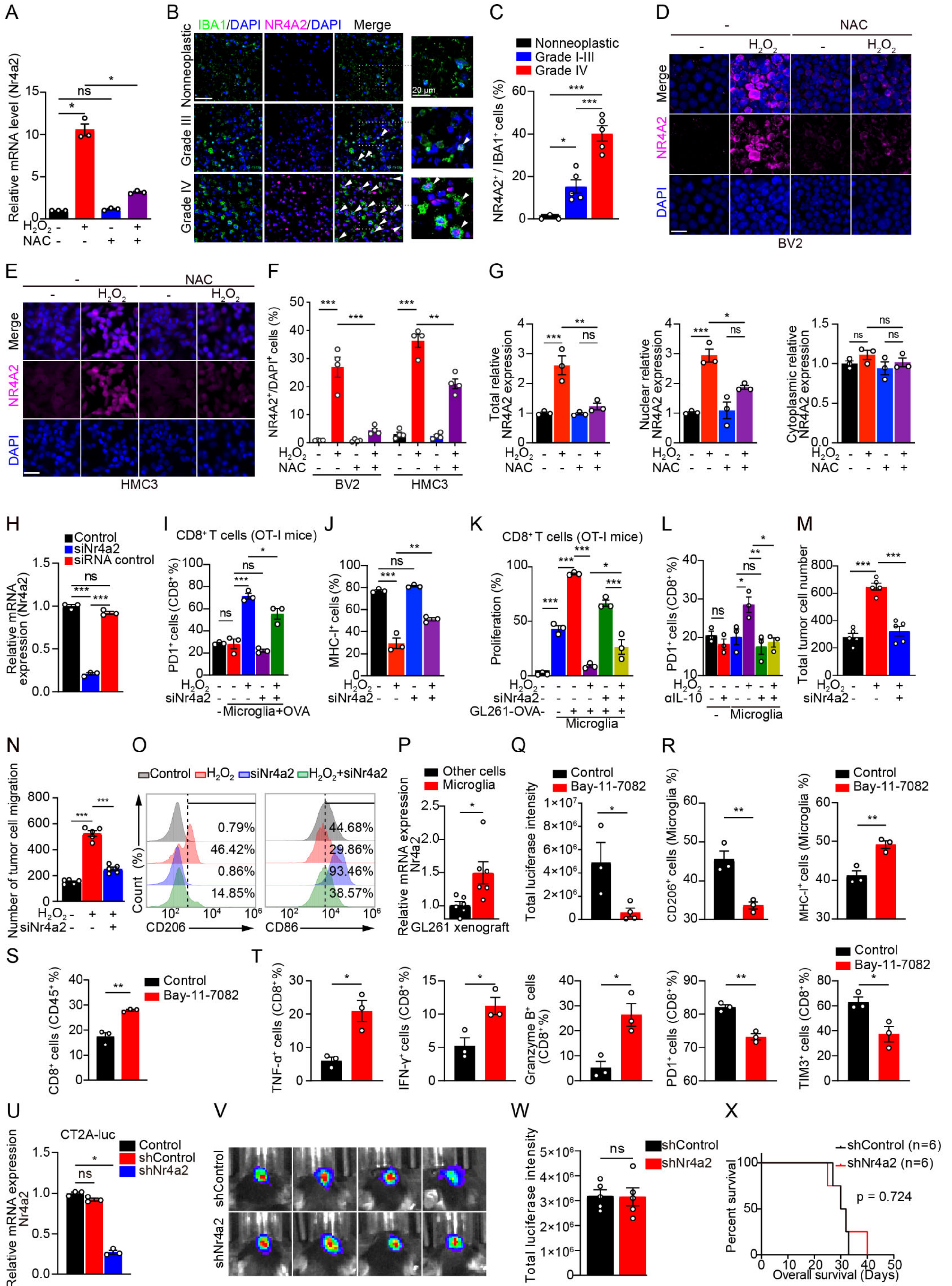

A
Relative mRNA level (Nr4a2)
ns
H2O2
NAC
B
IBA1/DAPI NR4A2/DAPI Merge
Nonneoplastic
Grade III
Grade IV
20 μm
C
Nonneoplastic
Grade I-III
Grade IV
NR4A2+ / IBA1+ cells (%)
D
NAC
H2O2
Merge
NR4A2
DAPI
BV2
E
NAC
H2O2
Merge
NR4A2
DAPI
HMC3
F
NR4A2+/DAPI+ cells (%)
H2O2
NAC
BV2
HMC3
G
Total relative NR4A2 expression
Nuclear relative NR4A2 expression
Cytoplasmic relative NR4A2 expression
H2O2
NAC
ns
H
Control
siNr4a2
siRNA control
Relative mRNA expression (Nr4a2)
ns
I
CD8+ T cells (OT-I mice)
PD1+ cells (CD8+ %)
ns
H2O2
siNr4a2
Microglia+OVA
J
MHC-I+ cells (%)
ns
H2O2
siNr4a2
K
CD8+ T cells (OT-I mice)
Proliferation (%)
H2O2
siNr4a2
GL261-OVA
Microglia
L
PD1+ cells (CD8+ %)
ns
H2O2
αIL-10
Microglia
M
Total tumor cell number
H2O2
siNr4a2
N
Number of tumor cell migration
H2O2
siNr4a2
O
Control
H2O2
siNr4a2
H2O2+siNr4a2
Count (%)
0.79%
46.42%
0.86%
14.85%
44.68%
29.86%
93.46%
38.57%
CD206
CD86
P
Other cells
Microglia
Relative mRNA expression Nr4a2
GL261 xenograft
Q
Control
Bay-11-7082
Total luciferase intensity
R
Control
Bay-11-7082
CD206+ cells (Microglia %)
MHC-I+ cells (Microglia %)
S
CD8+ cells (CD45+ %)
Control
Bay-11-7082
T
TNF-α+ cells (CD8+ %)
IFN-γ+ cells (CD8+ %)
Granzyme B+ cells (CD8+ %)
PD1+ cells (CD8+ %)
TIM3+ cells (CD8+ %)
Control
Bay-11-7082
U
CT2A-luc
Control
shControl
shNr4a2
Relative mRNA expression Nr4a2
ns
V
shControl
shNr4a2
W
Total luciferase intensity
ns
shControl
shNr4a2
X
shControl (n=6)
shNr4a2 (n=6)
p = 0.724
Percent survival
Overall survival (Days)

Supplementary Figure S3. Inhibiting nuclear translocation of NR4A2 hampers immunosuppressive functions and promotes antigen presentation capacities of microglia, related to Figure 4

(A) Relative expression levels of *Nr4a2* in primary microglia treated by H_2O_2 (500 μM) in absence or presence of NAC (5 mM) for 12 hours.

(B, C) Immunofluorescence staining for NR4A2 and microglia maker (IBA1) on different grades glioma tissues and nonneoplastic brain tissues. Scale bars, 50 μm (B). Quantification of NR4A2$^+$ microglia levels in respective group (C).

(D-F) Immunofluorescence staining for NR4A2 on BV2 cells (D) and HMC3 cells (E) cultured on glass coverslips. Scale bars, 20 μm. Quantification of marker-positive cell fractions is shown on the right (F).

(G) Quantification of NR4A2 protein expression in nucleus, cytoplasm and total cell lysates of microglia cells with respective treatment.

(H) Relative expression levels of *Nr4a2* in primary microglia transfected with siNr4a2 or siRNA control.

(I) Primary microglia were transfected by siNr4a2 for 48 hours. Nr4a2-knockdown microglia treated with H_2O_2 (500 μM) in the absence or presence of NAC (5 mM) for 12 hours. Quantitation of PD1 expression in CD8$^+$ T cells cocultured with treated Nr4a2-knockdown microglia pulsed with OVA peptide by flow cytometry.

(J) Quantitation of MHC-I expression in treated Nr4a2-knockdown microglia.

(K) Proliferation of CD8$^+$ T cells in treated Nr4a2-knockdown microglia by flow cytometry. Primary microglia transfected with siNr4a2 for 48 hours was treated with H_2O_2 (500 μM) in the absence or presence of NAC (5 mM) for 12 hours. Treated microglia was cultured with GL261-OVA cells (1:1) for 12 hours. Microglia purified by microbeads were co-cultured with CD8$^+$ T cells (1:10) of OT-I mice for 48 hours.

(L) Primary microglia were treated by H_2O_2 (500 μM) for 12 hours. Treated microglia were co-cultured with CD8$^+$ T cells (1:10) of for 48 hours in the absence or presence of αIL-10 (1 μg/ml). Flow cytometry was used to evaluate PD1 expression in CD8$^+$ T cells in each treatment group.

(M, N) NR4A2-knockdown inhibited the pro-tumoral functions of H_2O_2-challenged

microglia. Primary microglia transfected with siNr4a2 for 48 hours were treated by H_2O_2 (500 μM) in absence or presence of NAC (5 mM) for 12 hours. Conditional medium from treated primary microglia was used to incubate with glioma cells (GL261). Cell proliferation (M) and migration (N) of glioma cells were tested by cell counts and Transwell Chamber assays, respectively.

(O) Flow cytometry plot to test CD86 and CD206 levels of Nr4a2-knockdown microglia treated by H_2O_2 (500 μM) in the absence or presence of NAC (5 mM) for 12 hours.

(P) Relative expression levels of *Nr4a2* in primary microglia and others cells from glioma-bearing mice.

(Q) Quantification of tumor volume of glioma-bearing C57BL/6J mice treated by Bay-11-7082 based on bioluminescence (Figure 4M) (n=3 to 4 for each group).

(R, S) Flow cytometry analysis of CD206 and antigen presentation maker (MHC-I) of microglia (R) and percentage of $CD8^+$ T cells (S) in glioma tissue from glioma-bearing mice treated with Bay-11-7082 and control mice.

(T) Flow cytometry plot to evaluate cytotoxic functions and immune checkpoint of $CD8^+$ T cells in glioma tissue from glioma-bearing mice treated with Bay-11-7082 and control mice.

(U) Relative expression levels of *Nr4a2* in CT2A-luc transduced with shNr4a2 or shRNA control.

(V, W) Representative *in vivo* bioluminescence-based images of Nr4a2-knockdown CT2A-luc glioma-bearing C57BL/6J mice (V) and quantification (W) of tumor volume based on bioluminescence (n=5 per group).

(X) Survival curves of C57BL/6J mice implanted with Nr4a2- knockdown CT2A-luc and control (n=6 per group).

Data are shown as mean ± SEM. In (A), (C), (F-N) and (U) P value was calculated using one-way *ANOVA* analysis. In (P-T) and (W), P value was calculated using the two-tailed Student's *t* test. In (X), survival difference was calculated by log-rank test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.