Title:

Selective disruption of lipid peroxide homeostasis in intratumoral regulatory T cells by
targeting FSP1 enhances cancer immunity

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Abstract:
A burgeoning approach to treat cancer is the pharmacological induction of ferroptotic
cell death of tumor cells. However, the impact of disrupting anti-ferroptotic pathways in
the broader tumor microenvironment (TME), such as in immune cells, is still undefined
and may complicate treatments. Here, we show that Ferroptosis Suppressor Protein 1
(FSP1/*Aifm2*) is critically required for regulatory T cell (Treg) resistance to ferroptosis
and their immunosuppressive function within the TME. Compared to other canonical
ferroptosis regulators such as GPX4, GCH1, and NRF2, only FSP1 was induced upon T
cell activation. Deletion of *Aifm2* in all T cells, or Tregs specifically, enhanced tumor
control by selectively disrupting Treg immunosuppression within tumors without inciting
autoimmune pathology in mice. As opposed to deletion of *Gpx4* in all T cells, T cell
deletion of *Aifm2* did not impair antigen-specific CD8^{+} T cell responses. These results
reveal a unique opportunity for targeting a regulator of ferroptosis that can not only
directly target cancer cells, but also simultaneously enhance anti-cancer immune
responses without inciting autoimmunity.

Main Text:

Ferroptosis, an iron-dependent form of regulated cell death driven by unchecked
lipid peroxide accumulation, has emerged as a promising approach for treating therapy-
resistant cancers(*1–3*). Unlike apoptosis, ferroptosis can release danger-associated

molecular patterns (DAMPs) that activate immune responses(*4*, *5*). While glutathione

peroxidase 4 (GPX4) is the primary defense against ferroptosis, cancer cells often

express alternative protective ferroptotic regulators, such as ferroptosis suppressor

protein 1 (FSP1) and GTP cyclohydrolase 1 (GCH1), to resist GPX4 inhibition(*6–11*).

Regulatory T cells (Tregs), a suppressive subset of CD4$^+$ T cells, pose a major

barrier to effective cancer immunotherapy by inhibiting antitumor responses through

direct cell contact and the release of immunosuppressive molecules(*12–14*). Tregs are

uniquely adapted to the nutrient-deprived and hypoxic tumor microenvironment (TME)

by preferentially using oxidative phosphorylation (OXPHOS), importing lipids via CD36,

and utilizing lactate and fatty acids as alternative carbon sources for their

metabolism(*15–18*). Moreover, Tregs upregulate antioxidant pathways—including

glutathione synthesis and thioredoxin—to mitigate oxidative stress and preserve their

function in the TME(*19–21*). Although Tregs depend on GPX4 to prevent ferroptosis,

inhibiting GPX4 is not a viable strategy for cancer immunotherapy as its inhibition also

impairs effector CD8$^+$ and CD4$^+$ T cell survival(*22*, *23*).

In this study, we establish that FSP1/*Aifm2* is a critical effector protein for Treg

resistance to ferroptosis induction *in vitro* and Treg function *in vivo* in tumors. We found

that Tregs use FSP1 to prevent lipid peroxide accumulation despite having higher levels

of intracellular reactive oxygen species (ROS). *Aifm2*-deficiency was sufficient to

increase the sensitivity of Tregs to ferroptosis induction with GPX4 inhibition *in vitro*.

Aifm2-deficiency in Tregs decreased the expression of proteins essential for Treg

effector function, but did not impact effector T cell immune homeostasis or peripheral

tolerance in young or aged mice. Total ablation of *Aifm2* in all T cells did not disrupt

antitumor T cell responses but did diminish the immunosuppressive function of Tregs.

These findings are significant for the translation of pharmacological FSP1 inhibitors for

cancer as they show that FSP1 inhibition not only can directly sensitize cancer cells to

ferroptosis, but it may also enhance immune responses to cancer by selectively

blocking Treg function in the TME without generating autoimmune toxicity.

Results:

Tregs resist lipid peroxide accumulation and ferroptosis

To understand the regulation of ROS across different T cell populations, we

FACS-purified naive Tregs and conventional CD4$^+$ T cells (Tconv) for activation *in vitro*

and measuring their total ROS and lipid peroxide levels using fluorescent reagents

detected by flow cytometry **(Fig. 1A)**. In accordance with numerous studies, we

observed that Tregs had increased (~2-fold) intracellular ROS levels compared to Tconv

cells(*18*, *24*) **(Fig. 1B)**. Next, we directly measured lipid peroxidation by measuring the

amount of oxidized BODIPY C11 and observed that Tregs had decreased amounts of

lipid peroxides compared to Tconv cells **(Fig. 1C)**. While RSL3 increased oxidized C11-

BODIPY in both Tregs and Tconv cells, Tconv cells increased CD11-BODIPY^{OX}

significantly more than Tregs **(Fig. 1D)**. Importantly, only Ferrostatin-1 (Fer-1), a radical-

trapping antioxidant, but not the pan-caspase inhibitor Z-VAD-FMK or RIP1 kinase

inhibitor Nectrostatin-1 (Nec-1) treatments reversed CD11-BODIPY^{OX} elevation, as

expected **(Fig. 1D)**. Improved control of lipid oxidation ultimately made Tregs more

resistant to ferroptotic cell death with the inhibition of GPX4 across a series of

concentrations of RSL3 **(Fig. 1E)**. Decreased cell viability was confirmed to be due to

ferroptosis, as only treatment with Fer-1, but not other programmed cell death inhibitors,

was able to rescue cell viability **(Fig. 1F)**. Interestingly, the differences in sensitivity to

ferroptosis were not due to the expression of the essential Treg transcription factor

Foxp3, as *in vitro* induced Tregs (iTregs), were as sensitive to GPX4 inhibition as Tconv

cells **(Fig. 1E)**(*19*). To confirm these findings without activating or culturing T cells long

term *in vitro*, we isolated T cells from spleens of mice and treated them with RSL3 alone

or RSL3 and Fer-1 for 4 hours *ex vivo*. This revealed that while CD8$^+$ and CD4$^+$ Tconv

cells were highly sensitive to GPX4 inhibition, Tregs remained highly resistant to

ferroptosis *ex vivo* **(Fig. 1G)**. Therefore, despite having higher amounts of ROS, Tregs

reduce their accumulation of lipid peroxides, which likely increases their resistance to

ferroptosis.

FSP1 expression in Tregs confers resistance to ferroptosis

T cell activation increases levels of ROS, which can be tuned by the expression

of antioxidant genes to regulate cytosolic signaling pathways(*22, 25–29*). Analysis of

gene expression by RNA-sequencing of FACS-purified Tregs and Tconv cells that were

naive (CD44^{lo}CD62L^{hi}) versus four days after anti-CD3/anti-CD28 *in vitro* activation,

demonstrated that only *Aifm2* increased expression, but not other anti-ferroptotic

proteins, e.g. *Gpx4*, *Gch1*, or *Nfe2l2*, upon *in vitro* T cell activation **(Fig. 2A)**(*30*). In

addition, a comparison of freshly sorted *in vivo* activated (CD44^{hi}CD62L^{lo}) Tregs

compared to naive Tregs from mice showed a similar induction of *Aifm2* in activated

Tregs **(Fig. 2B)**(*30*). Moreover, direct comparison of *in vitro* activated Tregs versus

Tconv cells revealed a 2-3 fold greater induction of *Aifm2* and *Gch1* expression, and

reduced expression of *Nfe2l2* and *Ascl3*, in Tregs compared to Tconvs **(Fig. 2C)**. These

results demonstrate that *Aifm2*/FSP1 is induced during T cell activation, but that Tregs

induce higher expression of *Aifm2* and *Gch1* antioxidant genes with activation, which

may underlie their enhanced resistance to ferroptosis.

While GCH1 was shown to be crucial for T cell proliferative capacity, the role of

FSP1 in Treg biology is unexplored(*29*). To define the role of FSP1 in Tregs, we

crossed mice carrying LoxP-flanked *Aifm2^{fl}* alleles with *Foxp3-GFP-hCre* to specifically

delete *Aifm2* in Tregs (called Treg.*Aifm2$^{Δ/Δ}$* mice)(*31*). Treatment of *Aifm2*-deficient

Tregs with RSL3 to induce ferroptosis revealed that *Aifm2*-deficient Tregs now exhibited

significantly increased sensitivity to RSL3-mediated cell death compared to wildtype
Tregs (**Fig. 2D)**. Thus, FSP1 promotes Treg resistance to ferroptosis *in vitro*.
Finally, comparative gene expression analysis from human peripheral blood
mononuclear cells (PBMCs) confirmed that human Tregs that are activated (memory
Treg) also exhibit the highest expression of *Aifm2* compared both to naive Tregs as well
as all other activated T cell populations (**Fig. 2E-F**). These results indicate that
FSP1/*Aifm2* expression protects Tregs from ferroptosis and FSP1/*Aifm2* expression is
higher in both mouse and human activated Tregs compared to other T cell subsets.
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Treg-specific FSP1 deletion does not impact peripheral tolerance
To directly test whether Treg-specific deletion of *Aifm2* disrupted Treg function *in*
vivo, we analyzed lymphoid and nonlymphoid tissues from wildtype versus
Treg.*Aifm2*$^{\Delta/\Delta}$ mice at early (16 weeks) and late (28 weeks) stages of life. There were no
changes to the frequency of CD4$^+$ Tconv, CD8$^+$ T, or Tregs across lymphoid organs
(lymph node, spleen) at any age (**Fig. 3A and Fig. S1A-B**). However, in the organ
tissues examined (colon, skin, lung), while CD4$^+$ Tconv and CD8$^+$ T cell frequencies did
not change in Treg.*Aifm2*$^{\Delta/\Delta}$ mice, we did note a modest reduction in Treg frequency in
the lung and colon of Treg.*Aifm2*$^{\Delta/\Delta}$ mice **(Fig. 3A and Fig. S1C)**. Additionally, there
were no changes to the activation state of CD4$^+$ Tconv, CD8$^+$ T, or Tregs in all tissues
examined (**Fig. 3B and Fig. S1D**). These data suggest that Treg-specific *Aifm2*-
deficiency does not increase the prevalence of autoimmunity in mice.
Phenotyping of the Tregs from Treg.*Aifm2*$^{\Delta/\Delta}$ mice revealed no significant
changes in the expression of canonical lineage defining Treg markers (i.e. CD25,
Foxp3, and NRP1) in either lymphoid or organ tissues **(Fig. 3C and Fig. S1E)**.
However, there was a significant decrease in CD73 expression on colonic and lung
Tregs **(Fig. 3D-E).** CD73, in conjunction with CD39, acts as an ectonucleoside that
breaks down ATP to generate adenosine, a critical immunosuppressive molecule
generated by Tregs. Interestingly, both CD73 and CD39 expression were significantly
decreased in the lymphoid tissues of aged mice **(Fig. S1E)**. Altogether, these results
show that *Aifm2*-deficiency in Tregs does not dramatically impact the frequencies or
phenotype of Tregs in lymphoid or non-lymphoid tissues. Thus, *Aifm2*-deficiency in
Tregs does not lead to a systemic breakdown in immunotolerance or organ-specific
autoimmunity.
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FSP1-deficiency specifically in Tregs impairs immunosuppression in response to
cancer
Since the pharmacological inhibition of FSP1 in cancer cells is moving to clinical
trials, we next wanted to assess tumor Treg responses to the inhibition of FSP1. First,
we tested whether different subsets of T cells from tumors exhibited different levels of
resistance to ferroptosis induction, similar to experiments in Figure 1G. Here, we grew

MC38 colon carcinoma tumors subcutaneously for 14 days in wildtype mice, then
harvested tumors, made single cell suspensions, and treated all cells for four hours with
or without 1µM RSL3 to induce ferroptosis. Strikingly, Tregs again exhibited strong
resistance to the induction of ferroptosis with GPX4 inhibition as compared to Tconv
and CD8$^+$ T cells **(Fig. 4A)**. To test whether Treg-specific FSP1 activity promoted tumor
Treg resistance to ferroptosis and supported tumor growth, we compared MC38 tumor
growth in wildtype versus Treg.*Aifm2$^{\Delta/\Delta}$* mice **(Fig. 4B)**(*32*). Tumor growth was
significantly reduced in Treg.*Aifm2$^{\Delta/\Delta}$* mice **(Fig. 4C)**. While Treg.*Aifm2$^{\Delta/\Delta}$* mice did not
exhibit changes to Treg frequencies or tumor-specific CD8$^+$ T cells in tumors (**Fig. 4D**
and Fig. S2A), Treg.*Aifm2$^{\Delta/\Delta}$* mice did exhibit increased CD8 to Treg ratios in tumors,
indicative of a selective impact on Tregs compared to effector T cells in tumors with
FSP1-deficiency in Tregs (**Fig. 4D**). In addition, we did not observe altered expression
of the exhaustion markers PD-1 or LAG-3 in CD8$^+$ T cells in Treg.*Aifm2$^{\Delta/\Delta}$* mice (**Fig.**
S2B). Importantly, to demonstrate that the reduction in tumor growth was due to the
accumulation of lipid peroxides in FSP1-deficient Tregs, we treated wildtype and
Treg.*Aifm2$^{\Delta/\Delta}$* tumor-bearing mice with the radical trapping antioxidant liproxstatin-1
(Lip-1), a ferroptosis inhibitor that is functional *in vivo in mice(33)* (**Fig. 4E**). Lip-1
treatment restored tumor growth in Treg.*Aifm2$^{\Delta/\Delta}$* mice, while Lip-1 treatment had no
effect on tumor growth in wildtype mice (**Fig. 4F**). These results indicate that FSP1
activity in Tregs is required to control their accumulation of lipid peroxides within the
TME, and disruption of FSP1 activity can release immunosuppression by Tregs and
promote better tumor control.
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FSP1-deficiency in all T cells enhances T cell responses to infection and cancer
T cell deficiency for *Gpx4*- or *Gch-1* was recently shown to negatively impact T
cell responses in several disease settings(*23*, *29*, *34*). To test if *Aifm2*-deficiency also
impacts all T cell responses, which would occur with pharmacological inhibition for
cancer treatment, we generated *Aifm2*-deficient T cell mice (*CD4-Cre;Aifm2$^{fl/fl}$*, called
T.Aifm2$^{\Delta/\Delta}$) and infected these mice with *Listeria monocytogenes*, an intracellular
pathogen known for stimulating a robust CD8$^+$ T cell response (**Fig. S3A**)(*35*, *36*).
Importantly, *T.Aifm2$^{\Delta/\Delta}$* mice had no deficiency in bacterial control, as there was no
difference in bacterial CFUs recovered from spleens or livers of wildtype versus
T.Aifm2$^{\Delta/\Delta}$ six days post infection (**Fig. S3B**). Upon *Listeria* infection, we observed a
decrease in the frequency of Tregs as a percentage of CD4$^+$ T conv cells, as well as of
total CD45$^+$ immune cells, in *T.Aifm2$^{\Delta/\Delta}$* (**Fig. S3C**). The frequency of total CD8$^+$ T cells,
Listeria-specific CD8$^+$ T cells, or CD4$^+$ Tconv cell populations, however, did not change
(**Fig. S3D-F**).
Next, to test the impact of deleting *Aifm2* in all T cells, as would happen with a
pharmacological FSP1 inhibitor, in the setting of cancer, we implanted MC38 tumors
into wildtype versus *T.Aifm2$^{\Delta/\Delta}$* mice and measured tumor growth (**Fig. 5A**). Tumor

growth was significantly decreased in mice harboring *Aifm2*-deficient T cells (**Fig. 5B**).

While there was no change in the frequency of Tregs in MC38 tumors, there was an

increase in the CD8:Treg ratios in tumors from $T.Aifm2^{\Delta/\Delta}$ compared to wildtype mice,

as observed with Treg-specific *Aifm2*-deficiency (**Fig. 5C-D**)(*37, 38*). Assessment of

CD8^{+} T cell exhaustion in tumors by staining for both PD-1 and Lag-3 proteins showed

no changes in total CD8^{+} T cells **(Fig. 5E)**. However, tumor-specific CD8^{+} T cells, while

unchanged in total frequencies, exhibited decreased frequencies of PD-1^{+}Lag3^{+}

MuLV/H2K[b+] CD8^{+} T cells (**Fig. 5F**)(*39*). These results starkly contrast with the

consequences of T cell deficiencies in other antioxidant genes, i.e. *Gpx4* or *Gch1*,

where deficient T cells completely lost their capacities to control infections or cancer(*23,*

29). Instead, deficiency for FSP1/*Aifm2* in all T cells completely preserves CD8^{+} and

CD4^{+} Tconv cell function while potently disrupting the function of Tregs.

Discussion:

In this study, we demonstrate that FSP1 (*Aifm2*) plays a critical role, and unique

role amongst T cells, in sustaining Treg function within tumors. Unlike other ferroptosis

resistance factors such as GPX4, which are essential for proper T cell function during

infections or cancer, FSP1 appears to be selectively required for Tregs without

compromising effector T cell responses. We show that FSP1 expression is induced

during T cell activation, distinguishing it from other ferroptosis regulators like GPX4,

GCH1, or NRF2. Our data indicate that FSP1 supports Treg survival and suppressive

function by limiting lipid peroxide accumulation despite high levels of ROS generation in

Tregs. This suggests that increased FSP1 expression in Tregs may have evolved to

prevent sublethal oxidative damage that would otherwise kill or impair Tregs.

These findings reveal a novel paradigm in which targeting FSP1 may specifically

modulate Treg-mediated immunosuppression in cancer without affecting effector T

cells. In contrast to GPX4-targeted approaches, which can dampen overall T cell

responses and carry a narrow therapeutic window due to GPX4's essential role in

multiple tissues, inhibition of FSP1 appears to selectively compromise Treg function(*23,*

34, 40, 41). In addition, Tregs are sensitive to specific antioxidant pathway activity as

the genetic deletion of KEAP1 leads to loss of Treg lineage identity that leads to

spontaneous autoimmunity due to hyperactivation of NRF2 and uncontrolled induction

of antioxidant pathways(*42*). Our tumor models showed that genetic ablation of FSP1 in

T cells resulted in better tumor control, potentially due to a reduction in Treg

suppressive capacity and a concomitant decrease in canonical exhaustion markers

(e.g., PD-1, Lag3) on tumor-specific CD8^{+} T cells(*43*).

The implications of these findings are significant. By specifically targeting FSP1,

it may be possible to alleviate the immunosuppressive effects of Tregs within the TME,

thereby enhancing the efficacy of cancer immunotherapies. Moreover, FSP1 inhibition

exhibits great potential to synergize with immune checkpoint blockade (e.g., anti-PD-1

therapies) and cellular immunotherapies such as CAR T cells by preventing T cell
exhaustion and preserving cytotoxic function. PD-1 blockade has also been shown to
induce IFN-γ production by CD8$^+$ T cells, which induced ferroptosis in cancer cells
directly by impacting the lipid composition through Ascl4 expression(*44*). Thus, this dual
approach, targeting tumor cells directly through ferroptosis induction while
simultaneously modulating Treg function, could offer a much broader therapeutic
strategy for treating cancer.
Our study also underscores the importance of metabolic regulation in Treg
biology. Tregs exhibit a unique metabolic profile characterized by a reliance on oxidative
phosphorylation and fatty acid utilization, which supports their survival in the nutrient-
deprived, hypoxic TME(*15–17*). Despite high ROS levels due to these forms of
metabolism, Tregs maintain low lipid peroxide accumulation, in part by maintaining
higher expression of FSP1. Disrupting this balance through FSP1 inhibition leads to
functional defects in Tregs without appearing to cause their complete elimination in vivo,
which is preferable for preserving immune homeostasis.
In conclusion, our results position FSP1 as a Treg-specific ferroptosis regulator
and a promising therapeutic target for enhancing cancer immunity. By selectively
modulating FSP1 activity, it may be possible to reduce Treg-mediated suppression in
tumors, preserve effector T cell responses, and ultimately improve the outcomes of
cancer immunotherapy. Future studies should focus on translating these findings into
pharmacological interventions, as well as exploring the potential of FSP1-targeting
strategies in combination with existing immunotherapies to achieve synergistic effects.
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Materials and Methods:
Mice
CD4-Cre mice were obtained from Jackson laboratories (JAX:022071) and bred in
house. Foxp3-GFP-hCre mice were obtained from Jackson laboratories (JAX:023161)
and bred in house. *Aifm2^{fl}* mice were generated as described(*31*). For tumor studies,
syngeneic C57BL/6J mice were inoculated with 5.0×10^5 MC38 in PBS subcutaneously.
Tumor measurements were performed blindly across the entire experiment by a single
operator measuring three dimensions of the tumor with calipers three times per week.
For in vivo liproxstatin treatments (Selleck Chemicals), mice were injected
intraperitoneally with 10mg/kg of liproxstatin-1 dissolved in DMSO and diluted in
PEG300, Tween-80, and dH2O. All the experiments were conducted according to the
Institutional Animal Care and Use Committee guidelines of the University of California,
Berkeley.
273
274 **Cell lines**
275 MC38 cell lines were kindly provided by Dr. Jeff Bluestone's lab(*45*). All cell lines were
276 maintained in DMEM (GIBCO) supplemented with 10% FBS, sodium pyruvate (GIBCO),

277 10mM HEPES (GIBCO), and penicillin-streptomycin (GIBCO). Tumor cells were grown
278 at 37℃ with 5% CO_2.
279

280 **Listeria monocytogenes strains**
281 All strains of L. monocytogenes were derived from the wild-type 10403S strain. All
282 strains were cultured in filter-sterilized nutrient-rich Brain Heart Infusion (BHI) media
283 (BD Biosciences) containing 200 µg/mL streptomycin (Sigma-Aldrich).
284

Intravenous and Intratumoral Listeria infection
Overnight cultures were grown in BHI + 200 µg/mL streptomycin at 30℃. The following
day, bacteria were grown to logarithmic phase by diluting the overnight culture in fresh
BHI + 200 µg/mL streptomycin and culturing at 37℃ shaking. Log-phase bacteria were
washed and frozen in 9% glycerol/PBS. For intravenous infections, frozen stocks were
diluted in PBS to infect via the tail vein with $5x10^3$ CFU log-phase bacteria. The mice
were euthanized 6 days after intravenous injections and organs were collected for CFU
or flow analysis.
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Primary Treg and T cell culture
Spleens and lymph nodes were collected from Foxp3-GFP-hCre;R26$^{LSL-RFP}$; *Aifm2*$^{+/+}$,
Foxp3-GFP-hCre;R26$^{LSL-RFP}$; *Aifm2*$^{Δ/Δ}$, CD4-Cre;*Aifm2*$^{+/+}$ or CD4-Cre;*Aifm2*$^{Δ/Δ}$. Single
cell suspensions were generated and enriched for CD4+ T cells by negative selection
using EasySep magnetic bead kit (STEMCELL Technologies) and stained with anti-
mouse CD4 (RM4-5, BioLegend), anti-CD62L (MEL1-14, Biolegend), anti-CD8 (53-6.7,
Biolegend), anti-CD25 (PC61, BioLegend), anti-CD357 (GITR) (DTA-1, Biolegend).
Naïve Treg cells (CD4$^+$Foxp3GFP$^+$RFP$^+$CD62L$^+$ or CD4$^+$CD25$^+$GITR$^+$CD62L$^+$) and
naive CD4$^+$ T cells (CD4$^+$CD62L$^+$Foxp3GFP$^-$RFP$^-$ or CD4$^+$CD62L$^+$GITR$^-$CD25$^-$) were
sorted using an Aria Fusion sorter (BD Biosciences) with a 70µm nozzle. Cells were
activated with anti-CD3 and anti-CD28 coated beads (Dynabeads Mouse T-Activator
CD3/CD28, Invitrogen) at a ratio of 1:1 for Tconv or 1:3 ratio for Tregs (cell:bead). Cells
were kept at a concentration of 10^6 cells/ml in DMEM medium supplemented with 10%
FBS, non-essential amino acids, sodium pyruvate, L-glutamine, HEPES, and β-ME, and
cells cultured more than 24 hr were supplemented with 200–2,000 IU/ml recombinant
human IL-2 (NIH).
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Tissue Collection and preparation for Flow cytometry
Flow cytometry was performed on an BD LSR Fortessa X20 (BD Biosciences), CyTEK
Aurora (CyTEK Biosciences) or LSRFortessa (BD Biosciences) and datasets were
analyzed using FlowJo software (Tree Star). Single cell suspensions were prepared in
ice-cold FACS buffer (PBS with 2mM EDTA and 1% BS) and subjected to red blood cell
lysis using ACK buffer (150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM Na2EDTA, pH7.3). Dead

cells were stained with Live/Dead Fixable Blue or Aqua Dead Cell Stain kit (Molecular Probes) in PBS at 4℃. Cell surface antigens were stained at 4℃ using a mixture of fluorophore-conjugated antibodies. Surface marker stains for murine samples were carried out with anti-mouse CD3 (17A2, BioLegend), anti-mouse CD4 (RM4-5, BioLegend), anti-mouse CD8a (53-6.7, BioLegend), anti-mouse, CD44 (IM7, BioLeged), anti-mouse CD45 (30-F11, BioLegend), anti-H-2K^b MuLV p15E Tetramer-KSPWFTTL (MBL), anti-H-2K^b SIINFEKL Tetramer (NIH Tetramer Core) in PBS, 0.5% BSA. Cells were fixed using the eBioscience Foxp3/Transcription Factor staining buffer set (eBioscience), prior to intracellular staining. Intracellular staining was performed using anti-mouse Foxp3 (FJK-16S, eBioscience) at 4℃, according to manufacturer's instructions. Cells were resuspended in PBS and filtered through a 70-μm nylon mesh before data acquisition. Datasets were analyzed using FlowJo software (Tree Star).

Statistical Methods

p values were obtained from unpaired two-tailed Student's t tests for all statistical comparisons between two groups, and data were displayed as mean $\pm$ SEMs or mean $\pm$ SD. For multiple comparisons, one-way ANOVA or two-way ANOVA was used. For tumor growth curves, two-way ANOVA was used with Sidak's multiple comparisons test performed at each time point or by multiple regression analysis p values are denoted in figures by *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001.

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Acknowledgments:
We thank Hector Nolla, Alma Valleros, Kartoosh Heydari and Anita Wong Lin of the UC
Berkeley Cancer Research Laboratory Flow Cytometry Facility. This research was
supported by 1DP2CA247830-01 from the National Institutes of Health (NCI),
R01GM112948 from the National Institutes of Health, and C23CR5612 from the UC
CRCC Cancer Research Coordinating Committee. M.D. is a Pew-Stewart Scholar and a
St. Baldrick's Scholar with generous support from Hope with Hazel. J.G.C is a HHMI
Gilliam Fellow. J.A.O is a Chan-Zuckerberg investigator.
517

518 **Author Contributions:**
519 Conceptualization and Methodology, M.D., J.A.O., J.G.C.; Investigation, J.G.C., S.S.,
520 A.S., L.S., J.H., J.A.O., and M.D.; Writing – Original Draft, J.G.C., M.D., J.A.O.; Writing
521 – Review & Editing, J.G.C., M.D., J.A.O., S.S.; Supervision, M.D., J.A.O., J.G.C.;
522 Funding Acquisition, M.D., J.A.O.
523

524 **Resource Availability**
525 **Lead Contact**
526 Further information and requests for resources and reagents should be directed to and
527 will be fulfilled by the lead contact, Michel DuPage (dupage@berkeley.edu).
528

529 **Materials availability**
530 *Listeria* monocytogenes strains used in the study will be made available from the lead
531 contact upon request.
532

533 **Declaration of Interests:**
534 J.A.O. is a member of the scientific advisory board for Vicinitas Therapeutics and has
535 patent applications related to ferroptosis.
536

537 **Supplementary Materials**
538 Materials and Methods
539 Figures S1 to S3
540

Figure Legends:
Fig. 1 | Regulatory T cells (Tregs) naturally resist lipid peroxide accumulation and
ferroptosis. (A) Schematic for *in vitro* T cell assays. (B) Representative flow plots (left)
and quantification (right) of bulk reactive oxygen species (ROS) by CellROX^{TM} staining
in Tregs versus conventional CD4$^+$ T cells (Tconv) after activation and culture for 4 days
in vitro. Data pooled from 5 independent experiments. (C) Representative flow plots for
detection of oxidized C11-BODIPY in Tregs versus Tconv that were activated for 4 days
in vitro. (D) Quantification of oxidized C11-BODIPY in Tregs versus Tconv cells *in vitro*

+/- 1µM RSL3 in combination with 2µM Fer-1, 100µM Z-VAD-FMK, or 10µM Nec-1.
Data pooled from 3 experiments. (E) Cell viability of Tregs (nTregs), CD4$^+$ Tconv, and
induced Tregs (Foxp3$^+$ iTregs) treated with the indicated concentrations of RSL3 for 4
hours. Representative data from 2 independent experiments. (F) Cell viability of Tregs
versus CD4$^+$ Tconv treated *in vitro* +/- 1µM RSL3 in combination with 2µM Fer-1,
100µM Z-VAD-FMK, or 10µM Nec-1. Data pooled from 2 experiments. (G) Frequencies
of viable CD8$^+$ T (left), CD4$^+$ Tconv (middle), and Tregs (right) from spleen plotted as
the percent of CD45$^+$ cells +/- 1µM RSL3 and 2µM Fer-1. Data from n=7 mice. For all
plots, **P<0.05, **P<0.01, ***P<0.001 by two-way ANOVA (D-F) or one-way ANOVA
(G), or student t-test (B), mean ± s.d (B, D-F) or mean ± s.e.m (G).
559
Fig. 2 | FSP1 drives Treg resistance to ferroptosis. (A) Fold-change expression of
indicated anti-ferroptotic genes in naive (CD44^{lo}CD62L^{hi}) versus in vitro activated (+4
days with anti-CD3/CD28) Tregs. Data from n=5 mice. (B) Fold-change in expression of
indicated anti-ferroptotic genes from sorted naive (CD44^{lo}CD62L^{hi}) versus in vivo
activated (CD44^{hi}CD62L^{lo}) Tregs. (C) Fold change in expression of indicated
ferroptosis-related genes in 4 day in vitro activated Treg versus Tconv cells. Data from
n=5 mice. (D) Treatment of wildtype versus *Aifm2*-deficient Tregs with different
concentrations of RSL3 for 4 hours. (E) *Aifm2* expression from bulk RNA-Seq of human
PBMCs populations from Database of Immune Cell eQTLs, expression, epigenomics
(DICE). (F) Differential expression analysis of *Aifm2* expression across different PBMC
populations. For all plots, **P<0.05, **P<0.01, ***P<0.001 by one-way ANOVA (A-B),
student t-test (C), or two-way ANOVA (D), mean ± s.e.m (A-C) or mean ± s.d (D).
572
Fig. 3 | Deletion of *Aifm2* in Tregs does not disrupt peripheral tolerance in mice.
(A) Frequency of total CD8$^+$ T cells (left), CD4$^+$ Tconv cells (middle), and Tregs(right) as
a percentage of total CD45$^+$ immune cells in lymphoid tissues and organ tissues in 16
week mice. (B) Frequencies of activated (CD44^{hi}CD62^{lo}) CD8$^+$ T cells (left), CD4$^+$
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week mice. (C) gMFI of Foxp3, CD25, and NRP1 in Foxp3 Tregs across lymphoid
tissues and non-lymphoid tissues in 16 week mice. (D) Schematic of pathway for
conversion of ATP to Adenosine mediated by CD39 and CD73. (E) Representative
histograms and gMFI of CD73 and CD39 in wildtype versus *Aifm2*-deficient Tregs
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Treg.*Aifm2*$^{+/+}$ and n=6 for Treg.*Aifm2*$^{\Delta/\Delta}$ mice for 16 week mice. For all plots, **P<0.05,
**P<0.01, ***P<0.001 by two-way ANOVA (A, C, E) or student's t-test (B), mean ±
s.e.m.
586
Fig. 4 | Deletion of *Aifm2* in Tregs improves cancer immunity. (A) Schematic for
treating single cell suspensions from MC38 tumors +/- 1µM RSL3 (left). Frequencies of

viable CD8$^+$ (left), CD4$^+$ Tconv (middle), and Tregs (right) from tumor single cell

suspensions as a percentage of CD45$^+$ cells +/- 1µM RSL3 for 4 hours. Data from n=6

mice. (B) Experimental schematic for measuring tumor growth in wildtype versus mice

with *Aifm2*-deficient Tregs. (C) Growth of MC38 tumors in wildtype versus mice with

Aifm2-deficient Tregs. Data from n=5 for Treg.Aifm2$^{+/+}$ and n=6 from *Treg.Aifm2$^{\Delta/\Delta}$*. (D)

Frequency of Foxp3$^+$ Tregs as a percentage of total CD45$^+$ immune cells (left) and

CD8:Treg ratio (right) in MC38 tumors at D29. (E) Experimental schematic for

measuring tumor growth in wildtype versus mice with *Aifm2*-deficient Tregs with or

without 10mg/kg liproxstatin injected i.p daily. Data from n=3 for Treg.*Aifm2$^{+/+}$* + Vehicle,

n=4 for Treg.*Aifm2$^{+/+}$* + Lip-1, n=5 for Treg.*Aifm2$^{\Delta/\Delta}$*+ Vehicle, and n=5 for Treg.*Aifm2$^{\Delta/\Delta}$*

+ Lip-1. (F) Growth of MC38 tumors in wildtype versus mice with *Aifm2*-deficient Tregs

+/- Lip-1. For all plots, **P<0.05, **P<0.01, ***P<0.001 by student t-test (A, D) or two-

way ANOVA (C, F), mean ± s.e.m.

Fig. 5 | Total T cell deficiency for *Aifm2* preserves CD8$^+$ T cell function and

improves tumor immunity. (A) Experimental schematic for measuring tumor growth in

wildtype versus mice with *Aifm2*-deficient T cells. (B) Growth of MC38 tumors in

wildtype versus mice with *Aifm2*-deficient T cells. Data shown from n=5-6 mice/group

from one of two experiments. (C) Frequency of Foxp3$^+$ Tregs as a percentage total

CD45$^+$ immune cells in MC38 tumors at D29 from (B). (D) CD8:Treg ratio in MC38

tumors at D29 from (B). (E) Representative flow plot for PD-1 and LAG3 staining in total

CD8$^+$ T cells (left) and quantification of the frequency of PD-1$^+$Lag3$^+$ CD8$^+$ T cells in

MC38 tumors (right). (F) Frequency of tumor-specific (MuLV/H2K^{b+}) as a percentage of

CD8$^+$ T cells (left) and frequency of PD-1$^+$Lag3$^+$ of MuLV/H2K^{b+} CD8$^+$ T cells in MC38

tumors (right). For all plots, **P<0.05, **P<0.01, ***P<0.001 by two-way ANOVA (B) or

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Garcia Castillo J. et al. Fig. 1

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Fig. 4 | Deletion of *Aifm2* in Tregs improves cancer immunity. (A) Schematic for treating single cell suspensions from MC38 tumors +/- 1µM RSL3 (left). Frequencies of viable CD8$^+$ (left), CD4$^+$ Tconv (middle), and Tregs (right) from tumor single cell suspensions as a percentage of CD45$^+$ cells +/- 1µM RSL3 for 4 hours. Data from n=6 mice. (B) Experimental schematic for measuring tumor growth in wildtype versus mice with *Aifm2*-deficient Tregs. (C) Growth of MC38 tumors in wildtype versus mice with *Aifm2*-deficient Tregs. Data from n=5 for Treg.Aifm2$^{+/+}$ and n=6 from *Treg.Aifm2$^{\Delta/\Delta}$*. (D) Frequency of Foxp3$^+$ Tregs as a percentage of total CD45$^+$ immune cells (left) and CD8:Treg ratio (right) in MC38 tumors at D29. (E) Experimental schematic for measuring tumor growth in wildtype versus mice with *Aifm2*-deficient Tregs with or without 10mg/kg liproxstatin injected i.p daily. Data from n=3 for Treg.*Aifm2$^{+/+}$* + Vehicle, n=4 for Treg.*Aifm2$^{+/+}$* + Lip-1, n=5 for Treg.*Aifm2$^{\Delta/\Delta}$*+ Vehicle, and n=5 for Treg.*Aifm2$^{\Delta/\Delta}$* + Lip-1. (F) Growth of MC38 tumors in wildtype versus mice with *Aifm2*-deficient Tregs +/- Lip-1. For all plots, **P<0.05, **P<0.01, ***P<0.001 by student t-test (A, D) or two-way ANOVA (C, F), mean ± s.e.m.

Garcia Castillo, J. Fig. 5

Fig. 5 | Total T cell deficiency for *Aifm2* preserves CD8⁺ T cell function and improves tumor immunity. (A) Experimental schematic for measuring tumor growth in wildtype versus mice with *Aifm2*-deficient T cells. (B) Growth of MC38 tumors in wildtype versus mice with *Aifm2*-deficient T cells. Data shown from n=5-6 mice/group from one of two experiments. (C) Frequency of Foxp3⁺ Tregs as a percentage total CD45⁺ immune cells in MC38 tumors at D29 from (B). (D) CD8:Treg ratio in MC38 tumors at D29 from (B). (E) Representative flow plot for PD-1 and LAG3 staining in total CD8⁺ T cells (left) and quantification of the frequency of PD-1⁺Lag3⁺ CD8⁺ T cells in MC38 tumors (right). (F) Frequency of tumor-specific (MuLV/H2K^{b+}) as a percentage of CD8⁺ T cells (left) and frequency of PD-1⁺Lag3⁺ of MuLV/H2K^{b+} CD8⁺ T cells in MC38 tumors (right). For all plots, **P<0.05, **P<0.01, ***P<0.001 by two-way ANOVA (B) or student t-test (C, D, F), mean ± s.e.m.