Studying Membrane Cholesterol Efflux's Role in Tumor-Associated Macrophage Reprogramming and Tumor Progression


Citing this paper
Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights
Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the Research Portal

Take down policy
If you believe that this document breaches copyright please contact librarypure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Tumor-induced cholesterol efflux from macrophages drives IL-4 mediated reprogramming and tumor progression.

Pieter Goossens1,2,†, Juan Rodriguez-Vita1,3,†, Anders Etzerodt1,4, Marion Masse1, Olivia Rastoin1, Victoire Gourand1, Thomas Ulas5,6, Olympia Papantonopoulou5, Miranda Van Eck7, Nathalie Auphan-Anezin1, Magali Bebien1, Christophe Verthuy1, Thien Phong Vu Manh1, Martin Turner8, Marc Dalod1, Joachim L. Schultz5,6, Toby Lawrence1,9,10,*

1. Aix Marseille Univ, CNRS, INSERM, CIML, Marseille, 13009, France.
2. Department of Pathology, Cardiovascular Research Institute Maastricht, Maastricht University, 6229HX, The Netherlands.
3. Vascular Signaling and Cancer (A270), German Cancer Research Center (DKFZ), Heidelberg, 69120, Germany.
4. Department of Biomedicine, Aarhus University, Aarhus, 8000, Denmark
5. Genomics & Immunoregulation, Life and Medical Sciences Institute (LIMES), University of Bonn, Bonn, 53115, Germany.
6. PRECISE Platform for Single Cell Genomics and Epigenomics, German Center for Neurodegenerative Diseases and University of Bonn, Bonn, 53127, Germany
8. Laboratory of Lymphocyte Signaling and Development, The Babraham Institute, Cambridge, CB22 3AT, UK.
9. Centre for Inflammation Biology and Cancer Immunology, Faculty of Life Sciences and Medicine, King’s College London, SE1 1UL, UK.
10. Xinxiang Medical University, Henan Province 453003, China.
† These authors contributed equally.
* Lead contact/Corresponding author: Prof. Toby Lawrence, Centre for Inflammation Biology and Cancer Immunology, School of Immunology & Micribial Sciences, King’s College London, SE1 1UL, UK. Tel: +44(0)207 848 8630, Email: toby.lawrence@kcl.ac.uk.

**Running title:** Cholesterol efflux from TAM drives tumor progression
Highlights

- Monocyte-derived TAM gradually replace resident peritoneal macrophages in metastatic ovarian cancer
- Ovarian cancer cells promote membrane-cholesterol efflux in TAM
- Cholesterol-efflux depletes lipid rafts and increases IL-4 signaling in TAM
- Inhibition of ABC transporters reverts the tumor-promoting functions of TAM in ovarian cancer

eTOC blurb

Goossens et al. show that cancer cells scavenge membrane cholesterol from macrophages in tumors which reprogrammes them towards an immune-suppressive and tumor-promoting phenotype and makes them resistant to activation by anti-tumor cytokines.

Summary

Tumor-associated macrophages (TAM) have been shown to have important roles in the malignant progression of various cancers. However, macrophages also posses intrinsic tumoricidal activity, but rapidly adopt an alternative phenotype within tumors associated with immune-suppression and trophic functions supporting tumor growth. The mechanisms that promote TAM polarization remain poorly understood, these mechanisms may represent important therapeutic targets to block the tumor-promoting functions of TAM and restore their anti-tumor potential. Here we have characterized TAM in a mouse model of metastatic ovarian cancer. We show that ovarian cancer cells promote membrane-cholesterol efflux and depletion of lipid rafts from macrophages. Increased cholesterol efflux promoted IL-4 mediated reprogramming while inhibiting IFNγ-induced gene expression. These studies reveal an unexpected role for membrane-cholesterol efflux in driving the tumor-promoting functions of TAM, while rendering them refractory to pro-inflammatory stimuli. Thus, preventing cholesterol efflux in TAM may represent a novel therapeutic strategy to block pro-tumor functions and restore anti-tumor immunity.

Keywords

Tumor-associated macrophages, Ovarian cancer, Cholesterol efflux, Lipid rafts, IL-4 signaling
Introduction

There is now a wealth of clinical and experimental evidence that strongly links tumor-associated macrophages (TAM) with tumor progression, invasion and metastasis (Noy and Pollard, 2014). In the vast majority of published studies, increased numbers of TAM correlate with poor prognosis, but in some cases, specific TAM subsets have been associated with beneficial outcomes (de Vos van Steenwijk et al., 2013; Ino et al., 2013). Indeed, macrophages have been shown to possess intrinsic tumoricidal activity and promote the activation of cytotoxic lymphocytes (Bonnotte et al., 2001; Hagemann et al., 2008; Mytar et al., 1999), but they rapidly adopt an alternative phenotype within tumors, associated with immune-suppression and trophic functions that support tumor growth (Mantovani et al., 2008). However, the mechanisms that promote TAM reprogramming in the tumor-microenvironment remain poorly understood.

In mammals, macrophages are found in all tissues after birth and are endowed with trophic functions that contribute to organ development and remodelling (Pollard, 2009). Recent advances in genetic fate-mapping techniques have revealed that the majority of tissue-resident macrophages, at least in steady-state, develop from embryonic precursors and are maintained by local proliferation with little input from hematopoietic stem cells (HSC) in the bone marrow (Schulz et al., 2012). Subsequent studies have shown that embryonic macrophages can be gradually replaced by HSC-derived blood monocytes, to varying degrees depending on the specific context (Ginhoux and Guilliams, 2016). But the functional implications of these distinct developmental origins and certainly their respective contributions to tumor progression remain unclear. In a recent study, both tissue-resident macrophages of embryonic origin and monocyte-derived TAM were shown to contribute towards tumor growth in a mouse model of pancreatic cancer (Zhu et al., 2017).

The phenotype of tissue-resident macrophages is dictated by the tissue-specific signals in their respective niche (Gosselin et al., 2014; Lavin et al., 2014). However, during inflammation or tissue stress, monocyte-derived macrophages can be recruited into tissues and their functional reprogramming is dictated by the pathological context. It is now widely appreciated that macrophages follow a multi-dimensional model of activation states with distinct phenotypic and functional properties in response to different stimuli in the tissue microenvironment and can maintain considerable plasticity (Murray et al., 2014; Xue et al., 2014). Along these lines, TAM in various experimental models and human cancers have been shown to express uniques sets of gene patterns including the production of specific chemokines, cytokines and growth factors linked with tumor progression, such as CCL2, TNF, VEGF, basic fibroblast growth factor (bFGF) and matrix metalloproteinases (MMPs) (Kratochvill et al., 2015). Nevertheless, TAM are invariably reprogrammed towards a functional state that supports tumor growth and
immune-suppression and away from inflammatory phenotypes that could be associated with anti-tumor functions. The specific mechanisms that drive TAM accumulation and polarization in different tumors remain unclear. Several studies have shown that TAM are CSF-1 dependent, as are most tissue macrophages, and CSF-1 signaling has been suggested to be an important factor in their reprogramming towards pro-tumor functions (Martinez et al., 2006; Noy and Pollard, 2014). CSF-1 and IL-4 signaling in TAM was later shown to cooperatively promote growth of lung metastases in the MMTV-pyMT mouse model of mammary carcinogenesis (DeNardo et al., 2009). However, although primary tumor-development in this model was CSF-1 dependent (Lin et al., 2006), IL-4 signaling in TAM did not impact primary tumors (DeNardo et al., 2009). Subsequent studies showed that the development of lung metastases, but not primary tumors, in the same model critically requires the recruitment of CCR2-dependent monocytes (Qian et al., 2011). Suggesting that IL-4 signaling, specifically in monocyte-derived TAM, promotes metastatic disease in this model.

Here we have characterized TAM in a mouse model of metastatic ovarian cancer. We show that monocyte-derived TAM gradually replaced resident macrophages in this model and displayed an upregulation of cholesterol metabolism and reverse cholesterol efflux pathways during tumor progression. Further experiments revealed that ovarian cancer cells actively promoted plasma membrane cholesterol efflux from macrophages and the subsequent loss of cholesterol-rich membrane microdomains, or so called “lipid rafts”. Increased cholesterol efflux promoted enhanced IL-4 signaling in macrophages while inhibiting IFNγ-induced gene expression. IL-4 induced gene expression in TAM is associated with tumor-promoting functions, including increased arginine metabolism promoting immune-suppression and trophic functions, supporting invasion and metastasis. Whereas, IFNγ induced gene expression in TAM drives anti-tumor functions. We further demonstrate that IL-4 signaling and cholesterol efflux pathways in TAM significantly contribute to tumor progression in vivo. These studies suggest an important role for membrane-cholesterol efflux in driving IL-4 signaling and tumor-promoting functions of TAM in ovarian cancer, while rendering them refractory to reprogramming by anti-tumor cytokines. Therefore, preventing cholesterol efflux in TAM could represent a novel therapeutic strategy to block pro-tumor functions and restore anti-tumor immunity.

Results
Origins of TAM during ID8 tumor development.

High grade serous ovarian cancer (HGSC) is frequently associated with colonisation of the peritoneal cavity by cancer cells (George et al., 2016). ID8 cells are spontaneously transformed mouse ovarian surface epithelial cells (Urzua et al., 2016), when adoptively transferred by intra-peritoneal (i.p.) injection in syngeneic mice, these cells progressively develop a malignant ascites with tumor nodules throughout the peritoneal cavity (Hagemann et al., 2008), which is characteristic of HGSC. The peritoneal cavity is populated by two major subsets of serosal macrophages; large peritoneal macrophages (LPM), which are most abundant, and a minor population of small peritoneal macrophages (SPM) (Ghosn et al., 2010). Previous studies have shown that SPM and LPM have distinct developmental origins; SPM develop from blood monocytes which are derived from bone marrow progenitors, whereas LPM are derived from embryonic progenitors and are maintained independently of blood monocytes, retaining proliferative capacity for self-renewal (Yona et al., 2013). More recent studies have shown that LPM can be progressively replaced by long-lived bone marrow-derived macrophages that maintain self-renewal potential (Bain et al., 2016). To monitor the dynamics of peritoneal macrophages (PM) during ID8 tumor growth, we first characterized macrophage subsets by flow cytometry. SPM and LPM can be distinguished by F4/80 and MHCII expression; SPM are MHCII<sup>hi</sup> F4/80<sup>lo</sup> whereas LPM are F4/80<sup>hi</sup> MHCII<sup>lo</sup> (Fig.1A). LPM represent approximately 80% of PM in naïve mice, however, after seeding of ID8 cells in the peritoneal cavity, a significant population of F4/80<sup>int</sup> MHCII<sup>int</sup> PM rapidly accumulates (intPM; Fig.1A). Kinetic analysis of total cell numbers revealed that LPM numbers remain relatively constant throughout tumor progression, while intPM progressively accumulate and eventually become the dominant TAM population (Fig.1B).

To determine the dynamics of PM subsets during ID8 tumor growth, we performed fate-mapping studies with shielded radiation chimera mice. Radiation chimeras can be used to determine the contribution of bone marrow-derived progenitors towards cells in a given tissue. However, irradiation kills tissue-resident macrophages that then become replaced by monocyte-derived cells, thus to distinguish tissue-resident cells from monocyte-derived macrophages from the bone marrow, it is necessary to protect the tissue from the effects of radiation using lead shielding. To study the origins of PM subsets during ID8 tumor growth, we shielded the abdomen of host C57BL6 CD45.1 congenic mice during irradiation and then adoptively transferred a mixture of bone marrow cells from mice expressing both CD45.1 and CD45.2 (CD45.1/2) and Ccr2<sup>−/−</sup> mice, expressing only CD45.2. This allowed the distinction between host (CD45.1) and donor cells (CD45.1/2 or CD45.2), as well as their CCR2-dependency, CD45.2 single-positive cells being CCR2-dependent. Due to the low engraftment efficiency of Ccr2<sup>−/−</sup> bone marrow cells, Ccr2<sup>−/−</sup> donor cells were mixed at a ratio of 4:1 with...
competitor B6.CD45.1/2 cells. Five weeks after bone marrow engraftment, chimeric mice were injected with ID8 cells to track the contribution of bone marrow-derived cells to PM subsets (Fig.1C). CD45.1 and CD45.2 expression in TAM subsets was measured by flow cytometry a further 8 weeks after injection of ID8 cells, a total of 13 weeks after adoptive transfer of bone marrow cells, chimerism was normalised to blood monocytes. These experiments revealed that SPM and intPM were derived from CCR2-dependent bone marrow progenitors, with almost 100% chimerism after 8 weeks of tumor growth (Fig.1D,E). However, at this time point, LPM only showed approximately 30% chimerism, implying that LPM are more gradually replaced by bone marrow-derived cells during tumor development. To confirm the CCR2-dependency of intPM, we analyzed the accumulation of PM subsets in full CCR2 deficient mice (Ccr2^−/−), as expected, both SPM and intPM were drastically reduced in Ccr2^−/− mice bearing ID8 tumors, whereas CCR2 deficiency had little impact on LPM numbers (Fig.S1).

To confirm the continuous contribution of blood monocytes to SPM and intPM populations throughout tumor growth, we used a fluorescent fate-mapping approach. The chemokine receptor Cx3cr1 is expressed by blood monocytes (Geissmann et al., 2003) and previous studies have demonstrated the fate-mapping of monocyte-derived cells using knock-in mice that express a tamoxifen-inducible Cre-recombinase from the Cx3cr1 locus (Cx3cr1^{CreER}), crossed to mice expressing a ubiquitous lox-STOP-lox reporter cassette (Yona et al., 2013). As expected, we did not detect Cx3cr1 expression in steady-state LPM using the Cx3cr1^{CreER} reporter mice, however, high levels of Cx3cr1 expression were observed in SPM and intermediated levels in intPM (Fig.S1), reflecting the likely monocyte origins of these cells. We crossed Cx3cr1^{CreER} mice with Rosa26-Isl-tdRFP reporter mice (Cx3cr1^{CreER}:R26-tdRFP) and injected these mice with ID8 cells to track monocyte-derived cells during tumor growth. Six weeks after injection of ID8 cells, mice were given a single dose of 4-OHT by oral gavage (p.o.) and RFP expression in TAM subsets was measured by flow cytometry ten days later. These experiments showed strong RFP labelling in SPM and intPM within 10 days of 4-OHT administration, with very little labelling of LPM (Fig.1F,G). These data clearly demonstrated the contribution of blood monocytes to SPM and intPM during tumor growth, even within this short time frame.

**Transcriptional profiling of TAM.**
To evaluate the impact of the tumor-microenvironment on PM phenotype, we performed global gene expression analysis using microarrays on bulk PM from naïve mice and at different time points during tumor progression. We isolated naïve F4/80^hi PM and TAM at 5, 12 and 21 days during ID8 tumor development by flow cytometry (Fig.S2). Total RNA was extracted and samples were analyzed using MoGene 1.0st microarrays. RMA normalised data were filtered and analyzed for variations in gene expression. The heatmap in figure S2B shows the 1000
most variable genes in the dataset. To extract differentially expressed genes (DEGs) between naïve PM and TAM at the different time points, we used Anova with an adjusted p value and a threshold of 1.5 fold change (FC). We then used Gene Ontology (GO) enrichment analysis to identify pathways affected in TAM at different time points (Fig.1H,I). DEGs are represented by edges (green = up; blue = down) and individual GO terms are represented by nodes. GO terms that are similar, as indicated by the intersection of DEGs in a given GO term, are closer to each other. This generates clusters of similar GO terms indicating common biological processes within the cluster. This analysis revealed a major cluster of upregulated genes related to immunity in TAM after 5 days (Fig.1H), possibly reflecting a tumoricidal response triggered by resident PM in response to ID8 cells. However, after 21 days, when tumors had become more established, the gene expression profile of TAM more closely resembled the phenotype of naïve PM (Fig.S2). At this later time point, there was an upregulation of distinct gene clusters, including a large cluster of genes related to the innate immune response and tumor necrosis factor (TNF) signaling, in keeping with previous data showing an important role for TNF in this model (Charles et al., 2009; Hagemann et al., 2006), and also a distinctive cluster of genes associated with cholesterol metabolism and efflux (Fig.1I). To confirm the enrichment of genes related to cholesterol homeostasis, we merged several published genesets (Rayner et al., 2011) and known hallmarks to generate an extended gene list representing cholesterol homeostasis. This compiled geneset also showed a significant enrichment in TAM and among the up-regulated genes were known actors in cholesterol metabolism and efflux, including; Abcg1, Ldlr, Pparg, Hmgcs1, Hmgcr, Srebf2 (Fig.S2).

**Increased membrane cholesterol efflux in TAM.**

Changes in membrane cholesterol content have been shown to dramatically affect macrophage activation in response to pro-inflammatory stimuli, such as bacterial lipopolysaccharide (LPS) (Fessler and Parks, 2011). This is thought, at least in part, to be due to the depletion of cholesterol rich membrane micro-domains, also called lipid rafts, which act as signaling platforms for certain receptors. But membrane cholesterol influences multiple facets of membrane structure and dynamics that can also affect receptor signaling. To confirm the finding that cholesterol efflux pathways were upregulated in TAM, we sought to measure effects on cholesterol membrane content in TAM from ID8 tumor bearing mice. Cholesterol rich membrane micro-domains are commonly measured using cholera toxin B (CTB) staining, which binds to ganglioside GM1, the accumulation of which is linked with membrane cholesterol content. We isolated naïve PM and TAM at 5 and 21 days after injection of ID8 cells, stained the cells with Alexa Fluor 488-conjugated CTB and analyzed them by confocal microscopy. We observed that CTB staining was similar in naïve PM and TAM isolated at 5 days, but was significantly decreased in TAM after 21 days of tumor growth (Fig.2A,B),
indicating that the tumor-microenvironment may promote the depletion of cholesterol rich membrane micro-domains in TAM, in accordance with the upregulation of genes regulating cholesterol efflux in these cells (Fig.1I). To test if ID8 tumor cells had a direct effect on macrophage cholesterol efflux, we co-cultured ID8 cells with bone marrow-derived macrophages (BMDM) in vitro. After just one hour of co-culture, there was a significant decrease of CTB staining in BMDM (Fig.2C,D), indicating tumor cells actively promoted the depletion of membrane cholesterol in macrophages. To test if factors secreted by tumor cells were responsible for this effect, we incubated BMDM with conditioned medium obtained from ID8 cell cultures (ID8-CM). This also resulted in a rapid reduction in CTB staining, that was almost equivalent to the effects of methyl-β-cyclodextrin (MCD), which extracts cholesterol from cell membranes (Ostrom and Liu, 2007) (Fig.2E,F). Although CTB is commonly used to measure cholesterol rich membrane micro-domains, this is a rather indirect measure of membrane cholesterol. Another method to assess membrane cholesterol content exploits the highly ordered structure of cholesterol-rich membrane microdomains by the use of phase-sensitive fluorescent probes such as Laurdan and di-4-ANEPPDHQ (Owen et al., 2011; Sonnino and Prinetti, 2013). These molecules adapt their emission wavelength based on local membrane order, which is a direct reflection of cholesterol content, independently of membrane-associated proteins. To confirm our findings, we labelled macrophages with di-4-ANEPPDHQ and found a significant decrease of membrane order in the presence of ID8-CM (Fig.2G,H). These assays confirmed that the reduction in CTB staining observed after ID8-CM treatment, correlated with alterations in membrane order that reflect reduced levels of membrane cholesterol. Furthermore, we measured total cholesterol levels in macrophages cultured in the presence or absence of tumor cell-conditioned medium and observed a significant decrease of total cellular cholesterol (Fig.2I). Finally, to directly measure cholesterol efflux from macrophages, we loaded BMDM with thymidine (³H)-labelled cholesterol and measured its efflux into the culture media after addition of the apolipoprotein A1 (ApoA1). Membrane cholesterol efflux is mediated by the transfer of cholesterol to lipoproteins through ABC transporters (Zhao et al., 2010), in the case of ApoA1 this occurs through the transporter ABCA1. These assays showed that addition of ID8-CM significantly increased cholesterol efflux from macrophages, which was reversed in BMDM from ABCA1-deficient mice (Abca1⁻⁻), demonstrating that this was due to an increased efflux of membrane cholesterol (Fig.2J).

**Tumor cell-derived hyaluronic acid (HA) drives cholesterol efflux in macrophages.**

The studies described above showed that ID8 cells increased cholesterol efflux from macrophages. This effect could be recapitulated with conditioned medium but not with fixed cells (Fig.3A), indicating that cholesterol efflux is promoted by a secreted factor. In order to further characterize this factor, we exposed ID8-CM to a series of treatments, including ultra-
centrifugation, boiling (95°C for 5 min), repeated freeze/thaw cycles, DNAse and proteinase K, none of which had any impact on the ability of ID8-CM to deplete CTB staining in macrophages (data not shown). However, size fractionation of ID8-CM with cut-offs at 3, 10, 30 or 100 kDa, revealed that this activity was present in a fraction with a molecular weight above 100 kDa (Fig.3B). Several previous studies have shown that the extracellular matrix (ECM) component hyaluronic acid (HA) can be produced by tumor cells and has been linked with increased tumor progression (Chanmee et al., 2016). HA also forms high molecular weight oligomers (>100 kDa) with distinct biological activity (Gomez-Aristizabal et al., 2016; Kolapalli et al., 2016; Rayahin et al., 2015). Furthermore, receptors for HA are expressed by TAM, namely CD44 and Lyve-1 (Chanmee et al., 2016; Turley et al., 2002). To test the hypothesis that HA in ID8-CM contributed to the effects on membrane cholesterol content, we treated ID8-CM with hyaluronidase (HAse) to degrade HA. Indeed, ID8-CM treated with HAse was no longer able to deplete CTB staining in macrophages (Fig.3C). Conversely, when HA of different molecular weights was added to macrophages in normal culture medium, we observed a reduction of CTB staining with increasing molecular weight (Fig.3D). These experiments suggested that high molecular weight HA produced by ID8 cells promotes membrane cholesterol depletion in macrophages. Given that HA is an important component of ECM in many cancers, including EOC (Kolapalli et al., 2016), this suggests HA could affect the phenotype of TAM through membrane cholesterol depletion.

**Cholesterol efflux promotes IL-4 mediated macrophage reprogramming.**

Depletion of membrane cholesterol has been shown to profoundly affect macrophage activation in response to pro-inflammatory stimuli (Fessler and Parks, 2011; Pradel et al., 2009), which suggests that cholesterol efflux in TAM could affect their programming by signals in the tumor-microenvironment. In some instances, TAM have been shown to exhibit a tumor-promoting phenotype that can be driven by Th2 cytokines such as IL-4 or IL-13, and are skewed away from the pro-inflammatory and immunostimulatory activation state, for example induced by Th1 cytokines such as IFNγ (DeNardo et al., 2009; Murray et al., 2014). To test the effects of ID8 cells on macrophage reprogramming, we stimulated BMDM with IL-4 or IFNγ in the presence or absence of ID8-CM and measured the induction of IL-4 and IFNγ gene expression, respectively. ID8-CM pre-treatment profoundly increased the expression of IL-4 induced genes; Arg1, Retnla, Chi3l3 and Mrc1 (Fig.4A). In contrast, ID8-CM inhibited the IFNγ induced expression of Nos2 and Il12b (Fig.4B), as well as other IFNγ-regulated genes including Cxcl9, Cxcl10 and Ciita (Fig.S3), demonstrating that ID8-CM promoted macrophage programming towards an IL-4 induced pro-tumor phenotype. Similar results were obtained after co-culture of ID8 cells with BMDM or with IL-13 treatment, which also signals through the
IL4 receptor alpha chain (IL4RA) (Fig.S3). These effects were restricted to the high molecular weight (>100 kDa) fraction of ID8-CM (Fig.S3) and were not sensitive to freeze/thaw cycles, boiling, ultracentrifugation, DNase or proteinase K treatment (data not shown).

To evaluate the effects of membrane cholesterol depletion on macrophage reprogramming we used several mechanistically distinct treatments to induce cholesterol efflux, in comparison with ID8-CM; 9-cis-retenoic acid (9cRA) upregulates expression of ABC transporters and thereby induces cholesterol efflux (Ricote et al., 2004), whereas high-density lipoprotein (HDL) and ApoA1 strip cholesterol directly from the cell membrane (Zhao et al., 2010). Treatment of BMDM with 9cRA, HDL or ApoA1 resulted in similar levels of reduction in CTB staining as seen upon ID8-CM treatment (Fig.4C), as well as increased IL-4 induced gene expression, while inhibiting IFN\(\gamma\) induced genes (Fig.4D). In contrast, the addition of exogenous cholesterol to BMDM, reduced the effects of ID8-CM on IL-4 induced Arg1 and IFN\(\gamma\) induced Nos2 expression (Fig.S3).

These data suggested that membrane cholesterol depletion promotes IL-4 mediated macrophage activation and abrogates IFN\(\gamma\) signalling. To directly test the role of cholesterol efflux in macrophage reprogramming by ID8-CM, we used BMDM from mice with a combined myeloid deficiency in the ABCA1 and ABCG1 reverse cholesterol efflux transporters (Abca1/g1\(^{\Delta}\)Lyz2). Treatment with ID8-CM or other membrane cholesterol-depleting agents failed to reduce CTB staining in BMDM from Abca1/g1\(^{\Delta}\)Lyz2 mice (Fig.4E), which indeed reversed the increase in IL-4 induced gene expression by ID8-CM and the inhibition of IFN\(\gamma\) induced genes (Fig.4F), indicating that membrane cholesterol efflux through ABCA1 and/or ABCG1 promoted IL-4 mediated macrophage activation in the presence of ID8-CM.

**Tumor-induced macrophage reprogramming is STAT6 and PI3K dependent.**

To further characterize the mechanisms behind increased IL-4 induced gene expression in the presence of ID8-CM and how this may relate to cholesterol efflux, we analyzed IL-4 receptor signaling pathways. First, we observed no increase in the expression levels of the IL-4 receptor (IL4RA) on macrophages treated with ID8-CM (data not shown), but immunofluorescent staining illustrated an increased intracellular clustering of the receptor that suggested endosomal accumulation, which previously has been shown to promote downstream signaling (Gandhi et al.; Kurgonaite et al.) (Fig S4). We therefore measured activation of signaling pathways downstream of the IL-4 receptor. IL-4 induced gene expression is regulated by JAK-mediated phosphorylation of the STAT6 transcription factor. Treatment of BMDM with ID8-CM increased levels of activated STAT6 (pY-STAT6) in response to IL-4 (Fig.5A,D; Fig.S4) while reducing the accumulation of phosphorylated STAT1 (pY-STAT1), upon IFN\(\gamma\) activation (Fig.5B,E; Fig.S4). As expected, IL-4 induced gene expression in the presence of ID8-CM was
abolished in BMDM derived from STAT6 deficient mice (Stat6\(^{-/-}\)) (Fig.S4). IL-4 signaling also activates PI3K, which was recently shown to be an important pathway for the tumor-promoting functions of TAM (Kaneda et al., 2016a; Kaneda et al., 2016b), furthermore, increased PI3K signaling has been shown to promote IL-4 induced gene expression in macrophages (Rauh et al., 2005). To assess PI3K activation we measured phosphorylation of the downstream kinase Akt/PKB. We observed a marked increase in serine 473 phosphorylation of Akt (pS-Akt) in the presence of ID8-CM (Fig.5C,F; Fig.S4), this correlated with increased accumulation of phosphatidylinositol (3,4,5)-triphosphate (PIP\(_3\)), the product of PI3K activity, as measured by confocal microscopy (Fig.S4). To determine the contribution of PI3K to IL-4 mediated reprogramming in the presence of ID8-CM, we treated cells with the PI3K inhibitor LY294002. As expected, LY294002 treatment blocked the increase in pS-Akt by ID8-CM (Fig.5G) and also abrogated the increase in IL-4 induced Arg1 and Chi3l3 expression (Fig.5H,I; Fig.S4), indicating that PI3K activity was critical for ID8-CM induced reprogramming. To determine the role of cholesterol efflux in STAT6 and PI3K activation, we again treated macrophages with 9cRA and ApoA1 to deplete membrane cholesterol, both treatments resulted in similar increases in the accumulation of pY-STAT6 and pS-Akt (Fig.5J,K). Furthermore, macrophages lacking the ABCA1 and ABCG1 cholesterol efflux transporters failed to increase pY-STAT6 and pS-Akt upon treatment with ID8-CM (Fig.5L,M). In addition, increased pY-STAT6 and pS-Akt accumulation was restricted to the high molecular weight (>100 kDa) fraction of ID8-CM and could be reversed by HAse treatment (Fig.S4), indicating that HA-mediated cholesterol efflux promoted increased STAT6 and Akt activation.

The specific accumulation of pS-Akt in the presence of ID8-CM was intriguing, serine 473 phosphorylation of Akt is mediated by mammalian target for rapamycin complex 2 (mTORC2) (Jacinto et al., 2006), which is activated by PI3K through PIP\(_3\) accumulation (Liu et al., 2015). Interestingly, mTORC2 was also recently shown to promote IL-4 induced macrophage activation in response to metabolic stress (Huang et al., 2016). Thus, we hypothesised that ID8-CM induced PIP\(_3\) accumulation could activate mTORC2-mediated pS-AKT phosphorylation and increase IL-4 induced gene expression. In the absence of any specific mTORC2 inhibitors, to test the role of the mTORC complex we used rapamycin, which blocks mTORC1, and Torin which blocks both mTORC1 and mTORC2. Rapamycin treatment only partially inhibited ID8-CM induced pS-Akt accumulation in macrophages, however, Torin treatment completely inhibited ID8-CM induced pS-Akt phosphorylation in a dose-dependent manner (Fig.5N,O), suggesting that mTORC2 activity is required for ID8-CM induced pS-Akt accumulation in macrophages.

In summary, IL-4 induced macrophage activation or reprogramming in response to ID8-CM requires PI3K-mTORC2-Akt activity and is driven by STAT6.
IL-4 induced STAT6 and PI3K signaling in TAM drives tumor progression in EOC.

To test the relevance of these pathways for TAM and tumor progression in vivo, we revisited our characterisation of TAM in ID8 tumors. Our conclusions from the data presented in figure 1, was that monocyte-derived TAM gradually replaced resident macrophages during tumor progression and that TAM showed an enrichment for cholesterol efflux pathways (Fig.1I). However, these analyzes were performed on bulk TAM populations, including resident PM and monocyte-derived TAM. To refine our analysis and determine the specific gene expression signature of monocyte-derived cells, we isolated F4/80<sup>lo</sup> CCR2<sup>+</sup> monocytes (MN), alongside F4/80<sup>hi</sup> LPM, which were further divided into Tim4<sup>+</sup> and Tim4<sup>-</sup> subsets (Fig.S5). Tim4 was previously shown to be a marker for proliferative, self-renewing LPM (Rosas et al., 2014), whereas Tim4<sup>-</sup> F4/80<sup>hi</sup> cells represent monocyte-derived LPM, which are CCR2-dependent (Fig.1; Fig.S5). First, we collected these 3 populations from naïve mice and at different time points during tumor progression for microarray analysis. We performed a pairwise comparison between the 3 populations in naïve mice and extracted a specific gene signature for each subset, applying a 1.5 FC threshold and a p-value of 0.05. Using the Minimal method (pairwise[Mean(test)/Mean(ref)]), we identified sets of 553 genes specific for MN, 131 for Tim4<sup>+</sup> PM and 84 for Tim4<sup>-</sup> PM (Table S1). Given that CCR2 was the highest DEG between Tim4<sup>-</sup> and Tim4<sup>+</sup> populations, this strongly supported the monocytic origin of Tim4<sup>-</sup> cells, in keeping with our previous analysis (Fig.S5). We then used these gene sets to perform enrichment analysis (GSEA) with DEGs from the equivalent 3 subsets in ID8 tumor-bearing mice. This analysis showed a significant down-regulation of the naïve Tim4<sup>+</sup> PM gene signature and a strong enrichment of the MN and Tim4<sup>-</sup> gene signatures in Tim4<sup>+</sup> TAM (Fig.6A), supporting our conclusion that the tumor-microenvironment promotes the replacement of resident PM with MN-derived cells that acquire a resident-like phenotype, including expression of Tim4 (Fig.1; Fig.S5). To determine the specific genes associated with this phenotype, we extracted the leading edges (LEs) for this enrichment, that is the genes most strongly associated with the enrichment of the MN gene signature in Tim4<sup>+</sup> TAM. We identified 173 LEs that were enriched at all time points in Tim4<sup>+</sup> TAM (Fig.6B; Table S2), which we then used for Ingenuity Pathway Analysis (IPA). The most significant pathway associated with these genes was the IL-4 pathway (Fig.6B), suggesting that IL-4 in the tumor-microenvironment could be an important upstream regulator for the development of the monocyte-derived TAM phenotype.

To confirm the role of IL-4 signaling in tumor progression in vivo, we treated ID8 tumor-bearing mice with an IL-4 receptor blocking monoclonal antibody (αIL4ra) and monitored tumor progression. Treatment with αIL4ra significantly reduced ID8 tumor growth in vivo (Fig.6C), suggesting that IL-4 signaling is an important factor for tumor progression in this model. Furthermore, chimeric mice with hematopoietic deficiency in STAT6 (Stat6<sup>-/-</sup>) or PI3K (Pik3cd<sup>-/-</sup>)
also showed significantly reduced tumor growth (Fig.6D,E), indicating that both signaling pathways in tumor stromal cells are important factors for tumor progression. To evaluate the impact of these pathways on TAM phenotype in vivo, we sorted bulk TAM from Pik3cd-/- chimeric mice by flow cytometry and isolated RNA for microarray analysis. Expression of Arg1, Il10, Ccl2 and Stab1, which have previously been shown to upregulated in TAM, were significantly downregulated in macrophages from Pik3cd-/- chimeric mice compared to controls (Fig.S5), suggesting that PI3K activation contributes to the TAM phenotype. To further analyze the impact of PI3K activation on TAM, we generated a gene set from all DEGs between naïve PM and TAM from wild-type mice. Subsequent GSEA showed a significant enrichment for genes expressed by naïve PM in Pik3cd-/- cells (Fig.6F), confirming that PI3K activity contributes to the promotion of the TAM phenotype. In addition, there was no enrichment of genes associated with the IL-4 dependent TAM phenotype (Fig.6G), described above (Fig.6B; Table S2). These data indicated that PI3K is an important regulator of this gene set in TAM. Interestingly, using an established gene set for tumoricidal phenotype (GSE26912), which was enriched in naïve PM compared to TAM, we also observed an enrichment in Pik3cd-/- TAM compared to wild-type cells, suggesting that these cells retained a more tumoricidal phenotype in the absence of PI3K activation (Fig.S5). Finally, to evaluate the role of cholesterol efflux in TAM in vivo, we established ID8 tumors in mice with a myeloid-specific deletion of both ABCA1 and ABCG1 (Abca1/g1<sup>-/-</sup>Lyz2). ID8 tumor progression was significantly impaired in Abca1/g1<sup>-/-Lyz2</sup> mice compared to littermate controls (Fig.6H). Furthermore, microarray analysis of TAM sorted from these mice showed a significant downregulation of genes associated with the IL-4 dependent TAM phenotype and a positive enrichment for tumoricidal genes (Fig.6I; Fig.S5), reflecting the phenotype of PI3K deficient TAM.

Collectively, these data showed that IL-4 signaling in TAM plays an important role in tumor progression in this model. Furthermore, the PI3K pathway and increased cholesterol efflux, contribute significantly to the functional polarization of TAM and tumor progression in vivo.

Discussion

It is now well appreciated that tumor-associated macrophages (TAM) can play an important role in cancer progression. TAM contribute to tumor progression by various mechanisms, including immune-suppression and trophic functions, supporting angiogenesis, cell proliferation, invasion and metastasis. For example, increased expression of arginase I (Arg1) in TAM, depletes arginine which is required by activated T cells and consequently increases polyamine synthesis, which supports cancer cell proliferation. However, macrophages also possess intrinsic anti-tumor potential, through direct tumoricidal functions and orchestrating
anti-tumor immunity, which may be particularly relevant in response to therapy (Bonnotte et al., 2001; Hagemann et al., 2008; Mytar et al., 1999). But the mechanisms by which TAM become polarized towards pro-tumor functions remain poorly understood.

Here we have studied TAM in a mouse model of epithelial ovarian cancer (EOC), that reflects the peritoneal spread of high-grade serous ovarian cancer (HGSC). In mice, the peritoneal cavity contains a major resident macrophage population of embryonic origin (large peritoneal macrophages; LPM), as well as a minor population of monocyte (MN)-derived macrophages (small peritoneal macrophages; SPM). During tumor progression we showed that MN-derived TAM accumulate and gradually replace resident macrophages in the peritoneal cavity. We then analyzed the global changes in gene expression in TAM over time using microarrays and used pathway analysis to reveal changes in gene expression linked with different pathways and biological functions. At early time points, TAM displayed a more pro-inflammatory gene signature, which strongly distinguished them from naïve resident PM. However, in established tumors, TAM acquired a phenotype more closely resembling resident PM, which suggested a dynamic reprogramming of TAM phenotype during tumor progression.

Among the pathways upregulated in TAM from established tumors compared to naïve PM was a cluster of genes related to cholesterol metabolism and reverse cholesterol efflux. Reverse cholesterol efflux in macrophages is regulated by membrane cholesterol efflux transporters, such as ABCA1 and ABCG1. These transporters regulate the levels of cholesterol in the plasma membrane, which has a profound influence on macrophage responses to extracellular stimuli. For example, ABCA1 deficient macrophages accumulate cholesterol in the membrane and are hyperresponsive to pro-inflammatory stimuli, such as bacterial lipopolysaccharide (LPS) (Fessler and Parks, 2011; Pradel et al., 2009). This is thought to be due to the increase in cholesterol-rich membrane microdomains, also called lipid rafts, which are required to promote TLR4-signaling. However, previous studies have also shown that ABCA1-deficient macrophages are hyporesponsive to other stimuli, including IL-4 and IL-13 (Pradel et al., 2009). Interestingly, ABCG1 deficiency in macrophages was shown to increase their pro-inflammatory phenotype and reduce growth of subcutaneous tumors in mice fed on a high-fat diet (Sag et al., 2015), suggesting that cholesterol accumulation in tumor-associated macrophages can abrogate their pro-tumor functions.

Here, we showed that ovarian cancer cells actively promoted membrane cholesterol efflux in macrophages, which was associated with increased IL-4 signaling and inhibition of IFNγ-induced gene expression, resulting in transcriptional and functional reprogramming of TAM. Depletion of membrane cholesterol in macrophages increased PI3K activity and mTORC2-mediated Akt phosphorylation. Both PI3K and mTORC2 have previously been linked with IL-4 mediated macrophage activation in different contexts (Huang et al., 2016; Rauh et al., 2005). Furthermore, PI3K was recently shown to be a critical pathway to maintain the pro-tumor
functions of TAM (Kaneda et al., 2016a; Kaneda et al., 2016b). The exact mechanism by which membrane cholesterol regulates PI3K/mTORC2 activation remains to be elucidated. Perhaps cholesterol-rich membrane microdomains are required to recruit negative regulators of PI3K activity, such as the lipid phosphatase SHIP-1. Previous studies have suggested that SHIP may reside in detergent-resistant membrane fractions (Galandrini et al., 2002) and SHIP-1 is known to inhibit IL-4 signaling in macrophages (Rauh et al., 2005).

The distinct metabolic environment of tumors has long been suggested to influence the phenotype of tumor-infiltrating immune cells, rendering them hyporesponsive and contributing to immune-suppression. Cancer cells rely heavily on cholesterol, which they can scavenge from the tumor-microenvironment through upregulation of apolipoproteins and their receptors (Guillaumond et al., 2015; Podzielinski et al., 2013; Villa et al., 2016). This may lead to cholesterol depletion in tumor-stromal cells, and particularly TAM which express high levels of the ABCA1 and ABCG1 efflux transporters. Our in vitro studies suggest hyaluronic acid (HA) could be an important factor produced by cancer cells that promotes this process. HA is a major component of the extracellular matrix (ECM) in many human cancers, including ovarian cancer and in many cases the degree of HA accumulation strongly correlates with poor prognosis (Kolapalli et al., 2016; Sironen et al., 2011). Macrophages express at least two distinct receptors for HA; CD44 and Lyve1. Interestingly, CD44 signaling has previously been associated with PI3K activation in TAM (Lenart et al., 2017). Co-incidentally, PI3K also upregulates ABCA1 expression in macrophages (Chen et al., 2012; Okoro et al., 2016), potentially creating a feed-forward loop for enhanced cholesterol efflux and IL-4 mediated reprogramming.

In summary, we describe an important role for membrane cholesterol efflux in the regulation of macrophage activation state in the tumor-microenvironment. Depletion of membrane cholesterol renders macrophages hyperresponsive to pro-tumor signals, such as IL-4, but refractory to activation by the anti-tumor cytokine IFNγ. We propose that cholesterol efflux pathways may represent novel targets to abrogate the pro-tumor functions of TAM while retaining potentially beneficial anti-tumor effects in response to therapy.

**Limitations of this study**

Despite functional data demonstrating the contribution of IL-4 signaling to tumor progression in this model, IL-4 was not detectable in ascites from tumor-bearing mice, indicating this cytokine is produced at very low levels and consumed rapidly. This illustrates the significance of enhanced sensitivity of TAM to IL-4, which is increased by several orders of magnitude due to membrane cholesterol depletion. These studies are likely to be relevant to human ovarian
cancer, where IL-4 expression has been associated with poor clinical outcome (Candido et al.; Clendenen et al.; Munster et al., 1998). The source of IL-4 in this context remains unknown. While other cancer cells, including prostate, breast and bladder, have been shown to express IL-4 (Conticello et al., 2004), conditioned medium from ID8 cells alone was not sufficient to activate IL-4 signaling in macrophages. Other potential sources of IL-4 include CD4 T cells (DeNardo et al., 2009), eosinophils (Kratochvill et al., 2015), innate lymphocytes or macrophages themselves.

The precise mechanism by which the activity of ABC transporters is upregulated in TAM also remains to be elucidated. Cholesterol efflux was rapidly induced upon exposure to tumor-cell conditioned medium and no increase in Abca1 or Abcg1 mRNA was observed (data not shown), suggesting a post-transcriptional mechanism. However, cholesterol efflux was dependent PI3K activation associated with increased Akt serine 473 phosphorylation. Other studies have shown serine 473 phosphorylation of Akt regulates the translocation of ABC transporters to the plasma membrane (Huang et al.). This may offer a mechanistic explanation for increased ABC activity in TAM.

Acknowledgements

We thank Bernard Malissen (CIML, FR) for Stat6−/− mice. These studies were supported by grants to TL from: L'Agence Nationale de la Recherche (ANR); ANR-09-MIEN-029-01, ANR-10-BLAN-1302-01 and European Research Council; FP7/2007–2013 Grant agreement number 260753, and institutional funding from INSERM, CNRS and Aix-Marseille-Université. JRV was funded by Marie Curie actions IEF (No.234823). PG was funded by the French Ligue Nationale contre le Cancer (LNCC). AE was funded by the Novo Nordisk Foundation (NNF14OC0008781). Microscopy facilities are supported by ANR-10-INBS-04-01 France Bio Imaging. JLS is a member of the Excellence Cluster ImmunoSensation. The research leading to these results has received funding from the People Programme (Marie Curie Actions) of the European Union’s Seventh Framework Programme FP7/2077-2013 under REA grant agreement no. 317445 to JLS.

Author contributions
PG, JRV, AE and TL designed the experiments. PG, JRV, AE performed the experiments with help from OR, VG, CV, MB, NAA. Bioinformatics analysis was performed by MM, TPVM, MD, OP, TU and JLS. MVE performed cholesterol efflux assays. MT provided Pik3cd−/− mice. PG, JRV, AE and TL wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

References


**Figure Legends**

**Figure 1. Ontogeny and phenotype of TAM during ID8 tumor development.**

(A) Analysis of peritoneal macrophages (PM) by flow cytometry after intra-peritoneal (i.p.) injection of $10^6$ ID8-Luc cells. PM were gated as; live, single cells (FSC-H versus FSC-W), CD45.2+, Lin- (NK1.1 Ly6G CD5 CD19), CD11b+, CD64+. LPM were subsequently gated as F4/80hi MHCII- (blue), intPM as F4/80int MHCIIint (orange) and SPM as F4/80- MHCIIhi (green).

(B) Total numbers of LPM, intPM and SPM during tumor growth. (C) Shielded chimeras reconstituted with mixed bone marrow to analyze ontogeny of PM after engraftment with ID8-Luc cells. Host mice (CD45.1) were placed in protective lead shield with only the hind legs exposed, before irradiation with 9 Gy. The following day, mice were reconstituted with a mixture of bone marrow cells from Ccr2-/- (CD45.2) and CD45.1/2 congenic mice, at a ratio of 4:1. 5 weeks after reconstitution, mice were injected with ID8-Luc cells. A further 8 weeks after tumor inoculation, PM were collected for analysis. (D) Analysis of PM from chimeric mice by flow cytometry; CD45.1 and CD45.2 expression was analyzed on LPM (blue), intPM (orange) and SPM (green), as described above. (E) Proportion of chimerism was calculated relative to blood monocytes. (F,G) Fate mapping of Cx3cr1-expressing monocytes in ID8 tumor-bearing mice; Cx3cr1CreER.T26-tdRFP mice were inoculated with ID8-Luc cells, after 8 weeks 1 mg tamoxifen was administered by oral gavage and RFP expression was analyzed in PM subsets 10 days later. RFP expression within LPM, intPM and SPM is shown. (H,I) PM were collected from naïve mice and at 5 or 21 days after tumor inoculation by flow cytometry. Total cellular mRNA was extracted and global gene expression profiles analyzed using microarrays. Gene ontology enrichment was performed on the differentially expressed genes (DEGs) between
naïve PM and TAM at (H) 5 and (I) 21 days, respectively. Graphs are represented as mean ± SEM. See also Figures S1 and S2.

**Figure 2. Increased membrane cholesterol efflux in TAM.**

(A, B) Peritoneal macrophages were isolated from naïve mice (Ctrl) or at 5 and 21 days after inoculation with ID8 cells. Cells were seeded in chambered plastic slides and stained with Cholera Toxin B (CTB; green) and TO-PRO-3 (blue). (A) Representative images for all three groups are shown, (B) quantification of corrected total cell fluorescence (CTCF). (C, D) BMDM were co-cultured with ID8 cells for the indicated time points prior to CTB staining, (C) representative images for BMDM alone (Ctrl) and 3 hours after ID8 co-culture and (D) quantification of CTCF. (E-H) BMDM were incubated with ID8-CM overnight, or Methyl-β-cyclodextrin for 30 min, prior to CTB staining or di-4-ANEPPDHQ staining; (E) representative images for CTB staining in each condition and (F) quantification of CTCF. (G) Representative images of di-4-ANEPPDHQ staining and (H) quantification of relative changes in GP values (Owen et al., 2011). (I) Total cell cholesterol content was measured in BMDM with and without ID8-CM treatment, using the Amplex Red Cholesterol Assay kit. (J) BMDM from wild-type (WT) or Abca1−/− mice were loaded with 3H-labeled cholesterol and subsequently treated or not with ID8-CM in the presence of ApoA1, as an acceptor for cholesterol. 3H-Cholesterol was measured in the medium as a readout for cholesterol efflux. Graphs are represented as mean ± SEM.

**Figure 3. Tumor cell-derived hyaluronic acid (HA) oligomers deplete lipid rafts in macrophages.**

(A) BMDM were co-cultured with live or paraformaldehyde-treated (fixed) ID8 cells, prior to CTB staining, quantification of CTCF is shown. (B) High and low molecular weight fractions of ID8-CM were prepared using Centricon filters with 100 kDa pores, each fraction was compared with unfractionated ID8-CM for effects on CTB staining. (C) BMDM were treated with ID8-CM with and without hyaluronidase (HAse) treatment prior to CTB staining and quantification. (D) BMDM were incubated with HA oligomers of increasing molecular weights before CTB staining and quantification, ID8-CM was used as a positive control. Graphs are represented as mean ± SEM.

**Figure 4. Cholesterol efflux promotes IL-4 mediated macrophage reprogramming.**

(A, B) BMDM were treated with or without ID8-CM before stimulation with increasing concentrations of IL-4 or IFNγ for 8 hours. (A) Quantitative PCR (qPCR) analysis of IL-4 induced gene expression; Arg1, Chi3l3, Mrc1, Retnla and (B) IFNγ-induced expression of Nos2
and Il12b. (C) BMDM were treated with different cholesterol depleting agents; 9-cis-retinoic acid (9cRA), high-density lipoprotein (HDL) or apolipoprotein A1 (ApoA1), as well as ID8-CM; lipid raft density was subsequently measured by CTB staining, quantification of CTCF is shown. (D) IL-4 (20 ng/ml) induced Arg1 and IFNγ (20 ng/ml) induced Nos2 expression in BMDM treated with 9cRA, HDL or ApoA1, compared to ID8-CM. (E) BMDM from Abca1/g1fl/fl and Abca1/g1ΔLyz2 mice were incubated with ID8-CM, 9cRA, HDL or ApoA1 and lipid raft density was measure by CTB staining, quantification of CTCF is shown. (F) IL-4 induced Arg1 and IFNγ induced Nos2 expression in BMDM from Abca1/g1fl/fl and Abca1/g1ΔLyz2 mice with and without ID8-CM treatment. Graphs are represented as mean ± SEM. See also Figure S3.

**Figure 5. Tumor-induced reprogramming is STAT6 and PI3K dependent.**

(A-F) BMDM were treated with or without ID8-CM overnight before stimulation with either IL-4 or IFNγ for 20 min. Cells were then fixed and stained for p-STAT6, p-STAT1 or p-Akt and analyzed by confocal microscopy; representative micrographs for (A) p-STAT6, (B) p-STAT1 and (C) p-Akt staining are shown (green), nuclei are counterstained with TO-PRO-3 (blue). (D-F) Quantification of CTCF for (D) p-STAT6, (E) p-STAT1 and (F) p-Akt (NS – non stimulated). (G-I) BMDM were incubated with or without ID8-CM in the presence or absence of the PI3K inhibitor LY294002 (1.25 µM); (G) Quantification of p-Akt staining by confocal microscopy and (H-I) qPCR analysis of IL-4-induced Arg1 and Chi3l3 expression. (J-K) BMDM were incubated overnight with ID8-CM, 9cRA, HDL or ApoA1 before stimulation with IL-4 for 20 min; quantification of (J) p-STAT6 and (K) p-Akt by confocal microscopy. (L-M) BMDM from Abca1/g1fl/fl and Abca1/g1ΔLyz2 mice were treated with ID8-CM before stimulation with IL-4 and quantification of (L) p-STAT6 and (M) p-Akt by confocal microscopy. (N-O) BMDM treated with ID8-CM in the presence or absence of Rapamycin (Rapa) or Torin at the indicated concentrations; (N) quantification of p-Akt by confocal microscopy and (O) western blot analysis of Ser473 and Thr308 Akt phosphorylation (pAkt(Ser) and pAkt(Thr), respectively) in Rapamycin (R) and Torin (T) treated BMDM with and without ID8-CM treatment. Graphs are represented as mean ± SEM. See also Figure S4.

**Figure 6. IL-4 induced STAT6 and PI3K signaling in TAM drives tumor progression in vivo.**

(A) Generation of specific gene signatures for Tim4+ and Tim4– F4/80hi PM and F4/80lo CCR2+ monocytes (MN) using GeneSign (BubbleGUM) cell-specific signatures were assessed for enrichment using BubbleMap (Spinelli et al., 2015), based on the GSEA algorithm in pairwise comparisons of Tim4+ PM from naïve mice or ID8 tumor-bearing mice at different time points.
The bubble area is proportional to the absolute value of the normalised enrichment score (NES). The color intensity indicates the false-discovery rate (FDR), modified for multiple testing. The size and intensity of color increases with the enrichment of the gene signature from the matching cell population; the Tim4+ gene signature is enriched in naïve cells (red), whereas the Tim4− and MN gene signatures are enriched in TAM (blue). (B) Leading edges (LEs) of the MN gene signature enrichment at different time points (highlighted by colored boxes) were extracted and plotted in a Venn diagram. Ingenuity Pathway Analysis (IPA) was performed on the 173 common LEs, showing IL4 as the top upstream regulator. (C) ID8-Luc cells were injected i.p. and mice treated with either IL4Rα blocking antibody (anti-IL4Rα) or an isotype control antibody (Iso-ctrl) after 2 weeks; tumor growth was assessed at 4 weeks by ex vivo luciferase assay on peritoneal cells. (D-E) Cohorts of chimeric mice receiving either wild-type (WT), (D) Stat6−/− or (E) Pik3cd−/− bone marrow cells were injected with ID8-Luc cells and tumor growth assessed after 6 weeks. (F,G) TAM were isolated from Pik3cd−/− chimeric mice and WT controls after 6 weeks of tumor growth by flow cytometry. Microarray analysis was performed to assess geneset enrichment using GSEA. (F) Positive enrichment of the naïve PM gene signature in Pik3cd−/− TAM compared to WT cells and (G) negative enrichment of the IL-4 pathway signature of TAM (from B) in Pik3cd−/− cells. ES plots are shown on the left and the 25 most enriched genes on the right. NES, normalised enrichment score; FDR, false discovery rate. (H) Abca1/g1f/f and Abca1/g1Δlyz2 mice were injected with ID8-Luc cells and tumor growth assessed after 6 weeks. (I) GSEA with TAM isolated from Abca1/g1f/f and Abca1/g1Δlyz2 mice showing negative enrichment of the TAM associated IL4 pathway in Abca1/g1Δlyz2 mice. Graphs are represented as mean ± SEM. See also Figure S5.

**STAR Methods**

**Key Resources Table**

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibodies</td>
<td></td>
<td></td>
</tr>
<tr>
<td>anti-Nos2 (M-19)</td>
<td>Santa Cruz</td>
<td>sc-650</td>
</tr>
<tr>
<td>anti-Arg1 (N-20)</td>
<td>Santa Cruz</td>
<td>sc-18351</td>
</tr>
<tr>
<td>HRP-conjugated anti-Rabbit immunoglobulins</td>
<td>DAKO</td>
<td>P044801-2</td>
</tr>
<tr>
<td>HRP-conjugated anti-Mouse immunoglobulins</td>
<td>DAKO</td>
<td>P026002-2</td>
</tr>
<tr>
<td>anti-β-Actin (AC-74)</td>
<td>Sigma</td>
<td>A5316</td>
</tr>
<tr>
<td>anti-Akt (40D4)</td>
<td>Cell Signaling</td>
<td>2920</td>
</tr>
<tr>
<td>anti-pSer473-Akt (D9E)</td>
<td>Cell Signaling</td>
<td>4060</td>
</tr>
<tr>
<td>anti-pThr308-Akt (D25E6)</td>
<td>Cell Signaling</td>
<td>13038</td>
</tr>
<tr>
<td>anti-STAT1</td>
<td>Cell Signaling</td>
<td>9172</td>
</tr>
<tr>
<td>anti-pTyr701-STAT1</td>
<td>Cell Signaling</td>
<td>9171</td>
</tr>
<tr>
<td>Antibody Description</td>
<td>Vendor</td>
<td>Catalog Number</td>
</tr>
<tr>
<td>---------------------------------------------</td>
<td>----------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>anti-STAT6 (M-20)</td>
<td>Santa Cruz</td>
<td>sc-981</td>
</tr>
<tr>
<td>anti-pTyr641-STAT6</td>
<td>Cell Signaling</td>
<td>9361</td>
</tr>
<tr>
<td>anti-PI3</td>
<td>Cell Signaling</td>
<td></td>
</tr>
<tr>
<td>Anti-CD124</td>
<td>Novus Biologicals</td>
<td>NBPI-00884</td>
</tr>
<tr>
<td>anti-CD11b (M1/70)</td>
<td>BD Biosciences,</td>
<td>563553</td>
</tr>
<tr>
<td>anti-CD44 (IM7)</td>
<td>BD Biosciences, eBioscience, BioLegend, Life Technologies.</td>
<td>553775</td>
</tr>
<tr>
<td>anti-CD45.1 (A20)</td>
<td>BD Biosciences, eBioscience, BioLegend, Life Technologies.</td>
<td>553775</td>
</tr>
<tr>
<td>anti-NK1.1 (PK136)</td>
<td>BioLegend</td>
<td>108724</td>
</tr>
<tr>
<td>anti-Ly6G (1A8)</td>
<td>BD Biosciences</td>
<td>560600</td>
</tr>
<tr>
<td>anti-CD5 (53-7.3)</td>
<td>BD Biosciences</td>
<td>563194</td>
</tr>
<tr>
<td>anti-CD19 (1D3)</td>
<td>BD Biosciences</td>
<td>565076</td>
</tr>
<tr>
<td>anti-CD64 (X54-5/7.1)</td>
<td>BioLegend</td>
<td>139311</td>
</tr>
<tr>
<td>anti-Ly6C (AL-21)</td>
<td>BD Biosciences</td>
<td>553104</td>
</tr>
<tr>
<td>anti-MHCII (M5/114)</td>
<td>eBioscience</td>
<td>56-5321-82</td>
</tr>
<tr>
<td>anti-F4/80 (BM8)</td>
<td>BioLegend</td>
<td>123141</td>
</tr>
<tr>
<td>anti-Lyve-1 (ALY7)</td>
<td>eBioscience</td>
<td>53-0443-82</td>
</tr>
<tr>
<td>anti-rabbit-Alexa488</td>
<td>Invitrogen</td>
<td>A-10040</td>
</tr>
</tbody>
</table>

**Chemicals, Peptides, and Recombinant Proteins**

<table>
<thead>
<tr>
<th>Chemical Name</th>
<th>Vendor</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>LY294002</td>
<td>R&amp;D Systems</td>
<td>1130</td>
</tr>
<tr>
<td>Rapamycin</td>
<td>Merck</td>
<td>553211</td>
</tr>
<tr>
<td>Hyaluronic acid</td>
<td>Lifecore Biomedical</td>
<td>HA15M-1</td>
</tr>
<tr>
<td>Hyaluronidase</td>
<td>Sigma</td>
<td>H3506</td>
</tr>
<tr>
<td>Torin</td>
<td>Sigma</td>
<td>475991</td>
</tr>
<tr>
<td>Methyl-β-cyclodextrin</td>
<td>Sigma</td>
<td>C4555</td>
</tr>
<tr>
<td>High-density lipoprotein</td>
<td>Sigma</td>
<td>L8039</td>
</tr>
<tr>
<td>9-cis-retinoic acid</td>
<td>Sigma</td>
<td>R4643</td>
</tr>
<tr>
<td>Cholesterol-methyl-β-cyclodextrin</td>
<td>Sigma</td>
<td>C4951</td>
</tr>
<tr>
<td>Proteinase inhibitor cocktail</td>
<td>Sigma</td>
<td>P8340</td>
</tr>
<tr>
<td>Xylazine</td>
<td>Sigma</td>
<td>23076-35-9</td>
</tr>
<tr>
<td>PNPP</td>
<td>Sigma</td>
<td>333338-18-4</td>
</tr>
<tr>
<td>β-glycerophosphate</td>
<td>Sigma</td>
<td>G9422</td>
</tr>
<tr>
<td>DTT</td>
<td>Sigma</td>
<td>43819</td>
</tr>
<tr>
<td>Apolipoprotein A1</td>
<td>Sigma</td>
<td>A0722</td>
</tr>
<tr>
<td>Recombinant mouse IL4</td>
<td>Peprotech</td>
<td>214-14</td>
</tr>
<tr>
<td>Recombinant mouse IFNγ</td>
<td>Peprotech</td>
<td>315-05</td>
</tr>
<tr>
<td>Recombinant mouse M-CSF</td>
<td>Peprotech</td>
<td>315-02</td>
</tr>
<tr>
<td>Recombinant mouse IL13</td>
<td>Peprotech</td>
<td>210-13</td>
</tr>
<tr>
<td>Vybrant Alexa Fluor 488 Lipid Raft Labelling Kit</td>
<td>ThermoFisher Scientific</td>
<td>V34403</td>
</tr>
<tr>
<td>TRizol</td>
<td>ThermoFisher Scientific</td>
<td>15596018</td>
</tr>
<tr>
<td>di-4-ANEPPDHQ</td>
<td>ThermoFisher Scientific</td>
<td>D36802</td>
</tr>
<tr>
<td>TO-PRO-3</td>
<td>ThermoFisher Scientific</td>
<td>T3605</td>
</tr>
<tr>
<td>SYBR Green PCR Master Mix</td>
<td>ThermoFisher Scientific</td>
<td>4312704</td>
</tr>
<tr>
<td>IL4Rα neutralizing antibody</td>
<td>BD Biosciences</td>
<td>552508</td>
</tr>
</tbody>
</table>

**Critical Commercial Assays**

<table>
<thead>
<tr>
<th>Assay Name</th>
<th>Vendor</th>
<th>Catalog Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superscript IV Reverse Transcriptase</td>
<td>Thermo Fisher Scientific</td>
<td>18090050</td>
</tr>
<tr>
<td>Amplex Red Cholesterol Assay kit</td>
<td>Thermo Fisher Scientific</td>
<td>A12216</td>
</tr>
</tbody>
</table>

**Deposited Data**


<table>
<thead>
<tr>
<th>ID</th>
<th>Description</th>
<th>Source</th>
<th>Link</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSE126079</td>
<td>This paper</td>
<td><a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126079">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126079</a></td>
<td></td>
</tr>
<tr>
<td>GSE126080</td>
<td>This paper</td>
<td><a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126080">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126080</a></td>
<td></td>
</tr>
<tr>
<td>GSE126098</td>
<td>This paper</td>
<td><a href="https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126098">https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE126098</a></td>
<td></td>
</tr>
</tbody>
</table>

**Experimental Models: Cell Lines**

Bone marrow derived macrophages

ID8-Luc ovarian surface epithelial cell line

**Experimental Models: Organisms/Strains**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Strain</th>
<th>Institute</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57Bl/6</td>
<td>Charles River</td>
<td>JAX 000664</td>
</tr>
<tr>
<td>Abca1^{tm1,Jp} Abcg1^{tm1,Tall}</td>
<td>Jackson Laboratories</td>
<td>stock number 021067</td>
</tr>
<tr>
<td>Lyz2^{tm1(cre)Jlo}</td>
<td>Jackson Laboratories</td>
<td>stock number 004781</td>
</tr>
<tr>
<td>Stat6^{−/−}</td>
<td>Dr. Bernard Malissen</td>
<td>CIML, Marseille, FR</td>
</tr>
<tr>
<td>Abca1^{−/−}</td>
<td>Prof. Miranda Van Eck</td>
<td>Leiden University, NL</td>
</tr>
<tr>
<td>Cx3cr1^{cre/GFP}</td>
<td>Jackson Laboratories</td>
<td>stock number 005582</td>
</tr>
<tr>
<td>Cx3cr1^{cre/ERT2}</td>
<td>Dr. Stephan Jung</td>
<td>Department of Immunology, The Weizmann Institute of Science, Rehovot, Israel.</td>
</tr>
<tr>
<td>Pik3cd^{−/−}</td>
<td>Prof. Martin Turner</td>
<td>Babraham Institute, Cambridge, UK</td>
</tr>
</tbody>
</table>

**Oligonucleotides**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg1; F</td>
<td>CAGAAGAATGGGAAGAGTCAG</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Arg1; R</td>
<td>CAGATATGCAGGGAGTCCAC</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Chi3l3; F</td>
<td>TCACAGTCTGCGCAATCCTTCTG</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Chi3l3; R</td>
<td>TTGGTCTCTTAGGAGGGCTTCTC</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Ciita; F</td>
<td>CTCAAGCTTCAGGGGGCGACTT</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Ciita; R</td>
<td>GGAAACCATGGATCGGACCAAAACAC</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Cph; F</td>
<td>GGCAATAGGCTGGAAGCAGAGGACAC</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Cph; R</td>
<td>TTAGAGTTGACACAGTGCCAGGATG</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Cxcl9; F</td>
<td>TCCTTTTGGGGCATCCTTCT</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Cxcl9; R</td>
<td>TTCCCCTCTTTTGCTTTT</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Cxcl10; F</td>
<td>GGGCCATAGGGAAGCTTGGAA</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Cxcl10; R</td>
<td>GGATTAGGAGACCATCTCTGCTCATCA</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Il12b; F</td>
<td>GGAA GCACGCGACGCAGAATA</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Il12b; R</td>
<td>AACATTTGAGGAGAAGATAGGAAATG</td>
<td>This paper</td>
<td>N/A</td>
</tr>
</tbody>
</table>
**Materials**

The following materials were employed throughout the study: LY294002, Torin, Rapamycin (Merck); hyaluronic acid, hyaluronidase, methyl-β-cyclodextrin, apolipoprotein A1, high-density lipoprotein, 9-cis-retenoic acid and cholesterol-methyl-β-cyclodextrin (Sigma); recombinant mouse IL-4, IFNγ and M-CSF (Peprotech); IL4Rα neutralizing antibody (BD Biosciences).

**Mice**

C57Bl/6 mice were obtained from Charles River. All transgenic mouse strains were backcrossed to a C57Bl/6 background. Abca1tmJp Abcg1tmTall (Abca1/g1f/f) and Lyz2tm(cre)Ifo (Lyz2cre) mice were obtained from Jackson Laboratories. Stat6−/− mice were kindly donated by Dr. Bernard Malissen (CIML, Marseille, FR), Abca1−/− mice by Prof. Miranda Van Eck (Leiden University, NL) and Pik3cd−/− mice by Prof. Martin Turner (Babraham Institute, Cambridge, UK). Generation of shielded chimeras was performed as previously described (Scott et al., 2016); briefly, CD45.1 congenic mice were anaesthetised with Ketamine (150 mg/kg) and Xylazine (10 mg/kg) and placed in 6 mm thick lead cylinders, exposing only the hind legs. With the peritoneal cavity protected, mice were irradiated with 9 Gy and reconstituted with 10⁷ bone marrow cells from Ccr2−/− (CD45.2) and CD45.1/2 mice, at a ratio of 4:1. After 5 weeks, chimerism of blood leukocytes was assessed by flow cytometry. All mice were housed under specific pathogen-free conditions and animal experimentation was conducted in strict accordance with good animal practice as defined by the French animal welfare bodies relative...
to European Convention (EEC Directive 86/609) and approved by the Direction Départementale des Services Vétérinaires des Bouches du Rhônes.

**Cell Culture**

Bone marrow derived macrophages (BMDM) were obtained as previously described (Hagemann et al., 2008); briefly, femurs and tibiae from mice aged 8 to 10 weeks were flushed and cells collected by centrifugation at 450 g for 5 min at 4°C. Cells were resuspended in DMEM supplemented with L-glutamine (2 mM), penicillin (100 U/ml)/streptomycin (100 µg/ml) (Gibco), 10 % heat-inactivated FBS and 10 ng/ml recombinant mouse M-CSF (Peprotech) and cultured at a density of 10^6 cells/ml in non-tissue culture treated plastic dishes (BD Pharmingen) at 37°C and 5 % CO₂. After 7 days, adherent cells were collected and resuspended in complete DMEM containing 10 ng/ml M-CSF. The ID8-Luc ovarian surface epithelial cell line was kindly provided by Prof. Frances Balkwill (Barts Cancer Institute, London, UK). To obtain ID8 cell-conditioned conditioned medium (ID8-CM); 13.75 x10^6 cells in 25 ml were incubated for 72 hours in a 175 cm^2 flasks in DMEM containing 4 % of FCS. Medium was filtered through a 22 µM filter, aliquoted and stored at -80°C.

**Immunofluorescence and lipid raft staining**

BMDM were grown in Lab-Tek chambered slides (ThermoFisher Scientific) and fixed with 4 % PFA, permeabilised (0.1 % Triton-X100) and blocked in 5 % BSA with 10 mM glycine. The following primary antibodies were used for incubation during 90 minutes at 4°C; anti-pSTAT1, anti-pSTAT6, anti-pSer473-Akt, anti-pThr308-Akt, anti-PIP₃ (Cell Signaling) or anti-IL4Rα (Novus biologicals). After washing, anti-rabbit-Alexa488 (Invitrogen) and TO-PRO-3 (ThermoFisher Scientific) were added for 60 minutes. Lipid rafts were stained using the Vybrant Alexa Fluor 488 Lipid Raft Labelling Kit, following manufacturer’s instructions. Briefly, BMDM were grown and stimulated in Lab-Tek chambered slides. After washing with serum-free DMEM, they were incubated for 10 minutes with Alexa488-conjugated cholera toxin subunit B (CTB) at 4°C, followed by cross-linking with an anti-CTB antibody for 15 minutes at 4°C. Subsequently, the cells were fixed with 4 % Antigenfix (DiaPath) for 10 minutes on ice and nuclei were stained with TO-PRO-3. The di-4-ANEPPDHQ lipid raft staining protocol was adapted from the one described by Owen et al. (Owen et al., 2011). Briefly, culture medium was replaced with fresh, serum-free DMEM containing 2 µl of di-4-ANEPPDHQ (5 µM). Dishes were shaken to ensure good mixing. After 30 min incubation at 37 °C in a humidified 5 % CO₂ atmosphere, cells were fixed with 4 % PFA. Fluorescence was measured by confocal microscopy (Zeiss LSM780 or Leica SP5X) and analyzed with FIJI software. The corrected
total cell fluorescence (CTCF) was measured for each cell in at least 6 different fields of view per well.

**Cholesterol measurement**

Total cell cholesterol content was measured in BMDM using the Amplex Red Cholesterol Assay kit (ThermoFisher Scientific), according to the manufacturer’s instructions.

**Immunoblotting**

BMDM were lysed on ice in lysis buffer supplemented with proteinase inhibitor cocktail, PNPP, β-glycerophosphate and DTT. Separation by SDS-PAGE was followed by blotting on PVDF membrane. Blots were blocked with 5 % skimmed milk in TBS-0.05 %Tween20. The following primary antibodies were used; anti-Nos2, anti-Arg1 (Santa Cruz), anti-β-Actin (Sigma), anti-Akt, anti-pSer473-Akt, anti-pTyr701-STAT1, anti-STAT1, anti-pTyr641-STAT6 (Cell Signaling) and anti-STAT6 (Santa Cruz). Primary antibodies were incubated overnight at 4°C and appropriate HRP-conjugated secondary antibodies (DAKO) for 1 hour at room temperature. Chemoluminescence was detected by Pierce ECL Western Blotting Substrate (Thermo Scientific).

**Gene expression analysis**

Total cellular RNA was extracted from BMDM using TRIzol and cDNA was synthesised with cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer’s protocol. Gene expression was quantified using sequence specific primers in the presence of SYBR Green PCR Master Mix using an ABI 7900HT thermocycler (Applied Biosystems). Reactions were performed in duplicate or triplicate and Ct-values were normalised to the mean Ct-values of cyclophilin. Relative quantification of gene expression was calculated as 2ΔΔCt to controls. Primers used: Arg1; F-CAGAAGAATGGAAGAGTCAG, R-CAGATATGCAGGGAGTCACC. Chi3l3; F-TCACAGGTCTGGCAATTCTTCTG, R-TTTGTCTTATTAGGAGGCTTCTC Cph; F-GGCAAAATGCTGGACCAAACAC, R-TTAGAGTTGTCCACAGTCGGAGATG. Il12b; F-GGAA GCACGGCAGCAGAATA, R-AACTTGAGGGAGAAGTAGGAATGG. Ccl2; F-GCCAGCTCTC TCTTCTCCA, R-CCCCAAGACATGACAGGGAC. Nos2; F-CCCTCTTGATCTTGTGTGGA, R-CCACCCGAGCTCCTGGAAC. Retnla; F-GGTCCCAGTGCATATGGATGACCA TAGA, R-CACCTCCTTCACCTCGAGGGACAGTTGGAC. Cxcl9; F-TCTTTTGGGCTACCATCTTC, R-TTCTTCTTTGGGAGTTTCTGTT. Cxcl10; F-CCCTCTCCTTGGGAGCTTGAA, R GGATTCAGACATCTCTGCTCATCA. Ciita; F-TCAGCCTTAGGAGGACTTG, R-GACCTGGATCGTCGTCGAC.
**Ovarian cancer model**

One million ID8 cells were injected intraperitoneally in the different mouse strains using a 27G syringe. Mice were euthanised at the indicated times and peritoneal lavages were collected for cytometric analysis, ex vivo bioluminescence measurement and/or lipid raft staining. Briefly, 9 ml of ice cold PBS was injected intraperitoneally and after a careful massage to detach all the cells in the cavity, peritoneal fluid was collected through a 23G syringe. Tubes were weighed to determine the recovered lavage volume and the cell density was assessed using a Casy cell counter (Innovatis). Cells were centrifugated and resuspended in 1 ml cold PBS. One million cells from each peritoneal lavage were stained for flow cytometry. 50 µl of the 1 ml cell suspension obtained from peritoneal lavage was used for luciferase activity measurements. Cells were plated in a white 96-well plate and 50 µl luciferin was added to each well, Luminescence [photons/s] was measured for each well using the Mithras Microplate Reader (Berthold Technologies).

**Flow Cytometry**

Peritoneal lavage cells underwent a short NH₄Cl red blood cell lysis and were incubated at 4°C for 10 min with the 2.4.G2 antibody to block Fc receptors. The cells were stained with the indicated antibodies for 30 min at 4°C. Dead cells were gated out using SYTOX Blue dead cell stain (Life Technologies). After cell-surface staining, cells were fixed. Analysis was performed using an LSR-II flow cytometer or sorted using an Aria III cell sorter (both BD Biosciences) and data analysis was conducted with the FlowJo cytometric analytical software (Tree Star). Anti-CD11b (M1/70), anti-CD44 (IM7), anti-CD45.1 (A20), anti-CD45.2 (104), NK1.1, Ly6G, anti-CD5 (53-7.3), anti-CD19 (1D3), anti-CD64, anti-Ly6C (AL-21), anti-F4/80 and anti-MHCII (M5/114) were purchased from BD Biosciences, eBioscience, BioLegend, and Life Technologies.

**Microarray Analysis**

RNA samples were hybridised on Affymetrix Mouse 430 2.0 or MoGene 1.0 st chips. Samples were processed as follows: The biotinylated cRNAs were prepared according to a double amplification protocol using MessageAmp™ II aRNA Amplification Kit (Ambion). The images of the chips were generated with Affymetrix software AGCC version 3.2. The expression data was then extracted with the Affymetrix Expression Console version 1.1 software using the RMA (log2 scale) and MAS5 (linear scale) algorithms. Gene Set Enrichment Analysis (GSEA, Broad Institute) (Subramanian et al., 2005) was used to examine differentially expressed genes (DEGs). The output of GSEA is an enrichment plot (ES), a normalised enrichment score (NES) which accounts for the size of the gene set being tested, a p-value, and an estimated False Discovery rate (FDR). We computed P values using 1,000 permutations for each gene set and
corrected them with the false-discovery rate (FDR) method. When several probe sets were present for a gene, the mean of the probe set was used. Cell-specific gene sets were generated by performing pairwise comparisons between DEGs from different populations, applying a 1.5 FC threshold and a p value of 0.05, using the Minimal (pairwise[Mean(test)/Mean(ref)]) method. Sample correlation analysis was performed based on Pearson’s correlation coefficients using BioLayout Express3D (Theocharidis et al., 2009). GO enrichment analysis was applied using the Cytoscape plug-in BiNGO (v2.44) (Maere et al., 2005) with FDR q-value threshold of 0.05 as default. The Cytoscape plugins Enrichment Map (v1.1) (Merico et al., 2010) and Word Cloud (Oesper et al., 2011) were used to visualize the GO networks. Accession numbers for datasets are; GSE126079, GSE126080, GSE126098.

**Statistical Analysis**

Graphs were made and statistical analysis was performed using Prism software (Graphpad). All quantitative data are presented as mean ± SEM. Statistical significance was calculated using Student´s t-test, Wilcoxon-Mann-Whitney test, Chi-square test for contingency tables or One-Way ANOVA. P values <0.05 were considered as significant.
Figure 1

A. Plots showing the percentage of LPM, intPM, and SPM over time.

B. Graph showing the total cells (10^6) over time.

C. Diagram illustrating the procedure of irradiation (9 Gy lead shield) and the timeline for Ccr2^-/-:CD45.1/2 (4:1) and ID8.

D. Flow cytometry plots showing % RFP+ for LPM, intPM, and SPM under different conditions.

E. Bar graphs demonstrating % chimerism for WT and Ccr2^-/- over time.

F. Flow cytometry plots for Cx3cr1CreER:R26-tdRFP showing LPM, intPM, and SPM under Naive and ID8 conditions.

G. Graphs showing % RFP+ for LPM, intPM, and SPM under Naive and ID8 conditions.

H. Heatmap showing gene expression changes over 5 days.

I. Heatmap showing gene expression changes over 21 days.
Figure 2

A

B

C

D

E

F

G

H

I

J

**Figure 2**

A. Images showing cellular structures with green and blue fluorescence, labeled CTB and TOPRO3.

B. Bar graph showing percentage of CTCF CTB with Ctrl, 5, and 21 days, with significant differences indicated by ***.

C. Images with labels indicating different conditions, such as Ctrl 1 2 3 hours.

D. Bar graph showing percentage of CTCF CTB with Ctrl, 1, 2, and 3 hours, with significant differences indicated by ***.

E. Images labeled CTB TOPRO3.

F. Bar graph showing percentage of CTCF CTB with Ctrl, 1, and 2, with significant differences indicated by ***.

G. Images with di-4-ANEPPDHQ labeling.

H. Bar graph showing percentage of CTCF CTB with Ctrl, ID8-CM, and MCD, with significant differences indicated by ***.

I. Bar graph showing mg cholesterol/10^6 cells with Ctrl and ID8-CM, with significant differences indicated by ***.

J. Bar graph showing % H-cholesterol efflux with Ctrl and ID8-CM, with significant differences indicated by ***.
Figure 3

A. % CTCF CTB

B. % CTCF CTB

C. % CTCF CTB

D. % CTCF CTB
Figure 5

A  B  C
p-STAT6  p-STAT1  p-Akt

D  E  F
% CTCF p-STAT6  % CTCF p-STAT1  % CTCF p-Akt

G  H  I
% CTCF p-Akt (Ser) % CTCF p-Akt (Thr) t-Akt

J  K  L  M
% CTCF p-STAT6 % CTCF p-STAT1 % CTCF p-Akt

N  O
% CTCF p-Akt

Figure 5
Figure 6

A

Genechip normalized data
GeneSign
Cell-specific transcriptomic signatures

Legend
Circle area = NES
Enriched in first class
Enriched in second class
<0.01 <0.1 <0.5 NS (>1.0)

Color intensity = FDR

Tim4+ naïve vs Tim4+ 4 weeks

Tim4+ naïve vs Tim4+ 6 weeks

Tim4+ naïve vs Tim4+ 8 weeks

Pairwise comparisons

B

Leading edges
Tim4+ 4 weeks
32 13 19

Tim4+ 6 weeks
42 173 53

Tim4+ 8 weeks
51

Upstream Regulator p-value Target molecules

IL4 2.58E-009
Anxa2, Capg, Cd2, Cd44, Cita, Cxcl9, Il4r, Lgals1, Lgals3, Lrrc8c, Nabp1, Pfkp, Scd2, Sem4a4, Syk

C

D

E

Bioluminescence (AU)

0

25000

50000

75000

100000

125000

150000

WT

Stat6−−

0

40000

80000

120000

160000

WT

Pik3cd−−

0

500000

1000000

1500000

2000000

Naïve PM signature

Enriched in Pik3cd−− vs WT

NES: 1.52
FDR: 0.006

IL-4 pathway signature

Enriched in WT vs Pik3cd−−

NES: 1.13
FDR: 0.226

H

I

Bioluminescence (AU)

0

25000

50000

75000

100000

125000

150000

Abca1/g1f/f Abca1/g1ΔLyz2

Enriched in Abca1/g1f/f vs Abca1/g1ΔLyz2

NES: 2.54
FDR: <10

Legend

NES: Normalized Enrichment Score
FDR: False Discovery Rate

<0.01 <0.1 <0.5 NS (>1.0)

Color intensity = FDR

Circle area = NES

4.2 2.6 1.8 1.0

Leading edges
Tim4+ 4 weeks
Tim4+ 6 weeks
Tim4+ 8 weeks

Pairwise comparisons

Tim4+ naïve vs Tim4+ 4 weeks

Tim4+ naïve vs Tim4+ 6 weeks

Tim4+ naïve vs Tim4+ 8 weeks

Figures 6D and 6E show the bioluminescence readings for different conditions. Figure 6C and 6F demonstrate the naïve PM signature and its enrichment in Pik3cd−− compared to WT. Figure 6G highlights the IL-4 pathway signature and its enrichment in WT compared to Pik3cd−−. Additionally, Figure 6H and 6I illustrate the bioluminescence readings for Abca1/g1f/f versus Abca1/g1ΔLyz2, with NES and FDR values provided.
Supplemental Information

**Figure S1. CCR2-dependency of peritoneal macrophage subsets and TAM — related to Figure 1.**

Wild-type (WT) and Ccr2−/− mice were injected with 10⁶ ID8-Luc cells i.p. After 8 weeks, mice were euthanized and ascites was harvested by peritoneal lavage and analyzed by flow cytometry. (A) Peritoneal macrophages were gated as live cells; CD45.2+, Lin− (CD5, CD19, Ly6G, NK1.1), CD11b+ and CD64+. Large peritoneal macrophages (LPM) were subsequently gated as F4/80hi MHCII−, intermediate peritoneal macrophages (intPM) as F4/80+ MHCII+ and small peritoneal macrophages (SPM) as F4/80− MHCII+. (B) Total numbers of LPM, intPM and SPM in naïve and ID8-Luc injected mice were calculated. (C) Analysis of Cx3cr1 expression in LPM, intPM and SPM from Cx3cr1egfp/+ reporter mice bearing ID8 tumors. (D) Analysis of Lyve-1 expression in LPM, intPM and SPM from ID8-Luc injected mice by flow cytometry.

**Figure S2. Microarray analysis of TAM versus naive PM — related to Figure 1.**

(A) Gating strategy for sorting of TAM by flow cytometry; bulk TAM were gated as live cells, CD45.2+, CD11b+, Lin− (CD5, CD19, Ly6G, NK1.1), F4/80+ and CD64+. (B) Total RNA was extracted from TAM sorted from ascites at 5 (D5), 12 (D12) and 21 days (D21) of tumour growth and naive peritoneal macrophages (Ctrl). Gene expression was analyzed with MoGene 1.0st microarrays. RMA normalized data were filtered and analyzed for variations in gene expression and hierarchical clustering, the heatmap represents the 1000 most variable genes expressed. (C) Sample correlation analysis based on Pearson’s correlation coefficients using BioLayout Express3D. (D) Ingenuity Pathway Analysis (IPA) with DEGs between naive PM (Ctrl) and TAM at day 21 (D21). (E-F) GSEA comparing TAM with naive PM using a compiled cholesterol homeostasis geneset; (E) Heatmap representing significantly enriched genes in TAM (D21), and (F) the enrichment plot indicating normalized enrichment score (NES) and false discovery rate (FDR).

**Figure S3. ID8 cells promote M2-polarisation of macrophages — related to Figure 4.**

(A) BMDM were co-cultured with or without ID8 cells at a 1:1 ratio overnight, ID8 cells were removed by washing before stimulation with IL-4 or IFNγ for 8 hours and analysis of Arg1 and Nos2 expression by qPCR. (B) BMDM were treated with or without ID8-CM before stimulation with IL-13 (20 ng/ml) for 8 hours and Arg1 expression analysed by qPCR. (C) BMDM were stimulated with IL-4 after treatment with fractions of ID8-CM obtained after filtering with cut-offs at 3, 10, 30 and 100 kDa, Arg1 expression was analysed by qPCR after 8 hours. (D-E) BMDM treated with ID8-CM were incubated with and without cholesterol-methyl-β-cyclodextrin.
(Chl) to replenish membrane cholesterol before stimulation with IL-4 of IFNγ; Arg1 and Nos2 expression were analysed by qPCR or western blotting after 8 hours. (F) BMDM were treated with or without ID8-CM before stimulation with IFNγ for 8 hours. Cxcl9, Cxcl10 and Ciita gene expression was determined by qPCR. Data is represented as mean ± SEM of 3 independent experiments.

**Figure S4. Tumor-induced macrophage reprogramming is STAT6 and PI3K-dependent** – related to Figure 5.

(A,B) BMDM were treated with or without ID8-CM in the absence of cytokine stimulation. After fixation and permeabilization, they were stained for IL-4R and its subcellular location was imaged by confocal microscopy; (A) representative images are shown, (B) receptor clustering was quantified by plotting the mean aggregate size defined as areas larger than 0.2µm² with a mean fluorescence intensity higher than twice the background signal. (C-E) BMDM were treated with or without (C) ID8-CM either alone and with subsequent (D) IFNγ or (E) IL-4 stimulation; pAKT (ser473), pSTAT1 (Tyr701) and pSTAT6 (Tyr641) were measured by Western Blot at the indicated time points in minutes. Total Akt, STAT1, STAT6 or Actin were used as loading controls. Representative blots from at least 3 independent experiments are shown. (F) BMDM from wild-type (WT) and Stat6−/− mice were treated with and without ID8-CM before stimulation with IL-4 and Arg1 expression was measured after 8 hours by western blotting. (G) BMDM were treated with and without ID8-CM before stimulation with IL-4 and accumulation PIP3 was measured by confocal microscopy. (H) BMDM were incubated with or without ID8-CM in the presence or absence of the PI3K inhibitor LY294002 (1.25 µM) before stimulation with IL-4 or IFNγ; Arg1, Retnla, Chi3l3 and Nos2 expression were measured by western blotting after 8 hours. (I) BMDM were stimulated with IL-4 after treatment with fractions of ID8-CM obtained after filtering with cut-off at 100 kDa followed by quantification of p-STAT6 and p-Akt by confocal microscopy. (J) BMDM were incubated with ID8-CM with and without HAse treatment before stimulation with IL-4, p-STAT6 and p-Akt was subsequently measured by confocal microscopy.

**Figure S5. IL-4 signaling and TAM polarization in vivo – related to Figure 6.**

(A) Gating strategy for sorting of monocytes (MN), Tim4+ and Tim4− PM by flow cytometry. (B) Analysis of Tim4+ and Tim4− PM in Ccr2−/− mice. (C) Reduced expression of TAM marker genes in bulk TAM from Pik3cd−/− chimeras compared to wild-type (WT) mice at 21 days (D21). (D) GSEA using a tumoricidal gene signature (GSE26912) in naïve PM versus TAM after 21 days (D21). (E) GSEA using the most enriched genes in naïve PM from (D) as a geneset in TAM from WT versus Pik3cd−/− mice and Abca1/g1fl/fl versus Abca1/g1Δlyz2 mice; enrichment plots are
shown on the left and the 25 most enriched genes on the right. Normalized enrichment score (NES) and false discovery rate (FDR), are indicated. (F) Heatmap showing expression of IL-4 responsive genes in TAM from wild-type (WT) and Stat6ducer mice analysed by high-density quantitative PCR array.
A

B

C

D

WT

naïve

ID8

naïve

ID8

Ccr2⁺⁻

LPM

intPM

SPM

MHCII

Total cells (x10⁶)

F4/80

B

naïve

ID8

WT

Ccr2⁺⁻

LPM

intPM

SPM

C

SPM

intPM

LPM

Lyve-1

D

SPM

intPM

LPM

Lyve-1

Ccr2⁻⁻/⁻

ID8 naïve

ID8 naïve

Cx3cr1-egfp

Lyve-1
A) Live-Dead, FSC-A, CD45-2, Lin, CD64, Ctrl, D21, D12, D5, CD11b, CD64, Cholesterol pathway signature

B) Heatmap showing gene expression levels across different conditions.

C) Graph showing gene expression changes between Ctrl and D21.

D) Table showing p-values and number of molecules for different categories:

<table>
<thead>
<tr>
<th>Category</th>
<th>p-values</th>
<th># molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diseases and disorders</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>2.51E-06 - 1.11E-02</td>
<td>93</td>
</tr>
<tr>
<td>Cancer</td>
<td>2.68E-06 - 1.42E-02</td>
<td>205</td>
</tr>
<tr>
<td>Immunological disease</td>
<td>2.68E-06 - 1.36E-02</td>
<td>171</td>
</tr>
<tr>
<td>Molecular and cellular functions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipid metabolism</td>
<td>9.45E-07 - 1.40E-02</td>
<td>76</td>
</tr>
<tr>
<td>Small molecule biochemistry</td>
<td>9.45E-07 - 1.44E-02</td>
<td>105</td>
</tr>
</tbody>
</table>

E) Gene expression levels across conditions:

F) Cholesterol pathway signature is enriched in D21 vs CTL.
**Figure 3**

A) Representative images of IL-4Ra TOPRO3 staining in Ctrl and ID8-CM samples. Scale bar = 10 μm.

B) Bar graph showing IL-4Ra aggregate size (μm²) in Ctrl and ID8-CM samples.

C) Western blot analysis of p-Akt (Ser) and t-Akt in Ctrl and ID8-CM samples.

D) Western blot analysis of p-STAT1, STAT1, and Actin in Ctrl and ID8-CM samples.

E) Western blot analysis of p-STAT6 and STAT6 in Ctrl and ID8-CM samples.

F) Western blot analysis of Arg1, Actin, and other proteins in WT and Stat6⁻/⁻ samples.

G) Statistical analysis of % CTCF PIP3 in Ctrl and ID8-CM samples.

H) Western blot analysis of IL-4Ra aggregate size (μm²) in samples treated with IL-4 and/or LY294002.

I) Statistical analysis of % CTCF p-STAT6 and % CTCF p-Akt in samples treated with ID8-CM and/or IL-4.

J) Statistical analysis of % CTCF p-STAT6 and % CTCF p-Akt in samples treated with ID8-CM and/or IL-4 + Hase.
**Tumoridical signature**

NES: 1.19
FDR: 0.003

**PPL**

BMPR1A
FGF9
GHR
NKIRAS1
RYK
SFRP1
GKAP1
LDHB
CEBPD
TMEM45A
FBXO15
ZC3H14
HRSP12
BPGM
FZD4
HDAC7
P2RX1
PGRMC2
FOXO3
RAB28
LPL
ARG1
FICD
MAP1LC3A

dEnriched in Abca1/g1ΔLyz2 vs Abca1/g1f/f

**Pik3cd**

-1
0
1
2

Gene expression

**E**

WT
WT
WT
WT
Stat6+/−

+ID8

Abca1/g1ΔLyz2

vs Abca1/g1f/f

Enriched in **Pik3cd**−/− vs WT

Enriched in naïve vs D21

**D**

Tumoridical signature

NES: 1.19
FDR: 0.003

NES: 1.56
FDR: 0.001

NES: 2.51
FDR: 0.073

**B**

WT

Ccr2−/−