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GCN2 drives macrophage and MDSC function and immune suppression in the tumor microenvironment.

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Summary

GCN2 is an environmental sensor controlling transcription and translation in response to nutrient availability. While GCN2 is a putative therapeutic target for immuno-oncology, its role in shaping the immune response to tumors is poorly understood. Here we used mass cytometry,

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transcriptomics, and transcription factor binding analysis to determine the functional impact of GCN2 on the myeloid phenotype and immune responses in melanoma. We found myeloid-lineage deletion of GCN2 drives a shift in the phenotype of tumor-associated macrophages and MDSCs that promotes anti-tumor immunity. CyTOF and single cell RNA sequencing showed this was due to changes in the immune microenvironment with increased proinflammatory activation of macrophages and MDSCs and IFN γ expression in intratumoral CD8⁺ T cells. Mechanistically, GCN2 altered myeloid function by promoting increased translation of the transcription factor CREB-2/ATF4, which was required for maturation and polarization of macrophages and MDSCs in both mice and humans, while targeting *Atf4* by siRNA knockdown reduced tumor growth. Finally, analysis of cutaneous melanoma patients showed that GCN2-dependent transcriptional signatures correlated with macrophage polarization, T cell infiltrates, and overall survival. Thus, these data reveal a previously unknown dependence of tumors on myeloid GCN2 signals for protection from immune attack.

One Sentence Summary.

GCN2 is a key driver of macrophage and MDSC polarization in the tumor microenvironment causing T cell exhaustion.

INTRODUCTION

Interactions between cancer cells, T cells, and myeloid cells in the tumor microenvironment (TME) are a key determinant in tumor pathophysiology. In general, survival and responses to therapy correlate favorably with the extent of intratumoral CD8⁺ T cell infiltration (1); however, when the infiltrate is predominately myeloid, therapy responses and survival are reduced (2).

Myeloid function is governed by environmental signals driving commitment to a functionally polarized state (3). In the TME, several factors influence myeloid function including hypoxia (4, 5), pH (6), and nutrient depletion (7). General control nonderepressible (GCN)2 is a Ser/Thr kinase found in all eukaryotic organisms that is activated by deacylated tRNAs resulting from amino acid and glucose limitation (8). The principle substrate of GCN2 is the α subunit of eukaryotic translation initiation factor 2 (eIF2) α (9). After phosphorylation by GCN2, eIF2 α 's GDP/GTP exchange activity is reduced, abrogating cap-dependent translation. The resulting changes in mRNA translation alter the cellular phenotype regulating metabolism, autophagy, proliferation and survival (9). In T cells, GCN2 signals are associated with naïve T cell suppression, blocking entry into cell cycle and T cell receptor signal transduction, and promoting Foxp3⁺ regulatory T cell function (10). While data on GCN2 function in myeloid cells is limited, our laboratory has reported that GCN2 activation modulates macrophage (M ϕ) and dendritic cell (DC) responses in autoimmune disease promoting acquisition of an IL-10⁺TGF- β ⁺ phenotype (11).

Myeloid derived suppressor cells (MDSCs) are a mixed population of immature and highly immune-suppressive monocytic and granulocytic lineage cells elicited by cancer-driven myelopoiesis in mice and humans (12, 13). An essential molecular feature of MDSCs is

prominent expression of genes involved in the metabolism of L-arginine [i.e. Arg-1, inducible nitric oxide synthase (iNOS)]. MDSCs expanded by tumors are phenotypically distinct from tumor-associated M ϕ and dendritic cells (DCs), although MDSCs can differentiate into mature myeloid cells at the tumor site (14).

Tumor associated M ϕ s (TAMs) are a mature myeloid population originating from both monocytes or tissue resident M ϕ (15). TAMs often exhibit a suppressive phenotype with production of immune-regulatory factors suppressing innate and adaptive immunity and providing stromal support for tumor growth and metastasis (3). Like TAMs, MDSCs are potent suppressors of T cell function. In particular, the action of Arg-1 produced by TAMs and MDSCs in the TME has a profound effect on T cells reducing T cell receptor signal transduction and inhibiting cell cycle progression by downregulation of cyclin D3 (16, 17). This effect is the result of extracellular L-Arg consumption and subsequent activation of GCN2 in T cells. It is likely that GCN2 activity would impact myeloid behavior in the TME; however, there are no studies we are aware of that test this prediction. Therefore, we investigated the role of GCN2 in myeloid cell function in tumors. We found GCN2 deletion altered M ϕ and MDSC phenotype, causing an abrogation of suppressive function and enhanced anti-tumor CD8⁺ T cell immunity in vivo. This was due to altered gene expression and metabolism limiting polarization in M ϕ s and overall function in MDSCs. Thus, the data reveals that GCN2 is an essential driver of myeloid function in the TME shaping the tumor immune landscape.

RESULTS

Myeloid GCN2 function is required for tumor growth and T cell exhaustion

To test the functional role for myeloid GCN2 in tumor growth, we monitored tumor growth in mice with a myeloid-lineage deletion of GCN2 (B6. *Gcn2^{fl/fl} × Lyz2^{+/Cre}*, hereafter referred to as LysM–GCN2 cKO mice) or littermate control mice lacking Cre expression (*GCN2^{fl/fl}* mice). In control mice, B16F10 (B16) melanoma tumors grew rapidly, reaching a mean volume of 1300 mm³ at day (d) 25 post-implantation (fig. 1a). In contrast B16 tumor growth was markedly restricted in LysM–GCN2 cKO mice, where tumor weight and volume were reduced by 75% (fig. 1a). MC38 colorectal and EL4 lymphoma tumors showed similar changes with a 10-fold reduction in tumor size, suggesting myeloid GCN2 was required for tumor growth across several tumor types (fig. 1a). To determine if reduction of tumor growth in LysM–GCN2 cKO mice was associated with alterations in the cytokine milieu, we measured d17 whole B16 tumor mRNA for expression of cytokines by qPCR. Compared to control tumors, tumors from LysM–GCN2 cKO mice showed a 10-fold reduction in expression of the immune-suppressive cytokine interleukin (IL)-10 and increased in expression of inflammatory cytokines including *Ifng*, *Il1b*, and *Tnfa* (fig. 1b). Since tumor size differences at d17 may impact cytokine expression independent of GCN2 function, we also examined inflammatory cytokine expression in small tumors of equal size (75 mm³) from control and LysM–GCN2 cKO tumor-bearing mice. We observed a similar pattern of increased inflammatory cytokine expression in the absence of myeloid GCN2 function (fig. 1c) indicating loss of GCN2 increases local inflammation in the TME.

To gain better understanding of the impact myeloid GCN2 has on tumor immunity, we performed CyTOF analysis of d20 B16 tumors. Phenograph analysis (18) of the CyTOF data revealed 11 distinct intratumoral immune cell populations (fig. 1d). The majority of the immune infiltrate was TAMs resolving as two populations differentiated by expression of CD11c (populations 5 and 9, fig. 1d). Dendritic cells were present but represented a minor component of the infiltrate resolving as two distinct groups based on CD103 expression (populations 6 and 10, fig. 1d). Tumors from LysM–GCN2 cKO mice showed differences in the composition and activation state of the immune infiltrate compared to tumors from GCN2^{fl/fl} mice; this included an increase in CD8⁺ T cells, NK cells, and NKT cells and a trend towards decreased numbers of TAMs (fig. 1d). We observed both granulocytic (g) Ly6g⁺ and monocytic (m) Ly6G^{lo}Ly6C^{hi} MDSCs (fig. 1d); however, there were no differences in numbers between the two groups. Importantly, LysM–GCN2 cKO mice showed no change in bone marrow precursors or splenic macrophage phenotypes compared to GCN2^{fl/fl} mice (fig. s1a and fig. s1b) indicating changes in cellular composition were specific to the TME.

The cytokine expression data (fig. 1b and fig. 1c) suggested a more inflamed TME. In agreement with this, expression of MHCII and CD86 was increased in TAMs while there was a reduction in expression of CD206 and PD-L1 in LysM–GCN2 cKO tumors compared to controls (fig. 1f). Likewise, intratumoral CD4⁺ and CD8⁺ T cells had decreased expression of the exhaustion marker PD-1, the regulatory T cell transcription factors FoxP3, CD39 (19) and the Treg cell marker helios (fig. 1f) (20). Interestingly, gMDSCs exhibited a loss of suppressive phenotype with reduced expression of PD-L1, CD206, and CD39 while mMDSCs appeared more proinflammatory with increased expression of CD86 and MHC I and II (fig. 1f).

Intratumoral CD8⁺ T cell exhaustion is associated with tumor growth and resistance to immune destruction. In this vein, it had been hypothesized that increased expression of multiple exhaustion markers in CD8⁺ T cells is indicative of a deeply exhausted phenotype (21). Since our CyTOF data showed reduced PD-1 expression in intratumoral CD8⁺ T cells from LysM–GCN2 cKO mice, we examined expression of 4 key exhaustion markers in CD8⁺ T cells to determine if GCN2 impacted this deeply exhausted state. In agreement with the CyTOF data, flow cytometry showed that intratumoral CD8⁺ T cells from LysM–GCN2 cKO mice showed decreased surface expression of PD-1 and LAG3 and decreased expression of TIM3 and TIGIT (fig. 1g); importantly, in LysM–GCN2 cKO mice intratumoral CD8⁺ T cells positive for all 4 exhaustion markers (i.e. the deeply “exhausted” CD8⁺ T cells) were reduced 3-fold compared to controls (fig. 1g). Together, these data show loss of GCN2 in the myeloid infiltrate profoundly impacts the TME driving macrophage inflammatory maturation, which in turn promotes a more robust T cell infiltrate with reduced exhaustion and potentially improved effector maturation.

Myeloid GCN2 function is required for an immune suppressive transcriptional landscape in melanoma

Myeloid cells account for about 1% of the total cellularity in B16 tumors. Nevertheless, bulk tumor transcriptome analysis showed a substantial impact on RNA expression patterns with

>2900 transcripts showing significant changes in abundance ($\text{FDR} < 0.05$, $\log\text{FC} > +/ - 1$) in LysM–GCN2 cKO versus control tumors (fig. 2a). In particular, there was a decrease in expression of genes associated with immune suppression including *Ii10* and *Arg1*, as well as the transcript for the GCN2 responsive gene *Asns* (22, 23) (fig. 2a). In contrast, genes involved in T cell migration (*Selp1g*), macrophage scavenging (*Siglec1*) and adhesion (*Itgam*), and tumor inflammation (*Sepp1*) were increased in LysM–GCN2 cKO tumor-bearing mice compared to control tumors (fig. 2a). Ingenuity pathway analysis (IPA) of diseases and functions predicted these transcriptional changes in the tumor would result in increased cell death and morbidity, while pathways associated with cellular survival and viability were predominant in tumors from GCN2^{fl/fl} mice (fig. 2b). Thus, the loss of myeloid GCN2 function shifted the TME and gene expression patterns towards inflammation, T cell recruitment, and death of tumor cells.

We examined the impact of GCN2 deletion on specific myeloid populations in the TME. TAMs and MDSCs comprise a majority of myeloid cells in the tumor infiltrate (fig. 1c) and tracer experiments using a tomato reporter (24) showed TAMs and MDSCs exhibit active CRE-mediated excision, whereas only a minority of DCs showed reporter expression (fig. s1c). Thus, we focused on TAMs and MDSCs for further analysis.

We found that both TAMs and MDSCs from LysM–GCN2 cKO mice showed increased expression of inflammatory chemokines and their receptors, cytokines, toll-like receptors, and signal transduction machinery (fig. 2c, tables s1-3). Strikingly, this was also associated with reduced expression of key drivers of immune suppression (e.g. *Ii10*, *Ahr*) and regulatory polarization (e.g. *Chil3*, *Cd163*, *Marco*) in TAMs and *Vegfb*, *Ccl22* and *Retnla* in MDSCs (fig. 2c). Examination of FACS-enriched g- and mMDSC populations showed a module of proinflammatory cytokine and chemokine mRNAs upregulated in a concordant pattern in both populations, indicative of a more proinflammatory phenotype (fig. 2c). IPA analysis of TAM and MDSC RNAseq data predicted that GCN2 deletion altered homeostasis, reducing activity of retinoid X receptor (RXR) and epidermal growth factor signalling and increasing cholesterol biosynthesis (fig. 2d). RXRs are key nuclear receptors controlling the response to cholesterol and influencing immune suppressive function (25). Cumulatively, these data suggest GCN2 is a key driver of the immune suppressive phenotype in TAMs and MDSCs.

To examine the impact of GCN2 loss on the immune transcriptional landscape in the TME, we performed single cell (sc) RNA sequencing of the immune infiltrate from LysM–GCN2 cKO and GCN2^{fl/fl} B16 tumor-bearing mice. Similar to the results from CyTOF analysis (fig. 1c), phenograph analysis of the pooled data from both experimental groups showed that the infiltrate consisted of 11 clusters of cells. Examination of the top 10-expressed genes from each cluster using the ImmGen database (<http://www.immgen.org>) identified 2 clusters of CD8⁺ T cells [hereafter called CD8⁺ T cell (1) and CD8⁺ T cell (2)], B cells, and innate cells, including 2 clusters of TAMs, MDSCs, and conventional (c)DCs, plasmacytoid (p)DCs (fig. 3a, table s4). Interestingly, when we examined the relative population contribution from LysM–GCN2 cKO and GCN2^{fl/fl} B16 tumor-bearing mice, it was observed that the majority of CD8⁺ T cell cluster 1 was derived from the LysM–GCN2 cKO group, while CD8⁺ T cells cluster 2 was almost exclusively from the GCN2^{fl/fl} group (fig. 3b). Similarly, NK and NKT

cells were increased in the LysM–GCN2 cKO group compared to GCN2^{fl/fl} tumor infiltrates while myeloid cells appeared more equally distributed between the two experimental groups with a slight increase in the GCN2^{fl/fl} group (fig. 3b).

Upstream regulator pathway analysis of the two TAM and the MDSC clusters predicted that loss of GCN2 would increase activity of several proinflammatory effector cytokines and chemokines (fig. s3a). Analysis predicted the most upregulated cytokine pathways in the LysM–GCN2 cKO group included TNF α and IL-1 β (fig. s3a). We did not observe differences in *Tnfa* expression in the scRNAseq data set (fig. s3b); however, *Il1b* mRNA abundance was increased in LysM–GCN2 cKO tumors compared to controls (fig. 3c) suggesting a increase in IL-1 β -dependent inflammation may contribute to the observed anti-tumor effect of myeloid GCN2 deletion. To more deeply characterize the MDSC infiltrate, we performed scRNA sequencing on FACS-enriched CD11b⁺GR-1⁺MHCII^{low-neg} MDSCs from tumor-bearing mice. Phenograph and heatmap analysis identified 6 clusters of MDSCs: 2 gMDSC and 4 mMDSC (fig. s4a and fig. s4b). LysM–GCN2 cKO MDSCs were enriched for gMDSC cluster 2 and mMDSC cluster 1 while MDSCs from control tumors were enriched primarily for mMDSC clusters 1 and 4 (fig. s4c). IPA analysis predicted that IL-1 β would be most strongly impacted by the loss of GCN2 in gMDSC cluster 2 (fig. s4d). Accordingly, this cluster showed the highest overall expression of IL-1 β , and loss of GCN2 significantly (Pval 0.000002) increased *Il1b* in gMDSC cluster 2 (fig. s4e) showing that the loss of GCN2 skews the intratumoral MDSC infiltrate favoring a more granulocytic, proinflammatory phenotype.

Since the CD8⁺ T cell clusters segregated transcriptionally based on GCN2 (fig. 3b), we predicted they represent distinct functional states corresponding to the inflammatory environment of the TME. Heatmap analysis of the top 65 genes expressed across both clusters showed that CD8⁺ T cell cluster 2 (derived from the control tumors) had a resting/naïve phenotype with increased expression of T cell developmental factors (*Lef1*, *Tcf7*, *Klf2*, *Socs3*, *Slpr1*) lacking expression of activation or effector mRNAs (fig. 3d, table s5). In contrast, CD8⁺ T cell cluster 1 (derived primarily from the LysM–GCN2 cKO group) showed increased expression of activation and effector mRNAs (*Gzmk*, *Il2rb*, *Ccl5*, *Isg20*) (fig. 3d). In particular, the gene encoding interferon (IFN) γ was one of the most differentially expressed between the two clusters (fig. 3d) and intracellular FACS showed increased IFN γ ⁺ CD8⁺ T cells in LysM–GCN2 cKO tumors (fig. s3c). When we examined *Ifng* in all cells, we found expression was highest in CD8⁺ T cell cluster 1 as expected. We also observed *Ifng* message in NK and NKT cells (fig. 3e). Intracluster comparisons of *Ifng* between LysM–GCN2 cKO and GCN2^{fl/fl} tumor-bearing mice showed no differences within the populations (fig. 3e). However, given the increased presence of these cell populations in LysM–GCN2 cKO tumors (fig. 3b), the data show that myeloid GCN2 function represents a key barrier suppressing the accumulation of IFN γ ⁺ immune cells in the TME.

GCN2 is required for regulatory macrophage polarization and MDSC function

The in vivo data demonstrated that the loss of GCN2 altered TAM and MDSC phenotypes and promoted CD8⁺ T cell acquisition of effector function. However, to gain a better understanding of the cell-intrinsic role of GCN2, we directly tested the impact of GCN2 loss

on polarization and effector activity *in vitro*. We generated bone marrow-derived M ϕ (BMDM) from C57BL6/J (B6) and C57BL6/J. *Gcn2*^{-/-} (B6. *Gcn2*^{-/-}) mice polarized to either a classic inflammatory (iM ϕ) or alternatively activated regulatory (aaM ϕ) state. Superficial loss of GCN2 did not appear to impact polarization as flow cytometry showed that PD-L1, CD86, CX3CR1, MHCI, and MHCII levels were comparable between B6 and B6. *Gcn2*^{-/-} M ϕ (fig. 4a). However, loss of GCN2 enhanced iM ϕ transcriptional characteristics with a increase in expression (fig. 4b) and production (fig. 4c) of proinflammatory proteins. In contrast, aaM ϕ polarization was attenuated by GCN2 deletion with a decrease in *Arg1* and *Ccl22* mRNA and IL-10 protein (fig. 4b and fig. 4d). We tested if deletion of GCN2 would reduce the ability of aaM ϕ to suppress T cell proliferation. When CD8⁺ T cells were added to cognate peptide-pulsed DCs, there was proliferation with the majority of T cells undergoing 3-4 rounds of division after 72h of co-culture (fig. 4e). In contrast, when B6 aaM ϕ were added there was a reduction in proliferation with the majority of the T cells only undergoing one or two rounds of division (fig. 4e). Loss of GCN2 attenuated this effect and we observed that most T cells had undergone 3-4 rounds of division. Thus, the data shows GCN2 is required for the alternatively activated phenotype and plays a direct role in macrophage polarization.

To test the impact of GCN2 on MDSC function, we generated MDSCs from bone marrow by *in vitro* culture with IL-6 and GM-CSF as previously described (26). Deletion of GCN2 in MDSCs impacted the surface phenotype, with a 2-fold increase in CD86 expression, a 40% reduction in PD-L1, and a 30% reduction in IL4R α expression (fig. 5a). Moreover, GCN2 deletion reduced expression of a number of MDSC effectors, including a reduction in *Arg1* expression (fig. 5b) and arginase activity (fig. 5c). Consistent with our *in vivo* data, B6. *Gcn2*^{-/-} MDSCs showed increased IL-1 β message and protein (fig. 5d and fig. 5e), suggestive of a less suppressive phenotype.

C/EBP β is a key transcription factor for MDSC function (26). Since it was reported that GCN2-driven expression of C/EBP β is required for gluconeogenesis in the liver (27), we hypothesized GCN2 would be required for expression of C/EBP β in MDSCs. Analysis by qPCR and immunoblot showed C/EBP β was rapidly induced in MDSC cultures (fig. 5b and fig. 5f). However, *Cebpb* mRNA expression was reduced in the absence of GCN2 correlating with a lack of C/EBP β protein (fig. 5f) indicating GCN2 is required for C/EBP β induction during MDSC differentiation.

MDSCs are defined by the functional ability to inhibit T cell proliferation and maturation (13). Accordingly, addition of control MDSCs to CD8⁺ T cell cognate antigen-pulsed DC co-cultures strongly suppressed T cell proliferation (fig. 5g). In contrast, B6. *Gcn2*^{-/-} MDSCs showed a marked attenuation of their ability to suppress T cell proliferation (fig. 5g). Moreover, when we tested the ability of the T cells to kill target tumor cells, we found that T cells from cultures containing B6 MDSCs exhibited a complete abrogation of killing activity; whereas, T cells collected from cultures containing B6. *Gcn2*^{-/-} MDSCs killed all target tumor cells in the assay (fig. 5h), showing that GCN2 is required for functional maturation of MDSCs.

ATF4 is required for GCN2-dependent phenotype in macrophages and MDSCs

Translational induction of activating transcription factor 4 (ATF4) is a key mechanism mediating downstream effects of GCN2 (9). Thus, we asked if GCN2-dependent effects are contingent on ATF4 induction. We examined the pattern of expression in MDSC cultures and found ATF4 protein was induced within one day of culture initiation, in contrast to B6.*Gcn2*^{-/-} MDSCs that failed to induce ATF4 (fig. 6a). Arginase is a key suppressive effector utilized by MDSCs (13), and we observed a reduction of *Arg1* expression in B6.*Gcn2*^{-/-} MDSC cultures (fig. 5b). We tested whether increased expression of ATF4 would rescue *Arg1* expression and the immune suppressive phenotype in B6.*Gcn2*^{-/-} MDSCs. Introduction of an ATF4 expression vector (ATF4 OE) caused a 3-fold increase in *Arg1* expression in B6 MDSC cultures compared to controls (fig. 6b). Similarly, ATF4 overexpression increased *Arg1* expression 4-fold in B6.*Gcn2*^{-/-} MDSCs (fig. 6b). Importantly, overexpression of ATF4 rescued the ability of B6.*Gcn2*^{-/-} MDSCs to suppress T cell proliferation (fig. 6c) while, inhibiting *Atf4* by siRNA knock down reduced the ability of B6 MDSCs to suppress T cell proliferation by 50% (fig. 6c). These results show GCN2 is required for ATF4 induction in MDSCs and further that ATF4 is a key driver of MDSC immune suppressive activity.

Like MDSCs, deletion of GCN2 in aaMφs reduced ATF4 protein at baseline and after stimulation with LPS (fig. 6d) indicating ATF4 induction and overall expression is dependent on GCN2. When we knocked down ATF4 in macrophages using siRNA, the cytokine expression profile resembled B6.*Gcn2*^{-/-} Mφ, with an increase in expression of the proinflammatory cytokines in iMφ (fig. 6e), while in aaMφ, ATF4 knockdown decreased *Arg1* and *Ccl22* while increasing *Il1b* expression (fig. 6f). This implies that in Mφ, ATF4 induction serves as a feedback mechanism to limit proinflammatory function and promote regulatory polarization.

To gain a better understanding of the transcriptional response driven by ATF4, we investigated genome-wide ATF4-DNA binding in uMφ and aaMφ. For this, we performed chromatin immunoprecipitation-coupled deep sequencing (ChIP-Seq) (28). The analysis identified 91 ATF4-bound genomic sites (FDR < 0.1%) that were enriched in aaMφ versus uMφ (table s6). Moreover, ATF4-bound genomic regions were enriched near transcriptional start sites (TSS), suggesting the primary function is regulation of proximal promoter activity (fig. 6g). Overall, ATF4-bound DNA was low in uMφ compared to aaMφ (histogram, fig. 6h) implying increased function in alternative polarization conditions. Analysis of the transcriptional start sites within 1kb of the enriched regions identified known ATF4 target genes *Asns* and *Trib3* (fig. 6h); moreover, the majority of enriched ATF4 target gene loci were involved in protein synthesis including aminoacyl-tRNA synthetases (*Yars*, *Cars*, *Mars*, *Lars*, *Tars*, *Gars*, *Nars*, *Lars*), initiation factors/modulators of mTOR function (*Ei4ebp1*, *Trib3*, *Ddit4*), metabolic genes (*Pck2*, *Pfkfb*, *Atp9b*, *Ndufa4l2*) and regulators of inflammation (*Map3k3*, *Il4i1*, *Ctsc*) (fig. 6h).

These results imply that GCN2-driven ATF4 activity would impact metabolism. In macrophages, metabolism is closely related to functional polarization, and glycolysis is strongly upregulated in iMφ whereas aaMφ favor utilization of oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) (29). Much less is known about metabolism in

MDSCs but the emerging literature suggests both glycolysis and OXPHOS serve key roles in MDSC expansion and suppressive function (30, 31). When the transcriptomes of wild type TAMs from B16 tumors were examined, we identified expression of autophagy genes (*Atg2a*, *Atg9a*, *Atg101*) and genes involved in OXPHOS and FAO (*Ndufs3*, *ndufs5*, *Atp10d*, *Atp2a3*, *Cox4l2*, *Sdhb*, *Ndufs8*, *Ndufv1*, *Cox7a2l*, *Acss2*, *Acsf2*, *Ppargc11b*) (fig. s5a). However, GCN2 deletion reduced autophagy and OXPHOS/FAO transcripts and increased expression of glycolysis genes suggesting lack of GCN2 may shift M ϕ metabolism to a more glycolytic state (fig. s5a). In agreement with this, measurement of hydrogen ion flux and oxygen consumption by Seahorse showed that B6.*Gcn2*^{-/-} aaM ϕ cultures showed a drop (2-fold) in oxidative respiration compared to control M ϕ (fig. 6i and fig. s5b). In contrast, iM ϕ did not induce OXPHOS as robustly as aaM ϕ , and we did not observe differences between wild type and GCN2^{-/-} macrophage oxygen consumption rates (OCR) (fig. 6i). Gene expression in aaM ϕ cultures showed that loss of GCN2 impacted expression of several genes involved in oxidative respiration but did not impact genes associated with glycolysis (fig. s5c) suggesting GCN2 may drive OXPHOS metabolism in M ϕ . Supporting this observation, knockdown of ATF4 reduced aaM ϕ respiration, but had no impact on glycolysis (fig. 6j). Intratumoral MDSCs showed substantial differences in expression of genes associated with glycolysis and OXPHOS in the absence of GCN2 (fig. s6a), suggesting a shift to glycolytic metabolism. In vitro, B6.*Gcn2*^{-/-} MDSCs were less energetic compared to controls with a large decrease in OCR but minimal changes in glycolysis (i.e. ECAR) (fig. s6b). Cumulatively, the data suggest that GCN2 impacts metabolism in both M ϕ s and MDSCs and may regulate tumoral immunity, at least in part, by influencing metabolic utilization of the myeloid infiltrate.

MDSC function can be driven by ER stress-induced activation of the kinase PERK (32, 33). Since GCN2 and PERK both target eIF2 α (9), we tested if a GCN2-PERK crosstalk existed that could impact GCN2 function in MDSCs by measuring expression of CHOP (encoded by *Gadd153*), a stress protein downstream of both GCN2 and PERK. Loss of GCN2 had no impact on ER stress-driven *Gadd153* expression after treatment with tunicamycin; likewise, loss of PERK did not reduce *Gadd153* expression in the absence of tryptophan (fig. s6c). This suggests PERK and GCN2 function independently in MDSCs to drive an immune-suppressive phenotype.

ATF4 is required for tumor growth in vivo

Since we showed that ATF4 induction is a key functional node downstream of GCN2 that could be inhibited via siRNA interference, we next asked if administration of ATF4 siRNA in vivo would impact macrophage phenotype and tumor growth. We coupled ATF4 siRNA to nanoparticles optimized for in vivo nucleic acid delivery tagged with a fluorescent tracer to follow localization and uptake in vivo. Flow cytometric analysis showed that approximately 20% of the TAMs were positive for the nanoparticles, although there was minimal uptake by T cells or CD45^{neg} stroma (fig. 7a). TAMs that were positive for the siRNA-NPs showed substantial reduction of ATF4 mRNA detectable by qPCR correlating with increased *Ii1b* and *Ii6* mRNA levels (fig. 7b) suggesting knockdown of ATF4 altered TAM polarization in vivo. Strikingly, in mice that received siATF4-NP, tumor growth was inhibited while tumors in the siCTRL-NP treated group grew at the expected rate (fig. 7c). This outcome suggested

that: 1) Targeting a minority of TAMs in the TME is sufficient to impact tumor growth, and 2) that the GCN2-ATF4 circuit is an essential driver of tumor growth that it can be targeted for therapeutically meaningful inhibition of growth in established tumors.

Based on our observation that IL-1 β levels were increased in macrophages and MDSCs lacking GCN2 function, we predicted in myeloid GCN2 KO mice the decrease in tumor size was caused by higher local IL-1 β production. To test this, we treated tumor bearing LysM-GCN2 cKO and *GCN2^{fl/fl}* mice with IL-1 β blocking IgG. In agreement with our prediction, mice receiving IL-1 β blocking antibodies had an increase in tumor growth resulting in tumor size that was similar to control *GCN2^{fl/fl}* mice receiving irrelevant IgG (fig. 7d). This shows IL-1 β is a key driver of tumor-restricting inflammation in the absence of GCN2.

GCN2 activity correlates with human tumor macrophage polarization and outcomes in melanoma

The data presented above strongly supports the notion that GCN2 is a key driver of myeloid function in the TME. Thus we next asked if predictions generated by mouse modeling could be extended to humans. First we generated macrophages sorted from the monocyte fraction of healthy donor blood PBMC (PBM ϕ) (fig. s2b) knocking down either *GCN2* or *ATF4* message by siRNA examining the impact on the transcriptome in inflammatory or alternative regulatory polarization conditions. Similar to mouse, IPA pathway analysis predicted that knocking down either *GCN2* or *ATF4* would increase inflammatory pathway activity with the strongest impact on the IL-1 β pathway (fig. s7a and fig. s7c). In agreement with this, *IL1B* message was increased in the siGCN2 and siATF4 knock down groups in inflammatory (i)PBM ϕ cultures compared to controls (fig. 8a). In contrast, inflammatory effectors pathways not identified the IPA analysis (i.e. *TNFA*) or those predicted to be impacted with a low Z score (i.e. *IL6*) were not increased in the siGCN2 and siATF4 groups (fig. 8a). IPA analysis of alternatively activated (aa)PBM ϕ transcriptomes predicted inhibition of either GCN2 or ATF4 did not increase inflammatory pathway activity (fig. s7b and fig. s7d). Instead, in aaPBM ϕ , attenuation of GCN2-ATF4 signalling was predicted to reduce activity of key immune-regulatory pathways including IL-4, IL-13, and vascular endothelial growth factor (VEGF) (fig. s7b and fig. s7d). Moreover, for both iPBM ϕ and aaPBM ϕ knocking down ATF4 was predicted to negatively impact the EIF2AK4 (i.e. GCN2) pathway further implicating ATF4 in GCN2 function in macrophages. Supporting IPA predictions, siRNA knockdown of GCN2 or ATF4 caused a reduction of the key regulatory mRNAs for *CCL22*, *ARG1*, and *CD206* in a pattern analogous to mice (fig. 8b). Next, we FACS-sorted M ϕ (fig. s2c) from 5 melanoma tumors (TAM ϕ) and performed RNA sequencing comparing TAM ϕ to unpolarized PBM ϕ , which have low GCN2-ATF4 pathway activity. We interrogated the sequencing data using a list of mouse transcripts differentially expressed in TAMs from LysM-GCN2 cKO tumors to identify tumor M ϕ specific expression patterns that required GCN2 activity. By this approach, we were able to identify 51 GCN2-dependent transcripts that were common between human TAM ϕ and murine TAMs (fig. 8c), of these 33 were enriched in TAM ϕ included key drivers of immune suppression (*IL10*, *AHR*, *SOC33*), autophagy (*ATG3*, *ATG5*), and downstream effectors of GCN2-ATF4 (*DDIT3*) (fig. 8c, table s7). Cumulatively, the data indicates GCN2 function in human macrophages is analogous to mice and is required for macrophage polarization programs.

The results above show GCN2 is a crucial driver of human macrophage function required for enactment of transcriptional programs controlling acquisition of effector function in vitro. Based on this, we predicted that GCN2-dependent transcriptional control would impact disease outcomes in cutaneous melanoma. GCN2 is highly expressed in melanoma, however the expression level was not prognostic and there was no indications of increased expression or mutational burden contributing to disease (fig. s8), suggesting that absolute levels of GCN2 or altered function as a result of mutation is not a determinate factor in clinical outcomes. We next took the transcriptional data set from the in vitro knockdown of GCN2 in aaPBMφ identifying 60 genes that were induced (GCN2u; $p < 0.01$ and $\log FC > +0.5$) and 114 that were suppressed by GCN2 knock down (GCN2d; $p < 0.01$ and $\log FC < -0.5$) to generate a transcriptional signature to probe the cancer genome atlas (TCGA) (tables s8 and s9).

Since GCN2 suppresses inflammatory polarization and *IL1B* expression, we reasoned that transcripts enriched by GCN2 inhibition would correlate with inflammatory immune characteristics while transcripts that decreased when GCN2 function was reduced would correlate with immune suppression and a worse prognosis. To test this, we used CIBERSORT-derived estimates of proportions of immune cell subsets in melanoma (34) defined previously in a large pan-cancer analysis (35). Although we projected the GCN2d signature would promote regulatory immune cell accumulation, we did not find association with any immune cell signatures in the TCGA melanoma data sets. In contrast, the GCN2u signature correlated with immune cell phenotype in the tumors (fig. 8d). Positive correlations were identified for inflammatory and activated immune cell signatures including M1 macrophages, CD8⁺ T cells, activated NK cells, dendritic cells, and activated memory T cells (fig. 8d) suggesting GCN2 may actively suppress nascent inflammatory responses in the TME. Based on the CIBERSORT results, we hypothesized that the GCN2u signature would correlate with survival in melanoma. To test this, we analyzed 458 patients for overall survival stratifying them into quartiles based on expression of the GCN2u signature using a stratified Cox proportional hazards model with AJCC pathologic tumor stages as the strata. The patients with the lowest expression of the signature exhibited worse survival times with 50% mortality at 1500 days-post diagnosis while those in the top quartile (i.e. 25% of patients that showed highest expression of the GCN2u signature) had the best survival curves with 50% mortality at 4500 days-post diagnosis (fig. 8e). Thus, the data suggest in human melanoma GCN2 activity may be a negative prognostic factor for anti-tumor immunity and overall survival.

DISCUSSION

Myeloid cells are present at all stages of tumor growth (37), and based on patterns of arginase and IDO expression it would be predicted that GCN2 would be a prominent modulator of myeloid phenotype (38). In this study, we show that GCN2 is required for the suppressive phenotype and function of TAMs and MDSCs. Importantly, loss of GCN2 in the myeloid tumor infiltrate had a profound and complex effect on the overall tumor transcriptional program, growth, and immune infiltrate composition driving inflammation and reduction in tumor growth. These results underscore two key facts: **1**) the importance of myeloid cells in controlling tumor immune landscape, and **2**) that GCN2 is a fundamental

driver of myeloid phenotype in TME. It is hypothesized that GCN2 primarily impacts cellular phenotype by promoting ATF4-dependent expression of genes involved in amino acid biosynthesis, autophagy and cell death (39). Our data agrees with this prediction as we found ATF4 is required for suppressive M ϕ and MDSC identity in vitro. ChIP sequencing analysis of ATF4 binding in M ϕ showed that ATF4 preferentially binds to transcriptional targets in alternatively activated vs. unstimulated M ϕ ; however, the majority of gene targets identified were tRNA charging enzymes or other genes involved in amino acid synthesis and transport, genes of the mTOR pathway, or metabolism. These results are similar to ATF4-promoter interactions reported for other cell types (40) suggesting that GCN2-ATF4 may elicit a transcriptional response that is conserved across cell types. Importantly, the data implies GCN2 does not directly regulate cytokine production but impacts myeloid function by regulating metabolism or overall protein production.

Our data from both mouse and human experiments identified IL-1 β as a key cytokine suppressed by GCN2 activity. Ravindran et al. reported that GCN2-deficient antigen presenting cells (APCs) produced higher levels of IL-1 β with increased pathology in DSS-induced colitis (41). Similarly, we found in LysM-GCN2 cKO TAMs and MDSCs IL-1 β expression was increased. Most importantly, when we treated LysM-GCN2 cKO with anti-IL-1 β antibodies, tumor growth was restored to levels similar to control mice clearly showing that that IL-1 β is a major downstream effector pathway suppressed by GCN2. The role of IL-1 β in cancer progression is controversial. On the one hand IL-1 β signalling and inflammation has been proposed to be an early driver of tumorigenesis and growth (42). In contrast, evidence suggests IL-1 β secretion by pro-inflammatory M ϕ was essential for NK cell activation and tumoricidal activity (43). Moreover, a report by Spalinger et al. showed that myeloid knockout of protein tyrosine phosphatase non-receptor type 2 caused inflammasome activation and IL-1 β production in DSS-induced colitis (44). However, when colorectal tumors were induced in those mice by azoxymethane, tumor growth was reduced compared to controls. This suggests that the role of IL-1 β in tumorigenesis is context dependent, and that enhanced IL-1 β production by myeloid cells may potentiate anti-tumor T cell responses (44).

The CIBERSORT and TCGA correlation suggests the impact of GCN2 on the immune environment is primarily via suppression rather than activation of gene programs. Surprisingly, genes that were dependent on GCN2 for expression did not correlate with immune signatures and had no correlation with survival, in contrast to genes that increased in GCN2 knock down M ϕ . The transcripts suppressed by GCN2 (as shown in s table 9) are not directly involved in immune function per se suggesting that GCN2 may influence immunity in the human TME indirectly via metabolic control. Importantly, TAMs from melanoma patients showed increased expression of a number of transcripts that are impacted by GCN2 function in mice supporting the notion that GCN2 may serve an analogous role in the tumor microenvironment in humans.

GCN2 is downstream of several key immune-regulatory pathways, making it an attractive target for immune modulation therapy. Nevertheless, while our data shows loss of GCN2 function has a major impact on immune function and tumor growth in relatively small, transplantable tumor model systems; it remains to be seen if targeting GCN2 will be a

successful strategy against spontaneous tumorigenesis and more advanced disease. Currently there are no existing drugs that inhibit GCN2 but an inhibitor was recently reported to block GCN2 activity in the low nanomolar range (IC₅₀ of 2.4 nmol/L) (23). This compound had reasonable bioavailability and could target *ASNS^{lo}* AML when used in conjunction with aspariganse (23). This result showed that GCN2 could be targeted therapeutically in vivo. Moreover, as some tumors may rely on GCN2 to adapt to a low nutrient environment (22), GCN2 inhibition could serve a dual role as both an anti-cancer and immuno-oncologic agent. Yet caution must be taken as it is unknown what effects systemic GCN2 inhibition may have on normal physiology. In conclusion, our report shows, that GCN2 is a key driver of Mφ polarization in the TME revealing a key new target for cancer immune therapy.

Study Design

The aim of this study was to determine the impact of GCN2 on myeloid function in the tumor microenvironment. We characterized the effect of myeloid-lineage deletion of GCN2 on tumor growth and immune infiltrates by high dimensional cytometry (CyTOF), qPCR, and RNA sequencing at the bulk and single cell levels in a mouse model of melanoma. To determine the cell intrinsic role of GCN2 in myeloid function we conducted in vitro experiments characterizing the transcriptome by RNA seq, and DNA binding characteristics of its obligate downstream effector ATF4 in macrophage and MDSC cultures. We also characterized GCN2-dependent effects on metabolism by RNA seq, qPCR, and seahorse assay. The number of replicates is indicated in the figure captions. No animals were excluded from the studies.

MATERIALS AND METHODS

Mice

C57BL/6/J (*B6*), B6.Eif2ak4^{tm1.2Dron} (*B6.Gcn2^{-/-}*), B6.Eif2ak3^{flox/flox} (*Perk^{fl/fl}*), LysMCRE^{+/-}, B6.Eif2ak4^{flox/flox} (*Gcn2^{fl/fl}*), B6.SJL-*Ptprca^a Pepc^b*/BoyJ (CD45.1), B6.Cg-*Gt(ROSA)26Sor^{tm9(CAG-tdTomato)Hze/J}*(dtTomato), and C57BL/6j Tg(TcraTcrb)1100Mjb/J (OT1) mice were obtained from colonies maintained under specific pathogen-free conditions in the Princess Margaret Cancer Centre animal facility in accordance with Institutional Animal Care and Use Committee guidelines.

Human melanoma samples

All human tissues were obtained through a protocol approved by the University Health Network institutional review board. Patient information can be found in **supplemental table 11**.

Cell culture

B16F10, MC38, and EL4 cells obtained from ATCC were grown in complete DMEM medium (10% heat-inactivated FBS, 100 mg/mL streptomycin, and 100 mg/mL penicillin, Gibco). For splenocyte cultures and CTL activation assays medium was further supplemented with 0.02 mM 2-Mercaptoethanol (Gibco).

Bone marrow-derived macrophages and MDSCs (mouse) and PBMC-derived macrophages (human) were generated as previously described (26, 45). For further information see supplemental methods.

CTL assay

For lysis target cell preparation ovalbumin-antigen expressing EL4 tumor cells were labeled with 2 μ M CFSE, while the Ova^{neg} EL4 cells were labeled with 0.2 μ M CFSE. These two groups of differentially CFSE labeled target cells were mixed at 1:1 ratio, and added to cultures with activated, purified OTI cells to at an effector: target ratio of 1:1. After 8 hours, cultures were collected and the CFSE positive (high and low) cells were evaluated by flow cytometry. Specific lysis was calculated according to the previously described formula: $[1 - (\text{ratio of CFSE}^{\text{low}}/\text{CFSE}^{\text{high}} \text{ of naïve cells}) / \text{ratio of CFSE}^{\text{low}}/\text{CFSE}^{\text{high}} \text{ of activated cells}] \times 100$ (46).

T Cell Suppression Assays

We used either aaM ϕ or MDSCs sorted out of B16-OVA tumors. CD8⁺ T cells were isolated from OT1^{+/+}Thy1.1^{+/+} mice using the EasySEP mouse CD8⁺ T cell isolation kit (StemCell Technologies cat. # 19853). CD11c dendritic cells (DCs) were isolated from the spleen using anti-CD11c biotin antibody and the EasySEP positive selection kit (Stem Cell technologies, cat. # 18559). DCs were pulsed with OVA₂₅₇₋₂₆₈ peptide (Sigma, S7951) for 6 hrs. T cells were labeled with CFSE at a final concentration of 5 μ M. They were then incubated with OVA-pulsed DCs at a 1:10 ratio in the presence or absence of suppressive myeloid cells (MDSCs or aaM ϕ). T cell proliferation was determined by detecting CFSE dilution on gated CD3⁺CD8⁺Thy1.1⁺ cells by flow cytometry.

Tumor studies

For the tumor models, mice were injected subcutaneously with 2x10⁵ cancer cells and then monitored every other day for tumor growth. For information on nanoparticle-mediated ATF4 knockdown see supplemental methods.

RNA isolation and quantitative RT-PCR

RNA from cells was purified using RNeasy Plus RNA purification kits (Qiagen) and reverse transcribed using Quanta Bio qScript cDNA supermix. For qPCR reaction cDNA was amplified using the PerfeCTa SYBR green Supermix on a CFXConnect real time PCR detection system (Biorad). Results were analyzed using the accompanying software according to manufactures instructions. See **s Table 10** for full primer list).

Tumor Sample Preparation and Flow Cytometry

B16F10 tumors were digested using 100 U/ml collagenase IV and 50 u/ml DNase I in complete RPMI medium at 37C. For CYTOF experiments, the CD45⁺ population was enriched using StemCell EasySEP Biotin positive Selection Kit (Cat # 18559) after staining with anti-mouse CD45.2 biotin antibody (Cat. # 60118BT). The complete antibody list can be found in **supplementary table 10**. For analysis of intracellular cytokine production, cells were incubated with Golgi stop (eBioscience) for 4 to 5 hours and then washed and fixed/

permeabilized with perm/fix buffer (eBioscience). For flow cytometric analysis at least 10^5 events were collected on the LSR Fortessa flow cytometer (BD Biosciences). Data was analyzed by FlowJo (Tree Star, Inc.).

For human melanoma samples, fresh biopsies were minced and incubated in complete RPMI medium the presence of 100 U/ml collagenase IV and 50U/ml Dnase 1. They were incubated in GentleMACS Octo Cell Dissociator (Miltenyi Biotech) at 37°C with gentle dissociation. Media was then added to wash the cells and neutralize the reaction. Samples were then filtered twice, stained and sorted as described below.

For sorting experiments, mouse tumor cells were stained with anti-CD45.2, anti-CD11b and anti-Gr-1 (MDSCs), anti-CD45.2, antiCD11b, anti-Gr-1 and anti-F4/80 (TAMs). For isolation of human melanoma macrophages human anti-CD45, anti-HLA-DR, anti-Lin (CD3, CD19 and CD56), anti-CD14, anti-CD11b, and anti-CD163 mAbs were used on single cell suspensions generated as described above and the cells were sorted on a MoFlo (Beckman Coulter) cell sorter using the gating strategy illustrated in s figure 2c.

Immunoblotting

Western blot was done according to previously published methodology (47). For more information see supplemental methods.

RNA-seq

RNA samples were quantified by qubit (Life Technologies) and an Agilent Bioanalyzer assessed the RNA quality. All samples had RIN above 8. SMART-Seq v4 Ultra Low Input RNA Kit for Sequencing (Clontech #634894) was used per manufacturer's instructions for amplification of RNA and subsequent cDNA synthesis as previously described. For more information see supplemental methods.

RNA-Seq analysis

Raw fastq sequencing reads were aligned against the respective reference genome sequence (GRCm38/mm10 or GRCh37/hg19) using the STAR aligned v2.5.0c (48), discarding all non-uniquely aligned reads. For read counting per annotated gene, we have utilized the STAR function "--quantMode GeneCounts", counting reads matching exons of the Ensembl V75 Genes annotation. Further processing was performed with the R Bioconductor package edgeR v.3.14.0 (49) using non-stranded reads. Reads were normalized for intra- and inter-sample variances using the functions "calcNormFactors" and "estimateTagwiseDisp", resulting in counts-per-million (CPM) for each gene. Differential gene expression analysis was performed with the functions "glmQLFit" and "glmQLFTest", reporting p-value, false-discovery rate (FDR) and log2 fold-changes between any possible pair-wise comparison and gene.

ATAC-seq

To determine the chromatin accessibility, ATAC-seq was performed as previously described (45, 50). For additional information see supplemental methods.

CHIP-Seq

B6 uMΦs or aaMΦs BMDMs were cross-linked using 16% formaldehyde solution and then washed and scraped into PBS. Nuclei were then isolated, digested with micrococcal nuclease and then sonicated to release chromatin material using the SimpleCHIP Enzymatic Chromatin IP kit (Cell Signaling Technology, Cat. # 9003). Immunoprecipitation using ATF4 antibody (D4B8, Cell Signaling Technology Cat. # 11815) or anti-rabbit serum was performed overnight. Immunoprecipitated DNA was then purified and ran on a Bioanalyzer 2100 to check sample size and concentration. Libraries were then prepared using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England Biolabs, Cat. # E7645).

ChIP-Seq analysis

Raw fastq data of ChIP-Seq were processed with the hic-bench pipeline as previously described (51). For information on analysis see supplemental methods.

scRNA-Seq analysis

Tumors from 3 GCN2^{fl/fl} or 3 LysM-CRE cKO mice were digested, pooled and stained with anti CD45.2 antibody (see **s table 10**) and DAPI. Live CD45⁺ cells were FACS-sorted into buffer (PBS + 2% FBS), washed 2x with PBS + 0.04% BSA and then mixed with 10X Genomics Chromium single cell RNA master mix followed by loading onto a 10X Chromium chip according to the manufacturer's protocol to obtain single cell cDNA. Libraries were subsequently prepared and sequenced using the hiSeq 2500 sequencer (Illumina). For further information on scRNA sequencing analysis see supplemental methods.

ELISA

Bone marrow-derived macrophages were treated with either 100 ng/ml LPS alone for 5 hrs (for IL-6 and TNF- α), LPS + 5 mM ATP (for IL-1 β), or 100 ng/ml IL-4 overnight (for IL-10), and then supernatants were collected. ELISAs were performed using kits from Invitrogen (cat. # 88-7324 for TNF- α , cat. # 88-7064-88 for IL-6, cat. # 88-7013-88 for IL-1 β and cat. # 88-7105-88 for IL-10) according to manufacturers instructions. The plates were read using a Biotek plate reader at 450 nm wavelength.

Seahorse Assay

In vitro differentiated macrophages or MDSCs were plated in an Agilent Seahorse XF96 microplate at a density of 50,000-60,000 cells per well. In the case of MDSCs, the wells were pre-coated with 0.1% gelatin to allow cells to adhere to the bottom. Macrophages were either left unstimulated (uMΦs) or treated with 50 ng/ml LPS+ 50 ng/ml IFN γ (iMΦs) or 50 ng/ml IL-4+50 ng/ml IL-13 (aaMΦs) overnight. During that time, the Agilent Seahorse XFe96 cartridge was equilibrated overnight in a CO₂-free incubator according to the manufacturer's instructions.

Time-of-Flight mass cytometry (CyTOF)

Purified unconjugated antibodies were labeled with metal-tags at the SickKids Flow and Mass Cytometry Facility using the MaxPar Antibody Labeling kit from Fluidigm (catalog

#201300). Alternatively, directly conjugated antibodies were purchased from Fluidigm. CD45-enriched tumor single cell suspensions were stained with antibodies that did not perform well after fixation (indicated by * in the CyTOF antibody table) for 5 min at room temperature, washed with PBS and then pulsed with 12.5 μ M Cisplatin (BioVision) in PBS for 1 min prior to quenching with CyTOF staining media (Mg⁺/Ca⁺ HBSS containing 2% FBS (Multicell), 10mM HEPES (Corning), and FBS underlay). Cells were then fixed for 12 min at room temperature with transcription factor fixative (eBioscience, 00-5523-00), permeabilized and individual samples barcoded according to manufactures instructions (Fluidigm 20-Plex Pd Barcoding Kit, 201060), prior to being combined. Pooled samples were then resuspended in staining media containing metal-tagged surface antibodies and Fc block (CD16/32; in house) for 30 min at 4°C. Cells were then stained with metal tagged intracellular antibodies using Transcription Factor Staining Buffer Set (eBioscience, 00-5523-00), according to manufacturer's instructions. Cells were then incubated overnight in PBS (Multicell) containing 0.3% (w/v) saponin, 1.6% (v/v) paraformaldehyde (diluted from 16%; Polysciences Inc) and 1nM Iridium (Fluidigm). Cells were analyzed on a Helios mass cytometer (Fluidigm). EQ Four Element Calibration Beads (Fluidigm) were used to normalize signal intensity over time on CyTOF software version 6.7. FCS files were manually debarcoded and analyzed using Cytobank 6.2. For further information on analysis see supplemental methods.

Statistical analysis

Means, standard deviations, and unpaired Student's t test results were used to analyze the data. Tumor growth was analyzed using two-way ANOVA. TCGA survival was analyzed by Kaplan Meier Curve. When comparing two groups, a P value of 0.05 was considered to be significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. GCN2 is required for immune suppression and tumor growth.

A) B16F10, MC38, and EL4 tumor growth curves in LysM-GCN2 cKO and *GCN2*^{fl/fl} mice. N=7 mice/group. **B)** Quantitative (q) PCR analysis of cytokine mRNA levels in bulk B16 tumors from LysM-GCN2 cKO and *GCN2*^{fl/fl} mice collected on day 17. **C)** B16 tumors of equal size tumors (75 mm³) were collected from mice of the indicated genotype and cytokine message was analyzed by qPCR. **D)** UMAP and phenograph analysis of CD45⁺ infiltrate in B16 tumors showing the major immune subpopulations identified by CYTOF analysis. Data from 4 biological replicates were concatenated in the plots. **E)** Heatmap depicting median signal intensity (MSI) of indicated markers derived from CyTOF. Rows are scaled by Z score. **F)** Heatmap showing MSI of surface markers in TAMs (CD11b

⁺F4/80⁺), CD8⁺, and CD4⁺ T cells respectively. **G)** Plots of percentage CD8⁺ T cells in B16F10 OVA tumors expressing either one or all 4 markers indicated as determined by flow cytometry. *=pval<0.05, **=pval<0.01, ***=pval<0.001, ****=pval<0.0001 as determined by the unpaired Student's t test. For heatmaps each column represents an individual mouse. All experiments were repeated 3 times with similar results.

Figure 2. Increased inflammation signatures in the absence of myeloid GCN2 function.

A) Total B16 tumor transcriptome was analyzed on day 20 tumors by RNA sequencing. Volcano plot shows differential expression comparing LysM-GCN2 cKO or control tumor-bearing mice. Red dotted line marks significance threshold (FDR < 0.01, logFC > 1). **B)** Bar graph of IPA diseases and functions for analysis for tumor samples in (A). A Z score of > 2 was considered significant. **C)** Heatmaps showing differential expression (FDR < 0.01, logFC > 1) of selected pro-inflammatory or regulatory markers in whole tumors or FACS-sorted TAMs (CD11b⁺F4/80⁺), bulk MDSCs (CD11b⁺Gr1⁺MHCII^{neg}), gMDSCs (CD11b⁺Ly6G^{hi}Ly6C^{neg}MHCII^{neg}), or mMDSCs (CD11b⁺Ly6C^{hi}Ly6G^{lo}MHCII^{neg}). **D)** Plot of IPA

canonical pathway analysis comparing TAMs (left) or MDSCs (right) isolated from tumor-bearing mice of the indicated genotype. **= p val<0.01, ***= p val<0.001 as determined by the unpaired Student's t test. For heat maps each column represents an individual mouse. All experiments were repeated 2 times with similar results.

Figure 3. The immune transcriptional landscape in LysM-GCN2 cKO and GCN2^{fl/fl} tumors. Day 20 B16 tumors were collected and the CD45⁺ immune infiltrate was enriched. Then pooled samples of 3 mice/group were analyzed by scRNA sequencing. **A)** Heatmap showing relative expression of 10 most highly expressed genes across each population cluster identified by phenograph analysis. **B)** Overlay tSNE plot (left) and graph (right) showing frequency of cells in each cluster compared to the total population. **C)** Violin plots showing expression of selected cytokines and chemokines in TAM and MDSC clusters. **D)** Heatmap showing most highly expressed transcripts across the CD8⁺ T cell clusters. **E)** tSNE plot showing expression of *Ifng* in all clusters examined in **(B)** (left), total *Ifng* expression in all clusters (middle), and *Ifng* expression in each cluster (right). * = pval < 0.05, ** = pval < 0.01, *** = pval < 0.001, **** = pval < 0.0001 as determined by Seurat single cell analysis.

Figure 4. Cell intrinsic GCN2 is required for macrophage polarization and function.

A) Expression of surface markers in bone marrow-derived M ϕ of the indicated polarization state was determined by flow cytometry. MFI- geometric mean fluorescence intensity. **B)** M ϕ were polarized as described in Methods and mRNA for the transcripts indicated were measured by quantitative rtPCR (uM ϕ -unpolarized M ϕ , iM ϕ -inflammatory M ϕ , aaM ϕ -alternatively activated M ϕ). **C** and **D)** Culture supernatants from M ϕ cultured as described in (A) were tested for the indicated cytokines by ELISA. **E)** aaM ϕ cultures of the genotype indicated were tested for their ability to suppress T cell proliferation. OTI CD8⁺ T cells were labeled with CFSE and proliferation was estimated by CFSE dilution after 3 days of coculture with OVA 257-264 peptide pulsed CD11c⁺ DCs +/- aaM ϕ (1:5 M ϕ :T cell ratio) via FACS analysis. For the pie charts (bottom) n= 3 biological replicates per group.

*=pval<0.05, **=pval<0.01, ***=pval<0.001, ****=pval<0.0001 as determined by the unpaired Student's t test.

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Figure 5. MDSC function is attenuated in the absence of GCN2.

A) Quantitation of immune surface markers on MDSCs as determined by flow cytometry. MFI-geometric mean fluorescence intensity. **B)** Quantitative PCR analysis of indicated transcripts from cultures described in (A). Values are normalized to *Bactin*. **C)** Quantification of arginase activity in MDSC cultures. **D** and **E)** Measurement of IL-1 β expression and protein production by qPCR and ELISA respectively. **F)** Representative western blot showing C/EBP β isoform LAP and LIP expression in fresh bone marrow cells (FBM) and 4 day MDSC cultures. **G)** MDSCs cultures were tested for their ability to suppress T cell proliferation. **H)** Ovalbumin +/- EL4 tumor cells were labeled with CFSE, mixed at 1:1 ratio, and added to purified OTI cells activated in the presence or absence of MDSCs as described in (G). After 8 hours, numbers of CFSE positive (high and low) target cells were evaluated by FACS. *= p val<0.05, **= p val<0.01, ***= p val<0.001,

***=pval<0.0001 as determined by the unpaired Student's t test. Experiments were repeated 4 times with similar results.

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Figure 6. ATF4 is required for acquisition of an immuno-suppressive phenotype in aaMφ and MDSCs.

A) Representative Western blot showing ATF4 protein in MDSC cultures. **B)** *Arg1* quantification by qPCR in MDSCs overexpressing ATF4. **C)** Quantification of T cell suppression using MDSCs transfected with ATF4 siRNA (ATF4 KD) or ATF4 overexpression vector (ATF4 OE). **D)** Representative Western blot showing ATF4 expression levels in aaMφ stimulated with 50 ng/ml LPS. **E)** QPCR for cytokine mRNA expression in unpolarized (u) or inflammatory (i) Mφ transfected with ATF4 siRNA. **F)** Quantitative RT-PCR showing decreased regulatory marker and increased pro-inflammatory mRNA expression in alternatively activated (aa) Mφ transfected with ATF4 siRNA. **G)** Pie chart showing percentage of hits in different chromosomal regions identified by ATF4 ChIP

sequencing analysis. **H)** Comparative heatmap showing regions of ATF4 binding enrichment IN aaMΦs versus uMΦs. **I)** Quantification of glycolysis and oxidative respiration iMΦ and aaMΦ was determined by seahorse assay. ECAR-extracellular acidification rate, OCR-oxygen consumption rate. **J)** Quantification of glycolysis and oxidative respiration in aaMΦ cultures was assessed by seahorse assay. N= 5 biologic replicated/group. **=pval<0.01, ***=pval<0.001, ****=pval<0.0001, ns=not significant as determined by the unpaired Student's t test. All experiments were repeated 4 times with similar results.

Figure 7. ATF4 siRNA nanoparticle knock down decreases B16F10 tumor.

A) Plot showing uptake of AF647 tagged siRNA nanoparticles in tumor macrophages (CD11b⁺F4/80⁺), T cells (CD3⁺), or tumor/stromal cells (CD45^{neg}) was determined by FACS 3 hours after siRNA nanoparticle injection i/v. **B)** Plots showing ATF4, IL-1β and IL-6 mRNA levels of AF647⁺ TAMs was determined by flow cytometry. **C)** Plot showing growth curve of B16F10 tumors in mice treated with siRNA nanoparticles as indicated (left), and plot showing final tumor weights in these mice. **D)** Growth curve (left) and final tumor weight (right) for mice of the indicated genotype +/- administration of anti-IL-1β IgG or irrelevant control IgG as described in Methods. For **c** and **d** n=4 mice/group. * = p < 0.05,

=pval<0.01, *=pval<0.001, ****=pval<0.0001 as determined by the unpaired Student's t test. Experiments were repeated 3 times with similar results.

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Figure 8. GCN2 controls human macrophage polarization and influences tumor immune signatures and survival outcomes in melanoma.

A) Quantitative RT-PCR analysis of pro-inflammatory markers in unpolarized (uPBMφs) or inflammatory polarized (iPBMφs) PBMC-derived Mφ transfected with control, GCN2 or ATF4 siRNA respectively. **B)** Quantitative rtPCR analysis of regulatory markers in unpolarized or alternatively activated (aaPBMφs) PBMC-derived Mφ as described in (A). **C)** Heatmap showing selected differentially expressed genes in TAMs enriched by FACS from melanoma tumors (N=5) compared to uPBMφ. Each column represents a separate tumor/donor. **D)** Correlation between GCN2u gene signature and immune cell infiltrates using CIBERSORT analysis of TCGA melanoma datasets. **E)** Survival curves of melanoma

patients based on tiered expression levels of GCN2u gene signature show positive survival outcome is correlated with higher GCN2u gene expression.

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