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Graphical abstract

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Autophagic adaptation to oxidative stress alters peritoneal residential macrophage survival and ovarian cancer metastasis

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Introduction

Ovarian cancer frequently metastasizes to the peritoneal cavity as manifested by ascites fluid formation and a large number of tumor islets distributing to the peritoneum, omentum, and serosal surfaces of the viscer. The entire peritoneal cavity becomes an active tumor microenvironment, supporting ovarian cancer metastasis and progression (1). Tumor-associated macrophages (TAMs) constitute over 50% of cells in the peritoneal tumor implants and the ascites fluids in patients with peritoneal ovarian cancer metastasis. Macrophages are physiologically critical mediators of tissue homeostasis. However, TAMs can directly support tumor growth and suppress the tumor immune responses (2–4). In line with this, previous studies in mouse models have shown that peritoneal TAMs generally promoted ovarian cancer metastasis and inhibited immunity (5–7). These observations suggest that TAMs may be an ideal target for cancer immunotherapy (3). Following this thought, different approaches — including targeting TAM trafficking, switching their functions, and developing TAM-depleting antibody — have been tested in preclinical models (8). However, these approaches have failed to translate clinically (9–11). Furthermore, macrophages can uptake, process, and present antigens to T cells and promote antitumor immunity. Macrophages may directly mediate tumor killing and are associated with improved patient outcomes in some types of cancer (12–14). In addition, emerging evidence indicates that monocytes are not the sole and major source of tissue macrophages, and embryonically derived macrophages can form a distinct tissue macrophage subset (15, 16). It seems that embryonically derived macrophages and peripheral monocyte–derived macrophages may play
different and often conflicting roles in different types of tumor models (17–19). Therefore, the controversies among the previous studies beg an in-depth understanding of TAM ontogeny, phenotype, metabolism, and functional characteristics in vitro and in vivo, which is critical for eventually developing effective mechanism-informed, TAM-targeted cancer immunotherapy.

In this work, we focus on peritoneal residential macrophages in ovarian cancer models and patients with ovarian cancer. Previous studies have implicated that peritoneal residential macrophages may be distinguished from other tissue residential macrophages in gene profile and function (20–22). Notably, the majority of these studies are realized in steady-state and inflammatory disease models (23, 24). TAM subsets remain poorly understood in ovarian cancer peritoneal metastasis and progression (25). Based on the relative expression levels of F4/80 and MHC-II, previous studies have classified macrophages into 2 subsets, F4/80hiMHC-IIlo and F4/80loMHC-IIhi (5, 26). Here, based on Tim-4 expression, we observed 2 distinct peritoneal TAMs: Tim-4+ and Tim-4– peritoneal macrophages (Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.141115DS1). These cells expressed multiple macrophage markers, including CD68, CD206, colo-

dine-stimulating factor 1 receptor (or CD115), and tyrosine-protein kinase Mer (MerTK), but not Siglec-F, a marker for eosinophils and alveolar macrophages (Figure 1A). The levels of TAMs increased following tumor progression (Figure 1B).

Tim-4 is a marker for embryonically derived residential macrophages in the intestine (27, 28), skin (29), and heart (30). Interestingly, based on Tim-4 expression, we observed 2 distinct peritoneal TAMs: Tim-4+ and Tim-4– cells (Figure 1C). To explore whether Tim-4+ and Tim-4– peritoneal TAMs were phenotypically different populations, we compared the expression of a panel of macrophage-associated markers on Tim-4+ and Tim-4– peritoneal TAMs (Figure 1D). We noticed that Tim-4+ TAMs represented the majority of the F4/80hiMHC-IIlo subset while Tim-4– TAMs represented the majority of the F4/80loMHC-IIhi subset (Figure 1D and Supplemental Figure 1B). In addition, Tim-4+ and Tim-4– TAMs expressed comparable levels of CD206, PD-L1, and MerTK (Supplemental Figure 1C). Long-lived peritoneal residential macrophages selectively express transcriptional factor GATA6 and its downstream genes (20–22). We found that GATA6 was enriched in Tim-4+ TAMs but not Tim-4– TAMs (Figure 1D). Furthermore, Tim-4+ TAMs expressed high levels of several GATA6 downstream genes, including Aspa, Cd5l, Ptxd1, and Tgfb2, as compared with Tim-4– TAMs (20, 22) (Supplemental Figure 1D).

To gain comprehensive insight into the differences between Tim-4+ and Tim-4– TAMs, we performed transcriptional profiling on the paired Tim-4+ and Tim-4– TAM subsets isolated from ID8 tumor-bearing mice. Among 1037 differentially expressed genes in the paired Tim-4+ and Tim-4– TAM subsets, 267 and 770 genes were upregulated and downregulated, respectively, in Tim-4+ TAMs as compared with Tim-4– TAMs (Figure 1E). The top upregulated genes, including Lyz1, Vsig4, and Wnt2, were linked to F4/80hiMHC-IIlo residential macrophages (31), while the top downregulated genes, including Ccr2, Cd226, and Plxnd1, were associated with MHC-IIhi monocyte-derived macrophages (Supplemental Table 1) (32). Gene set enrichment analysis (GSEA) revealed an enriched F4/80hiMHC-IIlo residential macrophage gene signature (Figure 1F) (31) and a weak MHC-IIhi monocyte-derived macrophage gene signature (Figure 1G) (32) in Tim-4+ TAMs as compared with Tim-4– TAMs. Together, the data suggest that Tim-4+ TAMs were ontogenically, metabolically, and functionally distinct subsets in ovarian cancer. We next tested whether Tim-4+ and Tim-4– TAMs are ontogenically, metabolically, and functionally distinct subsets.

**Results**

**Tim-4 defines 2 distinct peritoneal macrophage subsets in ovarian cancer.** Ovarian carcinoma often metastasizes to the peritoneal cavity. However, peritoneal residential macrophages are poorly understood in ovarian cancer. To explore the nature of peritoneal TAMs in ovarian cancer, we established ID8 ovarian cancers in the mouse peritoneal cavity and studied TAMs in the peritoneal ascites fluids. Flow cytometry identified CD45+CD11b+CD90 B220 Gr1+ macrophages in the peritoneal cavity in ID8 tumor-bearing mice (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.141115DS1). These cells expressed multiple macrophage markers, including CD68, CD206, colo-

ne-stimulating factor 1 receptor (or CD115), and tyrosine-protein kinase Mer (MerTK), but not Siglec-F, a marker for eosinophils and alveolar macrophages (Figure 1A). The levels of TAMs increased following tumor progression (Figure 1B).

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**Tim-4+ TAMs migrate from peripheral monocytes without affecting tumor growth.** Based on our phenotypic and transcriptional profile on Tim-4+ and Tim-4– TAMs, we hypothesized that Tim-4+ TAMs were embryonically derived residential macrophages and Tim-4– TAMs were replenished from blood monocytes. In healthy mice (tumor free), Tim-4+ and Tim-4– TAM subsets remain poorly understood in ovarian cancer peritoneal metastasis and progression (25). Based on the relative expression levels of F4/80 and MHC-II, previous studies have classified macrophages into 2 subsets, F4/80hiMHC-IIlo and F4/80loMHC-IIhi (5, 26). Here, based on Tim-4 expression, we identified 2 phenotypically, transcriptionally, ontogenically, metabolically, and functionally distinct TAM subsets, Tim-4+ and Tim-4– TAMs, in an ovarian cancer peritoneal metastasis model, then extended our studies to patients with ovarian cancer.
for 85% and 15% of peritoneal macrophages in homeostasis (Supplemental Figure 2, A and B). After tumor inoculation, absolute numbers of Tim-4⁻ (Figure 2A) and Tim-4⁺ (Figure 2B) TAMs increased following tumor progression. Interestingly, the percentage of Tim-4⁺ TAMs in TAMs gradually shrank (Supplemental Figure 2A), while the percentage of Tim-4⁻ TAMs in TAMs moderately increased (Supplemental Figure 2B). Six to 8 weeks after tumor inoculation, there were approximately 50% each of Tim-4⁺ (Figure 2A) and Tim-4⁻ (Figure 2B) TAMs in mice.

Next, we explored the origin of Tim-4⁻ and Tim-4⁺ TAMs in 3 confirmatory settings. CCR2 mediates peripheral monocyte trafficking into the peritoneal cavity (32, 33). The first experimental setting was a pharmacological depletion model. We treated adult mice with sc-202525, a CCR2 antagonist, for 2 weeks. Treatment with sc-202525 resulted in reduced F4/80⁺MHC-II⁺ peritoneal macrophages and had no effect on F4/80⁺MHC-II⁻ macrophages (Supplemental Figure 2C) (33). Then, we inoculated ID8 tumor cells into the peritoneal cavity in these pretreated mice and treated them with sc-202525 continually for 6 weeks. We found comparable tumor volume (Figure 2C) and a similar quantity of Tim-4⁺ TAMs (Figure 2D) in mice receiving PBS and CCR2 antagonist. These data suggest that blockade of monocyte peritoneal trafficking has no obvious effect on Tim-4⁺ TAMs and ID8 tumor growth.
The second experimental setting was a genetic model. We compared peritoneal macrophages in CCR2-deficient (Ccr2−/−) and -sufficient (Ccr2+/+) mice. We observed a similar number of total peritoneal macrophages in tumor-free Ccr2−/− and Ccr2+/+ mice (Supplemental Figure 2D). However, the percentage (Supplemental Figure 2E) and absolute number (Supplemental Figure 2F) of Tim-4−, but not Tim-4+, peritoneal macrophages were reduced in Ccr2−/− mice. We inoculated ID8 tumor cells into the peritoneal cavity of Ccr2+/+ and Ccr2−/− mice. Again, we observed similar tumor growth (Figure 2E) in Ccr2−/− and Ccr2+/+ mice. As expected, Tim-4− TAMs remained limited in Ccr2−/− tumor-bearing mice as compared with Ccr2+/+ mice (Figure 2, F and G). These data suggest that monocyte trafficking deficiency has no obvious effect on ID8 tumor growth.

The third experimental setting was an adoptive immune cell transfusion system. We explored whether Tim-4+ TAMs could be directly differentiated from monocytes in the tumor microenvironment. We injected CD45.1+ monocytes into ID8 tumor–bearing cognate CD45.2 mice. Three days later, we observed that CD45.1+ donor TAMs were in the F4/80loMHC-IIhi population (Supplemental Figure 2G) and expressed no Tim-4 (Figure 2H). Together, these data suggest that circulating monocytes are a cellular source for Tim-4− TAMs, and Tim-4+ TAMs may be embryonic residential macrophages.

Tim-4+ TAMs are embryonically derived proliferative cells with protumor function. Both Tim-4+ and Tim-4− TAMs were increased following tumor progression. Tim-4− TAMs were from peripheral monocytes. We hypothesized that increased Tim-4+ TAM numbers are attributed to their local self-expansion in the tumor microenvironment. To test this, we compared the proliferative capacity of Tim-4+ macrophages in normal and tumor-bearing mice (21, 34). As expected, Tim-4+ TAMs exhibited increased cell proliferation as shown by more TAMs in the S/G2M phase, when compared with normal Tim-4− residential macrophages (Figure 3, A and B). As a confirmation, we injected BrdU into the peritoneal cavity in tumor-bearing mice for 3 hours and observed increased BrdU+ Tim-4+ TAMs (Supplemental Figure 3, A and B). Accompanied with this, we detected elevated expression of cell cycle regulator transcripts (Supplemental Figure 3C) and proteins (Figure 3C). Thus, Tim-4+ TAMs are locally proliferative residential macrophages.
Arginase-1 affects mitochondria fitness and mitophagy via mTORC1 in Tim-4+ TAMs. We next examined the mechanism by which Tim-4+ TAMs exhibited high mitophagy activity. GSEA revealed no enrichment of mitophagy-related genes in Tim-4+ TAMs compared with Tim-4- TAMs (Supplemental Figure 5A). This suggests that mitophagy-related autophagy genes may be regulated at posttranscriptional levels in Tim-4+ TAMs.
and Tim-4+ TAMs. Unc-51-like kinase 1 (ULK1) phosphorylation at Ser757 by mammalian target of rapamycin complex 1 (mTORC1) competes with phosphorylation at Ser317 by AMPK to inhibit the initiation of the canonical autophagy pathway (40). As compared with Tim-4– TAMs, we detected lower levels of ULK1 phosphorylation at Ser757 and similar levels of ULK1 phosphorylation at Ser317 in Tim-4+ TAMs (Figure 5A). We suspected that mTORC1 activity may be reduced in Tim-4+ TAMs. As expected, Tim-4+ TAMs manifested a weak mTORC1 activity as compared with Tim-4+ TAMs, as shown by low levels of phosphorylation of S6 kinase (Figure 5A), a well-characterized mTORC1 substrate (41). Amino acid sensing may determine mTORC1 activation (42). We explored a potential role of amino acids on mTORC1 activity in TAM subsets. We cultured TAM subsets in amino acid–free medium and observed a potent inhibition of mTORC1 activity in both Tim-4+ and Tim-4– TAMs (Figure 5A). We suspected that mTORC1 activity may be reduced in Tim-4+ TAMs. As expected, Tim-4+ TAMs manifested a weak mTORC1 activity as compared with Tim-4+ TAMs, as shown by low levels of phosphorylation of S6 kinase (Figure 5A), a well-characterized mTORC1 substrate (41). Amino acid sensing may determine mTORC1 activation (42). We explored a potential role of amino acids on mTORC1 activity in TAM subsets. We cultured TAM subsets in amino acid–free medium and observed a potent inhibition of mTORC1 activity in both Tim-4+ and Tim-4– TAMs. Interestingly, supplemental amino acids induced a robust mTORC1 activation in Tim-4+ TAMs but not in Tim-4– TAMs (Figure 5B). Intracellular arginine can sustain mTORC1 activation in regulatory T cells (43). To evaluate whether arginine is involved in maintaining mTORC1 activation in Tim-4+ TAMs, we treated Tim-4+ TAMs with different concentrations of arginine. As expected, exogenous arginine could induce and maintain mTORC1 activation in Tim-4+ TAMs (Figure 5C). Thus, arginine, as a key amino acid, might define different mTORC1 activities in Tim-4+ and Tim-4– TAMs.
Intracellular arginine is controlled by arginine uptake and metabolism. We detected comparable levels of arginine uptake in Tim-4+ TAMs and Tim-4– TAMs (Supplemental Figure 5B). Arginase-1 converts intracellular arginine to downstream metabolites, including ornithine and urea. Western blot revealed high levels of arginase-1 in total TAMs compared with normal peritoneal macrophages (Supplemental Figure 5C) (44). RNA-Seq data showed high levels of arginase-1 transcripts in Tim-4+ TAMs compared with Tim-4– TAMs (Supplemental Figure 5D), and comparable expression levels of other genes in polyamine biogenesis, including Odc1 and Srm, in Tim-4– and Tim-4+ TAMs (Supplemental Figure 5D). In line with this, flow cytometry analysis (Figure 5D) and Western blot (Supplemental Figure 5E) demonstrated superior levels of arginase-1 protein in Tim-4+ TAMs compared with Tim-4– TAMs. Notably, arginase-2 was not detectable in both TAM subsets (Supplemental Figure 5E). In addition, we detected higher levels of arginase activity in Tim-4+ TAMs than in Tim-4– TAMs (Figure 5E). Then, we treated Tim-4+ TAMs with N-hydroxy-nor-arginine (nor-NOHA), an arginase activity inhibitor, to block the arginine consumption. We observed that nor-NOHA stimulated mTORC1 activation in Tim-4+ TAMs (Figure 5F). Hence, weak mTORC1 activation may be attributed to high levels of arginase-1 and low levels of intracellular arginine in Tim-4+ TAMs. To further elucidate a role of arginine in mitochondrial fitness and mitophagy in Tim-4+ TAMs, we treated Tim-4+ TAMs with a combination of mitochondrial inhibitors oligomycin and antimycin A, which collapse ΔΨm (45), in the presence of arginine or nor-NOHA. Oligomycin and
antimycin A combination increased damaged mitochondria in Tim-4+ TAMs (Supplemental Figure 5F).

Interestingly, treatment with arginine or nor-NOHA resulted in an increase in damaged mitochondria in Tim-4+ TAMs, and this effect was abolished with rapamycin (Figure 5G). The data suggest that intracellular arginine mediates mitophagy inhibition via mTORC1 activity in Tim-4+ TAMs. Thus, arginase-1 fine-tunes mitochondria fitness and mitophagy in Tim-4+ TAMs.

**Autophagy deficiency results in loss of Tim-4+ TAMs in ovarian cancer.** Mitophagy degrades damaged mitochondria and removes ROS to prevent macrophage death (46). High levels of mitophagy in Tim-4+ TAMs might be important to support their survival in the tumor microenvironment. To test this possibility in vivo, we established ID8 tumor mouse models with autophagy deficiency in macrophages. FAK family-interacting protein of 200 kDa (FIP200) is one component of the ULK1-Atg13-FIP200-Atg101 complex and is essential for the induction of mammalian autophagy (47). We crossed mice with transgenic expression of Cre recombinase from the Lysozyme promoter with loxP-flanked FIP200 alleles (Fip200<sup>fl/fl</sup>) mice and deleted the loxP-flanked FIP200 alleles specifically in myeloid cells (called Fip200<sup>−/−</sup> here). Western blot showed a specific loss of FIP200 and an increase in autophagy receptor p62 in macrophages isolated...
from the peritoneal cavity and differentiated from bone marrow of Fip200<sup>−/−</sup> mice (Supplemental Figure 6A). We inoculated Fip200<sup>−/−</sup> mice with ID8 tumor cells and detected high levels of damaged mitochondria accumulated in Tim-4<sup>+</sup> Fip200<sup>−/−</sup> TAMs but not in Tim-4<sup>−/−</sup> Fip200<sup>−/−</sup> TAMs compared with their WT counterparts. The data additionally support the notion that Tim-4<sup>+</sup> TAMs, but not Tim-4<sup>−</sup> TAMs, relied on mitophagy in the tumor microenvironment in vivo (Figure 6A). To functionally understand if mitophagy is important to Tim-4<sup>+</sup> TAMs’ survival, we analyzed the impact of autophagy deficiency in TAM numbers and phenotype in tumor-bearing mice. We found that the percentage (Figure 6B) and number (Figure 6C) of total TAMs was decreased in Fip200<sup>−/−</sup> mice as compared with control mice. Interestingly, we observed a loss of Tim-4<sup>+</sup> TAMs, but not Tim-4<sup>−</sup> TAMs, in ID8 bearing Fip200<sup>−/−</sup> mice compared with the WT mice, as shown by the percentage (Figure 6D) and absolute number (Figure 6E) of Tim-4<sup>+</sup> and Tim-4<sup>−</sup> TAMs. To explore whether loss of Tim-4<sup>+</sup> TAMs is attributed to the tumor microenvironment, we analyzed peritoneal residential macrophage subsets in tumor-free mice. We found comparable numbers of Tim-4<sup>+</sup> residual macrophages in WT and Fip200<sup>−/−</sup> mice (Supplemental Figure 6B). The results suggest that loss of Tim-4<sup>+</sup> TAMs in Fip200<sup>−/−</sup> mice is related to tumor challenge.

Tim-4<sup>+</sup> macrophages are of embryonic origin. Tim-4<sup>+</sup> TAM pool may be determined by the balance between self-expansion and survival. Interestingly, we observed an increase in SG,M percentage in Tim-4<sup>+</sup> TAMs in Fip200<sup>−/−</sup> mice compared with WT mice. This indicates that loss of Tim-4<sup>+</sup> TAMs is unlikely due to a proliferative defect (Supplemental Figure 6C). T cell survival is impaired in the tumor microenvironment due to metabolic challenge (48, 49). We analyzed TAM death and survival. We observed increased annexin V (Figure 6F) and cleaved caspase-3 expression (Supplemental Figure 6D) in Fip200<sup>−/−</sup> Tim-4<sup>+</sup> TAMs as compared with WT cells. To understand if cell death is associated with intrinsic factor, we isolated CD45.2<sup>−</sup>Tim-4<sup>+</sup> residential macrophages from Fip200<sup>−/−</sup> mice and injected them into the peritoneal cavity in WT CD45.1<sup>+</sup> mice, and inoculated these mice with ID8 cells. We found 3- to 5-fold more apoptosis in Fip200-deficient CD45.2<sup>−</sup> Tim-4<sup>+</sup> TAMs compared with WT CD45.1<sup>−</sup> Tim-4<sup>+</sup> TAMs (Figure 6G and Supplemental Figure 6E). These data suggest that Fip200 deficiency results in Tim-4<sup>+</sup> TAMs’ death in the tumor microenvironment.

GATA6 may control peritoneal residential macrophage development and survival (20–22). We questioned if autophagy deficiency downregulated GATA6 expression in Tim-4<sup>+</sup> TAMs and affected their development in tumor. Expression of GATA6 and downstream genes was similar in WT and Tim-4<sup>+</sup> Fip200<sup>−/−</sup> TAMs (Supplemental Figure 6F). Given that damaged mitochondria accumulated in Tim-4<sup>+</sup> TAMs in Fip200<sup>−/−</sup> mice compared with WT mice (Figure 6A), we hypothesized increased mitochondria-related ROS may be responsible for Tim-4<sup>+</sup> TAM death. Indeed, we observed increased mitochondria-related ROS (Figure 6H) and DNA damage marker pH2A.X (Supplemental Figure 6G) in Tim-4<sup>+</sup> TAMs from Fip200<sup>−/−</sup> mice compared with WT mice. In addition, we treated the Fip200<sup>−/−</sup> tumor-bearing mice with ROS scavenger N-acetylcysteine (NAC) and analyzed Tim-4<sup>+</sup> TAM apoptosis. NAC treatment improved survival of autophagy-deficient Tim-4<sup>+</sup> TAMs (Figure 6I). To further understand if mitophagy supports TAMs’ survival by eliminating ROS in the tumor microenvironment, we compared the levels of cell apoptosis and ROS production between Tim-4<sup>+</sup> Fip200<sup>−/−</sup> TAMs and Tim-4<sup>+</sup> Fip200<sup>−/−</sup> TAMs because they showed different levels of damaged mitochondria accumulation (Figure 6A). We found a dramatic increase of apoptosis in Fip200-deficient Tim-4<sup>+</sup> TAMs as compared with Fip200-deficient Tim-4<sup>−</sup> TAMs (Figure 6J). In line with this, ROS accumulation was elevated in Tim-4<sup>+</sup> Fip200<sup>−/−</sup> TAMs but not in Tim-4<sup>−</sup> Fip200<sup>−/−</sup> TAMs (Figure 6K). Thus, autophagy deficiency causes a loss of Tim-4<sup>+</sup> TAMs via accumulated ROS in the ovarian cancer microenvironment.

Autophagy deficiency in macrophages supports T cell–mediated antitumor immunity. Given Tim-4<sup>+</sup> TAMs, but not Tim-4<sup>−</sup> TAMs, contributed to ovarian cancer growth (Figure 2 and Figure 3), we studied whether loss of Tim-4<sup>+</sup> TAMs affects ovarian cancer progression in autophagy-deficient mice. We found that FIP200 deficiency in macrophages slowed down tumor growth compared with WT mice (Figure 7A). In addition, we inoculated MC38 colon cancer cells into the peritoneal cavity of Fip200<sup>−/−</sup> mice. Again, FIP200 deficiency in myeloid cells resulted in a slower tumor growth compared with WT mice (Supplemental Figure 7). Furthermore, we analyzed T cell phenotype and cytokine profile in ID8 models. We detected an increase in the percentage of tumor-infiltrating CD90<sup>+</sup> T cells (Figure 7B) and Ki67<sup>+</sup> T cells (Figure 7C) in Fip200<sup>−/−</sup> mice compared with WT mice. Furthermore, we detected higher levels of IFN-γ (Figure 7D) and TNF-α (Figure 7E) expression in tumor-infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells in Fip200<sup>−/−</sup> tumor-bearing mice compared with WT tumor-bearing mice. Thus, autophagy deficiency in macrophages supports T cell–mediated antitumor immunity and slowed down tumor progression.
TAMs positive for complement receptor of the immunoglobulin superfamily are the human counterparts of murine Tim-4+ TAMs in ovarian cancer. In an effort to find the human equivalent of mouse Tim-4+ TAMs, we examined Tim-4 expression in TAMs in patients with ovarian cancer. Flow cytometry analysis identified CD45+CD3−CD14+ TAMs in primary ovarian tumor tissues, metastatic omentum, and ascites fluids in patients. We detected 3% of Tim-4+ TAMs with a commercially available monoclonal anti–human Tim-4 antibody in the human ovarian cancer microenvironment (Supplemental Figure 8A). The data suggest 2 possibilities: This anti–human Tim-4 clone may not be sensitive to detect membrane Tim-4 on human residential TAMs in ovarian cancer. Or, Tim-4 may not be an operational marker for human residential TAMs in ovarian cancer.

To explore potential markers for human ovarian cancer residential TAMs with similar features to mouse Tim-4+ TAMs, we assessed genes encoding transmembrane receptors in mouse Tim-4+ TAMs. V-set and immunoglobulin domain containing 4 (Vsig4) was among the most enriched genes in mouse Tim-4+ TAMs compared with Tim-4− TAMs (Figure 1E). Vsig4 encodes a protein that is also known as a complement receptor of the immunoglobulin superfamily (CRIG) (50). We found that the vast majority of CRIG+ TAMs were Tim-4+ TAMs in ID8 tumor–bearing mice (Supplemental Figure 8B). FACS analysis identified CRIG+ TAMs with membrane CRIG.
TAMs in patients with ovarian cancer (Supplemental Figure 8C). In addition, VSIG4 transcripts were highly correlated with the expression of the alternative macrophage marker CD163 in human ovarian cancer (Supplemental Figure 8D) (51). In line with our FACS data, TAMs highly expressed VSIG4 transcripts in human ovarian cancer (25). We wondered if human CRIg + TAMs could share genetic and biological features of mouse Tim-4+ TAMs. We first compared human ovarian TAM transcriptomes with the established signature of mouse Tim-4+ and Tim-4– TAMs (Figure 1E) by using the connectivity MAP (cMAP) (52), which generates scores (as scaled, dimensionless quantities) indicative of the degree of “closeness” of one cell subset to a defined signature gene set. Based on VSIG4 transcript levels from the RNA-Seq data in human ovarian cancer TAMs (25), cMAP analysis revealed that VSIG4 hi and VSIG4 lo human TAMs aligned closely with the gene expression patterns of mouse Tim-4+ and Tim-4– TAMs, respectively (Supplemental Figure 8E). In addition, principal components analysis could segregate the transcriptomic profiles of VSIG4 hi TAMs from VSIG4 lo TAMs (Figure 8A). It is reported that CRIg hi macrophages may represent peritoneal residential macrophages in humans as compared with CRIg lo macrophages (53). In line with this, GSEA revealed that CRIg hi peritoneal macrophages shared enriched upregulated genes (Figure 8B) and downregulated genes (Figure 8C) with VSIG4 lo TAMs (53). Furthermore, VSIG4 lo TAMs expressed enriched genes for lysosome organization and formation (Supplemental Figure 8F), while VSIG4 hi TAMs were highly enriched for genes for inflammatory response (Supplemental Figure 8G). The data suggest that, at the transcriptional level, VSIG4 hi TAMs represent CRIg hi residential macrophages in human ovarian cancer ascites.

To additionally characterize VSIG4 hi TAMs in human ovarian cancer, we used the Functional Annotation Clustering tool in Enrichr to identify significantly enriched pathways and gene ontologies in VSIG4 hi TAMs.
Based on the significantly upregulated genes, metabolic pathways and mitochondria activity were strongly enriched in VSIG4⁺ TAMs as compared with VSIG4⁻ TAMs (Figure 8D). Particularly, VSIG4⁺ TAMs were enriched with an OXPHOS gene set compared with VSIG4⁻ TAMs (Figure 8E). The data suggest that VSIG4⁺ residential TAMs may go through high levels of OXPHOS. In line with this, we observed more mitochondrial mass and high mitochondria-related ROS in CR1g⁺ TAMs compared with CR1g⁻ TAMs in human peritoneal ovarian cancer tissues (Supplemental Figure 8H). In addition, we observed that VSIG4⁺ TAMs were significantly enriched with autophagy-associated gene transcripts compared with VSIG4⁻ TAMs (Figure 8F), which might be due to the higher arginase-1 activity in CR1g⁺ TAMs compared with CR1g⁻ TAMs (Supplemental Figure 8I). Furthermore, high mRNA levels of VSIG4 were associated with poor survival (Figure 8G) in patients with ovarian cancer. Altogether, the results suggest that human ovarian cancer CR1g⁺ TAMs may be transcriptionally, metabolically, and functionally similar to the equivalent mouse Tim-4⁺ TAMs.

**Discussion**

Peritoneal residential macrophage subsets are poorly understood in ovarian cancer. Mouse macrophages are traditionally classified into 2 subsets: F4/80⁺MHC-II⁻ and F4/80⁻MHC-II⁺. This classification is arbitrarily based on the relative expression levels of F4/80 and MHC-II. Moreover, the expression levels of F4/80 and MHC-II are subject to environmental regulation. Hence, this classification does not serve in sorting specific TAM subsets to study their nature at the transcriptional, metabolic, and functional levels. To meet this challenge, using murine peritoneal ovarian cancer as a model, we found that Tim-4 expression explicitly defines 2 TAM subsets in the ovarian cancer–bearing peritoneal cavity: Tim-4⁺ and Tim-4⁻ cells. Interestingly, Tim-4⁺ and Tim-4⁻ TAMs are respectively enriched in F4/80⁺MHC-II⁻ and F4/80⁻MHC-II⁺ cells (31, 32). It has been reported that F4/80⁺MHC-II⁻ and F4/80⁻MHC-II⁺ cells are embryonically originated and are peripheral monocyte–derived cells, respectively (32, 33). Analogously, our complementary and confirmatory experiments reveal that Tim-4⁺ TAMs are embryonically originated and locally proliferative cells, while Tim-4⁻ TAMs are replenished from circulating monocytes. In further support of these findings, it has been reported that Tim-4 marks certain tissue residential macrophages, including heart, intestine, skin, and peritoneal cavity (21, 28, 30, 33, 54). Therefore, Tim-4 is a reasonable phenotypic marker to define TAM subsets in ovarian cancer.

We next explore a potential functional difference between Tim-4⁺ and Tim-4⁻ TAMs in mouse ovarian cancer. The CCL2 and CCR2 signaling pathway mediates peripheral monocyte trafficking into the tumor microenvironment (55). In line with this, we have observed that the levels of peritoneal Tim-4⁻ TAMs (monocyte-derived TAMs) are reduced in Ccr2⁻KO mice and in mice treated with CCR2 antagonist, as compared with controls. However, the reduction of Tim-4⁻ TAMs did not affect peritoneal tumor progression in either Ccr2⁻KO mice or CCR2 antagonist–treated mice. In contrast, we found that deletion of Tim-4⁻ TAMs (embryo-derived TAMs) results in reduced peritoneal tumor progression. In line with this, a recent study has reported a protumor role of omentum CD163⁺Tim-4⁻ TAMs in metastatic ovarian cancer (56). Thus, we suggest that embryo-derived TAMs (Tim-4⁻) and peripheral monocyte–derived TAMs (Tim-4⁺) are biologically different in peritoneal ovarian cancer progression, and specifically targeting Tim-4⁻ (but not Tim-4⁺) TAMs may be therapeutically beneficial to control ovarian cancer and/or other types of cancer peritoneal metastasis and progression.

In an effort to additionally characterize Tim-4⁺ and Tim-4⁻ TAMs, we have examined their major metabolic profiles. Compared with Tim-4⁻ TAMs, Tim-4⁺ TAMs presented higher levels of OXPHOS, higher levels of all mitochondrial DNA–encoded OXPHOS-related genes, and greater mitochondria mass and ROS production. These data indicate that Tim-4⁺ TAMs have experienced high levels of oxidative stress in the tumor microenvironment. In line with this possibility, Tim-4⁺ TAMs expressed high levels of active autophagy. To explore the relevance of autophagy activation in Tim-4⁺ TAMs, we genetically deleted autophagy gene FIP200 in myeloid cells. We observed that FIP200 deficiency abrogated peritoneal ovarian cancer progression and improved T cell–mediated antitumor immunity. Accompanied with this, we noticed a loss of Tim-4⁺ TAMs in FIP200-deficient mice bearing peritoneal ovarian cancer, but not Tim-4⁻ TAMs. This reinforces a protumor role of Tim-4⁻ TAMs. In support of this, FIP200 deficiency selectively results in high apoptosis of Tim-4⁻ TAMs due to accumulation of damaged mitochondria and mitochondria-related ROS. The data suggest that Tim-4⁺ TAMs, but not Tim-4⁻ TAMs, rely on mitophagy to survive in the tumor microenvironment. Interestingly, we found that Tim-4⁺ TAMs express high levels of arginase-1 and efficiently convert arginine to downstream metabolites, resulting in limited intracellular arginine. Sensing of amino acids, including arginine,
causes mTORC1 activation in immune cells (57). In line with this, we have detected low levels of mTORC1 activity in Tim-4+ TAMs. As mTORC1 is a negative regulator of classical autophagy through phosphorylating ULK1 (40), this may explain high mitophagy in Tim-4+ TAMs. Thus, Tim-4+ and Tim-4– TAMs are metabolically programmed to have distinct survival capacities and functions in the tumor microenvironment.

Given that Tim-4 protein is rarely expressed in human ovarian cancer–associated macrophages, Tim-4 cannot be used to define human counterparts of mouse Tim-4+ TAM subsets. CRIg (encoded by Vsig4), a macrophage signature marker, is coexpressed with Tim-4 in mouse F4/80hiMHC-IIlo peritoneal macrophages (21) and Lyve1hiMHC-IIlo interstitial macrophages (54). We have explored CRIg as a potential marker to define human ovarian cancer macrophage subsets. Similar to mouse Tim-4+ TAMs, transcriptome and functional analysis reveals enriched lysosome-related genes, OXPHOS pathway, and autophagy pathways in CRIg+ TAMs in human ovarian cancer. The data suggest that CRIg can be an operational marker to study TAM subsets in human ovarian cancer. In conclusion, based on Tim-4 (or CRIg in humans), we have identified 2 ontogenically, phenotypically, metabolically, and functionally distinct peritoneal macrophage subsets in ovarian cancer (see graphical abstract). We suggest that specifically targeting human CRIg+ TAMs may be a meaningful approach for treating peritoneal cancer metastasis.

**Methods**

*Mouse models.* Female 6- to 8-week-old Cdh5.2 C57BL/6 mice (Jackson Laboratory), Cdh5.1 C57BL/6 mice (Jackson Laboratory), Ccr2−/− mice (Jackson Laboratory), LysM-Cre C57BL/6 mice (Jackson Laboratory), and floxed FIP200 (Fip200flo/flo) C57BL/6 mice (58) were used for this study. Fip200flo/flo mice (“Fip200−/− mice”)...
were intercrossed with LysM-Cre mice to delete the loxP-flanked FIP200 alleles specifically in myeloid cells ("Fip200−/− mice"). All mice were bred in-house and maintained in specific pathogen–free conditions. All animal research performed was approved by the IACUC at the University of Michigan. MC38 colon carcinoma cells and ID8 ovarian cancer cells were used for this work. Mouse ovarian cancer cell line ID8 was originally from George Coukos, University of Pennsylvania, Philadelphia, Pennsylvania, USA, and mouse colon cancer cell line MC38 was originally from Walter Storkus, University of Pittsburgh, Pittsburgh, Pennsylvania, USA. ID8-luciferase cells (1.5–2 × 10⁶) were injected into the peritoneal cavity of WT mice. Tumor progression was monitored 1 to 2 times per week by Xenogen IVIS Spectrum in vivo bioluminescence imaging system (PerkinElmer). For NAC treatment, 4-week-old Fip200−/− tumor-bearing mice were I.P. injected with NAC in PBS at a dose of 150 mg/kg every other day. Two weeks after NAC treatment, apoptosis of TAMs was analyzed. For inhibiting monocyte migration, mice were I.P. injected with 50 μg/kg CCR2 antagonist (sc-202525, Santa Cruz Biotechnology) every other day for 2 weeks. Then pretreated mice were inoculated with 2 × 10⁶ ID8 tumor cells and continued with CCR2 antagonist injection for 6 weeks.

Human ovarian cancer tissues and ascites. Sixteen patients with high-grade serous ovarian cancer were recruited for this study. Ascites, omentum metastasis, and ovarian cancer tissues were collected from patients with informed consent according to the procedures approved by the Institutional Review Boards of the University of Michigan School of Medicine and the Henry Ford Health System. We used clinical samples from people who had received no prior anticancer therapies. Fresh tumor tissues and ascites were processed into single-cell suspensions for phenotype and functional studies.

Isolation of macrophages from the peritoneal cavity. Peritoneal Tim-4+ normal residential macrophages were enriched from 5–8 mL peritoneal elute fluid by PE–anti–Tim-4 antibody (clone RMT4-54, BD Biosciences) and anti-PE microbeads (Miltenyi Biotec). To isolate TAM subsets, most tumor cells and dead cells in peritoneal wash of tumor-bearing mice were first removed via density gradient centrifuge by overlaying 15 mL single-cell suspension on 20 mL of 75% Ficoll above 15 mL 100% Ficoll. After Ficoll, Tim-4 + TAMs were isolated by using the PE–anti–Tim-4 antibody and anti-PE microbeads from collected leukocyte mononuclear cells. Then, Tim-4– TAMs were enriched from Tim-4– cells through anti-F4/80/80 MicroBeads UltraPure (Miltenyi Biotec). Total TAMs were enriched from collected leukocyte mononuclear cells through anti-F4/80 MicroBeads UltraPure (Miltenyi Biotec). For Western blot and quantitative PCR (qPCR) analysis, 5 × 10⁶ fresh purified TAMs were directly used. In certain experiments, 5 × 10⁵ TAMs were seeded in 1 well of a 24-well plate for 2 hours, then treated with autophagy inhibitor chloroquine (MilliporeSigma). Media with/without amino acids were formulated with RPMI1640 (R8999-04A, US Biological) by supplementation or omission of amino acids. The medium was supplemented with 10% (v/v) dialyzed FBS. For flow cytometry, TAMs were incubated with 500 μL accutase (Life Technologies, Thermo Fisher Scientific) for 30 minutes at 37°C; then detached TAMs were washed and collected for staining.

Macrophage depletion and adoptive transfer. To deplete peritoneal resident macrophages, C57BL/6 mice were treated with 1 dose of clodronate-containing liposomes (100 μL each). Control mice were treated with same volume of control liposomes. After 2 weeks, mice were I.P. implanted with ID8 tumor cells. For competition experiments, donor Tim-4– peritoneal macrophages were isolated from several Fip200−/− CD45.2 congenic mice and pooled together. Then, 8 × 10⁶ purified cells were immediately admixed with 2 × 10⁶ ID8-luciferase tumor cells and injected I.P. into host CD45.1 mice. The cell death of CD45.1 and CD45.2 TAMs was detected 4 weeks later. To explore monocyte-derived macrophages in ID8 tumor–bearing mice, 1 million monocytes were enriched from bone marrow of CD45.1 mice and I.P. injected into 4-week tumor-bearing mice. Three days later, the phenotype of monocyte-derived TAMs was analyzed by flow cytometry.

FACS and analysis. Mouse single-cell suspensions were prepared from peripheral blood and from the peritoneal cavity and blocked with rat anti–mouse CD16/CD32 antibodies (eBioscience, Thermo Fisher Scientific) (1/200) for 10 minutes, pelleted by centrifugation (500g for 5 minutes; room temperature), subsequently labeled with fluorophore-conjugated anti-mouse antibodies at recommended dilutions for 30 minutes in a dark room, and washed with staining buffer. To quantitate the cells, 25 μL CountBright absolute counting beads (Thermo Fisher Scientific) were added to the samples. For proliferation assays, mice were injected with BrdU (200 μL/mouse) (Invitrogen, Thermo Fisher Scientific) I.P. 3 hours before sacrifice. Cytofix/Cytoperm kit was used to stain for BrdU (BD Biosciences). For detecting SG, M phase, transcription factor staining buffer set (eBioscience, Thermo Fisher Scientific) was used to stain for Ki67. PBS-diluted DAPI (1:1000) was used to stain for the DNA content. To quantitate blood monocytes, 100 μL of blood was obtained and incubated in red blood cell lysis buffer (BD Biosciences) for 10 minutes and
stained with fluorophore-conjugated antibodies for 30 minutes in a dark room. For apoptosis, cells were evaluated with an annexin V apoptosis detection kit (BD Biosciences). For autophagy quantification in TAM subsets, FlowCelect Autophagy LC3 Antibody-based Assay Kit (MilliporeSigma) was used. This kit disrupts the cell plasma membrane and extracts cytosolic LC-3 by flushing away during washing steps. LC-3 translocated into the autophagosome is protected from the extraction and remains intact inside autophagosome, thereby allowing its fluorescence to be measured by flow cytometry. Events were processed on LSR II and LSRFortessa flow cytometers (BD Biosciences), and data were analyzed with DIVA software (BD Biosciences). The antibodies used for flow cytometry are listed in Supplemental Table 2.

**Measurement of mitochondria content, mitophagy, and ROS staining.** To detect mitochondria activity of fresh TAMs, we first collected leukocyte mononuclear cells from peritoneal elute cells by density gradient centrifuge. Then, we stained leukocyte mononuclear cells with macrophage surface antibodies for 30 minutes. Following this, cells were washed and stained with mitochondria reagents (Life Technologies, Thermo Fisher Scientific) for 30 minutes at 37°C in RPMI-1640 without FBS. MitoTracker Green (100 nM) and MitoTracker Deep red (100 nM) were combined to detect the mitochondria mass. MitoSOX (5 μM) was used to check mitochondria-related ROS. To detected the effect of arginine on the induced mitophagy, TAMs were treated with oligomycin (10 μM) and antimycin A (4 μM) (control) in the presence or absence of rapamycin (100 nM). Meanwhile, arginine or arginase-1 inhibitor nor-NOHA (0.5 mM) was added into the culture for 24 hours. The levels of damaged mitochondria without any inhibitors were used as background control. The percentages of accumulated damaged mitochondria were normalized to the control group. After washing with PBS for 2 times, the stained TAMs were gated and detected via BD LSR flow cytometry.

**Extracellular flux analysis.** Analysis of the OCR was performed with a Seahorse XF96 Extracellular Flux Analyzer instrument. Sorted TAM subsets were seeded at 2 × 10^5 per well (96-well) in RPMI-1640 with 10% FBS and incubated for 1 to 2 hours. The media were removed and replaced with Seahorse assay media with 2 mM glutamine and 25 mM glucose. The plates containing cells were incubated for 1 hour at 37°C without CO₂. Extracellular flux analysis was performed at 37°C without CO₂ in the XF96 analyzer (Seahorse Bioscience) following the manufacturer’s instructions. Port additions and times are indicated in the figures. Oligomycin (1.25 μM), FCCP (0.5 μM), and rotenone (1 μM) plus antimycin A (1 μM) were injected where relevant, and OCR (pmol O₂/min) was measured in real time.

**Arginine uptake and arginase activity assay.** To detect the arginine uptake in TAM subsets, 5 × 10⁵ TAMs were seeded in a 24-well plate for 24 hours. Then, culture media with or without TAMs were collected to determine the amount of arginine in culture medium without TAMs to subtract the amount of arginine in culture medium with TAMs. To detect the arginase activity in TAM subsets, 5 × 10⁵ fresh TAMs were lysed in 10 mM Tris-HCl 7.4 buffer containing 0.4% (w/v) Triton X-100 and protease inhibitors. The arginase activity was measured by the Arginase Assay Kit (Abnova). The lysed cells were centrifuged at 14,000g at 4°C for 10 minutes, and the supernatants were plated onto a 96-well microtiter plate. l-arginine was converted to urea by a buffer containing a substrate and cofactor, and the absorbance of the samples was measured using a microplate reader at the wavelength of 430 nm.

**Real-time PCR and RNA-Seq analysis.** Total RNA was isolated from cells by column purification (Direct-zol RNA Miniprep Kit, Zymo Research) with DNase treatment. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) with poly-dT or random hexamer primers. qPCR was performed on cDNA using Fast SYBR Green Master Mix (Thermo Fisher Scientific) on a StepOnePlus Real-Time PCR System (Thermo Fisher Scientific). Fold changes in mRNA expression were calculated by the ΔΔCt method using Actb as an endogenous control. Results are expressed as fold change by normalizing to the controls. The primers used for qPCR are listed in Supplemental Table 2. The acquisition and analysis of RNA-Seq data were described previously (59). Total RNA was isolated from cells by column purification (Direct-zol RNA Miniprep Kit, Zymo Research) with DNase treatment. The Ribo-Zero Gold rRNA Removal Kit (Illumina) and TruSeq Stranded Total RNA Library Prep Globin kit (Illumina) were used to prepare the library for RNA-Seq. Sequencing was performed by the University of Michigan DNA Sequencing Core, using the Illumina Hi-Seq 4000 platform, paired end, 50 cycles. Quality of the raw reads data for each sample was first evaluated using FastQC (version 0.11.3). The Tuxedo Suite software package was used for alignment, differential expression analysis, and postanalysis diagnostics. In brief, reads were aligned to the reference transcriptome (hg19) using TopHat (version 2.0.13), and a second round of quality control was performed after alignment.
Cufflinks/CuffDiff (version 2.2.1) was used for expression quantification, normalization, and differential expression analysis. Locally developed scripts were used to format and annotate the differential expression data output from CuffDiff. Diagnostic plots were generated using the cummeRbund R package. Data were deposited in the NCBI’s Gene Expression Omnibus database (GSE157673).

**Western blot.** Cells were dissolved in RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) supplemented with protease inhibitor cocktail (Roche). The protein concentrations were determined by Bio-Rad protein assay reagent. The lysates were boiled for 5 minutes in 3× SDS sample buffer (0.5 M Tris-HCl pH 6.8, 30% glycerol, 3% SDS, 0.01% bromphenol blue) containing 3% β-mercaptoethanol and were analyzed by SDS-PAGE followed by Western blot using different antibodies, including arginase-1 (93668, Cell Signaling Technology), arginase-2 (55003, Cell Signaling Technology), CDK4 (11026-1-AP, Proteintech), CDK6 (3136, Cell Signaling Technology), cleaved caspase-3 (Asp175) (9661, Cell Signaling Technology), COX IV (4844, Cell Signaling Technology), cyclin D1 (2922, Cell Signaling Technology), cyclin D3 (2936, Cell Signaling Technology), FIP200 (17250-1-AP, Proteintech), LC3A/B (12741, Cell Signaling Technology), phospho-histone H2A.X (Ser139) (9718, Cell Signaling Technology), phospho-p70 S6 kinase (Thr389) (9205, Cell Signaling Technology), phospho-ULK1 (Ser317) (12753, Cell Signaling Technology), phospho-ULK1 (Ser757) (14202, Cell Signaling Technology), SDHB (ab14714, Abcam), SQSTM1/p62 (5114, Cell Signaling Technology), ULK1 (8054, Cell Signaling Technology), and β-actin (A5441, MilliporeSigma). Signals were detected by ECL reagents (Thermo Fisher Scientific). The protein expression levels were quantified with ImageJ (NIH) software and were normalized to 1 in specific control groups.

**Enrichment and gene ontology analysis.** GSEA was performed using the GSEA software downloaded from Broad Institute (60). The gene signatures for GSEA are listed in Supplemental Table 2. The function was used to compute the enrichment scores and simulated enrichment scores for each variable and signature. A comprehensive GSEA web server, Enrichr (61), was used for Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and gene ontology analysis in human TAMs. Important KEGG pathways and gene ontology terms were selected.

**Statistics.** Mann-Whitney U test was used to compare 2 independent groups. Student’s 2-tailed t test was used for paired samples. The Pearson correlation was used to analyze the association between 2 continuous variables. For multigroup comparisons, 1-way ANOVA with Dunnett’s multiple-comparisons test was used to identify group-specific differences. Statistical analysis was performed with GraphPad Prism 8 software (GraphPad Software, Inc.). P < 0.05 was considered significant.

**Study approval.** All patients provided informed written consent. All human samples were obtained in accordance with the following protocols approved by the Institutional Review Boards of the University of Michigan School of Medicine and the Henry Ford Health System (HUM00035663). All murine studies were performed under approval and in accordance with IACUC at the University of Michigan protocols (PRO00008278).

**Author contributions**
HX, IK, and WZ conceptualized the study; HX, IK, Weichao Wang, YB, SW, SG, Weimin Wang, LV, and JLG determined methodology and performed animal studies; XL and SL performed statistical and bioinformatics analysis; JRL, KM, RR, and AM performed pathological and clinical studies; HX and WZ wrote the original draft; WZ acquired funding; and IK and WZ supervised the study.

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