A Stat1 bound enhancer promotes Nampt expression and function within tumor associated macrophages

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Tumor associated macrophage responses are regulated by distinct metabolic states that affect their function. However, the ability of specific signals in the local tumor microenvironment to program macrophage metabolism remains under investigation. Here, we identify NAMPT, the rate limiting enzyme in NAD salvage synthesis, as a target of STAT1 during cellular activation by interferon gamma, an important driver of macrophage polarization and antitumor responses. We demonstrate that STAT1 occupies a conserved element within the first intron of Nampt, termed Nampt-Regulatory Element-1 (NRE1). Through disruption of NRE1 or pharmacological inhibition, a subset of M1 genes is sensitive to NAMPT activity through its impact on glycolytic processes. scRNAseq is used to profile in vivo responses by NRE1-deficient, tumor-associated leukocytes in melanoma tumors through the creation of a unique mouse strain. Reduced Nampt and inflammatory gene expression are present in specific myeloid and APC populations; moreover, targeted ablation of NRE1 in macrophage lineages results in greater tumor burden. Finally, elevated NAMPT expression correlates with IFNγ responses and melanoma patient survival. This study identifies IFN and STAT1-inducible Nampt as an important factor that shapes the metabolic program and function of tumor associated macrophages.
Performing RNA-Seq on TAMs from tumors grown in WT vs. with bone marrow-derived macrophages (BMMs) stimulated with IFNγ in macrophages and other immune cells during antitumor inflammation would reveal additional insight into the role of Nampt expression, yet has also been shown to increase to higher levels23. This probably reflects incomplete ablation due to the conflict between full deletion and cell survival. Thus far, no current model exists to study the relevance of inducible Nampt gene expression without altering baseline and essential functions. Such a model would reveal additional insight into the role of Nampt expression in macrophages and other immune cells during antitumor immunity.

Here, we searched for previously unknown mechanisms through which IFNγ signaling regulates TAM metabolism. By performing RNA-Seq on TAMs from tumors grown in WT vs. IFNγ receptor (IFNγR)-deficient mice, and through comparison with bone marrow-derived macrophages (BMMs) stimulated with LPS/IFNγ, we identify a subset of metabolic genes, including Nampt, as being strongly upregulated through an IFN-dependent process. We describe a mechanism through which Nampt is induced in both mouse and human myeloid cells, through direct STAT1 occupancy in vivo of a previously unrecognized conserved element we have identified in the first intron of Nampt, termed Nampt Regulatory Element-1 (NRE1), in contrast to other potential motifs in the Nampt promoter. Through deletion of NRE1 as well as pharmacological inhibition of NAMPT, we demonstrate that IFNs induce Nampt through NRE1, a process critical for aerobic glycolysis and expression of a subset of inflammatory genes. Using single-cell RNA-Seq, we analyze tumor infiltrating immune cells from mice specifically lacking the NRE1 region in hematopoietic cells and find inflammatory and metabolic defects in monocyte and APC populations, and defective control of tumor growth. Upon targeted deletion of this regulatory region of Nampt in specific myeloid cells including TAMs, we observe significant loss of the ability to control melanoma tumors. Finally, human clinical data from The Cancer Genome Atlas (TCGA) database reveals relationships between NAMPT expression, immune signatures of melanomas, and other tumors, inflammatory status, and patient survival. Overall, our study identifies STAT-dependent Nampt induction as a mechanism by which IFNs regulate macrophage metabolic shifts and consequently their function, and expands our understanding of how IFNγ promotes the function of TAMs in the TME.

Results

Nampt is upregulated by IFNs in TAMs through Jak/Stat1 signaling. To identify metabolic regulators of macrophage function during the response to IFNγ within the TME, we FACS-isolated TAMs from WT and IFNγR−/− mice challenged with syngeneic B16f10 melanoma tumors (Supplementary Fig. 1A), performed RNA sequencing, then compared metabolic genes from the KEGG pathway dataset to data generated from primary mouse BMMs stimulated in vitro with LPS/IFNγ. Twelve metabolic genes, including Nampt, were significantly upregulated, both within TAMs in the WT TME as well as by LPS/IFNγ stimulation in vitro (Fig. 1a). Nampt upregulation required intact IFNγR signaling in TAMs, and only stimulation with LPS/IFNγ (“M1” conditions), but not IL-4 (“M2” conditions), resulted in Nampt upregulation in BMMs (Fig. 1b). As expected, the stimulation of BMMs with LPS/IFNγ or IL-4 resulted in expression of either the pro-inflammatory M1 marker Nos2 (Nitric oxide synthase 2), or the anti-inflammatory M2 marker Arg1 (Arginase 1) by these populations, respectively (Fig. 1c). Nampt mRNA was induced in response to several inflammatory stimuli in primary BMMs, in particular those known to induce IFN autocrine/paracrine signaling (Fig. 1d). We noted the increased expression of Nampt at both the mRNA and protein levels using Northern and Western blots in BMMs stimulated with IFNγ alone (Fig. 1e, f).

Next, we wanted to determine the upstream signaling pathways required for the inducible regulation of Nampt in response to IFNγ. Upregulation of Nampt in response to IFNγ in primary BMMs occurred within an hour of stimulation and was sustained for at least 48 h (Supplementary Fig. 1B), suggesting the involvement of a transcription factor with the ability to rapidly upregulate the production of Nampt. Jak/Stat signaling is a known signaling pathway downstream of IFNγ that we hypothesized would be involved in regulating Nampt expression. To test this, we first utilized the Jak1/Jak2 inhibitor Ruxolitinib and observed a dose-dependent effect of this drug on Nampt expression in IFNγ-stimulated BMMs (Fig. 1g). We then deleted the STAT1 protein from RAW 264.7 cells, a mouse macrophage cell line, with CRISPR/Cas9 technology and found this
transcription factor is required for the IFNγ-inducible increase in Nampt mRNA and protein expression, but not for the baseline expression of Nampt (Figs. 1h, i). These Stat1-dependent effects on Nampt were in parallel to the canonical Stat1 target CXCL10 (Supplementary Fig. 1C, D). Further, increased expression of Nampt in response to LPS, IFNγ, the LPS/IFNγ combination, and IFNβ also required intact STAT1 signaling (Fig. 1j). As controls, the induction of Il6 was not significantly impacted by loss of STAT1 (Fig. 1k). The pro-inflammatory cytokine gene Il6 is strongly induced by LPS in a manner dependent on the TLR4-NF-κB pathway, but at a high LPS dose this induction does show some Stat1 dependence (Fig. 1k). Additionally, CXCL10
activation, which does directly require STAT1, was strongly decreased (Fig. 1l). Together, these data indicate a role for the Jak/Stat pathway in regulating NAMPT expression, and the requirement for Stat1 in Nampt responses to a spectrum of inflammatory stimuli.

NRE1 is an intronic STAT1 site regulating IFNγ-mediated Nampt induction. While we and others have noted a number of potential STAT1 DNA binding sites throughout the promoter region that might possibly account for the IFNγ inducible expression of Nampt, such short sequence motifs also pervade the genome in the absence of any functional relevance. However, bona fide Stat1-regulated genes also possess elements with similar consensus motifs that are not immediately distinguishable from non-functional sequences. Thus, we undertook a more thorough analysis of STAT1 activity in programming Nampt IFNγ-dependent induction. Using JASPAR to scan all potential regulatory regions of Nampt, we identified several STAT1 motifs within its first intron. We further observed elevated transcript levels based on RNA-Seq reads mapping to the first intron of Nampt in primary BMMs stimulated with LPS/IFNγ (Supplementary Fig. 2A), consistent with enhancer activity. To determine if this intronic region was regulating Nampt expression by functioning as a bona fide transcriptional enhancer, we utilized CRISPR/Cas9 constructs carrying sgRNAs directed against either boundary of this region of Nampt in RAW 264.7 cells, which we have termed NRE1 (Fig. 2A). This method created clonal populations of cells with deletions and mutations of NRE1, which we verified by Sanger sequencing of genomic PCR products (Supplementary Fig. 2B). NRE1 deletion clones expressed normal baseline levels of Nampt but had a diminished capacity to upregulate Nampt following IFNγ/LPS stimulation at both the mRNA and protein levels (Fig. 2B, c). This is also seen under IFNy only conditions (Supplementary Fig. 2C), with CRISPR/Cas9 deletion of Stat1 serving as a control for inducibility of Nampt. Importantly, northern blot analysis detected no shift in the size of Nampt mRNA isoforms in the NRE1 CRISPR/Cas9 clones compared to control clones, indicating normal splicing of the mutant locus (Supplementary Fig. 2D). Correlating with the loss of IFNy inducibility of Nampt in the absence of NRE1, NAD+ abundance in these cells as measured by liquid chromatography–mass spectrometry (LC-MS) was also significantly reduced (Supplementary Fig. 2E).

We next hypothesized that NRE1 contained a required binding site for STAT1 to program the inducible upregulation of Nampt during IFNγ stimulation. Analysis of the conservation of this region revealed dual adjacent STAT1 binding sites (SBS) common among multiple mammalian species, including humans (Fig. 2d). While there is not extensive homology extending throughout the 6kB NRE1 with any other area of the genome, well conserved minimal Stat1 binding motifs similar to these within NRE1 are readily identifiable in the regulatory regions of other IFNγ coordinately regulated genes. Chromatin immunoprecipitation (ChIP) of STAT1-associated DNA in RAW 264.7 macrophages revealed this region, located ~800 base pairs downstream of exon 1 of Nampt, had increased STAT1 occupancy in cells stimulated with IFNγ, which was dependent upon STAT1 expression (Fig. 2e). We saw only basal levels of STAT1 at the other putative SBS in NRE1 or the promoter region. Therefore, we designed a dual sgRNA CRISPR/Cas9 targeting construct (Stat1 BS CR) to delete the small (78 bp) region of NRE1 containing the dual STAT1 motifs (SBS), where correspondingly the highest increase in STAT1 binding was observed by ChIP (Fig. 2f). Cell clones with verified deletion and/or mutation of this region had defects in their capacity to induce Nampt in response to IFNγ (Fig. 2g). Cloning of this region into an ectopic luciferase reporter plasmid resulted in IFNγ responsive luciferase expression in RAW 264.7 macrophages, which was then abrogated by replacing the SBS located in this fragment with a scrambled sequence (Fig. 2h). These results demonstrate that NRE1 contains a SBS that contributes to the inducible upregulation of Nampt by IFNγ.

NAMPT enzyme inhibition blunts IFNγ-mediated inflammatory responses. We explored the function of NAMPT following its induction by IFNs in macrophages. RNA-Seq was performed on BMMs treated with IFNγ and the investigational drug FK866, a NAMPT inhibitor that has been shown to block production of nicotinamide mononucleotide (NMN), the immediate precursor to NAD in the salvage pathway. Results indicate that such inhibition of NAMPT causes decreased expression of inflammatory response, TNFa signaling, and also glycolytic pathways that are normally induced in response to IFNγ, as determined using GSEA (Fig. 3A). Defective induction of these pathways was rescued by further inclusion of NMN, the immediate product of NAMPT, to the otherwise identical experimental setting (Fig. 3B). The IFNγ response gene set itself is also altered markedly in the presence of FK866-inhibited NAMPT (Supplementary Fig. 3A vs. B). Addition of NMN partially restores induction of the IFNγ response gene set to that seen in IFNγ treatment alone (Supplementary Fig. 3C), suggesting that many aspects of the IFNγ program are impacted by NAD produced from NAMPT, including upregulation of glycolysis.

In order to analyze the dependence of NAMPT and NAD-dependent processes on M1 macrophage activation more quantitatively, we evaluated Il6, Il12b (IL-12p40), and several genes encoding other inflammatory factors in their responses to LPS/IFNγ. We observed that their induction was reduced upon
treatment with FK866 as measured by RT-qPCR, consistent with a role for NAMPT-produced NAD during responses by classically activated M1 macrophages (Fig. 3c). Among genes whose inducible expression was affected by FK866 was Ccr1, encoding a cell surface receptor important for immune cell recruitment to inflammatory sites\textsuperscript{27,28}.

Using flow cytometry, we found that CCR1 cell surface abundance levels were also reduced by FK866 in a dose-dependent manner (Fig. 3d). However, normal CCR1 levels could be restored by exogenous addition of NMN, even in the presence of increasing amounts of inhibitor. This effect was specific, because cell surface expression of the related receptor
**Fig. 2 IFNγ inducible expression of Nampt in macrophages through NRE1.** a Schematic of the first intron of Nampt, which contains the indicated predicted STAT1 binding sites, that we termed NRE1. CRISPR/Cas9-mediated deletion of this site was accomplished with the indicated constructs (CR1, CR2). b qPCR of Nampt expression in EV WT clones and NRE1 CR clones with and without LPS/IFNγ stimulation for 6 h. c Western blot of indicated cells treated with LPS/IFNγ for 24 h. Size markers are in kb. d Conservation of STAT1 binding site located within the first intron of Nampt. e Chromatin immunoprecipitation with anti-STAT1 antibody analyzed by qPCR of the indicated regions in RAW 264.7 Stat1 EV cells and Raw 264.7 Stat1 CR cells. f Schematic of the CRISPR mediated deletion of the STAT1 binding site (BS) located within NRE1 and the first intron of Nampt. g RT-qPCR expression measurement of RNA from Nampt mRNA or the STAT1 BS region of NRE1 from RAW 264.7 STAT1 BS CR cells and controls, with and without 6-h stimulation with IFNγ. h Oplophorus (Nanoluc) luciferase reporter constructs with no added sequence (EV), WT NRE fragment containing the STAT1 binding sites (SBS), or scrambled STAT1 binding sites (sbs-m) were transduced into RAW 264.7 cells treated with IFNγ or medium alone. Resulting luminescence was measured and normalized relative to a co-transduced Photinus luciferase positive control. For b, e, g, h, results presented are the average of N = 3 biological replicates, and are representative of at least three independent experiments. p values were determined by a two-tailed unpaired t-test:** p < 0.05, **p < 0.005, *** p < 0.0005, **** p < 0.00005. Error bars represent SEM. EV non-targeting CRISPR gRNA control. Uncropped images and raw scans provided in Source Data file in Supplementary information.

CCR2 was unaffected by these treatments in the same samples (Supplementary Fig. 3D). ELISA analysis of the secreted pro-inflammatory cytokine IL-6 from BMMs also showed blunted induction by LPS/IFNγ in the presence of inhibited NAMPT (Fig. 3e), while induced secretion of nitric oxide, a hallmark of macrophage inflammatory response, was also strongly reduced (Fig. 3f). To verify that the dosages of FK866 used in these assays were effective at reducing the IFNγ-stimulated high levels of NAD, but were also not causing toxicity effects, LC-MS-based chromatography–mass spectrometry (GC-MS) based metabolomics in BMMs treated with combinations of LPS/IFNγ and FK866, analyzing glycolytic and TCA cycle intermediates (Fig. 5b and Supplementary Fig. 5A, B). Metabolite levels measured in the presence of LPS/IFNγ only or LPS/IFNγ and FK866 together were normalized to those in medium only, or with FK866 only groups, respectively (Fig. 5b). Detected glucose catabolic intermediates produced upstream of the GAPDH enzyme-catalyzed step of glycolysis, including G-6-P and DHAP (see Supplementary Fig. 5Aa), accumulated to higher levels in M1-activated BMMs treated with FK866 relative to normal medium. We interpret this build up as due to GAPDH being the earliest enzyme in this pathway with a requirement for NAD, and that FK866-dependent reduction of NAD inhibits the normally efficient substrate conversion observed in activated control cells. However, those intermediate products downstream of GAPDH, including pyruvate and intracellular lactate, failed to accumulate to normal levels following inhibition of NAMPT. This pattern of blunted metabolite levels in M1-activated BMMs was also true for TCA cycle intermediates and products in the absence of normal NAMPT activity, at steps where NAD is additionally required. These metabolites include citrate, succinate, a molecule used for innate immune signaling, and also itaconate, the product of IRG1-catalyzed conversion of the TCA intermediate cis-aconitate, and which has been implicated in regulating macrophage inflammatory ROS production by inhibiting subsequent steps in the TCA cycle (see Supplementary Fig. 5A). Together, these results indicate an important role for NAMPT production of NAD during glycolysis in IFNγ-activated macrophages.

We next needed to verify that these metabolomic results indicating defects throughout respiration as well as glycolysis were also evidenced by overall metabolic phenotype consequences. We additionally sought to test whether solely IFNγ-induced metabolic reprogramming was similarly reliant on the same NAMPT-dependent production of NAD as was found using both LPS and IFNγ. Thus, we subjected IFNγ treated cells to oxygen consumption rate (OCR) analysis using mitochondrial stress (MST) tests. Our data indicate that IFNγ activation of BMMs increases OCR during mitochondrial ATP production, which is also dependent on full NAMPT activity (Supplementary Fig. 5D), in that this increase is dose-responsive.
Inhibition of glycolysis genes assayed individually by RT-qPCR (Fig. 5c). LPS/IFNγ treatment of BMMs bearing either floxed NRE1 alone (control) or Vav-Cre deleted homozygous NRE1 knockout alleles demonstrated a requirement for inducible Nampt for increased expression of glycolysis genes (Fig. 5d). To compare the abrogation of these responses by FK866 to those previously characterized during direct inhibition of glycolysis per se, we also measured gene expression following LPS/IFNγ treatment of BMMs in the presence of FK866 also showed impaired induction of glycolytic pathway genes assayed individually by RT-qPCR (Fig. 5c). LPS/IFNγ treatment of BMMs bearing either floxed NRE1 alone (control) or Vav-Cre deleted homozygous NRE1 knockout alleles demonstrated a requirement for inducible Nampt for increased expression of glycolysis genes (Fig. 5d). To compare the abrogation of these responses by FK866 to those previously characterized during direct inhibition of glycolysis per se, we also measured gene expression following LPS/IFNγ...
activation of WT BMMs in the presence of the hexokinase inhibitor 2-Deoxy-glucose (2-DG)\textsuperscript{31,32}. We observed reductions in glycolytic pathway gene activation (Fig. 5e) in addition to inflammatory factors induced by LPS/IFN\textgreek{G} (Fig. 5f). These results mimicked the effects of FK866, and confirmed the role of Nampt-supported glycolysis in promoting inflammatory gene expression in macrophages.

Stat1-induced Nampt supports myeloid function in a mouse melanoma model. In order to address the role of inducible expression of NAMPT in an in vivo cancer setting, mice bearing the homozygous NRE1 floxed allele with the broad hematopoietic lineage-specific deletion driver Vav-Cre, or their NRE1 floxed littermates lacking Cre, were inoculated subcutaneously with B16F10-ova melanoma cells\textsuperscript{33} to form flank tumors. Mice with
results are presented in Supplementary Data 2 and Supplementary enrichment for Biological Process terms was performed, and all both WT and NRE1-KO samples using Gene Ontology (GO) comprehensive expression analysis of all identi

Nampt differences in abundance where analyzed using the Seurat and in-house developed34,35 computational platforms. Figure 6b illustrates the identification of cell types from each genotype by multidimensional gene expression profiles using uniform manifold approximation and projection comparison plots35,36. Full descriptions of the marker genes used as classification criteria to assign clusters, including expression level, frequency, and significance are in Supplementary Data 1. A total of 18 single-cell clusters were identified and annotated by using transcriptome-wide similarities to known mouse immune cell subsets in the Immunological Genome Project (ImmGen) database37. Closely related cell populations were distinguished by using differential expression analyses leading to a variety of both lymphoid and myeloid cell subsets, including monocYTE populations differing in Nr4a1 and similar functionally relevant genes (consistent with classical and nonclassical monocyte phenotypes), granulocyte clusters classified by S100a9 status, macrophages classified by levels of Cxcl4, and dendritic cell types designated based on expression of several marker genes including cytokines and chemokines, and their receptors. Tumors from both genotypes have a similar distribution of immune cell types, notably there are no major differences in abundance of monocyte, macrophage, or dendritic cell lineages, with modest variation in relative cell quantities of certain other cell types (Supplementary Fig. 6A, B). For most myeloid and APC cell populations, the absence of NRE1 results in reduced Nampt expression (Fig. 6c), consistent with in vitro results using BMMs from these mouse lines. Further analysis using GSEA in two of these cell types, the Nr4a1 positive monocytes (consistent with a nonclassical monocyte profile) and mature dendritic cells, shows significant differences in gene expression profiles between WT and NRE1-KO. WT samples are enriched in several pro-inflammatory and pro-glycolytic pathways relative to NRE1-KO (Fig. 6d). Other cells in which Nampt is altered also show partial enrichment in some pathways including the pro-glycolytic MTORC1 signaling gene set (Supplementary Fig. 6E). Measurement of Nampt expression levels in all other cell clusters did not show significant differences in abundance where Nampt could be detected in substantial numbers of cells (Supplementary Fig. 6H). Also, comprehensive expression analysis of all identified clusters from both WT and NRE1-KO samples using Gene Ontology (GO) enrichment for Biological Process terms was performed, and all results are presented in Supplementary Data 2 and Supplementary Data 3. Overall, the above data suggest a scheme in which IFNγ/STAT inducible levels of Nampt support required metabolic and inflammatory responses by myeloid and APC cell types to curb malignancy. These differences were observed in mice with NRE1 deletion broadly in the hematopoietic compartment, encompassing many cell types with both positive and negative functions in tumor immunity, as well as in different stages of immune development. For instance, macrophages with a spectrum of pro- and anti-inflammatory characteristics38,39 coexist in the TME. Consistent with our other findings on the importance of NRE1 in bone marrow macrophage Nampt responses to IFNγ, the scRNAseq results directed us to hypothesize that NRE1 in TAM regulates antitumor function.

To test this more directly, we constructed mouse lines bearing the homozygous NRE1 floxed allele with the macrophage-selective deletion driver LysM-Cre. These or their NRE1 floxed littermates lacking Cre were again inoculated with B16F10-ova cells as above. Figure 6e shows that mice with blunted IFNγ-dependent induction of macrophage Nampt were significantly defective in their ability to control melanoma growth, by both growth rate and final tumor mass. Flow cytometric analysis of macrophages in these tumors again showed similar proportions regardless of genotype (Supplementary Fig. 6F), suggesting that TAM function rather than abundance is of importance. The requirement for macrophage NRE1 in tumor control may apply to other malignancies as well, as similar increases in tumor burden were observed using MC-38-ova colon adenocarcinoma derived subcutaneous flank tumors in the LysM-Cre NRE1 flox deletion context (Supplementary Fig. 6G). These observations are consistent with Nampt NRE1 as a critical point of control in TAMs during their IFNγ/STAT-driven antitumor program.

Nampt expression correlates with the IFNγ response and human melanoma outcome. Because malignant tumors are often infiltrated by innate immune cells of myeloid origin, and their polarization characteristics and functional status in this IFNγ-rich environment influences disease progression, we investigated the role of immune cell NAMPT and its IFNγ/STAT induction in the human melanoma TME. We first assessed several human macrophage cell lines, which all responded to IFNγ by upregulating NAMPT expression (Fig. 7a), in parallel with the positive control IFNγ target Cxcl10, leading us to investigate a role for this metabolic enzyme in regulating human tumor-associated immune responses.

Human melanoma tumor data from TCGA were analyzed for survival, degree of immune cell infiltration, and NAMPT expression level in tumor biopsies in the skin cutaneous melanoma (SKCM) collection. Long-term patient survival frequencies using the full available dataset were positively correlated with NAMPT expression, such that patients with higher than median expression showed statistically significant longer survival than those with below median expression in tumors, when analyzed using a Kaplan–Meier estimator (Fig. 7b).
In order to assess the correlation of NAMPT expression in tumor-associated immune cells, these data were further stratified with respect to both NAMPT expression levels and tumors with high immune infiltration (assessed by immune-related gene expression signatures as reported previously) vs. immune low (conversely high for the expression of tumor-related genes such as keratins). The 10-year survival data showed that the immune-low tumors were associated with a poor prognosis as expected, and that NAMPT expression level was not correlated with any difference in outcome. Conversely, while tumors scored as having a high level of infiltration by immune cells were similarly correlated with better disease outcomes, higher NAMPT expression was associated with a trend toward an increasingly improved prognosis within those immune rich tumors. Higher stringency
Fig. 5 NAMPT enzymatic function is required for LPS/IFNγ-induced glycolysis at GAPDH. a Glycolytic stress test showing secreted lactate as measured by ECAR (extracellular acidification rate) for BMMs treated with combinations of LPS/IFNγ and FK866. Error bars represent SEM for N = 10 biological replicates. b Glycolytic and TCA cycle intermediate metabolites in BMMs treated with combinations of LPS/IFNγ and FK866, showing reduction of inducible expression in the presence of FK866-inhibited NAMPT. c Expression of the same glycolytic gene set members as measured by RT-qPCR from the indicated macrophage RNAs when treated with combinations of LPS/IFNγ and FK866, showing reduction of inducible expression in the presence of FK866-inhibited NAMPT. d Expression of the same glycolytic gene set members as in c from Vav-Cre alone or Vav-Cre NRE1 fl/fl (NRE1-KO) macrophage RNAs treated with LPS/IFNγ, showing loss of induction in the absence of the NRE1. e Inducible glycolytic and f inflammatory gene expression is lost in the presence of the glycolysis inhibitor 2-Deoxy-D-Glucose. Results presented are the average of N = 3 biological replicates. Except for a, b, e, f, data are representative of at least three independent experiments. For b, f, results presented are the average of N = 3 biological replicates. p values were determined by a two-tailed unpaired t-test: *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00005. Error bars represent SEM.

Discussion

In mice, whole body deletion of Nampt results in embryonic lethality, while studies utilizing tissue-specific Cre and floxed Nampt alleles have demonstrated the requirement of Nampt for survival and function of various cell types15,43–45. However, to date it has been challenging to address the relevance of upregulated NAMPT during disease states without disrupting basal NAMPT functions. In our study, we were able to gain critical insight into the regulation and function of inducible NAMPT during macrophage activation by IFNγ through the identification and deletion of NRE1, which enabled us to begin to uncouple inducible from basal NAMPT function genetically. Our work points to an important role for increased Nampt in promotion of glycolysis and subsequently optimal inflammatory gene expression by TAM and other APC types.

Nampt is upregulated in a variety of human inflammatory diseases and cancers making it a gene of interest when considering improved therapeutic targets to treat such disorders13,46,47. However, the transcriptional regulation of Nampt within innate immune cells has been largely unstudied, with most work on Nampt transcriptional regulation limited to other cell types. In lung endothelial cells it was found that mechanical stress and exposure to LPS led to reduced promoter methylation and Stat5 dependent increased Nampt expression48,49. Nampt is also expressed by pancreatic β-cells to promote insulin secretion50 programmed by NCOA6, SREBP-1, and interaction with a sterol regulatory element within the promoter region of Nampt51. Nampt has also been shown to be transcriptionally regulated by Sir1, for which NAMPT-dependent NAD is a coenzyme, by interaction with an E-box element within the Nampt promoter52,53.

We have newly identified and functionally characterized a conserved SBS located within the first intron of Nampt. Its occupancy and activation by STAT1 in response to inflammatory signals is the first description of a functional transcriptional regulatory element for Nampt in activated innate immune cells. Previous analyses have implicated Nampt intron 1 in regulation by other factors in some other cell types52,53, or have correlated IFNγ/STAT1 regulation with the presence of possible Stat1 binding motifs in other locations near Nampt45. While this motif analysis identified potential binding sites upstream of Nampt, in the absence of functional binding data, the relevance of these motifs is inconclusive. In this work, we observed little or no Stat1 occupancy in the proximal promoter region including these potential sites, but identified strong binding of Stat1 in vivo to chromatin in intron 1. Future work will be needed to understand how Stat1 binding to NRE1 coordinates transcription of Nampt in combination with some of the other elements and factors mentioned above under different contexts and in distinct immune cell types. This will be of importance because Stat1 plays a major role not only in the response to IFNs, but also to a variety of other cytokines and growth factors34. Thus, we expect further study of the STAT1 mediated regulation of Nampt to yield important insight into the potential roles of Nampt in innate immune cell inflammation and the development, differentiation, and/or function of other immune or stromal cell types that reside in the TME.

Metabolic reprogramming of macrophages has been appreciated as an important factor for effective immune responses, including those that combat tumor growth, and our findings indicate that IFN/STAT1-induced NAMPT is a key component of this response. A majority of prior work on NAMPT and the NAD salvage pathway in macrophage inflammation have focused on the phenomenon of increased aerobic glycolysis under the influence of only LPS-based signaling56–57, while a few studies have started to address the role of IFNγ in pro-glycolytic transitions8. Although the de novo pathway of NAD biosynthesis has recently been shown to respond to inflammation and is a major contributor to NAD production in some contexts, NAMPT-mediated salvage synthesis of NAD is a critical pathway maintaining the NAD resource in many important cell types58,59. Our current study examined the impact of IFNγ driven classical
M1 macrophage activation on the upregulation of glycolysis and inflammatory gene expression. It defines an important role for the direct IFNγ/STAT-dependent induction of the salvage NAD synthesis pathway during this process. A recent study is in agreement with our findings in terms of induced Nampt supporting glycolysis, where NAD is needed at the GAPDH step in glycolysis under LPS-only induction in macrophages. However, our study clearly expands on this concept and is unique in that it examines the metabolic roles of Nampt induction directly by IFNγ-dependent Stat1 recruitment to NRE1 and the effects of this phenomenon in the context of the immune cells in the TME, including melanoma patients. However, we also do not rule out possible roles for NAMPT-dependent NAD in the regulation of other molecular mechanisms relevant to macrophage biology.
such as those involving Sirtuins56,60, PARPs61–63, and CD3847,64, and these will be explored in greater detail moving forward.

Our work also indicates that optimal induction of several macrophage inflammatory factors involves NAMPT regulation of glycolysis during the IFN response. One of these is the chemokine receptor CCR1, which functions within macrophages and other innate immune cells to promote homing to sites of inflammation, including invasion into tumors, and is vital to both immune cell homeostasis and the resolution of inflammation65. CCR1 in the TME may have both anti- and pro-tumorigenic activities, thus its characterization here as an inducible-Nampt-dependent factor and player in TAM recruitment is important. NRE1-mediated induction of Nampt is also important for other important inflammatory responses in macrophages, such as IL-6, IL-12p40, and mir155 expression, in addition to NO production, all of which play critical roles in modulating antitumor immunity4,33,66–69.

Nampt has previously been shown to mediate both cell intrinsic and extrinsic effects on cellular behavior. We observed decreased Nampt enzyme and target gene expression in NRE1-deficient, IFN-activated macrophages, indicating that STAT1/ NRE1-dependent induction of Nampt supports the inflammatory response. This, and the ability of FK866 to phenocopy these gene expression patterns yet be rescued by NMN, also point to a role for intracelluar as opposed to extracellular NAMPT (eNampt)70,71 in our system. However, we cannot rule out any contribution by eNampt within the scope of the current study. Additionally, there may be many other factors at play in the exogenous addition of downstream metabolites of an inhibited enzyme, such as availability at needed locations and concentrations on the subcellular scale. Inhibited Nampt may be incapable of carrying out other unknown functions unrelated to NMN production, or buildup of NMN precursors such as NAM in the presence of inhibited Nampt may lead to allostatic stress on gene regulatory processes. These possibilities will need to be evaluated in future work.

Nampt has been widely explored as a target for tumor chemotherapy18,19,22,46,47,58,72,73 and the inhibitor FK866 and other NAMPT-targeting drugs have been subjects of clinical trials, with the rationale of inhibiting NAD-dependent Warburg aerobic glycolysis of tumor cells19. However, gross interference with an enzymatic activity essential for normal cell viability is a significant drawback for any drug. Moreover, our experiments indicate that Nampt inhibitor treatment, even at doses without broad side effects, may be counter-therapeutic because of the critical roles for Nampt induction in supporting pro-inflammatory immune responses against malignant disease, either endogenous or especially in relation to cancer immunotherapies. Specific enhancement of Nampt induction in immune populations during interventional treatment may be one way to improve antitumor immune responses despite immunosuppressive stimuli in the TME. Assigning causality to the positive correlation between an immune-rich TME, inflammatory macrophage metagene signatures, high IFNγ-induced immune Nampt expression, and tumor control will require further investigation. Additionally, strong correlation of IFNγ-induced signatures with Nampt expression in multiple tumors suggests that this pathway of potential tumor control occurs more generally.

Ongoing research will also focus on understanding the roles of this pathway in other immune cell types, and in contexts where these cells promote autoimmune disease, pathogen clearance, or tumor surveillance. Along these lines, NAD metabolism was recently shown to play a role in mediating inflammation in a mouse model of inflammatory bowel disease, and the authors also demonstrated a role for enzymatic Nampt function in promoting inflammatory macrophage differentiation and the expression of inflammatory genes in mice treated with FK86674. Nampt has also been implicated in mediation of other inflammatory conditions including neuroinflammation, atherosclerosis, and arthritis75–77, and future work will take a closer look at the relevance of Stat1-induced Nampt in these settings.

Our observation that dysregulation of Nampt narrowly in macrophages has a stronger defect in tumor control than the phenotype of a broader hematopoietic deletion that includes macrophages as well as all other leukocyte lineages suggests additional complexity in the role of Nampt induction in immune cells. It may be the case that inducible Nampt function in negative regulatory cell types or otherwise during hematopoiesis is also important during the evolving antitumor response23,38,39. Myeloid cells in the TME may be characterized by a mixed continuum of tumoricidal or pro-tumorigenic signatures, leading to complex heterogeneity in their behavior and interactions, and suggesting that more narrowly focused interrogation of the functions of NRE1 and similar regulators will be informative in future work.

Taken together, a model is emerging whereby IFN/STAT1/ NRE1-upregulated Nampt functions to sustain NAD levels needed for optimal antitumor myeloid cell responses. As IFNs are key drivers of inflammation in a variety of contexts, our work suggests that a key function of IFNs is upregulation of NAD production via Nampt, which is needed to drive both aerobic glycolysis and pathways required to unleash an inflammatory program contributing to antitumor mechanisms. As we continue to understand the role of inducible Nampt and NAD during macrophage and other immune cell responses, novel therapeutic regimens will undoubtedly emerge.

Methods

Mice. All mice are on the C57BL6 genetic background. IFNγR knockout mice and their wild-type controls were obtained from The Jackson Laboratory (Bar Harbor, ME), cataloged as B6.129S7-Ifngr1<null>(Jax®)/J, Stock No: 002888. Conditional floxed NRE1 (NRE1 fl/fl) mice were custom generated by Biocytogen (Worcester, MA) using homologous recombination in C57BL6 ES cells. The final recovered mouse
The left NRE1 boundary (5′) loxP L containing cassette inserted into the genome at Chr12 between nt 32,821,032 and 32,821,033, and the right NRE1 boundary (3′) loxP R containing cassette at Chr12 between nt 32,827,113 and 32,827,114. A sequence file of the floxed locus is available on request. Male NRE1 fl/fl mice were then bred to Vav-iCre bearing females to generate hematopoietic specific NRE1 deleted mice hemizygous for Vav-iCre, and NRE1 floxed only or hemizygous Vav-iCre only controls. NRE1 deletion results in removal of 6080 bp of Nampt intron 1 in the target cell genome. A identical strategy to generate macrophage lineage-specific ablation of NRE1 instead used females carrying the LysM-Cre driver heterozygous with the WT Lyz2 (LysM) allele. Mice were sex and age matched in each individual experiment, in vivo studies used females, and ages ranged from 6 to 10 weeks old.

Mouse vivarium housing conditions are maintained with a 12-h light/dark cycle per day, temperature of 22 °C, and 20% relative humidity. All housing, husbandry, and experimental procedures using mice were performed with the approval of the
Fig. 7 Correlation between human immune NAMPT, malignancy, inflammation, and outcome. a qPCR of Nampt from the indicated human myeloid cell lines that were stimulated for 6 h with IFNy, with Hs CXCL10 induction as a control for induction. Data are the average of N = 3 biological replicates, and are representative of at least three independent experiments. p values were determined by a two-tailed unpaired t-test: *p < 0.05, **p < 0.005, ***p < 0.0005, ****p < 0.00005. Error bars represent SEV. b Kaplan–Meier plots of TCGA full 30-year (left) or first 10-year (right) human melanoma patient survival data showing curves from median-divided NAMPT expression, that is, from patients with tumors expressing the highest vs. lowest 50% of NAMPT expression. The right plot is from data that were further stratified with respect to both the highest vs. lowest 10% of NAMPT expression levels and by classifying tumor samples cytologically as containing high vs. low immune infiltration. **p values are reported within the figure panels for the difference between high and low NAMPT expression plots for either the keratin signature or immune signature-rich samples, and are from the log-rank (Mantel–Cox) test. e GSEA analysis for indicated pathways of gene expression data from TCGA melanoma transcriptomes in b. Gene rank order is determined by comparison of each gene’s expression between samples with high vs. low NAMPT levels. NES normalized enrichment score, pRMS adjusted p value: two-tailed, corrected for multiple comparisons using Benjamini–Hochberg method. b, c are based on data from a total of 463 patients. d Metagene signature analysis of NAMPT expression levels and macrophage subtypes in TCGA SKCM melanoma cohort, showing in immune rich tumors significant positive correlation of NAMPT abundance with inflammatory macrophage gene expression signatures (M1, middle). e Metagene signature analysis of NAMPT expression levels correlating with IFNy induced responses from TCGA datasets for multiple tumor types in comparison to SKCM, where the y-axis shows Pearson correlation values. For d, e, p values are two-tailed, from tests for positive slopes of correlations between the two variables.

Institutional Animal Care and Use Committee and the Comparative Medicine Center of the University of Utah.

Cell culture. Macrophage/myeloid cell lines including Raw 264.7, GM–641, Molm–14, and THP1 cells were cultured in D10 (DMEM media supplemented with 10% FBS, 1% Pen/Strep, and 1% L-glutamine). For generation of BMMs, bone marrow was isolated from the femurs and tibias of 6W WT or NRE1-KO mice and following RBC lysis were plated in cell culture plates at a concentration of 1–2 × 10^6 cells per cm^2 of growth area in D10 supplemented with 20 ng/ml M-CSF (Biologent Cat #576404 or eBioscience Cat #14-8983-80) as described. Cells were cultured 3 days before supplementing with additional media containing 20 ng/ml M-CSF, equivalent to half the amount of media added at day 0. Cells were utilized for experiments 6–7 days after differentiation with M-CSF. For the macrophage skewing RNA sequencing experiment, M1 skewed cells were stimulated with IFNy (50 ng/ml, eBioscience Cat #14-8311-63) and LPS (10 ng/ml) and M2 skewed cells were stimulated with IL-4 (100 ng/ml, eBioscience Cat #14-8041-80) for 6 h. In experiments with IFNy stimulation only, cells were stimulated with 50 ng/ml IFNy, unless otherwise indicated.

Stimulation treatments were with LPS (10 or 100 ng/ml), IFNy (50 ng/ml, eBioscience), IFNβ (10 or 50 ng/ml, Biologent), poly I:C (10 ng/ml), Flagellin (1 ng/ml), CpG class A (1 μM), or pam3csk4 (1 μg/ml), unless otherwise noted, and were all obtained from Invivogen. Experiments utilizing Raw 264.7 cells were done similarly, except where as indicated, they were pretreated in triplicate with Ruxolitinib (Invivogen) at indicated concentrations (1000, 100, 100, or 10 mM, or DMSO vehicle control) for 1 h prior to addition of IFNy. RAW 264.7 or BMM cells were stimulated with varied concentrations of the NAMPT inhibitor FK866 (10 nM–10 μM), or for 7 days with M-CSF, IL-4, and IFNy (10 ng/ml each). Analysis of RNA expression and protein expression via flow cytometry. Cells were not pretreated with the inhibitor prior to stimulating with IFNy. Where indicated, 100 μM NNM was added to cell culture at the beginning of each experiment to rescue the effects of FK866 on the depletion of NAD. For protein, enzyme or metabolite quantification, we transfected cells with the Stat1 BS CR and FK866 were added 24 h prior to analysis unless otherwise indicated. RNA measurements were done similarly except the times of treatments were 6 h.

RNA isolation and RT-qPCR. For RNA analysis of TAMS, WT, and IFNγR-deficient mice were injected subcutaneously in the hind flank with 1 × 10^6 B1610 melanoma cells. After 12 days, tumors were resected and processed into a single-cell suspension. Cells from tumors in three to four mice were pooled and sorted by flow cytometry based on the expression of CD45, CD11b, and F4/80. Sorted TAMS were then placed in QiaoZol and RNA was isolated with a miRNeasy kit from Qagen. Primary cell and cell culture lines were lysed with Qiazol reagent prior to isolation of total RNA with a miRNeasy RNA isolation kit (Qagen). In total, 100–400 ng of RNA was utilized in a cDNA synthesis reaction with qScript kit reagents (Quanta Biosciences) and was diluted to a final volume of 100–200 μl. Quantitative PCR (qPCR) was performed in a Lightcyler LC480 (Roche) or a QuantStudio 6 (Thermo) utilizing Promega GoTag qPCR master mix reagents or the equivalent, and qPCR primers listed in Supplementary Table 1. RT-qPCR data presented are representative of at least two independent experiments, with at least three biological replicates per condition, and two to three technical replicate PCR reactions per sample. Gene symbols for target genes analyzed are listed in Supplementary Table 2.

CRISPR/Cas9-mediated gene deletion. CRISPR/Cas9 lentivector infections were performed by utilizing Trans-IT 293 (Minus) to transfect 293T cells with the packaging plasmids pVSg and pPAX2 along with the CRISPR/Cas9 lentivector CRISPRv2 construct (Addgene plasmid #52961) containing one specific shortguide RNA (sgRNA) sequence and either a GFP or Puromycin selection marker, as described. To generate NRE1-KO cells, RAW 264.7 cells were transduced with two separately marked sgRNA expressing lentiviral constructs and selected by both puromycin resistance and GFP expression. These were labeled NRE1 CR. Clonal cell populations were obtained by plating single cells on a 96-well plate and the expression of NRE1 was quantified via RT-qPCR to verify knockout of NRE1. Deletion of the SBS within NRE1 in RAW 264.7 cells was performed similarly except two sgRNAs targeting sequences immediately flanking on either side of the SBS region (Stat1 BS CR) were expressed from one construct via a dual promoter system. This vector was designed at the University of Utah Mutation Generation and Detection Core, consisting of the U6 promoter for one gRNA and the H1 promoter for the second, all in a lentiCRISPRv2 derivative. Stat1 knockout RAW 264.7 cells were generated by targeting a single sgRNA to either exon 3 (Stat1 CR1) or exon 4 (Stat1 CR2) of the Stat1 gene. Controls for each of these cell lines were generated in a similar manner as described above with a CRISPR/Cas9 lentiCRISPRv2 empty vector plasmid that contained no sgRNA sequence with the exception of the controls for the SBS deletion within NRE1 which had a non-targeting sgRNA sequence. sgRNA sequences are described in Supplementary Table 1.

Genotyping of NRE1-KO cell lines. NRE1 CRISPR/Cas9 knockouts in Raw cells were generated by PCR using NRE1 5′ CRISPR Site F and NRE1 3′ CRISPR Site R primers (Supplementary Table 1) to amplify a 300 bp product from genomic DNA if the NRE1 region had been deleted. In the case where cells had a heterozygous genotype, PCR of the region surrounding the CRISPR/Cas9 binding sites was performed using NRE1 5′ CRISPR Site F and R, and NRE1 3′ CRISPR Site F and R primer pairs. PCR products above 6–8 colonies were analyzed by Sanger sequencing to identify mutations found in these regions.

Western blots. Cell pellets were lysed in RIPA buffer containing 150 mM Tris (pH7.4), 150 mM NaCl, 1% NP–40, 1 mM EDTA, and 1% SDS. Purified protein was separated via SDS-PAGE and transferred to a 0.45 μm nitrocellulose membrane. Antibody staining of NAMPT (Bethyl Laboratories), STAT1 (Santa Cruz sc-346), and ACTB (Santa Cruz sc-7778) was performed. HRP conjugated secondary antibody was detected with Amersham ECL reagent (GE) with Biorad GelDoc XR+ and ImageLab 6.1 software. Uncropped images of blots are included in the Source Data Supplementary file.

ChIP. ChIP was performed as previously described with modifications. 5 × 10^7 RAW 264.7 cells per sample in 15 cm tissue culture plates treated with 50 ng/ml Puromycin medium were crosslinked for 15 min at 37°C in PBS with 5 mM EDTA and 1% paraformaldehyde, quenched with glycine, and lysed at 0°C in ChIP Lysis Buffer (FA buffer containing 0.1% NP–40, 0.1% SDS, 2 mM MgCl_2, 1 mM DTT, and protease inhibitors, using zircon beads with a Mini-Bead Bacter 96 (Biospec Products)). Washed lysate pellets were resuspended in fresh ChIP Lysis Buffer and sonicated using a Biosortor XL bath sonicator (Diagnode). Soluble chromatin containing DNA sheared to 200 bp average length was immunoprecipitated with anti-STAT1 (sc-529, Santa Cruz) bound to Protein-G magnetic beads. Antibody staining of NAMPT (Bethyl Laboratories), STAT1 (Santa Cruz sc-346), and ACTB (Santa Cruz sc-7778) was performed. HRP conjugated secondary antibody was detected with Amersham ECL reagent (GE) with Biorad GelDoc XR+ and ImageLab 6.1 software. Uncropped images of blots are included in the Source Data Supplementary file.
that of the input chromatin to obtain a fraction of % IP value, then normalizing each ChIP signal to that for a constitutively transcribed unbound region26 within the Rps gene to normalize to cell counts. All ChIP-SEM data were from multiple independent experiments.

**Northern blots.** Total cellular RNA was electrophoresed in denaturing agarose gels, then transferred to a nylon membrane by passive transfer overnight with 20X SSC, UV crosslinked, and prehybridized at 42 °C for 1 h using UltraHyb (Invitrogen). Random-primer DNA probes or antisense RNA probes were radiolabeled with 32P according to manufacturer’s instructions (Thermo Fisher) and hybridized overnight before washing with SSC/SSD. Bound probes were visualized using a Phosphorimager (GE Healthcare) and Imagequant. Uncropped images of blots are included in the Source Data Supplementary file.

**Conservation of binding motifs.** For the analysis of species conserved STAT1 consensus binding elements of the Nampt NRE1 in Fig. 3d, sequences from corresponding regions of intron 1 from the listed mammals were downloaded from the most recent assemblies using the UCSC genome browser (http://genome.ucsc.edu). Segments of these conserved regions were aligned using ClustalX 2.1, with default parameters except Gap Opening and Extension penalties set to 5 and 3.33, respectively. Edited maf output files were then formatted with Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Stat binding sequence motifs (red box) were identified in the Nampt locus using the JASPAR transcription factor binding motif database (http://jaspar.genereg.net)26. Base numbers are from Mouse Nampt TSS+1.

**Luciferase assay.** A 280 bp fragment of the WT Nampt Intron 1 NRE1 containing bp 696–975 relative to +1 of the Nampt mRNAs and including STAT1 binding motifs was assembled into the Ophiolopsus luciferase (Nanoluc) vector pNL3.2 (Promega, GenBank JQ513370.1). A mutant version of this region was synthesized with the same length and G + C content but with the STAT1 motifs abolished based on Shufseq (EMBOSS: www.openbioinformatics.org), and was confirmed to have no STAT1 binding sequences using JASPAR (http://jaspar.genereg.net)26. In total, 250 ng of WT or mutant plasmid, pNL3.2 alone, or a TK-Nanoluc positive control plasmid were each transduced along with 250 ng pGL4.54 Phorinus luciferase reference plasmid into 5 x 10^4 RAW 264.7 macrophage cells using Lipofectamine LTX (Thermo Fisher) according to manufacturer’s directions for 8 h allowed to recover for 12 h in fresh medium, then treated with IFNγ or medium alone for 6 h. Lysates prepared from these cells were assayed for luciferase activity using the Nano-Glo Dual-Luciferase Reporter Assay System (Promega) in a Synergy H1M plate reader (Biotek).

**ECAR and OCR assay.** An Agilent Seahorse XF-96 Analyzer was used to perform the ECAR (extracellular acidification rate) or OCR assays under conditions of glycolytic (GST) or Mst in the Metabolic Phenotyping Core Facility at the University of Utah. BMMS obtained as above were plated on 96-well Seahorse XF Cell Culture Microplates the day prior to performing the assay. Seahorse XF Base Medium was supplemented with 1 mM glutamine, adjusted to a pH of 7.4 with 0.1 M NaaOH, and filtered through a 0.22 μm filter for use during the assay. For ECAR/GST, 10 mM Glu, 1 mM Oligomycin, and 50 mM 2-OG were, respectively, added to ports A, B, and C of the analyzer, then each solution was added at the corresponding time points for 5 min, supernatant aspirated, and cell pellets were snap-frozen in the dry state for liquid nitrogen. For OCR/MST, 1.5 mM Oligomycin, A, 1.5 mM FCCP, and 2.5 μM antimycin A + 1.25 μM rotenone were added as indicated.

**Mass spectrometry.** Metabolomics analysis was performed by the Metabolomics Core Facility at the University of Utah. Cells from the indicated treatment conditions were detached in ice cold PBS with 5 mM EDTA, collected, and centrifuged at 21,000 x g for 1 min, supernatant aspirated, and cell pellets were snap-frozen with liquid nitrogen. 3% methanol (MeOH) solution containing the internal standard d4-succinic acid (Sigma 293075) to give a final concentration of 80% MeOH was added to each cell pellet. Samples were then quickly vortexed, sonicated for 5 min at room temperature, then centrifuged at 20,000 x g for 10 min at 4 °C. Appropriate quality control samples were made in parallel. All GC-MS analyses were performed with an Agilent 7200 GC-QTOF and an Agilent 7693A automatic liquid sampler. Dried samples were suspended in 40 μl of 40 mg/ml O-methoxyamine hydrochloride (MOX) in pyridine and incubated for 1 h at 30 °C. Metabolites were identified and their peak area was recorded using MassHunter (Agilent). Metabolite identity was established using a combination of an in-house metabolite library developed using pure purchased standards, the commercially available NIST library, and the Fiehn library. For NAD+ measurement by liquid chromatography–mass spectrometry (LC-MS), cells were extracted using chilled solution of 100–90:10 methanol:water containing five nanograms of d9-carnitine (Cambridge Isotope Laboratories) as an internal standard. Each sample was vortexed for 30 s and centrifuged for 10 min at 20,000 RCF at 4 °C. The supernatant was removed to a fresh tube and dried overnight in vacuo. Immediately prior to LC-MS, the samples were reconstituted in water containing 10 mM of ammonium carbonate. Mass spectral analysis was performed using an Agilent 6550 UPLC-QTOF-MS system (Santa Clara, CA). A Hilocon iHILIC column (Umeå, Sweden) was used for fractionation using a linear gradient of 10:90 ACN:10 mM ammonium acetate buffer to 20:80 ACN:10 mM ammonium acetate buffer over 20 min at a flow rate of 0.2 ml/min. MS was performed in the positive mode with an AJS ESI source. Quantitative data analysis was conducted using Agilent MassHunter Quant software for NAD+. 

**RNA sequencing.** Total RNA isolated via miRNeasy Qiagen RNA isolation kit was submitted to the UW Utah genomics core facility, a cDNA library was prepared and Illumina HiSeq 2500 sequencing was performed as in27, with modifications. Briefly, RNA was quantified with a Qubit RNA HS Assay Kit (Fisher Scientific Q32835). RNA quality was evaluated with an Agilent Technologies RNA 9000 Chip (Agilent 5067-5579 and 5067-5580). Following on reactions on 100–500 ng total RNA, stranded libraries were prepared using the Illumina TruSeq Stranded Total RNA Kit with Ribo-Zero Gold (RS-122-2301 and RS-122-2302). Purified libraries were qualified on an Agilent Technologies 2200 TapeStation using a D1000 ScreenTape assay (5067-5582 and 5067-5583). Individual libraries were normalized to 1.0 μl and equal volume stocks were pooled in preparation for Illumina sequencing. Libraries (25 pm) were chemically denatured and applied to an Illumina HiSeq v4 single read flow cell, amplified and annealed to sequencing primers, and 50 cycle single read runs were performed using an Illumina HiSeq 2500 instrument (HSV2.2.28 and RNA v1.18.61). Corresponding reagent kits (FC-401-4003) were aligned to the mouse mm10 reference genome (mm10, M_musculus_Dec_2011, GRCm38). 

**sCRNaseq and syngeneic tumor experiments.** Mice were injected subcutaneously in the rear flank with 5 x 10^6 B16F10-ova melanoma or MC-38-ova COAD cells on day 0. Tumor size measurements were taken on the indicated days, and tumors were weighed upon sacrifice at day 14. Dimensions (mm) were measured using calipers, and size was estimated using the formula: (l x w)²/2, reported for Tumor Size. For further analysis, tumors were mechanically dissected and used for downstream analysis. 

The computational approach we used for annotating unknown cell clusters (Cluster Identity Predictor (CIPR)) was previously described28. In short, the CIPR algorithm compares the genome-wide expression signatures from unknown single-cell clusters to those of known reference cell types published by the ImmGen consortium. The reference dataset contains microarray expression profiles from mouse immune cell populations. By examining several genes simultaneously, CIPR calculates a similarity score for each single-cell cluster and known reference pairs to aid quickly and accurately annotate unknown clusters through CIPR-Shiny (v0.1.0), a user-friendly graphical interface available on the web (https://seeksh. bioinformatics.ucsc.edu/cipr/). The CIPR method is based on computational clustering algorithms to some cell cluster assignments relative to older established pipelines (such as SingleR package), and with less computing resource requirements. 

**GO analysis.** GO analysis was performed on differentially expressed gene sets in WT and NRE1 KO cell clusters in scRNAseq data. To this end, the FindMarkers() Seurat function was used to calculate upregulated genes in WT or NRE1 KO single-cell clusters through Wilcoxon Rank Sum test (default). The significance cutoff for subsequent GO analysis was set at the unadjusted p value of <0.05. These gene sets were then analyzed using the clusterProfiler R package against Biological Process GO terms. GO term enrichment results are presented in tabular format for each cluster (Supplementary Data 2 and Supplementary Data 3).

**Flow cytometry.** Flow cytometric analyses were performed on cultured cell lines and single-cell suspensions isolated from tumors, and carried out as described in85 with modifications. Cultured cells were treated with various reagents as described and collected off the plate via 5 mM EDTA in PBS in a non-enzymatic manner. Briefly, after mechanical disruption and dissociation of tumor tissue between the frosted ends of microscope slides, tumor cells were placed on an orbital shaker in Accustain (Innovative Cell Technologies) and incubated for 30 min at room temperature, followed by red blood cell lysis in ammonium-chloride-potassium buffer (Biogenoid), and filtration through a 0.45-micron filter. Prior to staining with fluorophore-conjugated antibodies (all from Biogenoid or Thermofisher), FC receptors were blocked using aCD16/32 antibody (1:200, Biogenoid) to reduce nonspecific staining. Staining was performed on ice for 15–30 min in PBS supplemented with 2% FBS and 0.1% Sodium Azide. Cells were stained with a combination of the following fluorophore-conjugated antibodies in Hanks’
balanced salt solution supplemented with 10% BSA, pyruvate, EDTA, and HEPEs: CD45 PE-cy7 (1:400), CD45 PB (1:400), Ly6c FITC (1:200) MHCCI PB (1:200), Gr1 PE (1:200), CD40 FITC (1:200), CD80 PerCp-cy5.5 (1:200), CD11b APC (1:200), all from Biolegend unless noted otherwise. For viability assays, Ghost Dye Red 780 (Tombo) stain was applied. The cells were then washed, and flow cytometry was performed using a BD LSRII Fortessa II (BD Biosciences). Data were subsequently analyzed using FlowJo software (Tree Star). Gating strategies are provided as Supplementary figure panels corresponding to the relevant figures. Sorting of GFP+ cells was completed on a FACS Aria III (BD Biosciences) in the flow cytometry core facility at the University of Utah.

Analysis of TCGA data: SKCM bulk RNA-seq data were downloaded from TCGA database using TCGAbiolinks R package. Raw counts were normalized using the voom approach and NAMPT expression was categorized at median or top bottom 10% to define NAMPT-high and NAMPT-low patients. In rare cases where both primary and metastatic samples are available from the same patient, metastatic database using TCGAbiolinks R package. Raw counts were normalized using the

Statistical analysis: T-Tests (two-tailed, unpaired) were performed when comparing differences between two experimental groups in in vitro experiments where noted in the figure legends. Two-way ANOVA was also used where noted. Wilcoxon test was used when calculating differential gene expression and comparing gene expression in scRNAseq data. GSEA profiles were evaluated using two-tailed

Data availability: scRNAseq and RNAseq data have been deposited in Gene Expression Omnibus (GEO) under the following accession codes: GSE113836, GSE118387, GSE123822, and GSE135814. TCGA data are available at https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga. All other data supporting the study are available within the article and Supplementary information files. Source data are provided with this paper.

Received: 5 May 2020; Accepted: 30 March 2021; Published: online 11 May 2021

References
1. Su, X. et al. Interferon-gamma regulates cellular metabolism and mRNA translation to potentiate macrophage activation. Nat. Immunol. 16, 838–849 (2015).
2. Artyushov, M. N., Sergushichev, A. & Schilling, J. D. Integrating immunometabolism and macrophage diversity. Immunometabolism 28, 417–424 (2016).
3. O’Neill, L. A. & Pearce, E. J. Immunometabolism governs dendritic cell and macrophage function. J. Exp. Med. 213, 15–23 (2016).
4. Siew, J. L., Gun, S. Y. & Wong, S. C. The sweet surrender: how myeloid cell metabolic plasticity shapes the tumor microenvironment. Front. Cell. Developmental Biol. 6, 1344 (2018).
5. Castro, F., Cardoso, A. P., Gonçalves, R. M., Serre, K. & Oliveira, M. J. Interferon-gamma at the crossroads of tumor immune surveillance or evasion. Front. Immunol. 9, 665 (2018).
6. Cosby, A. et al. Primary antitumor immune response mediated by CD4+ T cells. Immunity 22, 371–383 (2005).
7. Haabeth, O. A. W. et al. Inflammation driven by tumour-specific Th1 cells protects against B-cell cancer. Nat. Commun. 2, 240 (2011).
8. Sinha, P., Clements, V. K. & Ostrand-Rosenberg, S. Reduction of myeloid-derived suppressor cells and induction of macrophages facilitate the rejection of established metastatic disease. J. Immunol. 174, 636–645 (2005).
9. Møller, S. P. et al. Macrophage de novo NAD + synthesis specifies immune function in aging and inflammation. Nat. Immunol. 38, 1 (2018).
10. Kelly, B. & O’Neill, L. A. Metabolic reprogramming in macrophages and dendritic cells in innate immunity. Cell Res. 25, 771–784 (2015).
11. Langston, P. K., Shibata, M. & Horng, T. Metabolism supports macrophage activation. Front. Immunol. 8, 723 (2017).
12. Wang, T. et al. HIF1-a induced glycolysis metabolism is essential to the activation of inflammatory macrophages. Mediators Inflamm. 2017, 1–10 (2017).
13. Zhang, L. Q., Heruth, D. P. & Ye, S. Q. Nicotinamide phosphoribosyltransferase in human diseases. J. Bioanal. Biomol. 3, 13–25 (2011).
14. Samat, R. et al. Cloning and characterization of the cDNA encoding a novel human pre-B-cell colony-enhancing factor. Mol. Cell. Biol. 14, 1431–1437 (1994).
15. Rongvaux, A. et al. Nicotinamide phosphoribosyl transferase/pre-B-cell colony-enhancing factor/visfatin is required for lymphocyte development and cellular resistance to genotoxic stress. J. Immunol. 181, 4685–4695 (2008).
16. Pittelli, M. et al. Nicotinamide phosphoribosyltransferase (NAMPT) activity is essential for survival of resting lymphocytes. Cell Immunol. Cell Biol. 92, 191–199 (2014).
17. Bruzzone, S. et al. Catastrophic NAD+ depletion in activated T lymphocytes through NAMPT inhibition reduces demyelination and disability in EAE. PLoS ONE 4, e7609 (2009).
18. Hamann, M. & Schėmadina, I. FK866, a highly specific noncompetitive inhibitor of nicotinamide phosphoribosyltransferase, represents a novel mechanism for induction of tumor cell apoptosis. Cancer Res. 63, 7436–7442 (2003).
19. Yaku, K., Okabe, K., Hikosaka, K. & Nakagawa, T. NAD metabolism in cancer progression. Front. Oncol. 8, 300 (2018).
20. Wang, G. et al. PT3C neuroprotective chemicals function by activating the rate-limiting enzyme in NAD salvage. Cell 158, 1324–1334 (2014).
21. Lük, T., Malam, Z. & Marshall, J. C. Pre-B cell colony-enhancing factor (PEBF)/visfatin: a novel mediator of immune inflammation. J. Leukoc. Biol. 83, 804–818 (2008).
22. Garten, A., Petzold, S., Körner, A., Imai, S.-I. & Kiess, W. NAMPT: linking NAD biology, metabolism and cancer. Trends Endocrinol. Metab. 20, 130–138 (2009).
23. Travelli, C. et al. Nicotinamide phosphoribosyltransferase acts as a metabolic gate for mobilization of myeloid-derived suppressor cells. Cancer Res. 79, 1938–1951 (2019).
24. Kawai, T. & Akira, S. Toll-like receptors and their crosstalk with other innate immune sensors. J. Leukoc. Biol. 79, 114–122 (2006).
25. Dyver, D. P. et al. Chemokine receptor redundancy and specificity are context dependent. Immunity 50, 378–389 (2019).
26. Tannahill, G. M. et al. Sulfakinin is an inflammatory signal that induces IL-1β through HIF-1α. Nature 496, 238–242 (2013).
27. Lampropoulou, V. et al. Itaconate links inhibition of succinate dehydrogenase with macrophage metabolic remodeling and regulation of inflammation. Cell Metab. 24, 158–166 (2016).
28. Zhao, Q. et al. 2-Deoxy-d-glucose treatment decreases anti-inflammatory M2 macrophage polarization in mice with tumor and allergic airway inflammation. Front. Immunol. 8, 656 (2017).
29. Perrin-Cocom, L. et al. TL4R antagonist FP7 inhibits LPS-induced cytokine production and glycolytic reprogramming in dendritic cells and protects mice from lethal influenza infection. Sci. Rep. 7, 269 (2017).
30. Snook, J. P., Soedel, A. J., Ekiz, H. A., O’Connell, R. M. & Williams, M. A. Inhibition of SHP-1 expands the repertoire of antitumor T cells available to respond to immune checkpoint blockade. Cancer Immunol. Res. 8, 506–517 (2020).
31. Ekiz, H. A., Conley, C. J., O’Connell, R. M. CIPR: a web-based R/shiny app and R package to annotate cell clusters in single cell RNA sequencing experiments. BMC Bioinform. 21, 1–15 (2020).
32. Ekiz, H. A. et al. MicroRNA-135 coordinates the immunological landscape within murine melanoma and correlates with immunity in human cancers. JCI Insight 4, e126543 (2019).
33. Butler, A., Hoffman, P., Smibert, P., Papalexi, E. & Satija, R. Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nat. Biotechnol. 36, 411–420 (2018).
34. Heng, T. S. P., Painter, M. W. & Consortium, I. G. P. The Immunological Genome Project: networks of gene expression in immune cells. Nat. Immunol. 19, 1091–1094 (2018).
35. Murray, P. J. et al. Macrophage activation and polarization: nomenclature and experimental guidelines. Immunity 41, 14–20 (2014).
39. Martínez, F. O. & Gordon, S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* **6**, 13 (2014).

40. TGCA Network. Genomic classification of cutaneous melanoma. *Cell* **161**, 1681–1696 (2015).

41. Newman, A. M. et al. Robust enumeration of cell subsets from tissue expression profiles. *Nat. Methods* **12**, 453–457 (2015).

42. Thorsson, V. et al. The immune landscape of cancer. *Immunity* **48**, 812–830 (2018).

43. Stein, L. R. & Imai, S. Specific ablation of Namp in adult neural stem cells recapitulates their functional defects during aging. *EMBO J.* **33**, 1321–1340 (2014).

44. Stromsdorfer, K. L. et al. NAMPT-mediated NAD(+) biosynthesis in adipocytes regulates adipose tissue function and multi-organ insulin sensitivity in mice. *Cell Rep.* **16**, 1851–1860 (2016).

45. Liu, J. B. et al. NAMPT-mediated NAD(+) biosynthesis is essential for vision in mice. *Cell Rep.* **17**, 69–85 (2016).

46. Ohanna, M. et al. Pivotal role of NAMPT in the switch of melanoma cells toward an invasive and drug-resistant phenotype. *Genes Dev.* **32**, 448–461 (2018).

47. Garten, A. et al. Physiological and pathophysiological roles of NAMPT and NAD metabolism. *Nat. Rev. Endocrinol.* **11**, 535–546 (2015).

48. Sun, X. et al. The NAMPT promoter is regulated by mechanical stress, signal transducer and activator of transcription 5, and acute respiratory distress syndrome-associated genetic variants. *Am. J. Respir. Cell Mol. Biol.* **51**, 660–667 (2014).

49. Elangoovan, V. K. et al. Endotoxin- and mechanical stress-induced epigenetic changes in the transcription of the nicotinamide phosphoribosyltransferase promoter. *Pulm. Circ.* **6**, 539–544 (2016).

50. Revollo, J. R. et al. NAMPT/PREF/Viisfat regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. *Cell Metab.* **16**, 363–375 (2007).

51. Yoon, J., Lee, K. J., Oh, G. S., Kim, G. H. & Kim, S. W. Regulation of Nampt expression by transcriptional coactivator NCOA6 in pancreatic β-cells. *Biochem. Biophys. Res. Commun.* **487**, 600–606 (2017).

52. Nakahata, Y., Sahar, S., Astariya, G., Kaluzova, M. & Sassone-Corsi, P. Circadian control of the NAD+ salvage pathway by CLOCK-SIRT1. *Science* **324**, 654–657 (2009).

53. Ramsey, K. M. et al. Circadian clock feedback cycle through NAMPT-mediated NAD+ biosynthesis. *Science* **324**, 651–654 (2009).

54. Ramana, C. V., Chatterjee-Kishore, M., Nguyen, H. & Stark, G. R. Complex roles of Stat1 in regulating gene expression. *Oncogene* **19**, 2619–2627 (2000).

55. Liu, T. F. et al. Sequel actions of SIRT1–RELB–SIRT3 coordinate nuclear-oxidation during the acute inflammatory response. *Proc. Natl Acad. Sci. USA* **104**, 1604–1609 (2007).

56. Wallace, J. et al. Genome-Wide CRISPR-Cas9 Screen Identifies MicroRNAs That Regulate Myeloid Leukemia Cell Growth. *PLOS ONE* **11**, e0135689 (2016).

57. Voith, W. P. et al. A role for FACT in replication of nucleosomes at inducible genes. *PLOS ONE* **9**, e84092 (2014).

58. Simon, I. et al. Serial Regulation of Transcriptional Regulators in the Yeast Cell Cycle. *Cell* **106**, 697–708 (2001).

59. Parnell, E. J. et al. The Rts1 regulatory subunit of PP2A phosphatase controls expression of the HO endonuclease via localization of the Ace2 transcription factor. *J. Biol. Chem.* **289**, 35431–35437 (2014).

60. Rice, P., Longden, I. & Bleasby, A. EMBOSS: The European Molecular Biology Open Software Suite. *Trends Genet.* **16**, 276–277 (2000).

61. Stuart, T. et al. Comprehensive Integration of Single-Cell Data. *CELL* **177**, 1888–1902 (2019). e1821.

62. Hoffaker, T. B. et al. Antitumor immunity is defective in T cell–specific microRNA-155–deficient mice and is rescued by immune checkpoint blockade. *The J. Biol. Chem.* **292**, 18530–18541 (2017).

63. Corcoran, S. E. & O’Neill, L. A. J. IFH1a and metabolic reprogramming in inflammation. *J. Clin. Investig.* **126**, 3699–3707 (2016).

Acknowledgements

We would like to acknowledge the Flow Cytometry, Genomics, Metabolic Phenotyping, Metabolomics, and Mutation Generation and Detection (MGD) Core Facilities here at the University of Utah for their contributions to this work. Mass spectrometry equipment was obtained through NCIRR Shared Instrumentation Grants 1S10OD016232-01, 151OD018210-01A1, and 151OD021505-01. This work was supported by National Institutes of Health Grants T32 AI 138945-1 (to K.M.B.), AG047956-04, AI123106-01A1 (to W.P.V. and R.M.O.). We gratefully acknowledge the generous gifts of Dr. W.P. Young and Dr. R.M. Schrock for mass spectrometry equipment and access to the University of Utah for their contributions to this work. Mass spectrometry equipment was obtained through NCRR Shared Instrumentation Grants 1S10OD016232-01, 151OD018210-01A1, and 151OD021505-01. This work was supported by National Institutes of Health Grants T32 AI 138945-1 (to K.M.B.), AG047956-04, AI123106-01A1 (to W.P.V. and R.M.O.).

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T.B.H. and H.A.E. contributed equally to this work. T.B.H. designed and performed experiments, collected and analyzed data, and contributed to writing the manuscript. H.A.E. designed and performed experiments, collected and analyzed data, developed analytical tools, and contributed to writing the manuscript. C.B., M.C.R., M.C.N., K.M.B., C.B., M.C.R., M.C.N., K.M.B., A.M., T.L.M., and J.W.C. contributed to writing the manuscript. T.L.M. and C.B. provided analytical tools and analyzed data. J.E.C. developed analytical tools and analyzed data. C.B., M.C.R., M.C.N., K.M.B., and T.L.M. contributed to writing the manuscript. J.W.C. contributed to writing the manuscript. T.L.M. and C.B. provided analytical tools and contributed to writing the manuscript. W.P.V. and R.M.O. supervised the work, and contributed to writing the paper. All authors discussed the results and implications, gave technical support and conceptual advice, contributed to writing the manuscript, and jointly supervised this work. R.M.O. developed the study, gave technical support and conceptual advice, jointly supervised the work, and contributed to writing the paper. All authors discussed the results and implications, gave technical support and conceptual advice, and commented on the manuscript at all stages.

Competing interests

The authors declare no competing interests.
Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41467-021-22923-5.

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Peer review information Nature Communications thanks Antje Garten, Takashi Nakagawa, and the other, anonymous reviewer(s) for their contribution to the peer review of this work.

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