B. Quantification of frequency of CD8- naive CD44hiCD62Lhi (A) and CD44hiCD62Llo (B) cells from WT, Nr4a3−/−, Nr4a1−/− and gDKO mice as gated in Fig. 5A (n ≥ 5 biological replicates, 3 to 4-week-old gDKO and 5 to 6-week-old mice with the other genotypes were analyzed).
C. Splenocytes from DKO:WT = 1:5 chimera was stained to detect PD-1 expression on naive CD44
hi and on CD44
lo CD8+ T cells.
D. Quantification of %PD-1+ of naive and CD44
hi CD8+ cells as gated in C above. Lines connect donor genotypes within an individual chimera (n = 6 biological replicates from one chimera setup).
E. Splenocytes from CD8-cre, Nr4a3−/−, CD8-cre Nr4a1−/− and CD8-cre Nr4a1−/− Nr4a3−/− (cDKO) mice were stained as described for Fig. 5A to detect CD8+ T cell subsets. Plots are representative of 3 mice and correspond to quantification in Fig. 5H.
Graphs depict mean ± SEM. Statistical significance was assessed by one-way ANOVA with Tukey’s test (A, B) or two-tailed unpaired Student’s t-test with the Holm–Šidák method (D). **P < 0.01; ****P < 0.0001. NS, not significant.
Supplementary Figure 6. Supporting data for Figure 6

A. Splenocytes from 10 weeks post-transplant WT:WT = 1:1 and DKO:WT = 1:1 chimeras were stained as described for Fig. 6D to identify CD73^{FR4⁺} (anergic) T cells. Plots are representative of 3 mice.

B. Splenocytes from WT, Nr4a3^{−/−} and Nr4a1^{−/−} mice were stained as described for Fig. 6D. Plots are representative of 3 mice.

C, D, F, G. Quantification of frequency (C, D) and cell number (F, G) of CD73^{FR4⁺} T cells among naive (C, F) or memory (D, G) CD4⁺ cells in WT:WT = 1:1 chimera and DKO:WT = 1:1 chimera at indicated time points post-transplant (n ≥ 3 biological replicates from one chimera setup).

E. H. Quantification of frequency (E) and cell number (H) of CD73^{FR4⁺} T cells among total CD4⁺FOXP3⁺ T cells from WT, Nr4a3^{−/−} and Nr4a1^{−/−} mice (n = 3 biological replicates).

Graphs depict mean ± SEM. Statistical significance was assessed by two-tailed unpaired Student’s t-test with the Holm–Šidák method (C, D, F, G) or one-way ANOVA with Dunnett’s test (E, H). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. NS, not significant.
 Supplementary Figure 7. Supporting data for Figure 7
A. Gating strategy corresponding to Fig. 7A to identify splenic CD4+ T cell sub-populations for phosflow assay. Plots are representative of 3 mice.
B, C. Splenocytes from DKO:WT = 1:5 chimera were stimulated and stained as described in Fig. 7A. (B) Representative plots show naive and CD44+CD8- T cell gates. (C) Representative histograms show intracellular pErk expression in naive and CD44+CD8- T cells. Vertical dashed line shows the threshold of positive gate. Plots are representative of 6 mice.
D. Quantification of %pErk+ as described in C above (n = 3 biological replicates, representative of n = 2 independent experiments from one chimera setup).
E. Gating strategy for sorting naive and anergic CD4+ T cells from competitive chimeras. Splenocytes and lymph node cells from DKO:WT = 1:5 chimera were pooled, and CD4+ T cells were negatively isolated with MACS. Enriched CD4+ T cells were stained and pre-gated on live CD4+CD45.1 (WT) or live CD4+CD45.2 (DKO). CD25-CD44+CD62L+CD73- FR4-cells were sorted as “naive”, and CD25-CD44+CD62L+CD73- FR4+ cells were sorted as “anergic”.
F. Heatmap showing expression levels of anergy-related genes in each subset detected with RNA-seq as described in Fig. 7. Anergy-associated genes were identified as genes upregulated in WT anergic cells relative to WT naive cells identified with FDR < 0.05, CPM > 2, and fold change > 4.
G. Principal component analysis (PCA) plot of sorted cells assessed for the expression of anergy-related genes as described for F above. F, G, H, I. Splenocytes from DKO:WT = 1:5 chimera 12 (I) or 13 weeks (H) post-transplant were stained to detect PD-1 expression on naive (FOX3-CD44+CD62L+CD73+ FR4+) and anergic (FOX3-CD44+CD62L+CD73+FR4-)) CD4+ cells of each donor genotype. Shown are quantification of %PD-1+ (H) or MFI of LAG3 (I) of naive and memory anergic. Lines connect donor genotypes within an individual chimera (n = 3 biological replicates from one chimera setup). Graphs depict mean ± SEM. Statistical significance was assessed by two-tailed unpaired (D) or paired (H, I) Student’s t-test with the Holm–Šidák method. *P < 0.05; **P < 0.01; ***P < 0.0001; NS, not significant.
A. Heatmap showing expression levels of all primary response genes (PRGs) in sorted naive CD4+ T cells with or without TCR stimulation in vitro. PRGs were defined as described in Fig. 8A.

B. Principal component analysis (PCA) plot of naive CD4+ T cells with or without TCR stimulation in vitro assessed for the expression of PRGs as described for A above. Heatmap and PCA plot were generated using the ClustVis online tool (https://biit.cs.ut.ee/clustvis/).

C. Lymph node cells from DKO:WT = 1:1 chimera were cultured with 0.1 µg/ml of plate-bound anti-CD3 and treated as described for Fig. 8E. Cell surface was stained with CD4, CD8, CD45.1 and CD45.2, followed by permeabilization and intracellular staining for FOXP3 and IL-2. Shown is a representative histogram above. Heatmap and PCA plot were generated using the ClustVis online tool (https://biit.cs.ut.ee/clustvis/).

D. Lymph node cells from WT, Nr4a3−/− and Nr4a1−/− mice were mixed with CD45.1 lymph node cells, then cultured with 0.1 µg/ml of plate-bound anti-CD3 and stained as described for Fig. 8E. Plots are representative of 3 mice from one chimera setup.

E. Lymph node cells from 10 weeks post-transplant DKO:WT = 1:1 chimera were stimulated and stained as described in Fig. 8E. (E) Representative histograms show intracellular IL-2 in CD8+ T cells of each genotype. (F) Quantification of %IL-2+ in CD8+ T cells (data in E, F represent n = 3 biological replicates from one chimera setup). Graphs depict mean +/- SEM. Statistical significance was assessed by two-tailed unpaired Student’s t-test with the Holm–Šídák method. ****P < 0.0001.

F. Lymph node cells from CD8-cre, Nr4a3−/−, CD8-cre Nr4a1−/− or CD8-cre Nr4a1−/− Nr4a3−/− (cDKO) were mixed with CD45.1 lymph node cells, cultured with 1 µg/ml of plate-bound anti-CD3, and then treated and stained as described for Fig. 8E. Representative histograms show intracellular IL-2 in CD8+ T cells of each genotype. Plots are representative of 2 independent experiments.
Supplementary Figure 9. Supporting data for Figure 9

A. Splenocytes from DKO:WT = 1:5 chimera were harvested and stained with CD4, CD25, CD44, CD62L, CXCR5 and PD-1, then permeabilized and stained with FOXP3. Representative plots show FOXP3+ follicular helper T cells (Tfh) and FOXP3+ follicular regulatory T cells (Tfr) within CD4+CD44hiCD62LloCXCR5hiPD-1+ gate. Naive CD4+ T cell gate is shown for reference to define Tfh/Tfr gate. Donor genotype gates among each population are shown.

B. Ratio of CD45.2 to CD45.1/2 for Tfh or Tfr, normalized to naive CD4+ as gated in A above (n = 3 biological replicates from one chimera setup). Statistical significance was assessed by two-tailed unpaired Student’s t-test. **P < 0.01.

C, D, E, G, H. Anti-nuclear antibody (ANA) immunofluorescence images as described for Fig. 9A-D. Serum was collected from 12-week-old Nr4a1−/− (C), Nr4a3−/− (D) mice, from DKO:WT = 1:5 chimera at 6, 10, 12, or 14 weeks post-transplant (E), and from mb1-cre Nr4a1fl/fl Nr4a3−/− (mb1cre-cDKO) (G) or mb1-cre (H) non-competitive chimeras 40 weeks post-transplant. Images are representative of n = 3 (mb1-cre chimera and DKO:WT = 1:5 chimera), n = 5 (mb1-cre cDKO chimera) or n = 10 (Nr4a1−/− and Nr4a3−/) biological replicates.

F. Graph depict frequency of Hep-2 staining patterns in DKO:WT = 1:5 chimera from one chimera setup.
Supplementary Figure 10. Model. Roles for NR4A family in T cell tolerance and immune homeostasis

A. In WT, central and peripheral tolerance are intact. Nr4a1 and Nr4a3 mediate thymic negative selection and Treg homeostasis. Nr4a1 and Nr4a3 also play a role on induction and maintenance of anergy in periphery.

B. In Nr4a1−/− Nr4a3−/− (gDKO) mice, both thymic negative selection and Treg homeostasis are impaired, and highly autoreactive T cells escape to periphery. Autoreactive CD4+ T cells are activated in part because of Treg deficiency. Polyclonal B cells are activated by autoreactive T cells and produce autoantibody against a broad spectrum of self-antigens as detected by both cytoplasmic and nuclear Hep-2 staining pattern.

C. Treg compartment is reconstituted by WT donor BM in DKO:WT competitive chimeras. However, negative selection of self-reactive DKO thymocytes is still impaired. Self-reactive DKO T cells that escaped negative selection encounter self-antigens in the periphery, acquire features of anergy including expression of CD73 and FR4, yet exhibit a defect in peripheral tolerance. WT-origin Treg and cell-intrinsic peripheral tolerance mechanisms are insufficient to completely suppress autoreactive DKO T cells, which in turn drive anti-nuclear autoantibody production. Although Treg compartment restores some immune homeostasis (and tolerance to cytosolic antigens as detected by Hep-2 staining), autoimmunity is not suppressed due to role of NR4A Family in other T cell-intrinsic tolerance mechanisms.