**IMMUNOTHERAPY**

**Targeting monoamine oxidase A for T cell–based cancer immunotherapy**

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Monoamine oxidase A (MAO-A) is an enzyme best known for its function in the brain, where it breaks down neurotransmitters and thereby influences mood and behavior. Small-molecule MAO inhibitors (MAOIs) have been developed and are clinically used for treating depression and other neurological disorders. However, the involvement of MAO-A in antitumor immunity has not been reported. Here, we observed induction of the Maoa gene in tumor-infiltrating immune cells. Maoa knockout mice exhibited enhanced antitumor T cell immunity and suppressed tumor growth. MAOI treatment significantly suppressed tumor growth in preclinical mouse syngeneic and human xenograft tumor models in a T cell–dependent manner. Combining MAOI and anti–PD-1 treatments generated synergistic tumor suppression effects. Clinical data correlation studies associated intratumoral MAOA expression with T cell dysfunction and decreased patient survival in a broad range of cancers. We further demonstrated that MAO-A restrains antitumor T cell immunity through controlling intratumoral T cell autocrine serotonin signaling. Together, these data identify MAO-A as an immune checkpoint and support repurposing MAOIs for cancer immunotherapy.

**INTRODUCTION**

CD8 cytotoxic T cells are potent immune cells capable of recognizing and eradicating malignant cells; these immune cells are therefore attractive therapeutic targets for treating cancer (1–3). However, the antitumor responses of CD8 T cells can be severely restrained by negative-regulator (immune checkpoint) pathways that are particularly prevalent in the tumor immunosuppressive environment (4). To release this suppression and harness the antitumor potential of CD8 T cells, several immune checkpoint blockade (ICB) therapies have been developed over the past decade (5, 6). Blockade of the cytotoxic T lymphocyte–associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1)/programmed cell death 1 ligand 1 (PD-L1) inhibitory pathways have achieved remarkable clinical responses and revolutionized the treatment of many cancers; so far, the U.S. Food and Drug Administration (FDA) has approved these two ICB therapies for treating more than 10 different malignancies (5, 6). Despite these impressive successes, only a fraction of patients with cancer respond to CTLA-4 and PD-1/PD-L1 blockade therapies, and most responders suffer tumor recurrence due to the development of tumor immune evasion (7). These limitations of existing ICB therapies are thought to be largely caused by the presence of multiple immune checkpoint pathways, as well as the different roles of individual immune checkpoint pathways in regulating specific cancer types and disease stages (7). Thus, the identification of new immune checkpoints and the development of new combination treatments are a major focus of current cancer immunotherapy studies.

Monoamine oxidase A (MAO-A) is an enzyme that catalyzes the degradation of biogenic and dietary monoamines (8, 9). MAO-A is located on the outer membrane of mitochondria and, in humans, is encoded by the X-linked MAOA gene. MAO-A is best known for its function in the brain, where it regulates the homeostasis of key monoamine neuronal transmitters including serotonin, dopamine, epinephrine, and norepinephrine and thereby influences human mood and behavior (8, 9). Complete MAO-A deficiency in humans caused by a mutation of the MAOA gene leads to an excess of monoamine neuronal transmitters in the brain and results in Brunner syndrome, which is characterized by problematic impulsive behaviors and mood swings (10). Genetic association studies also identified several MAOA gene variants linked to altered MAO-A enzyme expression levels: Low-activity forms of the MAOA gene are associated with agression and hyperactivity disorders, whereas high-activity forms are associated with depression disorders (11, 12). Because of its link with aggressive and even violent behavior in men, a low-activity variant of the MAOA gene, MAOA-L, has previously received broad publicity and is popularly referred to as the “warrior gene” (13). On the other hand, small-molecule MAO inhibitors (MAOIs) have been developed and are clinically used for treating depression symptoms (14). However, MAO-A’s functions outside of the brain are largely unexplored. In particular, the involvement of MAO-A in antitumor immunity is unknown. Here, we investigated the role of MAO-A in regulating CD8 T cell antitumor immunity and evaluated the possibility of repurposing MAOIs for cancer immunotherapy using knockout (KO) and transgenic mice, preclinical mouse syngeneic and human xenograft tumor models, and clinical data correlation studies.
RESULTS
MAO-A deficiency enhances CD8 T cell antitumor immunity

To search for new drug targets regulating antitumor immunity, we grew B16-ovalbumin (OVA) melanoma solid tumors in C57BL/6j mice, isolated tumor-infiltrating immune cells (TIIs), and evaluated TII gene expression profiles using quantitative reverse transcription polymerase chain reaction (RT-PCR). Immune cells isolated from the spleen of tumor-bearing and tumor-free mice were included as controls. In addition to the classical immune regulatory genes, we detected significant changes in the expression of a group of genes typically classified as neuronal regulatory genes. In particular, we detected the induction of a Maoa gene in TII (Fig. 1A), suggesting that, along with its known function in the brain as a regulator of neuronal activity (8), it might also function in the tumor as a regulator of antitumor reactivity. We were especially interested to study whether MAO-A might regulate CD8 cytotoxic T cells, which play a critical role in immune response against cancer.

To test this, we began by studying MAO-A–deficient mice that carry a hypomorphic Maoa mutant (15). Although a degree of Maoa expression leakage in the brain was previously reported in these mice (15), analysis of their immune system showed a nearly complete ablation of Maoa mRNA and protein expression in the major immune organs including thymus and spleen (fig. S1, A and B). Since we focused on studying immune cells, here, we refer to these mice as Maoa-KO mice. Maoa-KO mice showed normal T cell development in the thymus and contained normal numbers of T cells in the periphery, compared with the wild-type control mice (Maoa-WT mice) (fig. S1, C to H). Before tumor challenge, these T cells carried a hypomorphic Maoa phenotype (CD25loCD69loCD44hiCD62Llo; fig. S1I). When challenged with tumors, compared with Maoa-WT mice, Maoa-KO mice exhibited significantly suppressed tumor growth in two syngeneic mouse tumor models, the MC38 colon cancer model and the B16-OVA melanoma model (Fig. 1, B to D). Flow cytometry analysis detected similar levels of tumor-infiltrating CD8+ T cells in Maoa-KO and Maoa-WT mice (fig. S2A). However, in Maoa-KO mice, these tumor-infiltrating T cells displayed an enhanced effector phenotype: They produced higher levels of effector cytokines and cytotoxic molecules [i.e., interferon-γ (IFN-γ) and IFN-γ (E and F)] and Granzyme B (G and H) production, as well as cell surface PD-1 expression (I and J). MFI, mean fluorescence intensity.

Fig. 1. MAO-A–deficient mice show suppressed tumor growth and enhanced CD8 T cell antitumor immunity. (A) qPCR analyses of Maoa mRNA expression in TII isolated from day 14 B16-OVA melanoma tumors grown in wild-type B6 mice (n = 3). Spleen (SP) cells collected from the tumor-bearing and tumor-free B6 mice were included as controls. A.U., artificial unit. (B to D) Syngeneic tumor growth in Maoa-WT and Maoa-KO mice. s.c., subcutaneous. (B) Experimental design. (C) MC38 colon cancer tumor growth (n = 4 and 5). (D) B16-OVA melanoma tumor growth (n = 5). (E to J) Flow cytometry analysis of tumor-infiltrating CD8 T cells isolated from day 14 B16-OVA tumors grown in Maoa-WT and Maoa-KO mice (n = 4). FACS plots and quantifications are presented, showing the measurements of intracellular IFN-γ (E and F) and Granzyme B (G and H) production, as well as cell surface PD-1 expression (I and J). MFI, mean fluorescence intensity. (K to M) scRNA-seq analysis of antigen-experienced (CD44+) tumor-infiltrating CD8 T cells isolated from day 14 B16-OVA tumors grown in Maoa-WT and Maoa-KO mice (10 tumors were combined for each group). (K) UMAP plots showing the formation of three major cell clusters. Each dot represents a single cell and is colored according to its cell cluster assignment. Gene signature profiling analysis identified cluster 1 cells to be proliferating/effectort-like CD8 T cells, cluster 2 cells to be terminally exhausted CD8 T cells, and cluster 3 cells to be progenitor exhausted CD8 T cells. (L) Bar graphs showing the cell cluster proportions. (M) Violin plots showing the expression distribution of Gzmb and Ifng genes. Each dot represents an individual cell. Representative of one (K to M), two (A), and three (B to J) experiments. Data are presented as the means ± SEM. ns, not significant; *P < 0.05 and **P < 0.01 by one-way ANOVA (A) or by Student’s t test (C, D, F, H, and J). P values of violin plots (M) were determined by Wilcoxon rank sum test.
Granzyme B; Fig. 1, E to H], and they expressed lower levels of T cell exhaustion markers (i.e., PD-1; Fig. 1, I and J).

To further investigate how MAO-A deficiency may affect the tumor-infiltrating CD8 T cell compartment, we isolated TILs from tumor-bearing Maaα-WT and Maaα-KO mice and performed a single-cell RNA sequencing (scRNA-seq) study. Uniform Manifold Approximation and Projection (UMAP) analysis of antigen-experienced (CD44hi CD62Llo) tumor-infiltrating CD8 T cells revealed the formation of three major cell clusters (Fig. 1K); signature gene profiling study (Fig. S2, B and C) and gene set enrichment analysis (GSEA; Fig. S2, D to F) identified cluster 1 cells as proliferating/effecter-like CD8 T cells, cluster 2 cells as terminally exhausted CD8 T cells, and cluster 3 cells as progenitor-exhausted CD8 T cells (16). Compared with their wild-type counterparts, Maaα-KO tumor-infiltrating CD8 T cells were enriched for proliferating/effecter-like (cluster 1) cells while decreased for exhausted (clusters 2 and 3), especially the progenitor-exhausted (cluster 3) cells (Fig. 1, K and L). Single-gene expression analysis also showed an overall enhanced expression of genes associated with cytotoxic T cell effector function (i.e., GzmB and Ifng; Fig. 1M) and genes associated with mitochondrial function that is a notable factor in T cell effector function (i.e., mitochondrial electron transport chain genes; fig. S3) (17), in tumor-infiltrating CD8 T cells isolated from Maaα-KO mice. Collectively, these scRNA-seq results suggest a possible role of MAO-A in regulating the generation/maintenance of active effector-like antitumor CD8 T cells. Together, these in vivo studies using MAO-A–deficient mice indicate that MAO-A is involved in regulating antitumor immunity, especially in regulating CD8 T cell antitumor effector functions.

MAO-A directly regulates CD8 T cell antitumor immunity

In our Maaα-KO mice tumor challenge study, MAO-A deficiency affected both immune and nonimmune cells. To determine whether MAO-A directly or indirectly regulates immune cells, we performed a pair of two-way bone marrow (BM) transfer experiments: In one experiment, we confined MAO-A deficiency comparison to immune cells by reconstituting CD45.1 wild-type recipient mice with BM cells from either CD45.2 Maaα-WT or Maaα-KO donor mice, followed by B16-OVA tumor challenge; in another experiment, we confined MAO-A deficiency comparison to nonimmune cells by reconstituting either CD45.2 Maaα-WT or Maaα-KO recipient mice with BM cells from CD45.1 wild-type donor mice, followed by B16-OVA tumor challenge (Fig. 2A). Successful reconstitution of immune cells, particularly T cells, was confirmed in both experiments (fig. S4, A to D). Suppressed tumor growth was observed only when MAO-A deficiency was confined to the immune cells, indicating that MAO-A affects tumor growth via directly regulating immune cell antitumor reactivity (Fig. 2, B and C).

To further study whether MAO-A directly regulates the antitumor reactivity of CD8 T cells, we bred Maaα-KO mice with OT1 transgenic (OT1-Tg) mice and generated OT1-Tg/Maaα-KO mice producing OVA-specific CD8 T cells deficient in MAO-A (fig. S5A). We isolated OT1 T cells from either the OT1-Tg or OT1-Tg/Maaα-KO mice (denoted as OT1-WT or OT1-KO T cells, respectively) and separately transferred these cells into CD45.1 wild-type mice bearing preestablished B16-OVA tumors (Fig. 2D and fig. S5B). In this experiment, MAO-A deficiency comparison was confined solely to tumor-specific OT1 T cells. Both OT1-WT and OT1-KO T cells actively infiltrated tumors and showed an antigen–experienced phenotype (CD44hi CD62Llo; Fig. 2E and fig. S5, C and D). However, OT1-KO T cells were more effective in controlling tumor growth, corresponding with their enhanced effector function and reduced exhaustion phenotype (Fig. 2F and fig. S5, E to J). Collectively, these in vivo studies demonstrate that MAO-A acts as an autonomous factor directly regulating CD8 T cell antitumor immunity.

MAO-A restrains the CD8 T cell response to antigen stimulation

Analysis of Maaα mRNA expression in tumor-infiltrating CD8 T cells showed an induction of the Maaα gene in these T cells compared with naïve CD8 T cells (Fig. 3A). Further analysis of tumor-infiltrating CD8 T cell subsets revealed that Maaα gene expression levels were positively correlated with the exhaustion and dysfunction status of these T cells: Compared with PD-1hi cells, PD-1lo “exhausted” T cells expressed higher levels of Maaα mRNA, and the PD-1hi cells coexpressing T cell immunoglobulin mucin 3 (Tim-3) and lymphocyte activation gene 3 protein (LAG-3), which were considered “most exhausted”, expressed the highest levels of Maaα mRNA (Fig. 3A) (18, 19). These in vivo data suggest that Maaα may be induced by tumor antigen recognition and then act as a negative-feedback regulator inhibiting CD8 T cell antitumor reactivity.

To test this hypothesis, we isolated CD8 T cells from Maaα-WT or Maaα-KO mice and then stimulated these T cells in vitro with anti-CD3, mimicking tumor antigen stimulation. We observed an induction of Maaα mRNA expression in Maaα-WT CD8 T cells, in agreement with previous reports; minimal Maaα mRNA expression was detected in Maaα-KO CD8 T cells, confirming their Maaα deficiency phenotype (Fig. 3B) (20). The induction of MAO-A protein expression in Maaα-WT CD8 T cells was also confirmed by Western blot analysis; the MAO-A isoenzyme, MAO-B, was not detected in CD8 T cells (fig. S6A). Compared with their Maaα-WT counterparts, Maaα-KO CD8 T cells showed an enhancement in almost all aspects of T cell activation, including cell proliferation (Fig. 3C), surface activation marker up-regulation (i.e., CD25; Fig. 3, D and E), effector cytokine production [i.e., interleukin-2 (IL-2) and IFN-γ; Fig. 3, F and G], and cytotoxic molecule production (i.e., Granzyme B; Fig. 3, H and I). Study of Maaα-KO OVA-specific OT1 T cells gave similar results, suggesting a general role of MAO-A in regulating CD8 T cells of diverse antigen specificities (fig. S7, A to D); study of Maaα-KO CD8 T cells stimulated with both anti-CD3 and anti-CD28 also yielded similar results, suggesting a general role of MAO-A in regulating CD8 T cell responses in the presence or absence of costimulation (fig. S6, B to I). To verify whether MAO-A deficiency directly contributed to the hyperresponsiveness of the Maaα-KO CD8 T cells, we performed a rescue experiment. We constructed a MIG [murine stem cell virus–internal ribosomal entry site–green fluorescent protein (MSCV-IRESGFP)]-Maaα retroviral vector, used this vector to transduce Maaα-KO CD8 T cells, and achieved overexpression of MAO-A in these T cells (Fig. 3, J to L). MAO-A overexpression significantly reduced the hyperactivation of Maaα-KO CD8 T cells and their expression of multiple effector genes (i.e., Il2, Ifng, and GzmB; Fig. 3, M to O). Together, these results indicate that MAO-A acts as a negative-feedback regulator restraining the CD8 T cell response to antigen stimulation.

MAO-A regulates CD8 T cell autocrine serotonin signaling

Next, we sought to investigate the molecular mechanisms mediating MAO-A restraint of CD8 T cell response to antigen stimulation. MAO-A is well known for its function in the brain where it breaks down neuron-produced serotonin, thereby regulating neuronal activity...
CD8 T cells have been reported to synthesize serotonin, and serotonin has been implicated as an accessory signal to enhance T cell activation by signaling through T cell surface serotonin receptors [5-hydroxytryptamine receptors (5-HTRs)] (20–22). We therefore postulated that MAO-A might regulate CD8 T cell activity through modulating T cell autocrine serotonin production and signaling (Fig. 4A).

To test this hypothesis, we cultured Maoa-WT and Maoa-KO CD8 T cells in vitro, stimulated them with anti-CD3 to mimic antigen stimulation, and then analyzed their autocrine serotonin signaling pathway. After antigen stimulation, Maoa-WT CD8 T cells up-regulated the expression of the Tph1 gene, which encodes the rate-limiting enzyme controlling serotonin synthesis, and also the expression of the Maoa gene, which would induce serotonin degradation, indicating the presence of an antigen stimulation–induced serotonin synthesis/degradation loop in CD8 T cells (Fig. 4, A to C). Considering the function of MAO-A, we speculated that MAO-A deficiency would not interfere with the serotonin synthesis arm but would impede the serotonin degradation arm, leading to enhanced secretion of serotonin by CD8 T cells. Compared with their wild-type counterparts, Maoa-KO CD8 T cells expressed comparable levels of Tph1 but secreted much higher levels of serotonin after antigen stimulation (Fig. 4, B and D). Pharmacological inhibition of MAO-A in Maoa-WT CD8 T cells using an established MAOI, phenelzine, recapitulated the serotonin overproduction phenotype of Maoa-KO CD8 T cells (Fig. 4E). Correspondingly, phenelzine treatment of Maoa-WT CD8 T cells recapitulated the hyperactivation phenotype of Maoa-KO CD8 T cells, shown by increased production of the effector cytokines IL-2 and IFN-γ (Fig. 4, F and G). Supplementing serotonin to Maoa-WT CD8 T cells resulted in T cell hyperactivation and elevated production of IL-2 and IFN-γ (Fig. 4, H and I), whereas blocking T cell surface 5-HTRs using the antagonist asenapine eliminated the cytokine production difference between Maoa-WT and Maoa-KO CD8 T cells (Fig. 4, J and K). Serotonin has been reported to enhance T cell activation by signaling through the mitogen-activated protein kinase (MAPK) pathway that cross-talks with the T cell receptor (TCR) signaling pathways (21). We compared the signaling pathways in Maoa-WT and Maoa-KO CD8 T cells after antigen stimulation and found that Maoa-KO T cells showed an enhancement of MAPK signaling [i.e., extracellular signal–regulated kinase (ERK) phosphorylation; Fig. 4L and fig. S8, B and C] and TCR downstream signaling [i.e., nuclear translocation of nuclear factor of activated T cells (NFAT), nuclear factor κB (NF-κB), and c-Jun transcription factors; Fig. 4M and fig. S8, D to I]; this enhancement was largely abrogated by blocking 5-HTRs (Fig. 4, L and M, and fig. S8, B to I). There are multiple 5-HTR family members; in mice, there are 13 5-HTR family members (21). Analyses of the expression of all 13 5-HTR genes in CD8 T cells before and after anti-CD3 stimulation revealed that these cells predominantly expressed 2 of the 13 5-HTR genes: Htr2b and Htr7; there was a further up-regulation of Htr7 after anti-CD3 stimulation (fig. S8J). These data suggest that serotonin signaling in mouse CD8 T cells may mainly be mediated through Htr2b and Htr7; in particular, Htr7 may mediate the activation-related signaling events. Collectively, these in vitro data suggest that MAO-A regulates CD8 T cell activation through modulating T cell autocrine serotonin production and signaling (Fig. 4A and fig. S8A).

To validate this working model in vivo, we directly measured intratumoral serotonin in Maoa-KO and Maoa-WT mice, as well as in wild-type mice treated or untreated with phenelzine. Consistent with the in vitro results, increased levels of serotonin were detected specifically in tumors collected from the Maoa-KO mice (Fig. 4N).
and phenelzine-treated Maoa-WT mice (Fig. 4O). Depletion of CD8 T cells in Maoa-WT mice largely abolished the phenelzine treatment–induced accumulation of serotonin in the tumor, indicating that tumor-infiltrating CD8 T cells are major producers of serotonin in the tumor and that this is negatively regulated by MAO-A (Fig. 4O and fig. S8L). There were no significant changes in serotonin levels in serum under any conditions (fig. S8, K and M), suggesting that MAO-A regulation of serotonin in the tumor is largely a local effect, resembling MAO-A regulation of serotonin in the brain (8, 9).

Together, these in vitro and in vivo data support a working model that MAO-A negatively regulates CD8 T cell antitumor immunity, at least partly, through modulating CD8 T cell autocrine serotonin signaling in the tumor.

**MAO-A blockade for cancer immunotherapy**

The identification of MAO-A as a new immune checkpoint negatively regulating CD8 T cell antitumor immunity marks it as a promising drug target for developing new forms of ICB therapy. Because of MAO-A’s well-characterized function in the brain, small-molecule MAOIs have been developed and clinically used for treating depression symptoms, making it a highly feasible and attractive approach to repurpose these established MAOI antidepressants for cancer immunotherapy (23). Some MAOIs cross-inhibit the MAO-A iso-enzyme MAO-B; however, only MAO-A effectively degrades serotonin, and all MAOIs exhibit their antidepressant function mainly through inhibiting MAO-A enzyme activity, thereby regulating serotonin signaling in the brain (14, 23). When tested in vitro, multiple MAOIs efficiently induced CD8 T cell hyperactivation (i.e., up-regulated expression of CD25, Granzyme B, IL-2, and IFN-γ; Fig. 5A and fig. S9, A to F). When tested in vivo in a B16-OVA melanoma prevention model, these MAOIs markedly suppressed tumor growth (Fig. 5, B and C). The MAOIs that we tested were phenelzine, clorgyline, and moclobemide, covering the major categories of established MAOIs classified on the basis of whether they are nonselective or selective for MAO-A and whether their effect is reversible (fig. S9A) (23). Among these MAOIs, phenelzine (trade name: Nardil) is clinically available in the United States (23). In the following studies, we chose phenelzine as a representative to further evaluate the cancer therapy potential of MAOI drugs.

First, we studied the efficacy of phenelzine in treating preestablished B16-OVA melanoma solid tumors and found that phenelzine treatment effectively suppressed tumor progression (Fig. 5, D and E).
This MAOI-induced tumor suppression effect was mediated by CD8 T cells, because no tumor suppression was observed when we depleted CD8 T cells in tumor-bearing B6 wild-type mice (fig. S10, A and B). Correspondingly, analysis of total tumor-infiltrating CD8 T cells under phenelzine treatment showed a hyperactivation phenotype of these T cells, evidenced by their enhanced production of effector molecules (i.e., IFN-$\gamma$ and Granzyme B; Fig. 5, F to I) and reduced expression of exhaustion markers (i.e., PD-1; Fig. 5, J and K); analysis of tumor antigen-specific CD8 T cells (i.e., OVA$^+$ T cells) showed the similar results (fig. S10, I to P). To further study the impact of phenelzine treatment on antitumor T cell exhaustion, we tracked the PD-1–positive subpopulation of tumor-infiltrating CD8 T cells over time; we detected a significant decrease of the PD-1–positive subpopulation in phenelzine-treated mice (fig. S10, C and D). Functional analysis showed an enhanced production of effector molecules (i.e., IFN-$\gamma$ and Granzyme B) in PD-1–positive subpopulations under phenelzine treatment (fig. S10, E to H). Therefore, MAO-A blockade could alleviate T cell exhaustion and improve the overall effector function of tumor-infiltrating CD8 T cells.

Next, we studied the potential of phenelzine treatment for combination therapy, particularly combining with other ICB therapies such as the PD-1/PD-L1 blockade therapy (Fig. 5L) (6). In the MC38 colon cancer model, which is sensitive to immunotherapy, phenelzine treatment completely suppressed tumor growth as effectively as the PD-1/PD-L1 blockade therapy (Fig. 5L) (6). In the MC38 colon cancer model, which is sensitive to immunotherapy, phenelzine treatment completely suppressed tumor growth as effectively as the PD-1/PD-L1 blockade therapy (Fig. 5L) (6).
anti–PD-1 treatment (Fig. 5M). In the B16-OVA melanoma model, which is less sensitive to immunotherapy, phenelzine treatment significantly suppressed tumor growth at a level comparable with the anti–PD-1 treatment; the combination of phenelzine and anti–PD-1 treatments yielded superior efficacy and totally suppressed tumor growth (Fig. 5N). These tumor suppression effects of phenelzine were likely mediated by its immune regulatory function, because phenelzine treatment did not affect the tumor cells themselves and did not suppress the growth of MC38 and B16-OVA tumors in immunodeficient NOD scid gamma (NSG) mice (fig. S11, A to D). Collectively, these syngeneic mouse tumor model studies provided proof-of-principle evidence for the cancer immunotherapy potential of MAOIs.

To explore the translational potential of MAO-A blockade therapy, we studied human CD8 T cells and confirmed that they also responded to MFOA in combination with anti–PD-1 treatment (Fig. 6A). To directly evaluate whether MAOI treatment could enhance human CD8 T cell antitumor efficacy in vivo, we used a human T cell adoptive transfer and human tumor xenograft NSG mouse model (24). NY-ESO-1, a well-recognized tumor antigen common in many human cancers,
was chosen as the model tumor antigen (24). An A375 human melanoma cell line was engineered to coexpress NY-ESO-1 and its matching major histocompatibility complex (MHC) molecule, human leukocyte antigen serotype A2 (HLA-A2), to serve as the human tumor target; the cell line was also engineered to express a dual reporter comprising a firefly luciferase and an enhanced green fluorescence protein (FG). ESO-T, human peripheral blood CD8 T cells engineered to express an NY-ESO-1 antigen-specific TCR (ESO-TCR; clone 3A1). ESOp, NY-ESO-1 peptide. (C) Experimental design to study the cancer therapy potential of MAOI treatment in a human T cell adoptive transfer and human melanoma xenograft NSG mouse model. (D) Tumor growth from (C) (n = 9 and 10). (E and F) Clinical data correlation studies. A TIDE computational method was used to study the association between the tumor-infiltrating CD8 T cell (also known as cytotoxic T lymphocyte, CTL) level and overall patient survival in relation to the intratumoral MAOA gene expression level. For each patient cohort, tumor samples were divided into MAOA-high (samples with MAOA expression one standard deviation above the average; shown in left survival plot) and MAOA-low (remaining samples; shown in right survival plot) groups, followed by analyzing the association between the CTL levels and survival outcomes in each group. The CTL level was estimated as the average expression level of CD8A, CD8B, GZMA, GZMB, and PRF1. Each survival plot presented tumors in two subgroups: CTL-high group (red) had above-average CTL values among all samples, whereas CTL-low group (blue) had below-average CTL values. A T cell dysfunction score (z score) was calculated for each patient cohort, correlating the MAOA expression level with the beneficial effect of CTL infiltration on patient survival. A positive z score indicates that the expression of MAOA is negatively correlated with the beneficial effect of tumor-infiltrating CTL on patient survival. The P value indicates the comparison between the MAOA-low and MAOA-high groups and was calculated by two-sided Wald test in a Cox-PH regression. (E) TIDE analysis of a colon cancer patient cohort (GSE29621; n = 65). z = 2.31; P = 0.0208. (F) TIDE analysis of a melanoma patient cohort receiving anti–PD-1 treatment (ENA PRJEB23709; n = 41). z = 2.16, P = 0.0305. Representative of two experiments. Data are presented as the means ± SEM. ***P < 0.001 by Student’s t test (A and D).
generated from single cells or sorted tumor-infiltrating CD8 T cells are needed to produce such information. Nonetheless, the present clinical data correlation studies identified MAO-A as a possible negative regulator of CD8 T cell antitumor function in a broad range of patients with cancer, including those receiving existing ICB therapies, suggesting MAO-A as a potential drug target for developing new forms of ICB therapy and combination therapy. Together, these preclinical animal studies and clinical data correlation studies suggest that MAO-A is a promising new drug target of T cell–based cancer immunotherapy and that repurposing of established MAOI antidepressants is a promising path to develop MAO-A blockade immunotherapy.

DISCUSSION

On the basis of our findings, we propose an "intratumoral MAO-A–serotonin axis" model to elucidate the role of MAO-A in regulating CD8 T cell antitumor immunity (fig. S14). Analogous to the well-characterized MAO-A–serotonin axis in the brain, where MAO-A controls the availability of serotonin in a neuron-neuron synapse, thereby regulating neuronal activity, the "MAO-A–serotonin axis" in a tumor controls the availability of serotonin in a tumor cell–T cell immune synapse, thereby regulating antitumor T cell reactivity (fig. S14). The resemblance is notable, considering that both the nervous system and the immune system are evolved to defend a living organism by sensing and reacting to environmental danger, externally and internally, including tissue traumas, infections, and malignancies (32). Despite their distinct anatomic structures, the nervous system has a fixed organization, whereas the immune system comprises mobile and dispersive cells; from an evolutionary point of view, it makes sense that some critical molecular regulatory pathways are preserved for both defense systems. Neurons and immune cells share a broad collection of signal transducers, surface receptors, and secretory molecules (32). In particular, many neurotransmitters and neuropeptides traditionally considered specific for neurons are expressed in immune cells, although their functions in the immune system are, to a large extent, still unknown (33). Studying this group of molecules and their regulatory circuits in tumor immunology thus may provide new opportunities for generating knowledge and identifying new drug targets for developing next-generation cancer immunotherapies; our current finding of this MAO-A–serotonin axis regulation of CD8 T cell antitumor immunity can be such an example.

Our study showed that Maoa expression was induced by antigen-TCR stimulation in CD8 T cells and, in turn, restrained T cell activation (Fig. 3). This negative-feedback loop qualifies MAO-A as an immune checkpoint and adds it to the expanding immune checkpoint family comprising PD-1/PD-L1, CTLA-4, Tim-3, LAG-3, T cell immunoreceptor with Ig and ITIM domains (TIGIT), V-domain Ig suppressor of T cell activation (VISTA), and others (4). However, MAO-A is unique in this group because it is already a well-established drug target due to its known function in the brain (23). Small-molecule MAOIs have been developed to block MAO-A activity, thereby regulating serotonin signaling in the brain, and were the first drugs approved for treating depression (23). In our study, we tested multiple clinically approved MAOIs (phenelzine, moclobemide, and clorglyline) and demonstrated their T cell–enhancing and tumor suppression effects in preclinical animal models, pointing to the possibility of repurposing these drugs for cancer immunotherapy (Fig. 5 and fig. S9).

Developing new cancer drugs is extremely costly and time consuming; drug repurposing offers an economic and speedy pathway to additional cancer therapies, because approved drugs have known safety profiles and modes of actions and thus can enter the clinic quickly (34). MAOIs were introduced in the 1950s and were used extensively over the subsequent two decades, but since then, their use has dwindled because of reported side effects and the introduction of other classes of antidepressant agents (23). However, these MAOI side effects were vastly overstated and should be revisited (23). For instance, a claimed major side effect of MAOIs is their risk of triggering tyramine-induced hypertensive crisis when patients eat tyramine-rich foods such as aged cheese (hence, "the cheese effects"); this concern led to cumbersome food restrictions that are now considered largely unnecessary (23). A transdermal delivery system for selegiline (Emsam) has also been developed that can largely avoid potential food restrictions (23). Therefore, interest in MAOIs as a major class of antidepressants is reviving (23), and repurposing MAOIs for cancer immunotherapy can be an attractive new application of these potent drugs.

Depression and anxiety are common in patients with cancer: Prevalent rates of major depression among patients with cancer are four times higher than the general population, and up to a quarter of patients with cancer have clinically significant depression and anxiety symptoms (35). Repurposing MAOIs for cancer immunotherapy thus may provide patients with cancer with dual antidepressant and antitumor benefits. A large majority of antidepressants, including MAOIs, selective serotonin reuptake inhibitors (SSRIs), serotonin modulators and stimulators (SMs), serotonin antagonists and reuptake inhibitors (SARIIs), and serotonin-norepinephrine reuptake inhibitors (SNRIs), all work through regulating serotonin signaling in the brain via inhibiting the various key molecules that control serotonin degradation, reuptake, and detection (36). Our study revealed the existence of a MAO-A–serotonin axis in tumors that regulates CD8 T cell antitumor immunity (fig. S14). It is plausible to postulate that the other key serotonin regulatory molecules that function in the brain may also function in the tumor regulating T cell antitumor immunity (36). A recent nationwide cohort study in Israel reported that adherence to antidepressant medications is associated with reduced premature mortality in patients with cancer (35). Another clinical study reported lymphocyte subset changes associated with antidepressant treatments in patients with a major depression disorder (37). Studying patients with cancer for possible correlations between antidepressant treatments, antitumor immune responses, and clinical outcomes therefore might yield valuable knowledge informing the immune regulatory function of antidepressants and instructing the potential repurposing of select antidepressant drugs for cancer immunotherapy.

In our study, we found that Maoa gene was highly expressed in tumor-infiltrating CD8 T cells, with the most "exhausted" cells (PD-1hiTim-3hiLAG-3hi) expressing the highest levels of Maoa, suggesting that these cells may benefit the most from the MAOI treatment (18, 19). We also found that MAO-A regulated CD8 T cell antitumor immunity, at least partly, through modulating the serotonin-MAPK pathway, which is nonredundant to other major immune checkpoint regulatory pathways, suggesting that MAOI treatment can be a valuable component for combination therapy (4). MAOI treatment synergized with anti–PD-1 treatment in suppressing syngeneic mouse tumor growth, and MAOA expression levels dictated patient survival in patients with melanoma receiving anti–PD-1 therapy (Figs. 5 and 6). Patients undergoing cancer treatment,
including traditional chemo/radio therapies and the new immuno-
therapies such as ICB therapies, often report incurred or exacerbated
depression symptoms; these central nervous system (CNS) side effects
are considered to be associated with treatment-induced immune
reaction and inflammation (38–40). Adding MAOIs with anti-
depressant function to a combination cancer therapy thus may both
improve antitumor efficacy and alleviate CNS side effects. Some
earlier studies reported that MAOIs can directly suppress the growth
in androgen-sensitive and castration-resistant prostate cancer cells,
prosumably through regulating cancer cell autophagy and apoptosis,
suggesting additional mechanisms that MAOIs may deploy to
target certain cancers (41, 42).

In summary, here, we identified MAO-A as an immune check-
point and demonstrated the potential of repurposing established
MAOI antidepressants for cancer immunotherapy. The notion that
MAOA, "the warrior gene," not only acts in the brain to regulate the
aggressiveness of human behavior but also acts in a tumor to con-
tral the aggressiveness of antitumor immunity is interesting. Future
clinical studies are encouraged to investigate the clinical correla-
tions between MAOI treatment and clinical outcomes in patients
with cancer and to explore the possibility of repurposing MAOIs for
combination cancer immunotherapy. Meanwhile, the immune regu-
lar function of MAO-A certainly goes beyond regulating CD8
T cells. We have detected MAO-A expression in other immune cells
(e.g., dendritic cells, macrophages, and regulatory T cells); and in
Maoa-KO mice, we have observed the hyperresponsiveness of
multiple immune cells in various mouse tumor models. It is also
likely that MAO-A regulates immune reactions to multiple diseases
beyond cancer, such as infections and autoimmune diseases. Studying
the roles of MAO-A in regulating various immune cells under
different health and disease conditions will be interesting topics for
future research.

MATERIALS AND METHODS

Study design

To search for new drug targets regulating antitumor immunity, we
isolated TILs from B16-OVA melanoma tumors and evaluated TII
gene expression profiles; we detected a significant change in Maoa
gene expression. To study MAO-A as an autologous factor regulating
antitumor CD8 T cell immunity, we conducted a series of in vivo
tumor experiments involving multiple syngeneic mouse tumor models
(i.e., B16-OVA melanoma and MC38 colon cancer models), Maoa-
KO mice, as well as BM and T cell adoptive transfer approaches.
Tumor growth was monitored, and the tumor-infiltrating CD8 T cells
were analyzed using flow cytometry and scRNA-seq. To investigate
the MAO-A regulation of CD8 T cell activation through modulat-
ing T cell autocrine serotonin, we stimulated Maoa-KO and Maoa-
WT CD8 T cells in vitro and compared their T cell activation, TCR
signaling, and serotonin secretion and signaling using flow cytometry,
enzyme-linked immunosorbent assay (ELISA), qPCR, and Western blot; serotonin modulation was also validated in vivo in the
B16-OVA melanoma model using high-performance liquid
chromatography (HPLC). To evaluate the potential of repurposing
MAOI antidepressants for cancer immunotherapy, we studied the
T cell regulatory and antitumor effects of MAOI treatment in vitro
in T cell culture and in vivo in B16-OVA and MC38 tumor models
alone or in combination with anti–PD-1 treatment. To explore the
translational potential of MAO-A blockade therapy, we examined
the MAOA gene expression in primary human CD8 T cells and studied
the therapeutic effects of MAOI treatment in a human T cell adopt-
ove transfer and human melanoma xenograft NSG mouse model.
Last, we conducted clinical data correlation studies and investigated
the correlation of intratumoral MAOA gene expression with CD8 T
cell antitumor activities and clinical outcomes in multiple cancer
patient cohorts spanning melanoma, colon cancer, lung cancer,
cervical cancer, and pancreatic cancer.

Mice

C57BL/6 (B6), B6.SJL-PtprcPepc b/Boyj (CD45.1), 129S-Maoa tm1Shih /?
(Maoa-KO) (15), C57BL/6-Tg (TcrαTcrβ)1100Mjbj (OT1-Tg), and
NOD.Cg-Prkd cscid Il2rg tm1Wjl /Sz (Nod scid gamma or NSG) mice
were purchased from the Jackson Laboratory (JAX; Bar Harbor).
The OT1-Tg mice deficient of Maoa (OT1-Tg/Maoa-KO) were gen-
erated at the University of California, Los Angeles (UCLA) through
breeding OT1-Tg mice with Maoa-KO mice. All animals were manu-
tained in the animal facilities at UCLA. Eight- to 12-week-old females
were used for all experiments unless otherwise indicated. All animal
experiments were approved by the Institutional Animal Care and
Use Committee of UCLA.

Cell lines

The B16-OVA mouse melanoma cell line and the PG13 retroviral
packaging cell line were provided by P. Wang (University of Southern
California, CA, USA) (43). The MC38 mouse colon adenocarcinoma
cell line was provided by M. Rosenberg (Yale University, CT, USA)
(44). The human embryonic kidney 293T and Phoenix-ECO retro-
viral packaging cell lines were purchased from the American Type
Culture Collection (ATCC). The A375-A2-ESO-FG human melanoma
cell line was previously reported (24, 25). The Phoenix-ECO-MIG,
Phoenix-ECO-MIG-Maoa, and PG13-ESO-TCR stable virus produc-
cell lines were generated in this study.

Viral vectors

The MIG retroviral vector was reported previously (45). MIG-Maoa
and Retro/ESO-TCR retroviral vectors were generated in this study.

Media and reagents

Adherent cell culture medium (denoted as D10 medium) was made
of Dulbecco’s modified Eagle's medium (DMEM; catalog no. 10013,
Corning) supplemented with 10% fetal bovine serum (FBS; catalog
no. F2442, Sigma-Aldrich) and 1% penicillin-streptomycin-glutamine
catalog (10.378016, Gibco). T cell culture medium (denoted as
C10 medium) was made of RPMI 1640 (catalog no. 10040, Corning)
supplemented with 1% FBS (catalog no. F2442, Sigma-Aldrich),
1% penicillin-streptomycin-glutamine (catalog (10.378016, Gibco),
0.2% Normocin (catalog no. ant-nr-2, InvivoGen), 1% Minimal
Essential Medium (MEM) Non-essential Amino Acid Solution
catalog (11140050, Gibco), 1% HEPES (catalog no. 15630080,
Gibco), 1% sodium pyruvate (catalog no. 11360070, Gibco), and
0.05 mM β-mercaptoethanol (catalog no. M3148, Sigma-Aldrich).

Cell culture reagents—including purified no azide/low endotoxin
(NA/LE) anti-mouse CD3ε (catalog no. 553057, clone 145-2C11),
purified NA/LE anti-mouse CD28 (catalog no. 553294, clone 37.51),
anti-human CD3 (catalog no. 56685, clone OKT3), and anti-human
CD28 (catalog no. 555725, clone CD28.2)—were purchased form
BD Biosciences. Recombinant human IL-2 (catalog no. 200-02) was
purchased from PeproTech.
In vivo depletion antibodies, including anti-mouse CD8α (catalog no. BE0061, clone RMP2.43) and its isotype control [rat immunoglobulin G2b (IgG2b), catalog no. BE0090], were purchased from BioXCell. In vivo PD-1–blocking antibody (catalog no. BE0146, clone RMP1-14) and its isotype control (rat IgG2a, catalog no. BE0089) were purchased from BioXCell.

MAOIs—including phenelzine (catalog no. P6777), moclobemide (catalog no. M3071), and clorglyline (catalog no. M3778)—were purchased from Sigma-Aldrich. Serotonin (catalog no. H9532) and serotonin receptor (5-HTR) antagonist asenapine (catalog no. A7861) were also purchased from Sigma-Aldrich.

Syngeneic mouse tumor models
B16-OVA melanoma cells (1 × 10^6 cells per animal) or MC38 colon cancer cells (3 × 10^6 per animal) were subcutaneously injected into experimental mice to form solid tumors. In some experiments, mice received intraperitoneal injection of MAOIs [i.e., phenelzine (30 mg/kg per day), moclobemide (50 mg/kg per day), or clorglyline (50 mg/kg per day)] to block MAO-A activity. In some experiments, mice received intraperitoneal injection of anti-mouse CD8α antibodies (200 µg per animal, twice per week) to deplete CD8 T cells; mice that received intraperitoneal injection of isotype antibodies were included as controls. In some experiments, mice received intraperitoneal injection of anti-mouse PD-1 antibodies (300 µg per animal, twice per week) to block PD-1; mice that received intraperitoneal injection of isotype antibodies were included as controls.

During an experiment, tumor growth was monitored twice per week by measuring tumor size using a Fisherbrand Traceable digital caliper (Thermo Fisher Scientific); tumor volumes were calculated by formula 1/2 × L × W^2. At the end of an experiment, TIIIs were isolated for analysis using qPCR, flow cytometry, and/or scRNA-seq. In some experiments, sera were also collected for serotonin measurement.

Two-way BM transfer B16-OVA tumor model
BM cells were collected from femurs and tibias of donor mice and transferred into the recipient mice through retro-orbital (r.o.) injection. Recipient mice were preconditioned with whole-body irradiation (1200 gray). For BM transfer experiments confining MAO-A deficiency comparison to nonimmune cells, WT or Maoa-KO BM cells were transferred into CD45.1 recipient mice (8 to 10 × 10^6 cells per recipient mouse). For BM transfer experiments confining MAO-A deficiency comparison to immune cells, WT CD45.1 BM cells were transferred into Maoa-WT or Maoa-KO recipient mice (8 to 10 × 10^6 cells per recipient mouse). After BM transfer, recipient mice were maintained on antibiotic water (Amoxil; Sigma-Aldrich), and centrifuged at 800 g at 25°C for 30 min with brake off. Cell pellets enriched with TIIIs were then collected and resuspended in C10 medium for further analysis.

In the experiments studying the Maoa gene expression in TIIIs, day 14 B16-OVA tumors were harvested from B6 wild-type mice to prepare TII suspensions. TII suspensions were then sorted using a FACSaria II flow cytometer (BD Biosciences) to purify immune cells (gated as DAPI− CD45.2+ cells), which were then subjected to qPCR and immunofluorescence analysis of Maoa mRNA expression.

In other experiments, TII suspensions prepared under indicated experimental conditions were directly analyzed by flow cytometry to study surface marker expression and intracellular effector molecule production of CD8 T cells (preigated as CD45.2+TCRβ+CD8+ cells).
**In vitro mouse CD8 T cell culture**

Spleen and lymph node cells were harvested from Mboaa-KO or Mboaa-WT (B6 wild-type) mice and subjected to MACS using the Mouse CD8 T Cell Isolation Kit (catalog no. 120117044, Miltenyi Biotec) following the manufacturer’s instructions. Purified mouse CD8 T cells were cultured in vitro in C10 medium, in a 24-well plate at 0.5 × 10^6 cells/ml medium per well, in the presence of plate-bound anti-mouse CD3ε (5 µg/ml) for up to 4 days. At indicated time points, cells were collected for flow cytometry analysis of surface marker expression and intracellular effector molecule production and for qPCR analysis of mRNA expression; cell culture supernatants were collected for ELISA analysis of effector cytokine production.

In experiments studying serotonin signaling, cells were cultured in C10 medium made of serotonin-depleted FBS that was pretreated overnight with charcoal-dextran (1 g per 50 ml of FBS; catalog no. C6241, Sigma-Aldrich). 1-Ascorbic acid (100 µM; catalog no. A4403, Sigma-Aldrich) was added to C10 medium to stabilize T cell–produced or –supplemented serotonin. In some experiments, cells were treated with MAOIs to block MAO-A activity; MAOIs studied were phenelzine (10 µM), moclobemide (200 µM), or clorgyline (20 µM). In some experiments, cells were supplemented with exogenous serotonin (10 µM) to stimulate serotonin signaling. In some experiments, cells were treated with serotonin receptor antagonist asenapine (10 µM) to block serotonin receptor signaling.

**In vitro human CD8 T cell culture**

Healthy donor human peripheral blood mononuclear cells (PBMCs) were purchased from the UCLA Center for AIDS Research (CFAR) Virology Core Laboratory. PBMCs were cultured in C10 medium in the presence of plate-bound anti-human CD3 (1 µg/ml) and soluble anti-human CD28 (1 µg/ml). After 5 days, activated CD8 T cells were sorted on the basis of surface markers (CD45^+ TCRαβ^- CD8^- ) using a FACSAria II flow cytometer (BD Biosciences). Naïve CD8 T cells were sorted from the same donors based on surface markers (CD45^+ TCRαβ^- CD8^- CD62L^hi CD45RO^- ) and were included as controls. The purified naïve and effector human CD8 T cells were then analyzed for MAOA mRNA expression by qPCR.

**In vitro OT1 T cell culture**

Spleen and lymph node cells were harvested from the OTI-Tg or OTI-Tg/Moaab-KO mice and then subjected to MACS sorting using the Mouse CD8 T Cell Isolation Kit (catalog no. 120117044, Miltenyi Biotec) following the manufacturer’s instructions. The purified OTI T cells (identified as CD8^+ TCR Vβ5^+ cells) were cultured in C10 medium, in a 24-well plate at 0.5 × 10^6 cells per ml medium per well, in the presence of plate-bound anti-mouse CD3ε (5 µg/ml) for up to 4 days. At the indicated time points, cells were collected for flow cytometry analysis of surface marker expression; cell culture supernatants were collected for ELISA analysis of effector cytokine production.

**MIG-Moaab retroviral vector and mouse CD8 T cell transduction**

The MIG-Moaab retroviral vector was constructed by inserting a codon-optimized Moaab complementary DNA (cDNA) [synthesized by Integrated DNA Technologies (IDT)] into the parental MIG retroviral vector (45). The vesicular stomatitis virus glycoprotein (VSVG)– pseudotyped MIG and MIG-Moaab retroviruses were produced using 293T virus–packaging cells following a standard calcium precipitation method (46, 47) and then were used to transduce Phoenix-Eco cells to generate stable cell lines producing ECO-pseudotyped MIG or MIG-Moaab retroviruses (denoted as Phoenix-Eco-MIG and Phoenix-Eco-MIG-Moaab cell lines, respectively). For virus production, Phoenix-Eco-MIG and Phoenix-Eco-MIG-Moaab cells were seeded at a density of 0.8 × 10^6 cells/ml in D10 medium and cultured in a 15-cm dish (30 ml per dish) for 2 days; virus supernatants were then harvested and freshly used for spin infection.

MACS-purified CD8 T cells isolated from the Moaab-KO mice were cultured in vitro and stimulated with plate-bound anti-mouse CD3ε (5 µg/ml) for 4 days. On days 2 and 3, cells were spin-infected with ECO-pseudotyped MIG or MIG-Moaab retroviral supernatants supplemented with polybrene (10 µg/ml; catalog no. TR-1003-G, Millipore) at 1321 g at 30°C for 90 min. On day 4, cells were collected for flow cytometry analysis of transduction efficiency and for qPCR analysis of effector gene expression.

**Retro/ESO-TCR retroviral vector and human CD8 T cell transduction**

The Retro/ESO-TCR vector was constructed by inserting into the parental pMSGV vector a synthetic gene encoding an HLA-A2–restricted, NY-ESO-1 tumor antigen–specific human CD8 TCR (clone 3A1) (24). VSVG-pseudotyped Retro/ ESO-TCR retroviruses were generated by transfecting 293T cells following a standard calcium precipitation protocol and an ultracentrifugation concentration protocol (48); the viruses were then used to transduce PG13 cells to generate a stable retroviral packaging cell line producing gibbon ape leukemia virus (GalV) glycoprotein-pseudotyped Retro/ ESO-TCR retroviruses (denoted as PG13-ESO-TCR cell line). For virus production, the PG13-ESO-TCR cells were seeded at a density of 0.8 × 10^6 cells/ml in D10 medium and cultured in a 15-cm dish (30 ml per dish) for 2 days; virus supernatants were then harvested and stored at −80°C for future use.

Healthy donor PBMCs were stimulated with plate-bound anti-human CD3 (1 µg/ml) and soluble anti-human CD28 (1 µg/ml) in the presence of recombinant human IL-2 (300 U/ml). By 24 hr, cells were spin-infected with frozen-thawed Retro/ESO-TCR retroviral supernatants supplemented with polybrene (10 µg/ml) at 660 g at 30°C for 90 min after an established protocol (25). Transduced human CD8 T cells (denoted as ESO-T cells) were expanded for another 7 to 10 days and then cryopreserved for future use. Mock-transduced human CD8 T cells (denoted as Mock-T cells) were generated as controls.

**In vitro A375-A2-ESO-FG human melanoma cell-killing assay**

The A375-A2-ESO-FG human melanoma cells (5 to 10 × 10^4 cells per well) were cocultured with either ESO-T cells or Mock-T cells at indicated ratios in C10 medium in a Corning 96-well clear bottom black plate (catalog no. 3603, Corning). At 24 hours, live tumor cells were quantified by adding d-luciferin (150 µg/ml; part no. 119222, Caliper Life Science) to cell cultures and reading out luciferase activities using an Infinite M1000 microplate reader (Tecan) according to the manufacturer’s instructions.

**Flow cytometry (FACS)**

Flow cytometry, also known as fluorescence-activated cell sorting (FACS), was used to analyze surface marker and intracellular effector molecule expression of T cells. Fluorochrome-conjugated monoclonal antibodies specific for mouse CD45.2 (clone 104), TCRβ...
kine (i.e., IFN-γ) staining was performed using the Fixation/Permeabilization Solution Kit (BD Biosciences). These cells were costained with surface markers to identify CD8 T cells (gated as TCRβ+CD8+ cells in vitro or CD45.2+TCRβ+CD8+ cells in vivo) or OT1 cells (gated as CD45.2+CD8+ cells in vivo). Stained cells were analyzed by using a MACSQuant Analyzer 10 flow cytometer (Miltenyi Biotec). A FlowJo software (Tree Star) was used to analyze the data.

**Enzyme-linked immunosorbent assay (ELISA)**

To study T cell cytokine production, MACS-purified mouse CD8 T cells were cultured in C10 medium under indicated experimental conditions for up to 4 days. At indicated time points, cell culture supernatants were collected for serum ELISA analysis using a commercial kit following the manufacturer’s instructions (SEU39-K01, Eagle Biosciences). The absorbance at 450 nm was measured using an Infinite M1000 microplate reader (Tecan).

**Western blots**

CD8 T cells purified from MaaOa-WT and MaaOa-KO mice were cultured in vitro in a 24-well plate at 0.5 × 10^6 cells per well for 2 days, in the presence of plate-bound anti-mouse CD3ε (5 μg/ml), with or without asenapine treatment (10 μM). Cells were then rested on ice for 2 hours and restimulated with plate-bound anti-mouse CD3ε (5 μg/ml) for 20 min. Total protein was extracted using a lysis buffer containing 20 mM HEPES (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% TritonX-100, and protease/phosphatase inhibitor cocktail (catalog no. 5872S, Cell Signaling Technology). Nuclear protein was extracted using the Nuclear Protein Extraction Kit (catalog no. P178833, Thermo Fisher Scientific). Protein concentration was measured using the Bicinchoninic Acid (BCA) Assay Kit (catalog nos. 575309, and 4370S, clone D13.14.4E), anti-mouse p-ERK1/2 (catalog no. 43705S, clone D13.14.4E), secondary anti-mouse (catalog no. 7074P2), and secondary anti-rabbit (catalog no. 7076P2). Actin (catalog no. hc-11100, Santa Cruz Biotechnology) was used as an internal control for total protein extracts, whereas Lamin A/C (catalog no. 214655, clone 3A7), and β-Actin were used as an internal control for total protein extracts. Signals were visualized with autoradiography using an enhanced chemiluminescence (ECL) system (catalog no. RPN2322, Thermo Fisher Scientific). The data were analyzed using an Image Lab software (Bio-Rad).

**High-performance liquid chromatography (HPLC)**

HPLC was used to measure intratumoral and serum serotonin levels as previously described (49, 50). Briefly, tumors and sera were collected from experimental mice at indicated time points and were snap-frozen using liquid nitrogen. Frozen samples were thawed and homogenized using methanol and acetonitrile by vortexing. Homogenized samples were centrifuged, and supernatants were collected to new tubes and evaporated under a stream of argon. Dried sample pellets were then reconstituted in HPLC running buffer and were ready for analysis. Serotonin concentration was quantified using a C18 column by reverse-phase HPLC (System Gold 166P detector, Beckman Coulter). For tumor samples, both intracellular and interstitial serotonin were analyzed.

**mRNA quantitative RT-PCR (mRNA qPCR)**

Total RNA was isolated using TRIzol reagent (catalog no. 15596018, Invitrogen, Thermo Fisher Scientific) according to the manufacturer’s instructions. cDNA was prepared using the SuperScript III First-Strand Synthesis SuperMix Kit (catalog no. 18080400, Invitrogen).
Thermo Fisher Scientific). Gene expression was measured using the KAPA SYBR FAST qPCR Kit (catalog no. KM4117, Kapa Biosystems) and the 7500 Real-time PCR System (Applied Biosystems) according to the manufacturer’s instructions. Ube2d2 was used as an internal control for mouse immune cells, and ACTIN was used as an internal control for human immune cells. The relative expression of the mRNA of interest was calculated using the $2^{ΔACT}$ method. Primer sequences are available in table S1.

**Single-cell RNA sequencing (scRNA-seq)**

scRNA-seq was used to analyze the gene expression profiling of TILs. Day 14 B16-OVA tumors were harvested from Maaoa-WT and Maaoa-KO mice to prepare TII suspensions (10 tumors were combined for each group). TII suspensions were then sorted using a FACSAria II flow cytometer to purify immune cells (gated as DAPI− CD45.2+ cells). Sorted TIIIs were immediately delivered to the Technology Center for Genomics & Bioinformatics (TCGB) facility at UCLA for library construction and sequencing. Briefly, purified TIIIs were quantified using a Cell Countess II automated cell counter (Invitrogen/Thermo Fisher Scientific). A total of 10,000 TIIIs from each experimental group were loaded on the Chromium platform (10x Genomics), and libraries were constructed using the Chromium Single Cell 3’ Library & Gel Bead Kit v2 (catalog no. PN-120237, 10x Genomics) according to the manufacturer’s instructions. Libraries were sequenced on an Illumina NovaSeq using the NovaSeq 6000 S2 Reagent Kit (100 cycles; 20012862, Illumina). Data analysis was performed using a Cell Ranger Software Suite (10x Genomics). Binary base call (BCL) files were extracted from the sequencer and used as inputs for the Cell Ranger pipeline to generate the digital expression matrix for each sample. Then, cell-ranger aggr command was used to aggregate the two samples into one digital expression matrix. The matrix was analyzed using Seurat, an R package designed for scRNA-seq. Specifically, cells were first filtered to have at least 300 unique molecular identifiers (UMIs), at least 100 genes, and at most 50% mitochondrial gene expression; only one cell did not pass the filter. The filtered matrix was normalized using the Seurat function NormalizeData. Variable genes were found using the Seurat function FindVariableGenes. The matrix was scaled to repress the sequencing depth for each cell. Variable genes that had been previously identified were used in principal components analysis (PCA) to reduce the dimensions of the data. After this, 13 principal components (PCs) were used in Uniform Manifold Approximation and Projection (UMAP) to further reduce the dimensions to two. The same 13 PCs were also used to group the cells into different clusters by the Seurat function FindClusters. Next, marker genes were found for each cluster and used to define the cell types. Subsequently, three clusters of antigen-experienced tumor-infiltrating CD8 T cells (identified by coexpression of Cd8a, Cd3d, and Cd44 marker genes) were extracted and compared between the Maaoa-WT and Maaoa-KO samples.

For GSEA, signal-to-noise ratio was used to rank the genes in correlation with indicated gene signatures. For each cluster, the average expression of each gene in this cluster and in all other clusters was calculated. Fold changes were calculated by comparing the average expressions. The genes were then ranked by fold changes from high to low. To compare our data to preannotated datasets, we used the CD8 T cell subtype marker genes published by Miller et al. (16). GSEA was performed on these marker genes against the ranked genes in our dataset.

**Tumor Immune Dysfunction and Exclusion (TIDE) computational method**

TIDE analysis was performed as previously described (26, 51) (http://tide.dfci.harvard.edu/query/). Briefly, this method was used to study the association between the tumor-infiltrating CD8 T cell [also known as cytotoxic T lymphocyte (CTL)] level and overall patient survival in relation to the intratumoral MAAoa expression level. For each patient cohort, tumor samples were divided into MAAoa-high (samples with MAAoa expression one standard derivation above the average) and MAAoa-low (remaining samples) groups, followed by analyzing the association between the CTL levels and survival outcomes in each group. The CTL level was estimated as the average expression level of CD8A, CD8B, GZMA, GZMB, and PRF1. Each survival plot presented tumors in two subgroups: “CTL-high” group had above-average CTL values among all samples, whereas “CTL-low” group had below-average CTL values. A T cell dysfunction score (z score) was calculated for each patient cohort, correlating the MAAoa expression level with the beneficial effect of CTL infiltration on patient survival. A positive z score indicates that the expression of MAAoa is negatively correlated with the beneficial effect of tumor-infiltrating CTL on patient survival. The P value indicates the comparison between the MAAoa-low and MAAoa-high groups and was calculated by two-sided Wald test in a Cox proportional hazards (Cox–PH) regression.

**Statistics**

A GraphPad Prism 7 software (GraphPad Software) was used for the graphic representation and statistical analysis of the data. Pairwise comparisons were made using a two-tailed Student’s t test. Multiple comparisons were performed using an ordinary one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparisons test or a two-way repeated measures ANOVA followed by Sidak multiple comparisons test. Data are presented as the means ± SEM, unless otherwise indicated. A P value of less than 0.05 was considered significant. ns, not significant; *P < 0.05, **P < 0.01, and ***P < 0.001. The P values of violin plots were determined by Wilcoxon rank sum test. The P values of comparison between survival plots were calculated by testing the association between TIDE prediction scores and overall survival with the two-sided Wald test in a Cox–PH regression.

**SUPPLEMENTARY MATERIALS**

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Fig. S1. Characterization of Mouse-KO mice (related to Fig. 1).

Fig. S2. MAO-A−deficient mice show suppressed tumor growth and enhanced CD8 T cell antitumor immunity (related to Fig. 1, E to M).

Fig. S3. MAO-A−deficient tumors indicate reduced MAO-A expression (related to Fig. 1, E to M).

Fig. S4. MAO-A directly regulates antitumor immunity (related to Fig. 3).

Fig. S5. MAO-A inhibition for cancer immunotherapy: Human T cell studies (related to Fig. 6).
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Targeting monoamine oxidase A for T cell–based cancer immunotherapy

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Repurposing antidepressants for cancer immunotherapy

Antibodies targeting immune checkpoints that restrain the antitumor effects of T cells are now a proven therapeutic approach for many forms of human cancer. The search for additional drug-targetable checkpoint molecules that regulate tumor immunity continues. Wang et al. identified monoamine oxidase A (MAO-A) as a gene up-regulated in tumor-infiltrating immune cells in mice. Tumor cells grew at a slower rate in mutant mice with decreased MAO-A expression. Inhibition of MAO-A activity by the antidepressant drug phenelzine enhanced the resistance of wild-type mice to tumor growth and exhibited synergy with anti–PD-1 treatment. These findings set the stage for further studies assessing the potential of repurposed MAO-A inhibitor drugs in the immunotherapy of cancer.