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Tumor immune evasion arises through loss of TNF sensitivity

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Immunotherapy has revolutionized outcomes for cancer patients, but the mechanisms of resistance remain poorly defined. We used a series of whole-genome clustered regularly interspaced short palindromic repeat (CRISPR)-based screens performed in vitro and in vivo to identify mechanisms of tumor immune evasion from cytotoxic lymphocytes [CD8+ T cells and natural killer (NK) cells]. Deletion of key genes within the tumor necrosis factor (TNF) signaling, interferon-γ (IFN-γ) signaling, and antigen presentation pathways provided protection of tumor cells from CD8+ T cell–mediated killing and blunted antitumor immune responses in vivo. Deletion of a number of genes in the TNF pathway also emerged as the key mechanism of immune evasion from primary NK cells. Our screens also identified that the metabolic protein 2-aminoethanethiol dioxygenase (Ado) modulates sensitivity to TNF-mediated killing by cytotoxic lymphocytes and is required for optimal control of tumors in vivo. Remarkably, we found that tumors delete the same genes when exposed to perforin-deficient CD8+ T cells, demonstrating that the dominant immune evasion strategy used by tumor cells is acquired resistance to T cell–derived cytokine-mediated antitumor effects. We demonstrate that TNF-mediated bystander killing is a potent T cell effector mechanism capable of killing antigen-negative tumors. In addition to highlighting the importance of TNF in CD8+ T cell– and NK cell–mediated killing of tumor cells, our study also provides a comprehensive picture of the roles of the TNF, IFN, and antigen presentation pathways in immune-mediated tumor surveillance.

INTRODUCTION

Cancer immunotherapies, such as checkpoint blockade, have had remarkable success in the clinic; however, acquired resistance often develops through mechanisms that are not well defined (1–3). Neoantigens presented through major histocompatibility complex (MHC)–I facilitated detection of tumor cells by cytotoxic CD8+ T cells and underpin the success of immune checkpoint blockade therapy (4–7). Thus, disruption of antigen presentation is a key mechanism of tumor immune evasion, demonstrated by loss-of-function mutations in the genes encoding β2-microglobulin (B2M), interferon-γ (IFN-γ) and Janus kinases (JAK1/2) in patients that fail to respond to immunotherapy (8–10).

Cytotoxic lymphocytes, such as CD8+ T cells and natural killer (NK) cells, promote antitumor immunity through a combination of direct perforin–dependent tumor cell killing and by increasing “tumor immune sensitivity” through the release of inflammatory cytokines such as IFN-γ and tumor necrosis factor (TNF), which act on both tumor and immune effector cells (11–13). However, the relative contribution of these additional mechanisms to tumor cell killing by cytotoxic lymphocytes is still unclear. Clustered regularly interspaced short palindromic repeat (CRISPR)–based loss-of-function screens are a powerful approach to identify genes that sensitize to, or protect tumors from, T cell– and NK cell–driven antitumor immunity. Here, we have used a series of whole-genome CRISPR-based screens to identify the genes and pathways that confer resistance to both CD8+ T cell– and NK cell–mediated killing. We found that suppression of cytokine signaling and antigen presentation are key mechanisms by which tumors evade attack by cytotoxic lymphocytes. In particular, we found that disruption of TNF-induced tumor cell death by CD8+ T cells was a major immune evasion mechanism.

RESULTS

Immune evasion occurs through loss of TNF, IFN-γ, or antigen presentation pathways

To uncover, in an unbiased manner, the genes and signaling pathways that regulate sensitivity to T cell–mediated attack, we first performed genome-wide CRISPR/Cas9 screening in vitro using two tumor lines—MC38 colon adenocarcinoma and B16 melanoma cells, both of which are responsive to IFN-γ, engineered to present the chicken ovalbumin (Ova) antigen on H-2Kb (MC38Ova, B16Ova), and recognized and killed by transgenic OT-I CD8+ T cells (fig. S1A). The expression of key molecules on the T cells and tumor cells was serially incubated with activated OT-I T cells, and the resulting population was sequenced to identify single-guide RNAs (sgRNAs) that provided resistance to T cell–mediated killing (Fig. 1A). In this model, anti–PD-1 (programmed death 1) treatment enhanced both

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Fig. 1. Immune evasion occurs through loss of TNF, IFN-γ, or antigen presentation pathways. (A) Experimental design of CRISPR/Cas9 screening. (B) OT-I T cell–MC38Ova killing and TNF secretion assays in the presence or absence of a neutralizing α-PD-1 antibody (50 μg/ml). (C) MC38Ova cells were subjected to three rounds of exposure to OT-I T cells, as described in (A), followed by sequencing for the top enriched genes. (D) The screen from (C) was carried out in parallel, in the presence of α-PD-1 (50 μg/ml), followed by sequencing for the top enriched genes. (E) Comparison of top scoring genes in screens described in (C) and (D). (F) Normalized sgRNA counts from the screen described in (C). unt, untreated. (G) Validation of screen hits using the indicated individual sgRNAs in MC38Ova upon an 18-hour exposure to OT-I T cells. (H) Protein network analysis of the screen hits from (D). (I) GO term analysis from the screen hits from (D). (J) The screen described in (A) was performed using B16Ova cells. After three rounds of exposure to OT-I T cells, the cells were sequenced for the top enriched genes. WT, wild type. (K) Protein network analysis from the top hits identified in (J). (L) GO term analysis from the top hits identified in (J). (M) The screen described in (A) was performed using MDA-MB-231 cells. After three rounds of exposure to HER2-directed human CAR T cells, the cells were sequenced for the top enriched genes.
OT-I T cell killing and cytokine production after recognition of MC38Ova cells (Fig. 1B). Parallel screens were conducted with the addition of an anti–PD-1 antibody to identify genes that confer resistance to checkpoint blockade therapy. Under these screening conditions, the surviving tumor cells were almost completely refractory to the increased cell death conferred by anti–PD-1 (fig. S1E).

Deletion of genes from three principal pathways—antigen presentation, IFN-γ signaling, and TNF signaling—provided protection from T cell killing (Fig. 1, C to F). Identification of Jak1, B2m, Ifngr1, and Tap1, key genes in antigen presentation and IFN-γ signaling, and recently identified in similar screens (14, 15), validated our approach. We also uncovered genes, such as Casp8 and Tnfrsf1a, as crucial T cell effectors using our genome-wide screening approach (Fig. 1, C to F). Other less well-characterized genes, such as Sactm1, Gosr1, and Ado, were also identified (Fig. 1, C to E). Subsequent depletion of selected genes from each pathway using two independent sgRNAs (fig. S2A) confirmed that they provided protection from T cell killing (Fig. 1G) without affecting MHC-I expression (Fig. 1H). Nonbiased gene interaction analysis using the top hits confirmed that the dominant immune evasion genes clustered in the TNF, IFN-γ, and antigen presentation pathways (Fig. 1I), and Gene Ontology (GO) term analysis classified antigen presentation, death receptor–mediated killing, and cytokine signaling as the key pathways affected (Fig. 1I).

Consistent with the screen carried out in MC38Ova cells, we identified Ifngr1/2, Jak1/2, and Stat1 as potent mediators of T cell killing using the B16Ova melanoma cell line (Fig. 1J), with enrichment of the IFN-γ and antigen presentation pathways but not TNF signaling (Fig. 1, K and L, and fig. S2C). To test a model system where antigen presentation is not required for T cell killing of target cells, we performed a genome-wide CRISPR screen using HER2-directed Cas9 knockins as crucial T cell effectors using our genome-wide screening approach (Fig. 1, C to F). We also uncovered genes, such as Casp8 and Tnfrsf1a, as crucial T cell effectors using our genome-wide screening approach (Fig. 1, C to F). Other less well-characterized genes, such as Sactm1, Gosr1, and Ado, were also identified (Fig. 1, C to E). Subsequent depletion of selected genes from each pathway using two independent sgRNAs (fig. S2A) confirmed that they provided protection from T cell killing (Fig. 1G) without affecting MHC-I expression (Fig. 1H). Nonbiased gene interaction analysis using the top hits confirmed that the dominant immune evasion genes clustered in the TNF, IFN-γ, and antigen presentation pathways (Fig. 1I), and Gene Ontology (GO) term analysis classified antigen presentation, death receptor–mediated killing, and cytokine signaling as the key pathways affected (Fig. 1I).

NK cell immune evasion also occurs through loss of TNF pathway members

NK cells also recognize and kill tumor cells through specific receptor-ligand interactions, without the need for neoantigen presentation (16). We therefore performed a whole-genome CRISPR screen using primary mouse NK cells and MC38 tumor cells as target cells to identify genes that confer resistance to immune-mediated killing in the absence of the need for antigen presentation (Fig. 2A). NK cells efficiently killed MC38 target cells and produced substantial amounts of both IFN-γ and TNF (Fig. 2, B and C). As seen in our immune evasion CRISPR screens using CD8+ T cells, deletion of genes involved in TNF-mediated cell death was also the predominant mechanism of immune evasion against NK cell–mediated killing (Fig. 2, D to F). These data demonstrated that well-characterized genes in this pathway, such as Casp8 and Tnfrsf1a, and previously poorly characterized genes, such as Ado, were crucial for both CD8+ T cell and NK cell antitumor effector function using two unbiased genome-wide CRISPR screens (Fig. 2G). This suggested that TNF derived from CD8+ T cells and NK cells upon target recognition plays an important role in mediating tumor cell death by these cells (Fig. 2H).

Immune evasion occurs independent of perforin-mediated killing

Notable in our results was that genes involved in direct CD8+ T and NK cell killing by the perforin-mediated granule-exocytosis pathway were not identified in any of our immune evasion screens. We subsequently repeated the screen using perforin knockout (Prf1−/−) OT-I T cells, which efficiently killed MC38Ova and B16Ova cells in the presence of antigen over 18 hours (Fig. 3A and fig. S1A). sgRNAs targeting the TNF signaling molecules Tradd, Casp8, and Fadd were heavily enriched using MC38Ova tumor cells as target cells and Prf1−/− OT-I T cells as effector cells (Fig. 3, B and H). Consistent with the data in Fig. 1, in a CRISPR screen using B16Ova as target cells and Prf1−/− OT-I T cells as effectors, sgRNAs targeting Ifngr1/2, Jak1/2, and Stat1 were also enriched (Fig. 1, C and K). In the absence of perforin-mediated tumor cell killing, Tnfrsf1a was revealed as a common T cell sensitivity gene between the two tumor types (Fig. 3, D and E), suggesting that, in the absence of perforin, the TNF pathway was engaged to kill B16 cells. Pathway interaction and GO term analysis confirmed that both TNF and IFN-γ signaling were the key T cell–mediated effector pathways affected (Fig. 3, F, G, I, and J).

T cell–derived cytokines drive immune evasion

Given the identification of cytokine networks in our tumor immune evasion screens, we performed 3’ RNA sequencing on MC38Ova cells exposed to T cells in the presence or absence of anti–PD-1, which resulted in robust changes in gene transcription (Fig. 4A). Gene set enrichment analysis (GSEA) identified preferential regulation of the genes that respond to TNF and IFN-γ signaling (Fig. 4, B and C). Incubation of MC38 cells with supernatant from the T cell–MC8Ova coculture (Fig. 4D) or recombinant TNF and/or IFN-γ (fig. S3) elicited a similar transcriptional signature to that identified after direct effector–target engagement. Furthermore, killing of MC38Ova and the breast tumor line E0771Ova using Prf1−/− OT-I T cells and coculture supernatant was abrogated in the presence of anti-TNF blocking antibody (Fig. 4E and fig. S4A) but not an anti-Fasl blocking antibody (fig. S4B). Finally, recombinant TNF, but not IFN-γ, was shown to directly kill MC38Ova and E0771Ova tumor cells (Fig. 4E and fig. S4A).

We next introduced a customized sgRNA pool, encompassing the top scoring 2000 guides identified in Fig. 1, into Cas9-expressing MC38Ova cells, which were then exposed to three independent rounds of TNF treatment. As expected, and consistent with our CRISPR screen using wild-type and Prf1−/− T cells as immune effectors, this screen also enriched for genes known to modulate TNF-induced cell death, including casp8, tradd, and tnfra, as well as uncharacterized genes in the TNF pathway, such as Ado (Fig. 4, F and G, and fig. S4C). We also sorted for cells that failed to up-regulate MHC-I upon IFN treatment. This revealed that the Jak1/2, Stat1, Tap1, and movie S2). TNF-mediated bystander killing was also confirmed in killing assays using chromium-labeled MC38Ova cells mixed in a 50:50 ratio with unlabeled MC38Ova cells, using perforin wild-type or
Fig. 2. NK cell immune evasion occurs through loss of TNF pathway members. (A) Experimental design of CRISPR/Cas9 screening for resistance to NK cell killing. (B) NK cell-MC38 killing assay (18 hours) at the indicated E:T ratios. (C) NK cells were cocultured with MC38 cells at the indicated E:T ratios. After 6 hours, cytokines were measured by cytometric bead array. (D) MC38 cells were subjected to three rounds of exposure to primary mouse NK cells, as described in (A), followed by sequencing for the top enriched genes. (E) Protein network analysis from the top hits identified in (D). (F) GO term analysis from the top hits identified in (D). TRAIL, TNF-related apoptosis-inducing ligand. (G) Comparison of the top scoring genes in screens described in (D) and the OT-I T cell screen described in Fig. 1C. (H) Schematic representation of immune evasion from NK attack. DISC, death-inducing signaling complex.
Fig. 3. Immune evasion occurs independently of perforin-mediated killing. (A) MC38 Ova/Vector chromium release assay (18 hours) at the indicated E:T ratios with Prf1−/− OT-I T cells. B16 Ova/Vector coculture (48 hours) with Prf1−/− OT-I T cells (2:1 E:T ratio). DAPI, 4′,6-diamidino-2-phenylindole. (B) MC38 Ova screen as in Fig. 1A, but using Prf1−/− OT-I T cells. (C) B16 Ova screen, as in Fig. 1J, but using Prf1−/− OT-I T cells. (D) Comparison of top enriched genes in screens described in (B) and (C). (E) Selected sgRNA counts from (B) and (C). (F) Protein network analysis of the top scoring genes from (B). (G) GO term analysis from the top scoring genes from (B). (H) Comparison of top enriched genes in screens described in Figs. 1C and 2C. KO, knockout. (I) Protein network analysis of the top scoring genes from (B). (J) GO term analysis of the top scoring genes from (B). (K) Comparison of top enriched genes in screens described in Fig. 1J and (C).
Fig. 4. T cell–derived cytokines drive immune evasion. (A) RNA-seq (triplicate samples) of MC38^Ova^ cells that were cocultured with OT-I T cells for 6 hours. (B) Volcano plot of top regulated genes from (A). (C) GSEA from (A). (D) MC38^Ova^ cells were treated with supernatant (6 hours) derived from an overnight MC38^Ova^ coculture with OT-I T cells (1:1 E:T ratio) followed by 3′ RNA-seq. GSEA is displayed. (E) MC38^Ova^ killing assay with Prf1^−/−^ OT-I T cells ± α-TNF or α–IFN-γ (25). MC38^Ova^ killing assay using supernatant from an MC38^Ova^/vec coculture with OT-I T cells ± α-TNF or α–IFN-γ (25 μg/ml). MC38^Ova^ killing assay upon TNF or IFN-γ treatment at the indicated concentrations for 18 hours. (F) Custom sgRNA pool screen in MC38^Ova^ cells. Cells were treated with TNF (1 ng/ml) three consecutive times. (G) Comparison of the top scoring genes from (F) to the OT-I T cell screen described in Fig. 1C. (H and I) MC38 and B16 cells carrying the custom gRNA library were treated with IFN-γ overnight and then cell-sorted for MHC-I negatives (repeated three times). Cells were then sequenced for gRNA enrichment. (J) Selected gRNAs from the MC38 screen in (H).
OT-I T cells were then overlaid, and killing was monitored by time-lapse microscopy. Percentage of MC38Vec PI-positive cells that occurred in the presence or absence of T cells ± α-TNF. Pooled experimental data, n = 3; *P < 0.05, Student's t test. (B) MC38Vec cells were labeled with chromium and then mixed (50:50) with unlabeled MC38Ova ± α-TNF (50 μg/ml). Prf1+/+ or Prf1−/− OT-I T cells were then added for 18 hours. *P < 0.05, Student's t test. (C) MC38Ova cells (1 × 10⁵) were injected subcutaneously into Prf1 knockout mice. Mice were treated with anti–PD-1 (200 μg) twice per week for the time span indicated. Tumor growth and overall survival are displayed.

Fig. 5. T cell–derived TNF kills tumor cells in a bystander effect. (A) MC38Ova cells were labeled with CFSE and MC38Vec with Cell Trace Violet and then mixed (50:50) in the presence or absence of neutralizing α-TNF (50 μg/ml). OT-I T cells were then overlaid, and killing was monitored by time-lapse microscopy. Percentage of MC38Vec PI-positive cells that occurred in the presence or absence of T cells ± α-TNF. Pooled experimental data, n = 3; *P < 0.05, Student's t test. (B) MC38Vec cells were labeled with chromium and then mixed (50:50) with unlabeled MC38Ova ± α-TNF (50 μg/ml). Prf1+/+ or Prf1−/− OT-I T cells were then added for 18 hours. *P < 0.05, Student's t test. (C) MC38Ova cells (1 × 10⁵) were injected subcutaneously into Prf1 knockout mice. Mice were treated with anti–PD-1 (200 μg) twice per week for the time span indicated. Tumor growth and overall survival are displayed.

Immune evasion occurs through loss of TNF signaling, IFN-γ signaling, or antigen presentation in vivo

To confirm our findings in vivo, Cas9-expressing MC38Ova cells were transduced with the customized 2000-sgRNA pool used in Fig. 4 (F and G), injected into NSG mice, and tumor-bearing mice were adoptively transplanted with OT-I T cells. Sequencing of tumors harvested 4 to 7 days after OT-I T cell transfer revealed enrichment in sgRNAs targeting genes involved in IFN-γ signaling (stat1), antigen presentation (tap1), and TNF signaling (caspase-8, tnfrsf1a, and ado) (Fig. 6, A and B, and fig. S5A). To validate these findings, we individually depleted an immune evasion gene from each pathway in MC38Ova cells and transplanted these into NSG mice that subsequently received adoptively transferred OT-I T cells. The proliferation in vitro and growth in vivo of these tumor lines were not significantly different (fig. S5B). However, depletion of tap1, tnfrsf1a, or jak1 significantly reduced the efficacy of adoptive cellular therapy (Fig. 6C), highlighting the importance of these three pathways in tumor immune evasion in vivo (Fig. 6D). We identified that low expression of at least two genes from each of these three pathways was associated with significantly poorer prognosis in patients with colorectal cancer (Fig. 6E).

Because we had identified Ado as important for tumor cell sensitivity to death induced by CD8+ T and NK cells, using both our in vitro and in vivo CRISPR-screening (Figs. 1E, 2D, 3H, 4G, and 6B), and because our targeted screen suggested that Ado modulates TNF sensitivity (Fig. 4F) but not IFN-γ signaling pathways (Fig. 4I), we investigated this gene further. Ado plays a role in the cysteine metabolism pathway, where it converts cysteamine to hypotaurine (17). To investigate the potential role of Ado in regulating the metabolic state of MC38 tumor cells, we first performed comprehensive metabolomic profiling of Ado control and knockout MC38 tumor cells (Fig. 7). We identified disrupted cysteine metabolism and polyamine synthesis pathways upon Ado depletion. We next performed transcriptional profiling of Ado control and knockout MC38 tumor cells, under both steady state and TNF stimulation, to investigate altered gene expression under steady-state Ado loss (Fig. 8A). The rapid induction of classical TNF-induced target genes was not affected by Ado depletion, suggesting that Ado was not directly involved in TNF-induced signal transduction (Fig. 8B). However, depletion of Ado in MC38Ova cells using two independent sgRNAs significantly impaired killing by OT-I T cells (Fig. 8C) and significantly reduced TNF-induced killing of MC38Ova cells (Fig. 8D and fig. S6A) but not granzyme B–induced killing (Fig. 8B). Depletion of Ado in two additional tumor lines, the mouse breast line EO771 and the human breast line AU565, also conferred decreased sensitivity to TNF-mediated cell death (figs. S6C and S7D). To determine whether deletion of Ado suppressed T cell killing in vivo, we injected Ado-depleted MC38Ova cells into NSG mice and monitored the efficacy of adoptive T cellular therapy. Ado-depleted knockdown effector OT-I T cells (Fig. 5B). Thus, upon antigen recognition, CD8+ T cells can efficiently kill antigen-negative bystander tumor cells through TNF. In support of this, we found that anti–PD-1 therapy also enhanced antigen immunity in perforin-deficient mice in vivo (Fig. 5C), an effect most likely due to enhanced TNF production upon checkpoint blockade, and enhanced TNF bystander killing in the absence of perforin.
tumors were significantly protected from the anti-tumor activity of T cells in vivo (Fig. 8E). Depletion of another uncharacterized molecule, Golgi SNAP receptor complex member 1 (Gosr1), also impaired killing of MC38Ova cells by OT-I T cells (fig. S7A) and impaired tumor control in vivo (fig. S7B). Reduced expression of Gosr1 was also found to be associated with poor prognosis in a number of cancer types (fig. S7C). These data confirmed that deletion of Ado or Gosr1 protected tumor cells from T cell killing, suppressed antitumor immune responses in an in vivo tumor model, and is associated with poor patient prognosis in different cancer types.

DISCUSSION

Using a series of whole-genome and customized CRISPR screens, we identified TNF, IFN-γ, and antigen presentation as the major pathways necessary for, and used by, CD8+ T cells to kill tumor cells in vitro and in vivo. Our study also found that TNF was a potent NK cell effector molecule and that the TNF-mediated apoptotic pathway was engaged to kill tumors in the context of NK cell attack. We did not find a role for IFN-γ in the context of NK cell–driven tumor immune evasion. These results suggest that tumor cells dampen the effects of IFN-γ signaling predominantly as a strategy to limit presentation of antigen.
to CD8+ T cells via MHC-I, not to minimize the potential cytostatic effects of this cytokine (18). Consistent with this, evasion from CAR T cell–mediated killing of tumor cells was mediated by selective loss of the CAR T cell target molecule (Her2) and not through any effects on the IFN-γ pathway. Thus, tumor cell suppression of these pathways may result in potent immune evasion and highlights the importance of cytokines in driving antitumor immunity.

Using similar genetic approaches, recent studies also identified genes within the IFN-γ and antigen presentation pathways as key immune evasion genes (14, 15). However, these genes were identified using a tumor cell line with preexisting resistance to TNF-induced cell death; thus, the global immune evasion network was not fully appreciated. Cell line selection for genome-wide screens is critical, because cell lines diverge in their sensitivity to different aspects of immune pressure. Here, we selected a variety of tumor cell lines for our immune-based screens to allow for a more comprehensive analysis of the pathways that are necessary for robust antitumor immune responses. We posit that our screens in B16 melanoma cells and MC38 colon cancer cells have provided extensive, but certainly not exhaustive, insight into immune evasion mechanisms. CRISPR screening itself is a powerful, nonbiased method for identifying genes that play an important role in a biological process. Here, we have used immune pressure on tumor cells to identify genes that, when deleted, provide resistance to immune cell killing. Although it is known that tumor cells frequently suppress expression of particular genes as an immune escape mechanism, including MHC-I, they often mutate key genes such as tp53 to provide a growth advantage. Gene mutations that reduce the efficacy of immunotherapy have recently been identified, including mutations in Janus kinases (JAK) providing resistance to anti–PD-1 therapy (9). Thus, a limitation of CRISPR screening that may arise as the effect of gene deletion, not genetic mutation, is investigated. However, because we detected JAK1/2 as key immune evasion genes in our screens, the phenotype that arises through CRISPR-based deletions can mimic loss-of-function mutations that arise in cancer patients. As previously reported (14, 15), our screens identified the IFN-γ and antigen presentation pathways, in addition to the TNF pathway, as important for antitumor immunity. The presence of genetic alterations in each of these pathways in cancer patients with poor prognosis (9, 19) provides confidence in the physiological significance of our discoveries. However, we recognize that other proteins and pathways important for immune cell–mediated tumor surveillance very likely exist and await further discovery and analysis.

Our screening approach revealed a somewhat unappreciated role for the TNF pathway, and bystander TNF-mediated cell death, in antitumor immunity. Cytotoxic lymphocytes use a variety of effector mechanisms to control tumors, including direct target cell killing via synapse-dependent granzyme delivery (20). However, our
Fig. 8. Ado loss drives TNF-dependent immune evasion. (A and B) Control or Ado knockout MC38 cells were left untreated or treated with TNF (10 ng/ml) for 1 or 6 hours, followed by 3′ RNA-seq. The top regulated genes are displayed with comparisons of control gRNA and Ado knockout (two individual gRNAs for each, performed in duplicate). Representative box plots for the top three TNF-induced genes are displayed. (C) Ado was targeted in MC38 Ova with two individual sgRNAs. Prf1+/+ OT-I T cells were then added, and cell death was measured at 18 hours. Data are pooled from three independent experiments. (D) Cells from (C) were treated with TNF (1 ng/ml). At 18 hours, cell death was measured by PI uptake. Data are representative of three independent experiments. (E) Control or Ado-depleted MC38Ova cells were injected subcutaneously into NSG mice. On day 12, and again on day 16, 5 × 10^6 Prf1+/+ OT-I T cells were adoptively transferred. (F) Schematic representation of bystander immune evasion. *P < 0.05, Student’s t test. (G) TCGA data analyses of Ado expression and disease-free survival for different cancer types.
results suggest that this is not a process disengaged by tumors to avoid T cell– and NK cell–mediated death. Instead, we propose a model whereby the cytotoxic synapse with a tumor cell triggers TNF and IFN-γ production from the T or NK cell (21, 22). If the tumor is intrinsically TNF-sensitive, then TNF will initiate apoptosis of surrounding tumor cells in a bystander effect (22–25), which may be a more effective way to kill a larger tumor cell mass. However, as a consequence, tumor cells may acquire resistance to bystander T or NK cell attack, having never been the subject of direct recognition through the cytotoxic synapse (9, 10, 22, 26). Many tumor cell lines are intrinsically resistant to TNF treatment in vitro, but the molecular mechanism that determines this sensitivity threshold is not fully understood. The resistance to TNF-mediated killing may be due to the relative expression levels of TNF receptor–associated anti-apoptotic proteins such as cFLIP, cIAPs, and caspase-8 (27–29). Our screens revealed that loss of caspase-8 was the most frequently targeted TNF receptor complex component that drives immune eva- sion. Caspase-8 is frequently lost or functionally mutated in several tumor types (19, 30, 31) and represents an attractive protein for tumor cells to target, because loss of its enzymatic activity entirely eliminates the apoptotic potential of TNF, but not the nuclear factor κB (NFκB) activation signal, which can promote cell proliferation and survival (27).

Our study also identified a number of genes not previously implicated in antitumor immunity or resistance to immunotherapy. These included Gosr1, a trafficking membrane protein that transports proteins among the endoplasmic reticulum and Golgi apparatus (32). Our results demonstrated that loss of Gosr1 facilitates resistance to T cell–mediated tumor control both in vitro and in vivo. Although our study did not address the mechanism behind this, Gosr1 was identified as a prognostic marker in a number of cancer types and highlights the power of unbiased screens to identify new markers of resistance to antitumor immunity and immunotherapy. One gene encoding an uncharacterized protein that scored highly in our screens was Ado. Deletion of Ado provided protection against TNF-mediated cell death in a number of tumor lines and provided resistance to immune-mediated tumor control in vivo. Depletion of Ado did not affect transcriptional responses to TNF but did result in disrupted cysteine metabolism and polyamine synthesis pathways. Polyamine pathway metabolites can modulate sensitivity to TNF-induced cell death (33), and this may provide a functional link between deletion of Ado and immune evasion through loss of TNF-mediated cell death, although this remains to be formally demonstrated in our experimental systems. This finding highlights the potentially underappreciated role of cysteine metabolism in TNF biology. Further studies will be required to determine exactly how loss of Ado inhibits TNF-induced apoptosis through deregulated cellular metabolism and how this affects tumor cell survival. We also found that deletion of two other genes encoding proteins associated with TNF signaling and cell death, sequestosome-1 (Sqstm1) (34) and spermatogenesis-associated protein 2 (Spata2) (35, 36), resulted in resistance to T cell– or NK cell–mediated killing. Spata2, in particular, was identified in a number of recent studies as a key molecule required for TNF-induced, receptor-interacting protein kinase 1 (RIPK1)–dependent apoptosis (36). In addition to direct inhibition of TNF-mediated apoptosis through death receptor signal- ing, these findings provide an additional pathway for tumors to target to evade cell death mediated by cytotoxic lymphocytes.

Our study, and those previously reported (14, 15), identified the IFN-γ and antigen presentation pathways as important for antitumor immunity, but our additional finding that the TNF pathway is targeted as an immune evasion mechanism may be important therapeutically. Agents that sensitize tumor cells to TNF-induced apoptosis, such as smac mimetics, are currently undergoing clinical trials for a variety of solid and hematological cancers (37, 38). Thus, combining immunotherapies, such as anti–PD-1 (which enhances T cell TNF production), with smac mimetics may yield potent antitumor responses (11, 39). Similarly, using smac mimetics to increase the cytotoxic potential of adoptive cellular therapies is an exciting possibility. Understanding the major effector pathways used by cytotoxic lymphocytes through functional screens, such as those presented here, is critical for the generation of future targeted immune-based therapies.

**MATERIALS AND METHODS**

**Study design**

All animal studies were conducted under a protocol approved by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee and according to their guidelines.

**Antibodies and reagents**

Neutralizing antibodies used were as follows: anti–TNF (BioLegend) clone MP6-XT22, anti–PD-1 clone RMP1-14, and anti–IFN-γ clone R4-6A2 (BioXCell). Recombinant TNF and IFN-γ were from PeproTech.

**Cells and cell lines**

The cell lines MC38 and B16, and derivatives expressing chicken Ova, were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin (Gibco) and incubated at 37°C in 5% CO2. OT-I T cells from wild-type or perforin-deficient (Prf1−/−) mice were activated from spleens with the chicken Ova peptide SIINFEKL. Activated T cells were used on days 5 to 10 and had a typical effector phenotype (CD8+CD69+CD25−CD62L−CD44+). OT-I T cells and NK cells were cultured in RPMI supplemented with 10% FCS, 1-glutamine, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, Hepes, β2-mercaptoethanol, and interleukin-2 (100 IU/ml).

**Time-lapse microscopy**

MC38Ova cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) or CellTrace Violet for 20 min and then washed and mixed together in a 50:50 ratio. Cells were then seeded into each well of an eight-well chamber slide (Ibidi, Munich, Germany) and incubated overnight at 37°C/10% CO2. OT-I T cells were then added to adherent targets, in medium containing 100 μM propidium iodide (PI). Chamber slides were mounted on a heated stage within a temperature-controlled chamber maintained at 37°C, and constant CO2 concentrations (5 or 7%) were infused using a gas incubation system with active gas mixer (“The Brick”; Ibidi). Optical sections were acquired through sequential scans or brightfield/differential interference contrast on a TCS SP5 confocal microscope (Leica Microsystems, Deerfield, IL) using a 40 Å (numerical aperture, 0.85) air objective and Leica LAS AF software. Image analysis was performed using MetaMorph Imaging Series 7 software (Universal Imaging, Downingtown, PA).

**3’ mRNA sequencing**

RNA extraction, library preparation, and data analysis were performed as described previously (1). Briefly, total RNA was isolated as per...
the manufacturer’s instructions (NucleoSpin RNA Extraction kit, Macherey-Nagel, Bethlehem, PA). Subsequently, the RNA was analyzed on the TapeStation (Agilent TapeStation 2200), and only RNA with RNA integrity number values greater than 9 were used for downstream library preparation. The QuantSeq 3’ mRNA Library Prep Kit (Lexogen) was used to prepare libraries. The 3’ mRNA sequencing libraries were sequenced single-end 75 base pairs (bp) on the NextSeq 500 (Illumina). The resulting reads were demultiplexed using CASAVA 1.8, and sample quality control was performed using FastQC (Babraham Bioinformatics, Babraham Institute). The reads were trimmed using cutadapt (v1.7) and subsequently aligned to the mouse reference genome (GRCm38/mm10) using HISAT2 (v2.1.0), after which read counting was performed using FeatureCounts from the Subread package (v1.5.0). Differential gene expression analysis was performed using Voom-LIMMA. GSEA was performed using GSEA (v3.0).

**Genome-wide and targeted CRISPR screens**

Genome-wide CRISPR/Cas9 screens were performed using MC38, MC38Ova, B16Ova, and MDA-MB-231 cells transduced with mCherry-Cas9 (FUCas9Cherry). The mouse cell lines MC38-Cas9, MC38Ova-Cas9, and B16Ova-Cas9 cells were transduced with the Brie genome-wide sgRNA library (40), and the human cell line MDA-MB-231 was transduced with the Brunello genome-wide sgRNA library. Transductions were performed at a multiplicity of infection of 0.3 to ensure integration of single sgRNA constructs per cell. After transduction, the cells were selected with puromycin (5 μg/ml) for 5 days. The immune evasion screening was started at 7 days after transduction, after which the T0 time point was taken as a reference. The MC38Ova-Cas9 cells were cocultured with activated Prf1+/+ and Prf1−/− OT-I T cells, or NK cells, as effectors to target (E:T) ratios of 1:20 and 1:10, respectively. The B16Ova-Cas9 cells were cocultured with Prf1+/+ and Prf1−/− OT-I T cells at E:T ratios of 1:5 and 1:1, respectively. MDA-MB-231 cells were cocultured with HER2-directed CAR T cells at an E:T ratio of 1:5. The tumor cells were exposed to a total of three hits with either OT-I T or NK cells, after which the pellets were snap-frozen. An untreated control was taken along as a reference for all cell lines. Genomic DNA extraction was subsequently performed using the DNeasy Blood & Tissue Kit (Qiagen), and libraries were prepared as described previously (41). The libraries were subsequently multiplexed and run on the NextSeq 500 (Illumina) generating 75-bp single-end reads. After demultiplexing with CASAVA (v1.8), the vector-derived sequence reads were removed, and only reads of exactly 20 bp were extracted using cutadapt (v1.7). Subsequently, MAGeCK (v0.5.6) was used to count the reads and perform gene/sgRNA enrichment and statistical analysis (42). The resulting data were visualized using the R package ggplots2. The targeted CRISPR library was composed of the top scoring genes from the MC38 screens, and the libraries were custom-cloned from oligo pools obtained from CustomArray Inc.

**In vivo CRISPR screens**

MC38Ova cells expressing Cas9 mCherry were transduced with a custom-cloned immune evasion library into pLenti-puro sgRNA vector as described above. After puromycin selection for 7 days, 1 × 10^6 cells were implanted subcutaneously into recipient NSG mice. When tumors reached 30 to 50 mm^2, mice were injected with 5 × 10^5 SINFEKL-activated OT-I T cells (days 6 to 8 after activation). Four days after mice received either a single dose (Prf1+/+) or two doses (Prf1−/−) of OT-I T cells, tumors were isolated. Genomic DNA isolation and library preparation were conducted as described above.

**Metabolomics profiling**

Control or Ado knockout cells were maintained in full growth medium. Medium was aspirated, and wells were washed with normal saline. Cells were snap-frozen by addition of liquid nitrogen to cell culture plates immediately after washing. For metabolite extraction, ice-cold MeOH/CHCl_3 (9:1, v/v) containing 13C-sorbitol and 13C,15N-valine internal standards was added. Cells and metabolite-containing supernatants were collected, and insoluble material was pelleted by centrifugation at 16,000g for 5 min. Polar metabolites were prepared for analysis by gas chromatography–mass spectrometry (GC-MS) as previously described (43) and analyzed using a Shimadzu GC-TQ8040 instrument with metabolites identified and quantified using the Shimadzu Smart Database. Primary and secondary amines were identified and quantified relative to authentic standards, using a Shimadzu LC-TQ8050 instrument after derivatization of amine-containing metabolites, as previously described (44). Raw peak areas of individual metabolites were normalized to the total peak area in each sample. Adjusted data are presented as fold change in metabolite abundance relative to control.

**T cell killing assays**

Specific OT-I T cell killing was measured using chromium release assays at the indicated target to effector (T:E), as previously described in detail (45).

**SUPPLEMENTARY MATERIALS**

immunology.sciencemag.org/cgi/content/full/3/23/eaar3451/DC1

Fig. S1. Additional validation and control for CRISPR screen.

Fig. S2. Confirmation of CRISPR gene deletion.

Fig. S3. Transcriptional analysis of cytokine-treated tumor cells.

Fig. S4. Additional cytokine treatment data.

Fig. S5. Further adoptive T cell therapy screen data.

Fig. S6. Validation of Ado knockout TNF response in additional tumor lines.

Fig. S7. Confirmation of Gosr1 as an immune evasion gene.

Movie S1. Time-lapse imaging of T cells killing MC38Ova or MC38Vec cells in the presence of anti-TNF antibody.

Table S1. Raw data.

Table S2. CRISPR screen data, B16 perforin knockout OT-I screen.

Table S3. CRISPR screen data, B16 OT-I screen.

Table S4. CRISPR screen data, MC38 OT-I screen.

Table S5. CRISPR screen data, MDA CAR T cell screen.

Table S6. CRISPR screen data, OT-I IgG versus T0.

Table S7. CRISPR screen data, OT-I PD-1 versus T0.

**REFERENCES AND NOTES**


Discovery and characterization of a second mammalian thiol dioxygenase, cysteamine, identifies Ptpn2 as a cancer immunotherapy target.


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Tumor immune evasion arises through loss of TNF sensitivity

Killing without poking holes

Given the success of T cell–centric cancer immunotherapies, there is considerable interest in understanding exactly how tumors evade this form of therapy. Kearney et al. carried out a series of genome-wide CRISPR screens to identify mechanisms of tumor immune evasion from cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. They found IFN-γ signaling and antigen presentation to be critical for CTL-mediated killing of cancer cells and uncovered TNF signaling as a key effector mechanism for both CTL and NK cell antitumor activity. The same immune evasion mechanisms arose upon screening with perforin-deficient CTLs, suggesting that tumors evade the immune system by dampening the effects of cytokines, not direct killing via perforin.