NR4A nuclear receptors restrain B cell responses to antigen when second signals are absent or limiting

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Antigen stimulation (signal 1) triggers B cell proliferation and primes B cells to recruit, engage and respond to T cell help (signal 2). Failure to receive signal 2 within a defined time window results in B cell apoptosis, yet the mechanisms that enforce dependence on co-stimulation are incompletely understood. Nr4a1-3 encode a small family of orphan nuclear receptors that are rapidly induced by B cell antigen receptor stimulation. Here, we show that Nr4a1 and Nr4a3 play partially redundant roles to restrain B cell responses to antigen in the absence of co-stimulation and do so, in part, by repressing the expression of BATF and, consequently, MYC. The NR4A family also restrains B cell access to T cell help by repressing expression of the T cell chemokines CCL3 and CCL4, as well as CD86 and ICAM1. Such NR4A-mediated regulation plays a role specifically under conditions of competition for limiting T cell help.

B cells are unable to mount productive immune responses if they encounter self-antigen (signal 1) in the absence of T cell co-stimulation (signal 2) and this serves to enforce self-tolerance1. Although antigen recognition is not sufficient, it is nevertheless essential for B cells to recruit, engage and respond to T cell help2-5. In addition to driving cell cycle entry and the metabolic remodeling required to sustain an initial round of proliferation, antigen modulates the expression of chemokines and chemokine receptors that position antigen-activated B cells in proximity to T cells in secondary lymphoid organs4,5. Antigen stimulation also upregulates cell surface molecules required for engagement of T cell help, such as intercellular adhesion molecule 1 (ICAM1) and CD86, and primes B cells to respond to CD40 ligand (CD40L) and cytokines supplied by T cells, in part by initiating transcription of Myc6-8. However, if B cells fail to recruit T cell help within a restricted window of time, they trigger apoptosis, become anergic or revert to a naive-like state, depending on the strength and duration of antigen stimulation4,5. Nevertheless, the mechanisms that normally restrain antigen-activated B cells and enforce their dependence upon co-stimulation are incompletely understood. Here, we identify a role for the nuclear receptor 4A (NR4A) family in this process.

Nr4a1-3 encode a small family of orphan nuclear receptors (NUR77, nuclear receptor related-1 protein (NURR1) and neuron-derived orphan receptor 1 (NOR-1)) that are induced by antigen stimulation in lymphocytes and are thought to function as ligand-independent, constitutively active transcription factors5. In addition, NR4A family members trigger apoptosis via a cytosolic interaction with B cell lymphoma 2 (BCL-2)6-8. Structural homology and an overlapping expression pattern raise the possibility of functional redundancy among Nr4a gene products6. Indeed, the Nr4a genes are highly upregulated in thymocytes undergoing negative selection, in regulatory T cells (Treg cells) and in anergic or exhausted T cells, where they play a collectively tolerogenic role, yet loss of multiple family members is necessary to fully reveal these functions6-9. Similarly, germline deletion of both Nr4a1 and Nr4a3 in mice (but neither one alone) leads to rapid development of a severe myeloproliferative disorder10.

We previously showed that Nr4a1 expression scales with the extent of antigen stimulation in vitro and in vivo, marks naturally occurring self-reactive B cells10-13 and imposes a novel layer of B cell tolerance by mediating competitive elimination of self-reactive B cells13. However, the function of Nr4a genes in response to acute B cell antigen receptor (BCR) stimulation is unknown, and redundancy among the family members has never been explored in B cells.

Here, we used germline and conditional mouse models to show that NUR77/Nr4a1 and NOR-1/Nr4a3 restrain B cells that receive signal 1 (antigen) in the absence of signal 2 (co-stimulation). In contrast, receipt of co-stimulatory signals bypasses inhibition by the NR4A family. Through unbiased gene expression profiling, we identified a novel set of transcriptional targets of the NR4A family that are enriched for BCR-induced primary response genes, including basic leucine zipper ATF-like transcription factor (BATF), which in turn regulates MYC and B cell proliferation. Unexpectedly, we also identified a role for the NR4A family

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in restraining B cell access to T cell help by repressing expression of the T cell chemokines C–C motif chemokine ligand 3 (CCL3) and CCL4, as well as CD86 and ICAM1, and showed that this regulation is relevant under conditions of B cell competition for limiting amounts of T cell help.

**Results**

**NUR77/Nr4a1 expression scales with BCR stimulation.** Nr4a1–3 are rapidly but transiently induced by BCR stimulation (Fig. 1a–c). Nr4a1 is the most highly expressed family member in B cells with or without BCR stimulation; Nr4a3 transcript is approximately sixfold less abundant than Nr4a1; and Nr4a2 transcript is minimally detectable even after BCR stimulation (fragments per kilobase of transcript per million mapped reads (FPKM) <10) (Fig. 1d). We previously characterized a bacterial artificial chromosome (BAC) transgenic (Tg) reporter (NUR77–EGFP) in which enhanced green fluorescent protein (EGFP) is under the control of the regulatory region of Nr4a1 but does not perturb endogenous Nr4a1 expression (Fig. 1e; www.gensat.org)17. As with endogenous Nr4a1 transcript, endogenous NUR77 protein also exhibited rapid induction.
and a relatively short half-life, peaking between 2 and 4 h after BCR stimulation (Fig. 1f)\(^6\). Although the induction of NUR77–EGFP mirrors that of endogenous NUR77, EGFP protein has a relatively long half-life in vivo (approximately 20–24 h), and consequently accumulates over time (Fig. 1g).

To assess the regulation of NUR77 by bona fide antigen, we stimulated IgH ECL BCR Tg reporter B cells with the cognate model antigen hen egg lysozyme (HEL) (affinity = 2 x 10\(^{9}\) M\(^{-1}\)), as well as variants with much lower affinity (HEL 2x (R73E and D101R; affinity = 8 x 10\(^{10}\) M\(^{-1}\)) and HEL 3x (R73E, D101R and R21Q; affinity = 1.5 x 10\(^{10}\) M\(^{-1}\)))\(^9\). Induction of NUR77–EGFP and endogenous NUR77 scales not only with concentration, but also with affinity of antigen (Fig. 1h and Extended Data Fig. 1a,b).

To probe reporter induction in vivo, we took advantage of the B1-8i BCR model system in which the VH186.2 heavy chain is knocked into the heavy chain locus, and recognizes the hapten 4-hydroxy-3-nitrophenylacetyl (NP) when paired with endogenous \(\lambda\)1 light chains\(^6\). After adoptive transfer of vital dye-loaded B1-8i reporter splenocytes, hosts were immunized with NP-conjugated proteins. NP-binding \(\lambda\)-1 donor B cells robustly upregulated EGFP and diluted vital dye after 72 h, while non-NP-binding \(\lambda\)-1 B cells did not (Fig. 1i–k). These data confirm that EGFP is upregulated in antigen-specific B cells in response to immunization in vivo\(^6\).

NUR77/Nr4a1 restrains expansion of BCR-stimulated B cells. Since Nr4a1 is the most highly expressed family member in B cells, we used Nr4a1\(^{−/−}\) mice to dissect the role of the NR4A family in acutely antigen-activated B cells\(^6\). We co-cultured either Nr4a1\(^{−/−}\) or Nr4a1\(^{−/+}\) CD45.2\(^{−}\) lymphocytes with congenically marked wild-type CD45.1\(^{−}\) lymphocytes and anti-immunoglobulin M (anti-IgM). After 72 h Nr4a1-deficient B cells exhibited a competitive advantage in the presence (but not absence) of BCR stimulation and this was not attributable to CD45 allotype (Fig. 2a,b)\(^6\). Early biochemical events triggered by BCR ligation, such as intracellular calcium entry and activation of the phosphoinositide 3-kinase and extracellular-signal-regulated kinase (ERK) pathways, were unchanged in Nr4a1\(^{−/−}\) B cells, suggesting that altered BCR signal transduction does not account for this advantage (Extended Data Fig. 1c–f). This competitive advantage was suppressed in the presence of B cell-activating factor (BAFF), strongly suggesting that it specifically reflects a survival advantage (Fig. 2a). Importantly, this advantage is not attributable to differences in BAFF receptor (BAFFR) expression or BAFF sensitivity (Extended Data Figs. 1g–i and 2a,b). Although a competitive survival advantage was not evident until 48 h of co-culture, Nr4a1\(^{−/−}\) B cells exhibited reduced activated caspase 3 expression relative to the wild type by 24 h after BCR stimulation (Fig. 2c and Extended Data Fig. 2c). Both caspase 3 activation and this competitive advantage were suppressed in the presence of BAFF at every time point assayed (Fig. 2d and Extended Data Fig. 2d). These data suggest that NUR77 mediates BCR-induced apoptosis of B cells beginning at early time points, and this translates into a competitive survival advantage at later time points.

We observed that NUR77 also restrains proliferation of BCR-stimulated B cells, even in the presence of BAFF, suggesting that this is independent of apoptosis (Fig. 2e,f). This phenotype was Nr4a1 gene dose dependent and—as predicted for a negative feedback regulator—increasing NUR77 expression reduced the slope of the dose–response curve (Fig. 2f.g and Extended Data Fig. 2e). Importantly, this proliferative advantage was evident in both mixed and unmixed cultures of either purified B cells or total lymphocytes, suggesting that it reflects a cell-intrinsic function for NUR77 in B cells (Fig. 2f.g and Extended Data Fig. 2f–h). In addition, the BCR repertoire did not account for these phenotypes (Extended Data Fig. 2i,j).

To further exclude a B cell-extrinsic contribution of NUR77 to these phenotypes, we generated competitive bone marrow chimeras by reconstituting lethally irradiated hosts with a 1:1 mixture of congenically marked CD45.1\(^{−}\) Nr4a1\(^{−/+}\) and CD45.2\(^{−}\) Nr4a1\(^{−/−}\) bone marrow. Ex vivo cultures of lymphocytes from these chimeras recapitulated the proliferative and survival advantage of Nr4a1\(^{−/−}\) B cells (Fig. 2h)\(^6\).

Finally, we took advantage of a conditional allele of Nr4a1 (distinct from the germline-deficient Nr4a1 allele used above; Fig. 2i) to generate mice in which Nr4a1 was deleted either early or late during B cell development, using mb1-cre or CD21-cre, respectively\(^6,23\). Competitive bone marrow chimeras of mb1-cre N77\(^{−}\) lymphocytes, CD21-cre Nr4a1\(^{−}\) lymphocytes or cre controls (each mixed with congenically marked CD45.1\(^{−}\) lymphocytes) recapitulated the survival and proliferative advantage of Nr4a1-deficient B cells to a comparable extent (Fig. 2i,k and Extended Data Fig. 2k,l). Together, these data reveal a B cell-intrinsic role for NUR77/Nr4a1 in restraining

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**Fig. 2 | NUR77/Nr4a1 promotes BCR-induced cell death and restrains BCR-induced proliferation of B cells in vitro in a cell-intrinsic manner.**

**a,b.** Lymphocytes from either Nr4a1\(^{−}\) or Nr4a1\(^{−/−}\) CD45.2\(^{−}\) mice were mixed in a 1:1 ratio with CD45.1\(^{−}\) lymphocytes, CTV loaded and co-cultured in the presence of the indicated doses of anti-IgM (20 ng ml\(^{-1}\) BAFF) for 72 h. Cells were then stained to detect CD45.1, CD45.2, B220 and CTV via flow cytometry. Displayed are the ratios of CD45.2\(^{−}\) B cells (Nr4a1\(^{−}\) (knockout (KO); a) or Nr4a1\(^{−/+}\) (b)) relative to co-cultured CD45.1\(^{−}\) wild-type B cells, normalized to the input ratio. c,d. Lymphocytes were harvested from CD45.2\(^{−}\) Nr4a1\(^{−/+}\) or Nr4a1\(^{−/−}\) mice, mixed 1:1 with CD45.1\(^{−}\) wild-type lymphocytes and co-cultured with the given doses of anti-IgM (20 ng ml\(^{-1}\) BAFF for either 24 or 48 h. The graphs depict the percentage of B cells expressing activated caspase 3 (aCas3p3), as assessed by intracellular staining via flow cytometry in samples without (c) or with the addition of BAFF (d). e,f. Lymphocytes were cultured as in a and b above. The representative histograms in e show CTV dilution in stimulated (+6.4 µg ml\(^{-1}\) anti-IgM) Nr4a1\(^{−/+}\) (red) and Nr4a1\(^{−/−}\) (blue) B cells. The graph in f depicts the division index of co-cultured Nr4a1\(^{−/+}\) CD45.2\(^{−}\) and Nr4a1\(^{−/−}\) CD45.1\(^{−}\) B cells under all of the conditions assayed. g. Lymphocytes from Nr4a1\(^{−/−}\), Nr4a1\(^{+/−}\) and Nr4a1\(^{−/+}\) mice (littermate progeny of an intercross betweenNr4a1\(^{−/−}\) parents) were co-cultured in vitro with the indicated doses of anti-IgM for 72 h. Cells were then stained to detect B220 and CTV via flow cytometry. The graphs depict the division index of each genotype. h. Competitive bone marrow (BM) chimeras were generated by reconstituting lethally irradiated CD45.1\(^{−}\) wild-type mice with 1:1 mixtures of either Nr4a1\(^{−/+}\) or Nr4a1\(^{−/−}\) CD45.2\(^{−}\) bone marrow mixed with wild-type CD45.1\(^{−}\) bone marrow. At 8 weeks post-reconstitution, lymphocytes were harvested, loaded with CTV and incubated for 72 h with 5 µg ml\(^{-1}\) anti-IgM. CTV dilution in B220\(^{−}\) B cells was determined via flow cytometry and the graph depicts the division index for n = 2 or n = 3 biological replicates. i. Lymphocytes from mb1-cre, Nr4a1\(^{−/−}\) and mb1-cre Nr4a1\(^{−}\) mice were stimulated with PMA and ionomycin for 2 h. Endogenous NUR77 expression was assessed in B220\(^{−}\) B cells and CD20\(^{−}\) T cells by intracellular staining via flow cytometry. The histograms are representative of more than three biological replicates for each condition. j,k. Lymphocytes from mb1-cre, mb1-cre Nr4a1\(^{−}\), CD21-cre and CD21-cre Nr4a1\(^{−}\) mice were mixed in a 1:1 ratio with wild-type CD45.1\(^{−}\) lymphocytes, CTV loaded and co-cultured with the given doses of anti-IgM for 72 h. In j, the ratio of cre\(^{−}\) or cre\(^{−}\)Nr4a1\(^{−}\) CD45.2\(^{−}\) B cells relative to co-cultured CD45.1\(^{−}\) wild-type B cells is displayed, normalized to the input ratio. The graph in k depicts the division index of each genotype. Data depict n = 3 biological replicates for all panels except h, as noted above. Mean ± s.e.m. are displayed for all graphs. Statistical significance was assessed by two-tailed unpaired Student’s t-test with (a–d) or without the Holm–Šidák method (h), or by two-way ANOVA with Tukey’s test (f, g, j and k). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
both B cell survival and proliferation in response to BCR stimulation that is independent of development and repertoire.

**NUR77 only limits B cell expansion in the absence of signal 2.** Next, we determined how co-stimulatory signals regulate NUR77 expression in B cells. Neither BAFF nor interleukin-4 (IL-4) could induce NUR77–EGFP expression, while both CD40 and the Toll-like receptor 4 ligand lipopolysaccharide (LPS) could do so, suggesting that the reporter is sensitive to canonical nuclear factor-κB signaling (Extended Data Figs. 2b and 3a–c). As with BCR stimulation alone, we found that NUR77 expression peaked at 2–4 h, irrespective of co-stimulatory input (Extended Data Fig. 3a–c).

TLR ligands can serve as mitogenic stimuli for B cells either in isolation or together with BCR stimulation. We found no survival or proliferative advantage for Nr4a1−/− B cells at any dose across a very broad titration of either LPS or CpG (Fig. 3a–e and Extended Data Fig. 4a). We further found that BCR-activated Nr4a1−/− B cells lose their advantage with superimposed high doses of LPS or CpG (Fig. 3f–g and Extended Data Fig. 4b,c).

T cells deliver essential co-stimulatory signals to B cells that synergize with antigens to promote B cell survival and proliferation. We found that both anti-CD40 and IL-4, when added to anti-IgM, eliminate the competitive advantage of BCR-activated Nr4a1−/− B cells (Fig. 3f–i). While TLR ligands are usually delivered in...
Fig. 3 | NUR77/Nr4a1 represses survival and proliferation of B cells that receive signal 1 in the absence of signal 2. Lymphocytes from CD45.1+ Nr4a1+/+ and CD45.2+/Nr4a1−/− mice were mixed in a 1:1 ratio and loaded with CTV before co-culture for 72 h in the presence of various stimuli, as described. Cells were then stained to detect CD45.1, CD45.2, B220 and CTV via flow cytometry. a, The histograms depict CTV dilution in B cells stimulated (from left to right) with either 10 μg/ml−1 anti-IgM alone, 5 μg/ml−1 LPS, 0.2 μM CpG or anti-IgM + 20 ng/ml−1 BAFF + 10 ng/ml−1 IL-4, and are representative of more than three biological replicates. b, d, f, h, i. Displayed are the ratios of CD45.2+/− B cells relative to CD45.1+− B cells, normalized to the input ratio, following stimulation with different doses of LPS (b) or CpG (d), different combinations of anti-IgM, anti-CD40 and LPS (f) or different doses of anti-IgM ± IL-4 (h), c, e, g, i. The graphs depict the division indices of CD45.1+/− Nr4a1+/− (red) and CD45.2+/− Nr4a1−/− (blue) B cells (blue), following the same treatments as shown in b, d, f, h and i, respectively. In b–f, doses of 10 μg/ml−1 anti-IgM (b–g), 1 μg/ml−1 LPS and anti-CD40 (f and g) and 10 ng/ml−1 IL-4 (h and i) were used. The data depict n = 3 biological replicates for all panels. Means ± s.e.m. are displayed for all graphs. Statistical significance was assessed by one-way ANOVA with Dunnett’s (b, d, f, h) or Sidák’s test (c, e, g, i) or two-tailed unpaired Student’s t-test with the Holm–Šidák method (c, e, g, i). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

Compensation and redundancy among the NR4A family in B cells. Although NUR77/Nr4a1 plays a non-redundant role in B cells, the NR4A family exhibits functional redundancy in other immune cells, most strikingly in Treg cells13,25. Therefore, we next sought to unmask redundant NR4A functions in B cells. In the absence of NUR77 expression, Nr4a2 and Nr4a3 exhibited modestly enhanced induction in response to BCR stimulation (Fig. 5a and Extended Data Fig. 5a–c). NUR77 may also repress its own expression; we generated Nr4a1−/− NUR77−/−EGFP BAC Tg mice and observed enhanced EGFP transcript and protein induction in response to BCR stimulation in the absence of endogenous NUR77 expression (Extended Data Fig. 5d–e). Importantly, although Nr4a2 was modestly upregulated in Nr4a1−/− B cells, absolute transcript abundance

Conjunction with antigen stimulation (for example, bacteria or virus), B cells typically experience a physiologic time delay in vivo between antigen encounter and recruitment of T cell help. To mimic this delay, we systematically varied the time between initial BCR ligation and the addition of anti-CD40. We found that the addition of signal 2 at early time points eliminated the advantage of Nr4a1−/− B cells, but this advantage persisted if provision of signal 2 was substantially delayed (Extended Data Fig. 4d). These data collectively suggest that NUR77 selectively represses survival and expansion of B cells that receive signal 1 (antigen) alone, and may help to render B cells dependent on rapid receipt of signal 2 within a fixed time window following antigen encounter.

NUR77 limits antibody responses in the absence of co-stimulation. Next, to test in vivo the relevance of these in vitro observations, we probed immune responses to a T cell–independent BCR stimu-

lers induced by NP–LPS or the T cell–dependent immunogen NP–KLH (NP hapten conjugated to keyhole limpet hemocyanin) were comparable irrespective of NUR77 expression (Fig. 4c–e). Affinity maturation in response to NP–KLH was also unaffected (Fig. 4f). These data suggest that, similar to in vitro assays, NUR77 selectively represses B cell responses to antigen in the absence of signal 2 and does so in a B cell-intrinsic manner in vivo (see model; Extended Data Fig. 4f).
Fig. 4 | NUR77/Nr4a1 selectively restrains humoral immune responses to T cell-independent type II immunogens in vivo. a, Nr4a1+/− and Nr4a1−/− mice were immunized intraperitoneally with 100 μg NP–Ficoll and anti-NP IgG3 titters were determined via ELISA at serial time points. The data in a are representative of n = 5 biological replicates and n = 3 independent experiments. b, Mb1-cre and mb1-cre × Nr4a1fl/fl (Nr4a1 cKO) mice were immunized with 100 μg NP–Ficoll and anti-NP IgM titters were determined via ELISA at serial time points. The data in b are representative of n = 5 biological replicates. c,d, Nr4a1+/− and Nr4a1−/− mice were immunized intraperitoneally with 100 μg NP–LPS and anti-NP IgG3 (c) and IgM (d) titters were determined via ELISA at serial time points. The data in c and d are representative of n = 4 biological replicates. e,f, Nr4a1+/− and Nr4a1−/− mice were immunized intraperitoneally with 100 μg NP–KLH, admixed 1:1 with alum, and anti-NP IgG1 titters were determined via ELISA at serial time points. ELISA plates were coated with either NP(29–85)–BSA (e) or NP(1–15)–RSA (f) in order to detect total and high-affinity NP-specific antibodies, respectively. The data in e and f are representative of n = 4 biological replicates and n = 2 independent experiments. Means ± s.e.m. are displayed for all graphs. Statistical significance was assessed by two-tailed unpaired Student’s t-test with the Holm–Šídák method (a–f). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

remained quite low relative to Nr4a3, even after BCR stimulation (Fig. 5a). We therefore hypothesized that loss of Nr4a1 and Nr4a3 gene products (NUR77 and NOR-1, respectively) should eliminate virtually all NR4A function in B cells and reveal any redundancy.

To test this hypothesis, we generated a germline deletion of Nr4a3 using CRISPR-mediated non-homologous end joining on the Nr4a1fl/fl genetic background. Two independent founders were selected for further breeding and study. These lines exhibited 115- and 112-base pair deletions, respectively, that resulted in a frameshift and premature stop codon (Extended Data Fig. 5f). Although Nr4a3 transcript abundance was unaltered and evaded nonsense-mediated decay in Nr4a3−/− B cells (Extended Data Fig. 5g,h), little protein was produced; residual truncated protein expression—detected using a polyclonal antibody raised against amino acids 200–300 of Nr4a3—was minimally inducible and of extremely low expression is unlikely to compensate for the loss of Nr4a1 and Nr4a3 in the B cell compartment (Fig. 5d). Together, these data suggest that our cDKO model eliminates NR4A function in B cells without disrupting B cell development and immune homeostasis.

NUR77/Nr4a1 and Nr4a1/Nr4a3 restrain BCR-induced expansion. We found that Nr4a3−/− B cells also displayed a competitive survival and proliferative advantage relative to co-cultured wild-type B cells in response to BCR ligation, but this was not as pronounced as the advantage enjoyed by Nr4a1−/− B cells in parallel assays (Fig. 5e,f and Extended Data Fig. 7a–c). Next, we took advantage of mb1-cre Nr4a1fl/fl (Nr4a1 cKO forthwith) and Nr4a3−/− mice in order to generate an allelic series of mice with varying numbers of functional Nr4a alleles in the B cell compartment (Fig. 5g–j). We found that BCR-induced survival and proliferation of B cells was enhanced in proportion to the number of deleted Nr4A alleles (Fig. 5g–j and Extended Data Fig. 7d,e).

Because Nr4a3 is deleted in the germine in cDKO mice, we wanted to rule out cell-extrinsic contributions to these phenotypes. To do so, we generated competitive chimeras and showed that cDKO B cells indeed exhibited a cell-intrinsic advantage that was lost with the addition of signal 2 (Extended Data Fig. 7f–k). We conclude that Nr4a1 and Nr4a3 play partially redundant roles in restraining the survival and proliferation of B cells in response to BCR stimulation in vitro.

The NR4A family restrain the expression of BCR-induced target genes. Since proximal BCR signal transduction is unaltered in Nr4a1−/− B cells, we next sought to define transcriptional targets of NUR77 that might account for its negative regulatory role. To
do so, we assessed global transcript abundance via RNA sequencing (RNA-Seq) in Nr4a1−/− and Nr4a1+/+ B cells stimulated with anti-IgM for 2 h. We sought to capture the genes regulated by Nr4a1, as described for Nr4a1, Nr4a2, and Nr4a3 mice, as determined by CD25 expression and intracellular staining for Foxp3 in splenic CD4+ T cells. The plots are representative of n = 5 biological replicates. d, Purified B cells were isolated from whole lymph nodes via MACS purification and stimulated for the indicated times with PMA and ionomycin for 2 h. Subsequently, whole-cell lysates were blotted with antibodies to detect NOR-1, MYC, pERK and GAPDH. The red arrow indicates the presence of low-abundance truncated NOR-1 protein.

A fraction of the naive B cell transcriptome is rapidly upregulated in response to antigen stimulation. Transcripts that were overinduced in Nr4a1−/− B cells (including Nr4a2 and Nr4a3; Fig. 5a) were highly enriched for such BCR-induced PRGs (Fig. 5b). In contrast, this was not the case for genes that were downregulated in Nr4a1−/− B cells (Supplementary Data 1). This suggests that NR77 imposes negative feedback regulation downstream of antigen encounter to restrain the expression of a subset of PRGs, and we focused our attention on these genes.
To further prioritize this list for follow-up, we filtered differentially expressed PRGs on the basis of statistical significance and fold change, which served to identify a limited number of genes, many of which are known to play important roles in mediating humoral immune responses (Fig. 6a)\(^1\)\(^2\)\(^8\)\(^9\)\(^10\). Of these, the most differentially induced genes we identified encoded the chemokines CCL3 and CCL4 (also known as MIP-1x and MIP-1β, respectively) and the transcription factor BATF (Fig. 6c–e)\(^1\)\(^2\)\(^3\)\(^4\). We found that expression of CCL3 and CCL4 transcripts was rapidly and transiently induced in wild-type B cells, but both the peak and duration of transcription were increased markedly in the absence of NUR77 (Fig. 6f,g). This in turn correlated with increased CCL3 and CCL4 chemokine secretion by Nr4a1\(^{−/−}\) B cells after 48 h of in vitro BCR stimulation (Fig. 6h,i). In contrast, Nr4a3\(^{−/−}\) B cells exhibited minimal change in CCL3 and CCL4 transcript induction (Fig. 6j,k).

To cooperatively restrain the expression of a small subset of other PRGs that play important roles in orchestrating humoral immune responses.

### Negative regulation of MYC by NUR77/Nr4a1 via BATF. Not only did Nr4a1\(^{−/−}\) B cells proliferate more in response to BCR stimulation, but cell size was markedly increased relative to the wild type after 24 h of stimulation (Fig. 7a). MYC protein levels have been shown to correlate with and direct cell growth, metabolic reprogramming and the proliferative potential of naive and germinal center B cells in a dose-dependent manner\(^10\)\(^–\)\(^19\). Indeed, we found that MYC protein expression was markedly overinduced in BCR-stimulated Nr4a1\(^{−/−}\) and cDKO (but not Nr4a3\(^{−/−}\)) B cells in a cell-intrinsic manner (Fig. 7b and Extended Data Fig. 9a–c). Moreover, MYC protein was robustly upregulated in both Nr4a1\(^{−/−}\) and Nr4a1\(^{−/−}\) B cells with the addition of IL-4, and this could help account for how the provision of signal 2 might bypass negative regulation by NUR77 (Fig. 7b). Therefore, we hypothesized that negative regulation of MYC contributes to Nr4A-dependent repression of B cell proliferation in response to signal 1 in the absence of signal 2. However, overinduction of Myc transcript in Nr4a1\(^{−/−}\) B cells after BCR stimulation was subtle at early time points, becoming more marked only by 5 h (Fig. 7c). Similarly, MYC protein overinduction in Nr4a1\(^{−/−}\) B cells was only detectable at relatively late time points (24 h) after BCR stimulation (Fig. 7b,d,e). These data suggest that Myc may be an indirect transcriptional target of the NRA4 family.

In contrast with MYC, the activator protein 1 (AP-1) family member BATF is the most robustly upregulated transcription factor in Nr4a1-deficient B cells at early time points (2 h) after BCR stimulation. We identified consensus NRA4-binding motifs in open chromatin regions (that is, putative cis-regulatory elements) 20 kilobases (kb) downstream of Batf in mature follicular B cells (ImmGen assay for transposase-accessible chromatin using sequencing (ATAC-seq) data; Extended Data Fig. 9d), suggesting that Batf may be a direct transcriptional target of the NRA4 family. Since BATF can cooperate with interferon regulatory factor 4 (IRF4) to induce Myc expression\(^10\), and Batf-deficient B cells exhibit defective clonal expansion\(^10\), we hypothesized that NUR77 regulates MYC in part...
via modulation of BATF (see model; Fig. 7f). To test this hypothesis, we sought to rescue BATF overinduction in NUR77-deficient B cells by generating Nr4a1+/− Batf+/− mice. We found, as expected, that hemizygosity for Batf reduced the expression by approximately 50% and resulted in a near-normalization of BATF protein expression in Nr4a1-deficient B cells after BCR stimulation
(Fig. 7g and Extended Data Fig. 9e). As predicted by our model (Fig. 7f), MYC overinduction was partially rescued (Fig. 7h). Across genotypes, we observed a correlation between BATF/MYC expression and B cell proliferation (Fig. 7g–j). These data suggest that NUR77 represses MYC in part via repression of BATF, but other mechanisms may also contribute to enhanced proliferation of Nr4a1−/− B cells.
**Fig. 8** | NUR77/Nr4a1 restrains B cell competition for T cell help under limiting conditions. B cells were purified from splenocytes harvested from N4a1+/−, B1-8i Tg CD45.1/2+ and Nr4a1−/− B1-8i Tg CD45.2+ mice via bench-top negative selection, mixed 1:1 and loaded with CTV. A total of 2 × 10^6 to 3 × 10^6 B cells were then adoptively transferred into OTII splenocytes into either wild-type or CD40L−/− hosts. Host mice were then immunized intraperitoneally 1 d later with 100 µg NP–OVA/alum, followed by spleen harvest on day 4. A schematic of the experimental design is depicted in Extended Data Fig. 10a,b. a, Representative flow plots gated on total donor B cells depicting CTV dilution among NP-binding donor B cells in individual recipients (as a proxy measure of T cell help). Means ± s.e.m. are displayed above. 

b–d. Adoptive transfers into wild-type hosts were performed as described above. Mice that received excess T cell help (OTII = HI) received donor B cells co-transferred with 2 × 10^6 OTII splenocytes. The graphs depict CTV dilution among donor NP-binding B cells (b), expansion of NP-binding donor B cells (c) and the ratio of Nr4a1−/− relative to Nr4a1+/− donor NP-binding B cells normalized to the ratio in unimmunized hosts (d) (n = 5 unimmunized recipients; n = 3 independent experiments). e–g. Adoptive transfers into CD40L−/− hosts were performed as described above. Mice that received limited T cell help (OTII = LO) received donor B cells co-transferred with 5 × 10^5 splenocytes harvested from OTII mice, and mice that received excess T cell help (OTII = HI) received donor B cells co-transferred with 2 × 10^6 splenocytes. The graphs depict CTV dilution among donor NP-binding B cells (e), expansion of NP-binding donor B cells (f) and the ratio of Nr4a1−/− relative to Nr4a1+/− donor NP-binding B cells, normalized to the ratio in unimmunized hosts (g) (n = 5 unimmunized recipients; n = 3 independent experiments). h, Graph depicting the correlation between the ratio of Nr4a1−/− relative to Nr4a1+/− donor NP-binding B cells (as plotted in d and g above) and CTV dilution of NP-binding donor B cells in individual recipients (as a proxy measure of T cell help). Means ± s.e.m. are displayed for all graphs. Statistical significance was assessed by one-way ANOVA with Tukey’s (b–d) or Šidák’s test (e–g), or Pearson’s correlation coefficient (h). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.
NUR77 limits B cell responses when T cell help is limiting. Although we identified a role for the NR4A family in restraining B cell expansion in response to BCR stimulation (signal 1) without co-stimulation (signal 2), the most differentially induced genes in Nr4a1−/− B cells were Ccl3 and Ccl4, which encode chemokines that serve to recruit T cell help via CCR5 (refs. 32,33). In addition, RNA-seq analysis identified additional putative NUR77 target genes that are well recognized to facilitate engagement of T cell help for B cells, including Cd86 and Icam1 (refs. 34). Cd86 protein was over-induced in the absence of Nr4a1 but not Nr4a3, and this was recapitulated in cDKO B cells in a cell-intrinsic manner (Extended Data Fig. 10a,b). Similarly, Icam1 was overinduced in antigen-stimulated Nr4a1−/− B cells (Extended Data Fig. 10c,d). Collectively, these data suggested that the NR4A family may play a role in T cell-dependent immune responses.

Although NUR77/Nr4a1 expression did not influence B cell responses to co-stimulatory signals in vitro and in vivo, we reasoned that access to T cell help (signal 2) in these assays was not limiting. We hypothesized that Nr4a1-deficient B cells might exhibit a competitive advantage in the context of a limiting supply of T cell help. To test this hypothesis, we undertook adoptive transfer of antigen-specific Nr4a1+/+ or Nr4a1−/− B cells harboring the B1-8i heavy chain Tg. By co-transferring varying numbers of ovalbumin (OVA)-specific OTII splenocytes in conjunction with donor B cells into either wild-type or CD40L−/− hosts, we could manipulate the supply of antigen-specific T cell help (Extended Data Fig. 10d,e). As expected, we observed a profound defect in antigen-specific B cell proliferation in CD40L−/− hosts that could be rescued with adoptive transfer of antigen-specific T cells (Fig. 8a).

Although we observed robust vital dye dilution and expansion of adoptively transferred antigen-specific B cells in wild-type hosts after immunization with NP–OVA, this response increased dramatically with the provision of excess T cell help, suggesting that endogenous OVA-specific T cells are limiting (Fig. 8b,c). Indeed, we observed a striking competitive advantage for Nr4a1−/− NP-specific donor B cells in immunized wild-type hosts that was completely lost with the provision of excess T cell help (Fig. 8d). We next sought to reproduce this finding in CD40L−/− mice where endogenous T cell help is defective (Fig. 8a). Upon transfer of either no, limiting or excess numbers of OTII splenocytes into CD40L−/− hosts, the amplitude of donor B cell expansion scaled with the amount of antigen-specific T cell help (Fig. 8e,f). Again, we observed a clear competitive advantage for antigen-specific Nr4a1−/− B cells, specifically under conditions where T cell help was limiting, that was lost with the provision of excess T cell help (Fig. 8g).

Finally, we took advantage of heterogeneity in the amplitude of B cell responses in individual host mice to look for a correlation between the supply of T cell help and any competitive advantage enjoyed by Nr4a1−/− B cells. To do so, we used vital dye dilution of antigen-specific donor B cells in individual hosts as a proxy measure of the supply of T cell help. Indeed, we identified a clear inverse correlation ($r^2=0.7$) between the supply of T cell help and the competitive fitness of antigen-specific Nr4a1−/− donor B cells (Fig. 8h) and Extended Data Fig. 10g). These data suggest that NUR77 mediates a negative feedback loop downstream of antigen stimulation, and may play a role not only in T cell–independent responses, but also in T cell-dependent immune responses, specifically in competitive settings where T cell help is limiting.

Discussion
Co-stimulatory signals must be received within a limited span of time in order to divert antigen-stimulated B cells from apoptosis/anergy, but there is a physiologic delay between the initial antigen encounter by B cells and acquisition of T cell help34. Recent work described gradual accumulation of intracellular calcium in antigen-activated B cells accompanied by progressive mitochondrial dysfunction and loss of glycolytic capacity35. This process may serve as a molecular timer that imposes a ‘point of no return’ after which B cells can no longer be rescued from apoptosis by co-stimulation36. Our data suggest that induction of Nr4a gene expression represents a novel molecular strategy that limits B cell responses to antigen and helps to enforce dependence on co-stimulation. We hypothesize that the balance between NR4A-mediated apoptosis and pro-survival signals generated by BCR ligation may help to modulate the time window within which T cell help must be engaged to avoid a terminal fate. Consistent with such a model, Nr4a1−/− B cells exhibit an advantage relative to wild-type B cells when co-stimulatory signals are delayed.

Since the initial increase in the glycolytic capacity of antigen-activated B cells depends on MYC induction, we propose that the NR4A family may also contribute to a gradual loss in glycolytic capacity and metabolic dysfunction over time by suppressing MYC induction37. Conversely, upregulation of MYC by co-stimulation may help to explain how second signals circumvent the inhibitory effects of the NR4A family. Additional mechanisms may also contribute to this phenomenon; for example, post-translational modifications triggered by antigen and/or co-stimulatory signals could regulate subcellular localization or protein-binding interactions of NR4A family members to influence downstream effector functions such as apoptosis or target gene transcription38–40.

Several NR4A target genes play a specific role in T cell–B cell interactions. Most notably, secretion of the chemokines CCL3 and CCL4 (MIP-1α and MIP-1β) by B cells can induce migration of activated T cells via CCR5 (refs. 41,42). Cd86 is a ligand for C2D8 and has a very well-established role in T cell co-stimulation, while Icam1 and Icam2 facilitate T cell–B cell conjugate formation during T cell–dependent immune responses and are critical for efficient B cell expansion in this context43. C69 functions to promote the retention of activated lymphocytes in secondary lymphoid organs via negative regulation of Sip1, and thereby facilitates cognate T cell–B cell interactions44.

Other important mediators of the humoral immune response are also negatively regulated by the NR4A family. Although the zinc finger transcription factors early growth response 2 (EGR2) and EGR3 have tolerogenic and regulatory functions in the setting of chronic antigen stimulation, like EGR1, they are also important for proliferation in response to acute antigen stimulation45. BATF heterodimerizes with JUN to bind cooperatively with IRF4 at so-called AP-1–IRF4 composite elements (AICEs)46,47, and as a result BATF plays pleiotropic roles as a transcriptional regulator in immune cells47,48,49.

Importantly, we also identified a subset of genes that are downregulated in Nr4a1−/− B cells. Although these targets are not enriched for PRGs, they may nevertheless be functionally relevant. For example, the transcription factor c-MYB is important for B cell development and for BAFF-dependent B cell survival50. It will be important to explore this and other NR4A targets in future studies.

Since NR4A expression scales with the intensity of antigen stimulation, we predict that the NR4A family may disproportionately restrain the most strongly antigen-activated B cell clones in a polyclonal repertoire. Indeed, although B cell clones compete for clonal dominance and limited resources (T cell help) during the primary immune response51, low-affinity B cell clones do enter (and persist in) the germinal center52, suggesting that some physiological mechanism must exist to restrain high-affinity B cell clones from completely monopolizing T cell help53. Future work will test the hypothesis that the NR4A family may serve to limit immunodominance and preserve clonal diversity during evolving humoral immune responses.

Our data suggest that the NR4A family imposes a novel negative feedback loop downstream of antigen stimulation that renders B cells highly dependent on co-stimulatory input, and restrains
strongly activated B cells from monopolarizing limiting quantities of T cell help. Although no endogenous ligand has been identified for the NR4A family, small-molecule agonist and antagonist ligands for NUR77/Nr4a1 have been described[20,21]. One could envision treating B cell-mediated autoimmune disease with a selective NUR77 agonist. Conversely, an antagonist compound could boost T cell–independent B cell responses and serve as a novel universal adjuvant. Our work identifies a molecular mechanism by which both self- and foreign-reactive B cells are regulated and may be therapeutically manipulated.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41590-020-0765-7.

Received: 30 March 2020; Accepted: 17 July 2020; Published online: 31 August 2020

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**Methods**

Mice. NUR77–EGFP and IgHEL (MD4) mice were previously described. Nra4−/− mice were generously shared by P. Chambon at the University of Strasbourg and C. Hedrick at the La Jolla Institute for Immunology. MB1-cre, CD21−/−, CD19−/−, B6.TCRI−−/−, OTII, Cd40l−−/−, Nr4a1−/−, B1−−, Batf−−, C57BL6 and CD45.1−−/− mice were from The Jackson Laboratory. Nra4−/− mice were obtained via electroporation of guide RNA and Cas9 messenger RNA. In brief, Cas9 protein and Nr4a1 mRNA guide RNAs (80−mer) were mixed and electroporated into C57BL6/Nra4−/− zygotes. Nra4−/− exon 3 (containing start ATG) was targeted for deletion. A total of 15 founder lines with the targeted deletion were identified through a screen for PCR amplicon size and confirmed via sequencing of cloned PCR products. Two founder lines (2 and 3) harboring a 115- and 112-base pair deletion. A total of 15 founder lines with the targeted deletion were identified at the University of Michigan. Complete culture media was prepared with RPMI-1640 supplemented with 5% l-glutamine (Corning–Gibco), penicillin-streptomycin (Life Technologies) and 10% heat-inactivated fetal bovine serum (Omega Scientific).

Flow cytometry and data analysis. After staining, cells were analyzed on a Fortessa (Becton Dickinson). Data analysis was performed using Flowjo software (versions 9.9.6 and 10; Tree Star). The proliferative indices ‘division index’ and ‘percentage divided’ were calculated using FlowJo. Statistical analysis and graphs were generated using Prism version 6 (GraphPad Software). The statistical tests used throughout the study are listed at the end of each figure caption. Student's unpaired 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–Sidak method. One- or two-way analysis of variance (ANOVA) with follow-up Tukey’s, Dunnett’s or Sidák tests were performed when more than two groups were compared with one another. Means ± s.e.m. are displayed in all graphs. The statistical analysis of RNA-seq data is described separately below. Throughout the figures, *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001.

Intracellular staining to detect pERK, pS6, NUR77, MYC, BATF, EGR2, IRF4 and activated caspase 3. Either immediately ex vivo or following in vitro stimulation, cells were fixed in a final concentration of 2% paraformaldehyde for 10 min, permeabilized on ice with 100% methanol for 30 min (or at −20°C overnight) and, following washes and rehydration, stained with primary antibody (for surface markers) or lineage markers and secondary antibodies (if needed) for 40 min at 20°C.

FOXP3 staining. FOXP3 staining was performed utilizing a FOXP3/Transcription Factor Staining Buffer Set (ebioscience) in conjunction with an APC anti-FOXP3 antibody (ebioscience), per the manufacturer’s instructions.

Intracellular calcium flux. Cells were loaded with 5 μg ml−1 Indo-1 AM (Life Technologies) and stained with lineage markers for 15 min. Cells were rested at 37°C for 2 min, and Indo-1 fluorescence was measured by fluorescence-activated cell sorting immediately before and after stimulation to determine intracellular calcium.

Live/dead staining. LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen) was used. The reagent was reconstituted (per the manufacturer's instructions) and diluted 1:1,000 in phosphate-buffered saline (PBS), and the cells were stained at a concentration of 2×10^6 cells per 100 μl in ice for 10 min.

Vital dye loading. Cells were loaded with CellTrace Violet (CTV; Invitrogen), per the manufacturer's instructions, except at 5×10^6 cells per ml rather than 1×10^6 cells per ml.

In vitro B cell culture and stimulation. Spleenocytes or lymphocytes were harvested into single-cell suspension, subjected to red cell lysis using ammonium chloride potassium buffer in the case of spleenocytes (± CTV loading, as described above) and plated at a concentration of 2×10^6 cells per 200 μl in round-bottom 96-well plates in complete RPMI-1640 media for 3–7 days. Cells were stimulated with stimuli as described, and unstimulated cells were stained to exclude dead cells, as above, in addition to surface or intracellular markers for analysis by flow cytometry, depending on the assay.

Immunoblot analysis. Thymocytes and purified splenic/lymph node b cells (protocol described below) were harvested from mice and stimulated in complete media ± phorbol myristate acetate (PMA)/ionomycin for 20 h at 37°C. Following stimulation, cells were lysed with 1% NP-40, and centrifuged for 15 min at 20,000g to remove cellular debris. The supernatants were denatured at 95°C for 5 min in sodium dodecyl sulfate sample buffer with 2.5% β-mercaptoethanol. Lysates were run on Tris–Bis (pH 6.8) thick gradient gels (Invitrogen) and transferred to polyvinylidene fluoride membranes with a Mini-PROTEAN Tetra cell (Bio-Rad). Membranes were blocked for 1 h with 3% skimmed milk in Tris-buffered saline with Tween 20 (pH 7.6), then probed with the primary antibodies listed above, overnight at 4°C. The next day, membranes were incubated with HRP-conjugated secondary antibodies. Blots were developed utilizing a chemiluminescent substrate (Western Lightning Plus–ECL; Perkin Elmer) and visualized with a ChemiDoc Touch Imaging system (Bio-Rad).

Immunizations. Mice were immunized via intraperitoneal injection of 200 μl immunogen diluted in PBS. For T cell-dependent immunizations, 100 μg NP(24)–KLH, NP(17)–OVA or NP(29)–KLH (Oligo BioScience Technologies) was diluted in PBS and emulsified in anhydrolgel 1% adjuvant (Accurate Chemical and Scientific Corporation).

**Other antibodies and reagents.** Recombinant HEL, HEL 2x (R73E and D101R) and HEL 3x (R73E, D101R and R21Q) proteins were a gift from W. Cheng at the University of Michigan. Complete culture media was prepared with RPMI-1640 + l-glutamine (Corning–Gibco), penicillin-streptomycin L-glutamine (Life Technologies), 10 mM HEPES buffer (pH 7.2–7.5; Life Technologies), 55 mM β-mercaptoethanol (Gibco), 1 mM sodium pyruvate (Life Technologies), non-essential amino acids (Life Technologies) and 10% heat-inactivated fetal bovine serum (Omega Scientific).

**Live/dead staining.** LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen) was used. The reagent was reconstituted (per the manufacturer’s instructions) and diluted 1:1,000 in phosphate-buffered saline (PBS), and the cells were stained at a concentration of 2×10^6 cells per 100 μl in ice for 10 min.

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For NP-Ficoll immunization, 100 μg NP(53)–Ficoll (Biosearch) was diluted in PBS. For NP–LPS immunization, 100 μg NP(0.6)–LPS (Biosearch) was diluted in PBS. For serum antibody titers, mice were bled before immunization and then serially every 7 d for either 21 or 28 d in total, and titers were
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and injected intravenously with \(10^6\) donor A mixed with \(10^6\) donor B bone marrow. Host mice were irradiated with \(530\) rads and stopped with \(1\) N sulfuric acid. Absorbance was measured at \(450\) nm using a \(5,5'\)-tetramethylbenzidine (Sigma–Aldrich) for high-affinity anti-NP IgG1. 96-well plates (Costar) were coated with \(1\) µg/ml NP(29)–BSA (Biosearch). For statistical robustness, a reference dataset of PRGs was extracted from publicly available RNA-seq data generated following 2 h anti-IgM stimulation of primary mouse B cells (GEO accession number: GSE146747). Statistically significant PRGs (greater than fivefold upregulated by BCR stimulation) were identified using Cufflinks/Cuffdiff suite version 1.3.1 (GSE61608_Differential_Expression_summary.txt).

B cell ATAC-seq analysis. Publicly available follicular mature B cell ATAC-seq data (ImmGen.org) were accessed to identify all consensuses NR4A DNA-binding motifs located within open chromatin regions that were <100 kb from the Batf gene. The closest, most prominent peak was identified ~20 kb downstream of 3' Batf exons, and tracks were displayed using UCSC Genome Browser.

Quantitative PCR (qPCR). Either total lymphocytes or MACs-purified B cells were cultured at \(37^\circ\)C with varying stimuli and harvested into TRIzol (Invitrogen), then stored at \(-80^\circ\)C. RNA was extracted via phenol phase separation. Complementary DNA was prepared with a SuperScript III kit (Invitrogen). qPCR reactions were run on a QuantStudio 12K Flex thermal cycler (ABI) using SYBR Green detection with the primer sets as follows: Batf: forward: 5'-CACAAAGGACCGAAGTGGGCA-3'; reverse: 5'-ACCTGTTGTACCTGGTCGGA-3'; Ccl3: forward: 5'-ACCATGACACTCTGGACAAC-3'; reverse: 5'-CGATGAACTGGCTGAACTCT-3'; Ccl4: forward: 5'-AAACCTTAAACCCGGACAC-3'; reverse: 5'-GAGAGAACAGGACGATTTGG-3'; Nr4a1: forward: 5'-AGCTTCTCCTACATCGGAGAG-3'; reverse: 5'-CCACATTAAACAGGACCAG-3'; Eg2: forward: 5'-TTGACGAGATGAAAGGATTG-3'; reverse: 5'-CAGATGGGAGCCGGAGAATG-3'; Ccl3: forward: 5'-ACAAAGGAGGAGGAGGAGGAG-3'; reverse: 5'-TGACTCTCTTGAGGTTGTTG-3'; Nr4a1: forward: 5'-GCTGTAGCTGGCAAAATTG-3'; reverse: 5'-GGAAACAGGAGCACTGATCT-3'; Nrf2: forward: 5'-TGAATGAGAGAACGCGAAC-3'; reverse: 5'-TGCTGTAATCTCGAGGAGAGA-3'; Nr4a3: forward: 5'-AACAAGGAGGAGCGACTCCACC-3'; reverse: 5'-CTGGTGTCCCTTAAGAATGTC-3'. Data were normalized within each sample to Gapdh and further normalized to the unstimulated ice sample in each graph using the ddCT method.

NANOPAC and bioinformatic. Sample preparation, RNA-seq and data processing. Single-cell suspensions were generated from pooled splenocytes and lymphocytes, as described above. B cells were purified via negative selection using a MACS kit (Miltenyi Biotech), following the manufacturer's instructions. B cells were stimulated with anti-IgM F(ab')2 (10 µg/ml) for 2 h and subsequently pelleted via centrifugation. Supernatant was then removed and the pellet was frozen at \(-80^\circ\)C. Quadruplicate biological replicate samples of stimulated \(N{r}4{a}1^{+/+}\) and \(N{r}4{a}1^{-/-}\) were then sent to the Q3 Solutions commercial laboratory for RNA-seq preparation, sequencing and analysis. In brief, RNA samples were converted into complementary DNA libraries with the Illumina TruSeq Stranded mRNA sample preparation kit, then sequenced on an Illumina sequencing platform. After sequencing, quality control analysis and gene and isoform quantification were performed according to the Q3 Solutions in-house RNAseq pipeline. After quality control analysis, samples were aligned using STAR software version 2.4, and quantification of FPKM was performed using RSEM version 1.2.14. Fastq and RSEM FPKM data are publicly available (GEO accession number: GSE146747).

Statistical analysis for PRG enrichment. To identify antigen-induced B cell PRGs with statistical robustness, a reference dataset of PRGs was extracted from publicly available RNA-seq data generated following 2 h anti-IgM stimulation of primary mouse B cells (GEO accession number: GSE14608). Statistically significant PRGs (greater than fivefold upregulated by BCR stimulation) were identified using Cufflinks/Cuffdiff suite version 1.3.3 (GSE61608_Differential_Expression_summary.txt).

B cell ATAC-seq analysis. Publicly available follicular mature B cell ATAC-seq data (ImmGen.org) were accessed to identify all consensuses NR4A DNA-binding motifs located within open chromatin regions that were <100 kb from the Batf gene. The closest, most prominent peak was identified ~20 kb downstream of 3’ Batf exons, and tracks were displayed using UCSC Genome Browser.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Raw and processed data files for the RNA-seq analyses (corresponding to Fig. 6a,b) have been deposited in the NCBI Gene Expression Omnibus under accession code GSE146747 and are provided in Supplementary Data 1. All other data that support the findings of this study are available from the corresponding author upon request.

Source data are provided with this paper.

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Acknowledgements

We thank our funders: NIAID 5T32AI070334-28 (C.T.), the HHMI Medical Research Fellows program (J.H.), NIAMS R01AR69520 (J.Z.) the Rheumatology Research Foundation Innovative Research Award (J.Z.), and the Uehara Memorial Foundation Research Fellowship (R.H.). A.M. holds a Career Award for Medical Scientists from the Burroughs Wellcome Fund, is an investigator at the Chan Zuckerberg Biohub and is a recipient of The Cancer Research Institute Lloyd J. Old STAR grant. A.M. has received funds from the Innovative Genomics Institute and Parker Institute for Cancer Immunotherapy.

Author contributions

C.T., R.H., J.L.M., V.V., K.H., M.N., J.H., J.F.B. and J.Z. conceived of and designed the experiments. C.T., R.H., J.L.M., V.V., K.H., M.N., J.H., J.F.B., J.G. and C.H. performed the experiments. C.T., R.H., J.L.M., V.V., K.H., M.N., J.H. and J.Z. analyzed the data. J.L.M.,
Z.L. and A.M. generated the Nr4a1−/− mice. C.T. and J.Z. wrote the manuscript. C.T., R.H., J.L.M., M.N., J.F.B. and J.Z. edited the manuscript.

Competing interests
A.M. is a co-founder of Arsenal BioSciences and Spotlight Therapeutics and serves on their boards of directors and scientific advisory boards. A.M. has served as an advisor to Juno Therapeutics, was a member of the scientific advisory board at PACT Pharma and was an advisor to Trizell. A.M. owns stock in Arsenal BioSciences, Spotlight Therapeutics and PACT Pharma. The Marson Laboratory has received sponsored research support from Juno Therapeutics, Epinomics, Sanofi, GlaxoSmithKline, Gilead and Anthem Blue Cross Blue Shield. J.Z. serves as a scientific consultant for Walking Fish Therapeutics.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41590-020-0765-7.
Supplementary information is available for this paper at https://doi.org/10.1038/s41590-020-0765-7.
Correspondence and requests for materials should be addressed to J.Z.
Peer review information Peer reviewer reports are available. L. A. Dempsey was the primary editor on this article and managed its editorial process and peer review in collaboration with the rest of the editorial team.
Reprints and permissions information is available at www.nature.com/reprints.
Extended Data Fig. 1 | NUR77 does not alter proximal BCR signal transduction or BAFFR expression. a, b, NUR77-EGFP lymphocytes were treated as in Fig. 1h except for only 2 h. Endogenous NUR77 (A) and EGFP (B) in B220 + B cells was assessed via flow. c, Splenocytes from Nr4a1+/+ and Nr4a1−/− mice were loaded with Indo-1 dye and stained to identify CD23+B cells. Samples were collected on a flow cytometer for 20 s, and then for 3 m following stimulation with either anti-IgM or anti-IgD. Intracellular calcium is depicted for CD23+B cells. d, e, Splenocytes from Nr4a1+/+ and Nr4a1−/− mice were stimulated with anti-IgM for 5 m, fixed, permeabilized, and then stained for either pErk (B) or pS6 (C) followed by staining to identify CD23+B220+B cells. f, Splenocytes from CD45.1+Nr4a1+/+ and CD45.2+Nr4a1−/− mice were mixed 1:1, and then either stimulated with 10 μg/mL anti-IgM or media alone for 4 h. After stimulus wash-out and 15 m rest, cells were re-stimulated with anti-IgD for 5 m and processed as in B, C to detect intra-cellular pErk. g, h, i, Lymphocytes from CD45.1+Nr4a1+/+ and CD45.2+Nr4a1−/− mice were mixed 1:1 and co-cultured for 72 h with anti-IgM+/− BAFF 20 ng/ml as described. Cells were then stained to detect CD45.1, CD45.2, B220, and BAFFR. G. Plots depict surface expression of BAFFR in B and T cells either ex vivo or after culture +/− anti-IgM. H. Graph depicts BAFF MFI in B220 + B cells. I. Graph depicts ratio of CD45.2+Nr4a1−/− relative to CD45.1+Nr4a1+/+ B220+B cells after 72 h culture normalized to input ratio. Data in C-F reflect N = 2 biological replicates and in G-I reflect N = 3 biological replicates. Mean +/- SEM displayed for all graphs. Statistical significance was assessed with two-way ANOVA with Tukey’s (H); two-tailed unpaired student’s t-test with Holm-Sidak (I). **p < 0.01, ****p < 0.0001.
Extended Data Fig. 2 | NUR77 restrains survival and proliferation of BCR-stimulated B cells in a cell-intrinsic manner. a, Graph depicts CD45.2 + B cell number corresponding to Fig. 2a,b samples. b, Lymphocytes from NUR77-EGFP mice were stimulated with anti-IgM + /- 20 ng/mL BAFF for 24 h. Graph depicts GFP MFI of live B cells. c, d, Graphs depict the ratio of CD45.2 + Nr4a1+/− or Nr4a1−/− B cells relative to co-cultured WT CD45.1 + B cells, normalized to the input ratio, and correspond to Fig. 2. C, D samples. e, Lymphocytes from Nr4a1+/−, Nr4a1+/+ and Nr4a1−/− mice were stimulated with 10 μg/mL anti-IgM for 2 h. Graph depicts MFI of endogenous NUR77 expression in CD23 + B cells. f-h, Either MACS-purified B cells or total lymphocytes from CD45.2 + Nr4a1+/− and CD45.1 + Nr4a1−/− mice were co-cultured with anti-IgM for 72 h. f, Histograms depict CTV dilution after culture with 6.4 μg/ml anti-IgM. g, Graph depicts ratio of Nr4a1−/− CD45.2 + relative to co-cultured WT CD45.1+ purified B cells, normalized to the input ratio. h, Graph depicts division index of co-cultured purified B cells. i-j, Lymphocytes from either Nr4a1+/− or Nr4a1−/− CD45.2 + IgHEL Tg mice were treated as in Fig. 2a. i, Graph depicts ratio of CD45.2 + B cells of each genotype relative to co-cultured CD45.1+ WT B cells, normalized to the input ratio. j, Graph depicts division index of CD45.2 + B cells for each genotype. k, l, Lymphocytes from either mb1-cre, mb1-cre Nr4a1fl/fl, or Nr4a1−/− mice were mixed 1:1 with CD45.1+ lymphocytes and treated as in Fig. 2a. k, l, Graphs depict total B cell number (K) and division index (L) of each genotype. Data in this figure depict N = 3 biological replicates. Mean +/- SEM displayed for all graphs. Statistical significance was assessed with two-tailed unpaired student’s t-test with Holm-Sidak (A-D, G-I); two-way ANOVA with Tukey’s (K, L). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Extended Data Fig. 3 | Regulation of NUR77 expression by co-stimulation. a-c, Lymphocytes from WT mice were stimulated with the given doses of the indicated stimulus over a time course. Graphs depict endogenous NUR77 expression in B cells as determined via intracellular flow staining. Graphs in this figure depict N=3 biological replicates for all panels. Mean +/- SEM displayed for all graphs.
Extended Data Fig. 4 | NUR77 restrains B cell expansion in the absence of signal two. a–c, Lymphocytes from CD45.1+ Nr4a1++ and CD45.2+ Nr4a1-- mice were mixed 1:1, loaded with CTV, and co-cultured for 72 h with the given stimulus. Cells were then stained to detect CD45.1, CD45.2, B220, CTV via flow cytometry. a, Representative histograms depict CTV dilution of Nr4a1++ and Nr4a1-- B cells stimulated with: 10 μg/mL LPS, 2 μM CPG, or 10 μg/mL anti-IgM + either 1 μg/mL anti-CD40 or 10 ng/mL IL-4. b, Graph depicts ratio of CD45.2+ Nr4a1-- B cells relative to co-cultured CD45.1+ Nr4a1++ B cells, normalized to the input ratio. c, Graph depicts % of B cells that divided at least once as determined by CTV dilution. d, Mixed co-cultures were generated as described above in (D–F) in the presence of low dose anti-IgM 1 μg/mL. Co-stimulation with 1 μg/mL anti-CD40 was added at the indicated timepoint, and cells were harvested for analysis after a total of 72 h in culture each condition. Shown are representative histograms depicting CTV dilution of live B cells of each co-cultured genotype. Histograms are representative of 4 biological replicates.

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Extended Data Fig. 5 | Compensatory expression of Nr4a genes in Nr4a1−/− B cells and generation of Nr4a3−/− mice. a-d, Lymphocytes from Nr4a1+/+ and Nr4a1−/− mice harboring NUR77-EGFP BAC Tg were stimulated with 10 μg/mL anti-IgM for the indicated times. qPCR was performed to determine relative expression of Nr4a1, Nr4a2, Nr4a3, and GFP transcripts. Samples were also subjected to surface staining in parallel to detect % B cells via flow cytometry. Mean % B cells for Nr4a1+/+ samples was 37.6 ± 0.15 (SEM), and for Nr4a1−/− samples was 33.5 ± 1.02 (SEM). N = 3 biological replicates for all conditions. Nr4a1+/+ samples correspond to data in Fig. 1a–c.

e, Lymphocytes from reporter Nr4a1+/+ and Nr4a1−/− mice were stimulated with the given doses of anti-IgM for 24 h. Graph depicts GFP MFI in B cells as determined by flow cytometry.

f, Left: Schematic showing the extent of nucleotide deletion in exon 3 of the Nr4a3 gene harboring ATG translation initiation site, resulting in the introduction of a premature stop codon. Right: Representative PCR showing absence of the WT Nr4a3 gene and the presence of a truncated product in Nr4a3−/− mice.

g, h, MACS-purified B cells from Nr4a1+/+, Nr4a1−/−, and Nr4a3−/− splenocytes were stimulated with 10 μg/mL anti-IgM for the indicated times. qPCR was performed to determine relative expression of Nr4a3 (G) and Nr4a1 (H) transcripts.

i, Thymocytes from WT, Nr4a1−/−, and Nr4a3−/− mice were incubated +/− PMA and ionomycin for 2 h. Subsequently whole cell lysates were blotted with Ab to detect NUR77, NOR-1, MYC, and GAPDH. Red arrow indicates presence of low abundance truncated NOR-1 protein in Nr4a3−/− mice. Graphs in this figure depict N = 3 biological replicates for all panels. Mean +/− SEM displayed for all graphs. Statistical significance was assessed with two-tailed unpaired student’s t-test with Holm-Sidak (A-E); two-way ANOVA with Tukey’s (G, H). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Extended Data Fig. 6 | B and T cell development and homeostasis in NR4A-deficient mice. a–c, Splenocytes from WT, Nr4a1−/− and Nr4a3−/− mice were stained to detect T cell subsets. a, Plots depicting gating. CM = central memory. EM = effector memory. b, c, Graphs depict T cell subsets. d, Thymocytes and splenocytes from WT, Nr4a1−/− and Nr4a3−/− mice were permeabilized and stained to detect Foxp3. Graph depicts % Tregs in CD4+ gate. e–g, Splenocytes from WT, Nr4a1−/− and Nr4a3−/− mice were stained to detect B cell subsets. e, Plots of B220+ cells (top row) to identify marginal zone (MZ). Bottom row depicts B220+ subsets after exclusion of MZ. f, g, Graphs depict B cell subsets (F) and IgM/IgD MFI on B220+CD23+ splenocytes (G). h, i, Non-competitive chimeras were generated by reconstituting lethally irradiated CD45.1+ WT mice with BM from either CD45.2+ mb1-cre mice (cre chimera), or CD45.2+ cDKO chimera for 8-10 weeks. Graphs depict splenic T cell subsets as gated in A. N = 5 chimeras / genotype. j, k, Splenocytes from from mb1-cre, Nr4a1cko, and cDKO mice were stained to detect B cells. Graphs depict % B220+ cells (J) and IgM/IgD MFI on B220+ splenocytes (K). l, Graph depicts splenic subsets from chimeras above (H, I) as gated in E. m, n, Irradiated CD45.1+ recipients were reconstituted with 1:1 BM mix from donor A (CD45.1/2 WT mice) and donor B (either CD45.2+ mb1-cre control or CD45.2+ cDKO mice) for 10-12 weeks. Graphs depict relative reconstitution of B cell developmental subsets in the BM and spleen. N = 5 for chimeras each. Panels depict N = 3 biological replicates except N = 5 got chimeras (H, I, L, M, N). Mean ± SEM for all graphs. Statistical significance was assessed with two-way ANOVA with Tukey’s (B-D, F, G, K); two-tailed unpaired student’s t-test with Holm-Sidak (H, I, L); one-way ANOVA with Tukey’s (J). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | NUR77/Nr4a1 and NOR-1/Nr4a3 redundantly restrain BCR-induced B cell expansion. a–c, Samples correspond to those described in Fig. 5e,f. a, Histograms depict CTV dilution in CD45.2+ B cells (black histogram) and co-cultured CD45.1 WT B cells (shaded gray histogram), and are representative of at least 3 mice/genotype. b, Shown is the ratio of CD45.2+ WT, Nr4a1−/− or Nr4a3−/− B cells relative to co-cultured CD45.1+ WT B cells (+ 20 ng/ml BAFF), normalized to the unstimulated condition. c, Graph depicts division index for each genotype. d, e, Samples correspond to those described in Fig. 5i,j, cultured in the presence of 20 ng/ml BAFF. d, Shown is the ratio of each CD45.2 + B cell genotype relative to co-cultured CD45.1+ WT B cells, normalized to the unstimulated condition. e, Graph depicts division index for each genotype. f–k, Competitive bone marrow chimeras were generated as described in Extended Data Fig. 5h, i. 10-12 weeks after reconstitution, lymphocytes were harvested from chimeras, CTV loaded and cultured with given stimuli (anti-IgM doses +/- 20 ng/ml BAFF +/- 10 ng/ml IL-4). N = 5 chimeras of each genotype were analyzed. f, g, h, Graphs depict ratio of CD45.2+ cre+ or cDKO B cells relative to CD45.1/2 WT B cells from each chimera, normalized to unstimulated condition. i, j, k, Graphs depict division index for CD45.2+ cre+ or cDKO B cells from each chimera. Graphs in this figure depict N = 3 biological replicates for panels (D–E) except (F–K) as noted above which represent N = 5 chimeras each. Mean +/- SEM displayed for all graphs. Statistical significance was assessed with two-way ANOVA with Tukey’s (B–E); two-tailed unpaired student’s t-test with Holm-Sidak (F–K). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Extended Data Fig. 8 | Validation of NuR77 targets in BCR-stimulated B cells. a, Relative expression of Batf transcript assessed by qPCR in purified B cells corresponding to Extended Data Fig. 5a–c samples. b, Lymphocytes from Nr4a1+/- and Nr4a1-/- mice were stimulated with anti-IgM for 24 hours. Graph depicts MFI of BATF in B cells. c, Lymphocytes from competitive bone marrow chimeras (as in Extended Data Fig. 6m,n) were stimulated with anti-IgM for 24 h. Graph depicts MFI of BATF in CD45.2+ Cre-only or cDKO B cells. d, Graph depicts FPKM of Cd69 corresponding to Fig. 6a. e, Lymphocytes from CD45.1+ Nr4a1+/- and CD45.2+ Nr4a1-/- mice were co-cultured with anti-IgM for 24 h. Graph depicts MFI of CD69 on B cells. f, Relative expression of Cd69 transcript assessed by qPCR in purified B cells corresponding to Fig. 6j, K samples. g, Experiment performed as in Fig. 6o. Graph depicts MFI of CD69 on B cells. h, Relative expression of Cd69 transcript assessed by qPCR in purified B cells corresponding to Fig. 6l,m. i, Experiment performed as in Fig. 6q. j, Experiment performed as in C above. Graph depicts MFI of CD69 on B cells. k-o, Experiments performed as in H-J, but Egr2 transcript and protein assayed instead. p, Experiment performed as in C above. Graph depicts MFI of IRF4 in B cells. q, Model: Nr4a1 and Nr4a3 feedback to restrain expression of other primary response genes (PRGs) induced by BCR stimulation. Data in this figure depict N = 3 biological replicates for all panels except in C, D, J, K, O, P which have N = 4. Mean +/− SEM displayed for all graphs. Statistical significance was assessed with two-tailed unpaired student’s t-test with Holm-Sidak (A-E, J-L, O, P); two-way ANOVA with Tukey’s (F-I, M, N). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
**Extended Data Fig. 9 | Regulation of MYC and BATF by NUR77.**

a, Experiment performed as in Fig. 6o. Graph depicts MFI of intracellular MYC expression on B cells as determined via flow cytometry.

b, Experiment performed as in Fig. 6q. Graph depicts MFI of intracellular MYC expression on B cells as determined via flow cytometry.

c, Experiment performed as in Extended Data Fig. 8c with competitive chimeras described in Extended Data Fig. 6m,n. Graph depicts MFI of intracellular MYC expression on B cells as determined via flow cytometry.

d, Publicly available ATAC-seq data (Immgen.org) was accessed to identify all consensus NR4A DNA binding motifs located within OCR < 100 kb from Batf gene in follicular mature B cells. The closest, most prominent peak was identified -20kB downstream of 3’ Batf exons, and tracks were displayed using UCSC genome browser.

e, Lymphocytes from Batf+/+, Batf+/-, and Batf−/− mice were stimulated with the given doses of anti-IgM for 24 h. Graph depicts intracellular BATF expression in B cells as determined via flow cytometry. Data in this figure depict N = 3 biological replicates. Mean +/- SEM displayed for all graphs. Statistical significance was assessed with two-way ANOVA with Tukey’s (A, B); two-tailed unpaired student’s t-test with Holm-Sidak (C). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Extended Data Fig. 10 | NUR77 modulates access to T cell help under competitive conditions. a, Experiment performed as in Fig. 6o. Graph depicts MFI of surface CD86 expression on B cells as determined via flow cytometry. b, Experiment performed as in Extended Data Fig. 8c with competitive chimeras described in Extended Data Fig. 6m,n. Graph depicts MFI of surface CD86 expression on B cells as determined via flow cytometry. c, d, Lymphocytes harvested from CD45.1+Nr4a1+/+ and CD45.2+Nr4a1−/− mice were mixed 1:1 and co-cultured with the given doses of anti-IgM for 24 h (C) or 48 h (D). Graphs depict MFI of ICAM-1 surface expression on each genotype of B cells as determined via flow cytometry. e, f, Schematic of adoptive transfer experimental design for Fig. 8. B cells were purified from splenocytes harvested from Nr4a1+/+ B1-8 T g CD45.1/2 and Nr4a1−/− B1-8 T g CD45.1/2 mice via bench-top negative selection, mixed 1:1, and loaded with CTV. 2×10^6–3×10^6 B cells were then adoptively transferred +/- OTII splenocytes into either WT or CD40L−/− hosts. Host mice were then immunized IP one day later with 100 μg NP-OVA/alum, followed by spleen harvest on d4. g, Graph depicts adoptive transfer data from experiments described in E, F above and presented in Fig. 8h with addition of unimmunized controls and CD40L−/− hosts that did not receive adoptive transferred OTII cells (shaded gray points). Data in this figure depict N=3 biological replicates except B (N=4) and G where individual biological samples are plotted. Mean +/- SEM displayed for all other graphs. Statistical significance was assessed with two-way ANOVA with Tukey’s (A); two-tailed unpaired student’s t-test with Holm-Sidak (B-D). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

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☐ For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted Give P values as exact values whenever suitable.

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☐ Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection

Flow cytometry data was collected on Dual LSRFortessa.
RNAseq: Illumina sequencing platform
ELISA: absorbance was measured at 450 nm using spectrophotometer (Spectramax M5, Molecular Devices).
Western blots were visualized with a Chemi-doc Touch imager (Bio-Rad).
cPCR reactions were run on a QuantStudio 12K Flex thermal cycler (ABI) using SYBR Green detection

Data analysis

Flow cytometry data was analyzed using Flowjo (v9.9.6 and v10) software (Treestar Inc.).
Statistical analysis and graphs were generated using Prism v6 (GraphPad Software, Inc).
RNAseq data: After sequencing, QC analysis and gene and isoform quantification was performed according to the Q2 solutions in-house RNAseq pipeline. For RNAseq data, samples were aligned using STAR software version 2.4 and quantification of FPKM was performed using RSEM version 1.2.24. EdgeR platform was used to identify genes that are differentially expressed (DEG) between wild-type and Nr4a3−/− B cells after 2 h stimulation. For ATACseq analysis, publicly available follicular mature B cell ATAC-seq data [immgen.org] was accessed to identify all consensus NR4A DNA binding motifs located within OCR <100 kb from Baf6 gene. The closest, most prominent peak was identified -20 kb downstream of 3’ Baf6 exons, and tracks were displayed using UCSC genome browser.
Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
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Raw and processed data files for the RNA-seq analyses (corresponding to Fig. 6a, b) have been deposited in the NCBI Gene Expression Omnibus under accession number GSE146747 and are provided in "Supplemental Data 1.xlsx". All other source data that support the findings of this study are available from the corresponding author upon request.

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Please select the one below that is the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | For all studies samples size were defined on the basis of past experience in the laboratory. In vivo studies, between 4 and 5 mice per group were used, dependent on the experimental manipulation and additionally based on historical variance within each of the assays and the relevant readouts. For ex vivo studies, 3-5 mice/biological group were used as cell donors. No statistical methods were used to determine the sample size. |
| Data exclusions | No data were excluded |
| Replication | At least two independent experiments and typically three or more - as noted in figure legends - were performed for all key observations, and all experiments were reliably reproduced. The reproducibility was based on magnitude and consistency of measurable differences between samples. For RNA seq data analysis and metabolite measurements, the experiments were conducted once with 4 mice/genotype. |
| Randomization | For adoptive transfer experiments, age-sex matched animals were used. Animals were randomly assigned to experimental groups. |
| Blinding | Experiments were not performed in a blinded fashion. Blinding was not relevant to our study, since we are studying and comparing the property of known cell types and genotypes using objective data measurements (flow staining, ELISA e.g.). |

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a | Involved in the study |
| ☑ X Antibodies | ☑ X ChiP-seq |
| ☑ X Eukaryotic cell lines | ☑ X Flow cytometry |
| ☑ X Palaeontology | ☑ X MRI-based neuroimaging |
| ☑ X Animals and other organisms | ☑ X Clinical data |
| ☑ X Human research participants | ☑ X Clinical data |

Antibodies

| Antibodies used | Antibody / Manufacturer / Clone / Catalog # / dilution |
|-----------------|------------------------------------------------------|
| FOXP3 | Intra-cellular FOXP3 staining |
| Transcription factor staining buffer set ebiscience 00-5523-00 n/a |
| FOX3 Ab conjugated to APC ebiscience FJK-16s 17-5773-82 1:100 |
| Abs for intra-cellular staining |
| 8ATF Ab Cell Signaling Technologies C705 8638 1:400 |
Commercial available antibodies were purchased from widely used vendors which perform validation. Primary antibodies were validated by vendor for use in mice and for flow cytometry. Validation statements, citations, and antibody profiles for all primary antibodies are found on vendor website. Our lab also validates antibodies by comparing staining patterns to those reported in publications that use the same clone. The antibodies used in this study are commonly used by the field. In addition, we used Nr4a1/- and Batf/- animals to validate specificity of intra-cellular staining with Nur77 and Batf antibodies respectively.
Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research.

Laboratory animals

NUR77-EGFP and gHEL (MD4) mice were previously described (25,31). Nr4a1fl/fl mice were generously shared by Pierre Chambron and Catherine Hedrick (15). M6b1-cre, CD21-cre, OTII, CD40L−/-, Nr4a1−/-, B1-8i, Batf3−/-, C57BL/6, and CD45.1+ Boyl mice were from The Jackson Laboratory (32-38). TCRalpha−/- were obtained from the Weiss lab (39). Nr4a3−/- mice were generated via electroporation of rRNA and Cas9 mRNA. In brief, Cas9 protein (400M) and Nr4a3 gRNAs (800M) were mixed and electroporated into C57BL/6 zygotes. The sequence of Nr4a3 exon 3 (containing start ATG) targeted for deletion is shown in Fig 4E. 15 founder lines with the targeted deletion were identified through screening for PCR amplicon size, and confirmed via sequencing of dPCR products. Two founder lines were chosen for further analysis and were backcrossed for at least 4 generations onto the C57BL/6J genetic background. All other strains were fully backcrossed to the C57BL/6J genetic background for at least 6 generations. Mice of both sexes were used for experiments between ages of 6-10 weeks. All mice were housed in a specific pathogen-free facility at UCSF according to University and National Institutes of Health guidelines. All references here correspond to those in the manuscript. Animal facilities followed a 12hr light / 12hr dark cycle with temperatures of 65-75°F (~18-23°C) and 40-60% humidity.

Wild animals

No wild animals were used in this study.

Field-collected samples

No field-collected samples were used in this study.

Ethics oversight

All mice were housed in a specific pathogen-free facility at UCSF according to University and National Institutes of Health guidelines.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
☒ All plots are contour plots with outliers or pseudocolor plots.
☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation

Mouse cells were taken from spleens or lymph nodes of euthanized animals. Organs were meshed on cell strainers. ACK lysis was performed to remove RBCs from spleen samples. Where indicated, B cells were purified using bench-top negative selection as described. Culture condition depended on experimental detail outlined in the methods section and in figure legends. Viability marker was included in all flow cytometry experiments involving cell culture to exclude dead cells.

Instrument

Flow cytometry data was collected on Dual LSRFortessa.

Software

Flow cytometry data was analyzed using FlowJo (v6.9.6 and v10) software (TreeStar inc.). Statistical analysis and graphs were generated using Prism v6 (GraphPad Software, Inc).

Cell population abundance

No cell sorting was performed. Bench top negative selection for B cells was undertaken and purity was >92% in all cases as assessed by flow cytometric staining with B cell markers.

Gating strategy

FSC-A vs SSC-A was used to initially gate splenocytes. FSC-H vs FSC-A was used in this gate to determine the singlets. FSC-H vs Live dead marker was used in the singlet gate to exclude dead cells. Further gating depended on experimental design and were clearly described in the manuscript.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

October 2018

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