Endothelial Rbpj is essential for the education of tumour-associated macrophages

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Abstract

Epithelial ovarian cancer (EOC) is one of the most lethal gynaecological cancers worldwide. EOC cells educate tumour-associated macrophages (TAMs) through CD44-mediated cholesterol depletion to generate an immunosuppressive tumour microenvironment (TME). In addition, tumour cells frequently activate Notch1 receptors on endothelial cells (ECs) to facilitate metastasis. However, little is known whether the endothelium would also influence the education of recruited monocytes. Here, we report that canonical Notch signalling through RBPJ in ECs is an important player in the education of TAMs and EOC progression. Deletion of *Rbpj* in the endothelium of adult mice reduced infiltration of monocyte-derived macrophages into the TME of EOC and prevented the acquisition of a typical TAM gene signature. This was associated with stronger cytotoxic activity of T cells and decreased tumour burden. Mechanistically, we identified CXCL2 as a novel Notch/RBPJ target gene. This angiocrine factor regulates the expression of CD44 on monocytes and subsequent cholesterol depletion of TAMs. Bioinformatic analysis of ovarian cancer patient data showed that increased CXCL2 expression is accompanied by higher expression of CD44 and TAM education. As such, EOC cells employ the tumour endothelium to secrete CXCL2 in order to facilitate an immunosuppressive microenvironment.

18

Introduction

High grade serous ovarian cancer is the deadliest type of all gynaecological cancers¹. The high mortality rate is due to the fact that most women have already developed peritoneal metastasis at the time of diagnosis. Epithelial ovarian cancer (EOC) cells can directly infiltrate the peritoneal cavity to seed metastases, a process called transcoelomic metastasis². Metastatic EOC cells initially reside in the omentum, where they undergo certain adaptations allowing them to spread throughout the whole peritoneal cavity.

1 Importantly, this peritoneal microenvironment is so immunosuppressive that even the
2 infiltration of effector T cell does not *per se* correlate with better prognosis³.

3 Peritoneal spread of tumour cells is accompanied by monocyte-derived macrophage
4 (MN-derived macrophages) recruitment, which eventually become the most abundant
5 myeloid cell type⁴ and are a major contributor to the immunosuppressive TME in EOC³.
6 Upon recruitment from blood to the tumour, monocytes differentiate into macrophages which
7 are further educated by the TME. Eventually, tumour-associated macrophages (TAMs)
8 strongly promote the progression of metastatic ovarian cancer⁵. However, little is known
9 about the contribution of other stromal cells to the peritoneal spread of EOC cells and to
10 macrophage education.

11 Monocytes must cross the vascular endothelial barrier before infiltrating peritoneal
12 organs or the peritoneal fluid. Endothelial cells (ECs) not only form tubes for the transport of
13 blood, but also produce soluble factors controlling the differentiation and function of adjacent
14 cells. These angiocrine functions are highly context and organ specific^{6,7}. In cancer, ECs
15 provide angiocrine factors which influence tumour progression⁸. Therefore, we hypothesized
16 that monocytes are influenced by ECs, for instance during transmigration, while infiltrating
17 into peritoneal tissues.

18 Notably, tumours can alter the transcriptome of ECs^{9,10} and this may also influence
19 transmigrating monocytes. For example, endothelial Notch signalling activity is frequently
20 higher in tumours like EOC and in the metastatic niche compared to ECs from non-
21 tumorous tissue¹¹. Notch signalling is a highly conserved cell-to-cell communication
22 system. Ligand binding induces cleavage of the transmembrane Notch receptors releasing
23 the intracellular domain (ICD) which enters the nucleus to alter gene transcription. This
24 canonical signalling pathway relies on the DNA-binding protein RBPJ, which turns into a
25 transcriptional activator upon binding of a Notch receptor ICD¹². Sustained endothelial
26 Notch1 signalling is associated with increased myeloid cell infiltration and metastasis¹¹. The
27 Notch pathway in ECs is a major regulator of angiogenesis, metabolism, angiocrine

1 functions and tumour cell transmigration^{11,13-18}. Although EOC cells do not necessarily have
2 to cross the endothelial barrier to spread throughout the peritoneum we hypothesized that
3 endothelial Notch signalling could still influence EOC progression via transmigrating myeloid
4 cells.

5 Here, we provide insights into the essential role of RBPJ in ECs for the recruitment of
6 monocytes to the microenvironment of metastatic EOC and their proper education into pro-
7 tumoural TAMs.

8

9 **Results**

10 **Deletion of *Rbpj* in endothelial cells reduces EOC progression**

11 Metastatic EOC cells seed initially in the omentum and later spread within the
12 peritoneum. During these steps, tumour cells undergo transcriptional changes that allow
13 them to further grow and colonise (**Fig. 1a**). The latter is strongly influenced by MN-derived
14 macrophages¹⁹. To determine the contribution of canonical Notch signalling in ECs to
15 myeloid cell infiltration and EOC progression, we used the tamoxifen-inducible VE-Cadherin
16 (Cdh5) Cre^{ERT2} strain to delete *Rbpj* specifically in ECs of adult mice (*Rbpj*^{ΔEC}). This mouse
17 model is well established and leads to robust gene recombination in ECs of several
18 organs^{14,15,17,18} (**Fig. 1b**). Under physiological conditions, there were no differences in blood
19 vessel density in the larger omentum upon *Rbpj* deletion in ECs compared to controls
20 (**Suppl. Fig. 1a,b**). However, upon intraperitoneal injection of ID8 cells mimicking metastatic
21 EOC, the omenta showed evidence of tumour nodule growth (**Fig. 1c**) and had significantly
22 higher vessel density in *Rbpj*^{ΔEC} mice compared to control animals (**Fig. 1d**). The density of
23 endothelial coverage with α-smooth muscle actin-positive mural cells was, however,
24 unchanged (**Suppl. Fig. 1c**). Despite increased vessel density, tumour burden in omenta of
25 *Rbpj*^{ΔEC} mice was significantly lower than that in their littermate controls (**Fig. 1e**). Moreover,

1 peritoneal spread of tumour cells was significantly reduced as well in *Rbpj*^{ΔEC} mice (**Fig. 1f**)
 2 concluding that deletion of *Rbpj* in ECs reduces EOC progression and metastasis.

3

4 **Deletion of endothelial *Rbpj* decreases monocyte-derived macrophage recruitment**

5 Next, we analysed the immune cell compartment of our EOC model since it is a
 6 major contributor to tumour progression. High Notch signalling activity in ECs induces
 7 VCAM1 expression, which promotes leukocyte extravasation^{11,20}. However, VCAM1
 8 expression was unchanged between *Rbpj*^{ΔEC} and their controls littermates (**Suppl. Fig. 2a**).
 9 Nevertheless, whole-mount staining of omenta revealed that there was a reduction in
 10 immune cells inside tumour nodules of *Rbpj*^{ΔEC} mice compared to controls (**Fig. 2a** and
 11 **Suppl. Fig. 2b**). Interestingly, tumours in control mice contained more cells with a large
 12 vacuolated cytoplasm, reminiscent of macrophages. Therefore, we analysed this cell
 13 population in greater detail. Tumour-bearing omenta from *Rbpj*^{ΔEC} mice contained less
 14 CD11b⁺ cells within tumour nodules, although this reduction was not statistically significant
 15 (**Fig. 2b**). In order to better determine the macrophage subpopulations responsible for the
 16 observed decrease in CD11b⁺ cells; we studied the myeloid and macrophage populations in
 17 the peritoneal cavity to understand whether RBPJ in ECs could play a role in their
 18 composition. After four weeks of tumour growth, there were no significant differences in the
 19 total amount of myeloid cells or macrophages (**Fig. 2c,d**). However, we found decreased
 20 numbers of small peritoneal macrophages (SPMs), which are MN-derived macrophages
 21 characterized as MHC-II^{hi}/F4/80^{low} (**Fig. 2e**) and CCR2⁺/Tim4⁻ (**Fig. 2f**). Notably, in naïve
 22 (tumour-free) conditions, endothelial *Rbpj* deletion had no effect on peritoneal macrophage
 23 populations (**Suppl. Fig. 2c-f**). These data suggest that the tumour-driven recruitment of
 24 monocytes into the peritoneum is impaired upon endothelial deletion of *Rbpj*.

25 To further dissect endothelial chemotaxis in a more simplified *in vitro* system, we
 26 switched to the human system and measured transmigration of human CD14⁺ monocytes

1 through a monolayer of human ECs in a transwell insert. Chemotaxis was stimulated by SK-
2 OV-3 human ovarian cancer cells below the insert (**Fig. 2g**). *RBPJ* deletion in human
3 umbilical vein ECs (HUEVECs) resulted in significantly decreased monocyte transmigration
4 rates compared to control HUEVECs (**Fig. 2h**). This further suggests that canonical Notch
5 signalling in ECs is important to facilitate monocyte recruitment into the TME.

6

7 **Canonical Notch signalling in ECs regulates monocyte recruitment through CXCL2**

8 We have previously analysed the transcriptional induction of chemokines in HUVECs
9 upon Notch1 ICD (N1ICD) overexpression¹¹. Based on this, we transduced *RBPJ*-deficient
10 HUVEC and control cells with N1ICD to determine which of those transcriptional changes
11 require *RBPJ*. The chemokines that were induced by N1ICD through RBPJ were *CCL1*,
12 *CCL21*, *CXCL12*, and *CXCL2* (**Fig. 3a**) and we excluded those that were not induced
13 through RBPJ (**Suppl. Fig. 3a-b**). Next, we measured the mRNA expression levels of these
14 chemokines in peritoneal adipose tissue obtained from *Rbpj*^{ΔEC} mice. This revealed that
15 there was lower *Cxcl2* expression in peritoneal adipose tissue from *Rbpj*^{ΔEC} mice compared
16 to littermate controls, whereas the other chemokines analysed were not changed (**Fig. 3b**).
17 Analysis of the same cytokines in endothelial-specific N1ICD mice^{11,15}, exposed that higher
18 endothelial Notch1 signalling activity led to higher *Cxcl2* expression in peritoneal adipose
19 tissue (**Fig. 3c**).

20 *In silico* analysis showed that the mouse and human *Cxcl2* gene contains RBPJ-
21 binding sites in the promoter region (**Fig. 3d**). Consistently, cultured human ECs secreted
22 higher CXCL2 protein levels after being transduced with N1ICD expressing adenovirus
23 compared to GFP-transduced control cells (**Fig. 3e**).

24 These data revealed that canonical Notch signalling induces CXCL2 expression in
25 ECs. CXCL2 is known to attract granulocytes but also to a lesser extend monocytes²¹. As
26 such, it poses as an interesting endothelial Notch target, which could mediate the observed

effects on monocyte recruitment into the TME. To evaluate whether CXCL2 was capable of attracting monocytes, we performed transwell chemotaxis experiments and observed that recombinant CXCL2 induced monocyte chemotaxis (**Fig. 3f**). Next, we silenced *CXCL2* expression in ECs using shRNA, which led to an about 50% reduction of CXCL2 protein expression (**Fig. 3g**). Compared to non-silencing control, this reduction of CXCL2 levels was already capable of reducing the numbers of monocytes transmigrating through ECs towards SK-OV-3 cells (**Fig. 3h**). In summary, the data implicate that the endothelial RBPJ/CXCL2 axis contributes to monocyte recruitment into the peritoneum during transcoelomic metastasis.

***Rbpj* in ECs is necessary for tumour-mediated TAM education**

Recruited monocytes differentiate into macrophages which are further educated by tumour cells to become tumour-promoting macrophages. Macrophage education in EOC is facilitated by hypersensitivity towards IL4, which is induced by tumour cell-mediated cholesterol depletion⁴. We sought to understand whether lack of *Rbpj* in ECs could influence macrophage phenotypes. To assess this, we isolated newly recruited MN-derived macrophages (CD11b⁺/F4/80⁺/CCR2⁺) (**Fig. 4a**) and obtained their transcriptomic profile by microarray analysis. Gene set enrichment analysis (GSEA) comparing MN-derived macrophages from *Rbpj*^{ΔEC} and control tumour-bearing mice revealed that *Rbpj* in ECs is necessary to acquire the typical phenotype of TAM in this model of metastatic EOC (**Fig. 4b**). As such, tumour cells could not fully educate MN-derived macrophages in mice lacking endothelial *Rbpj*.

Transcriptomic profiling via Ingenuity Pathway Analysis (IPA) determined that IL4 was the most downregulated signalling pathway in MN-derived macrophages obtained from *Rbpj*^{ΔEC} tumour bearing mice (**Fig. 4c**). IPA and pathway analysis showed that genes important for cholesterol synthesis were downregulated in MN-derived macrophages from *Rbpj*^{ΔEC} mice (**Fig. 4d,e**). We then employed a cholesterol homeostasis gene set from TAMs

1 obtained 21 days after tumour injection⁴, representing EOC-induced cholesterol metabolism
2 in TAMs. GSEA showed that this gene set was significantly enriched in newly recruited
3 macrophages coming from control tumour-bearing mice (**Fig. 4f**), indicating that *Rbpj* in ECs
4 is necessary for cholesterol depletion in TAMs.

5 Cholesterol depletion is mediated by tumour cell-secreted high molecular weight
6 hyaluronan (HMW-HA). The interaction with HA receptors, such as CD44, in macrophages
7 leads to cholesterol efflux through ABC transporters⁴. In order to understand whether *Rbpj* in
8 ECs could be important for CD44 expression in monocytes, we co-cultured human
9 monocytes with HUVECs lacking *RBPJ* or with respective controls. Monocytes co-cultured
10 with *RBPJ*-deficient HUVECs, had less CD44 on their membrane than those incubated with
11 control HUVECs (**Fig. 4g**). Next, when incubating bone marrow-derived macrophages
12 (BMDMs) with conditioned medium (CM) from immortalized mouse cardiac endothelial cells
13 (MCECs) lacking *Rbpj* (CRISPR-Cas9 mediated), macrophages expressed less *Cd44* (**Fig.**
14 **4h**) and *Mmp9* (**Fig. 4i**), a known CD44 target gene. This indicates that a secreted
15 angiocrine factor regulated by the transcription factor RBPJ in ECs is necessary for the
16 regulation of CD44 in macrophages. To understand whether this would also happen *in vivo*,
17 *Rbpj*^{ΔEC} and control mice were injected with thioglycolate to induce MN-derived macrophage
18 recruitment in the absence of tumour cells. We found that MN-derived macrophages in
19 *Rbpj*^{ΔEC} mice expressed significantly less *Cd44* than those in control mice (**Fig. 4j**),
20 confirming that RBPJ in EC is essential for *Cd44* regulation in MN-derived macrophages *in*
21 *vivo*.

22 Considering the important role that CXCL2 had in monocyte recruitment in *Rbpj*^{ΔEC}
23 tumour bearing mice and that higher level of CXCL2 in serum is associated not only with
24 myeloid cell infiltration into the TME, but also with worse prognosis for EOC patients²², we
25 decided to analyse its role in regulating CD44 expression. Indeed, when stimulating BMDMs
26 with CXCL2, *Cd44* expression was increased (**Fig. 4k**). Moreover, since CD44 is a
27 membrane-bound receptor, we analysed by immunofluorescence whether also its cellular
28 localization could be altered. We observed that CXCL2 stimulation of BMDMs increased the

1 presence of CD44 on the plasma membrane, which would consequently increase its
2 accessibility to hyaluronic acid (**Fig. 4I** and **Suppl. Fig. 4**).

3

4 **The TAM gene signature is enriched in human ovarian carcinoma samples with high** 5 **CXCL2 expression**

6 The data presented so far indicate that the angiocrine factor CXCL2, which is under
7 transcriptional control of Notch/RBPJ signalling, preconditions monocytes to be educated by
8 tumour cells in the TME. In order to evaluate the relevance of this, we analysed *CXCL2* and
9 *CD44* mRNA expression levels in publicly available data sets from the Cancer Genome Atlas
10 (TCGA). Remarkably, the stratification of ovarian cancer patients in 25% upper (*CXCL2*^{hi})
11 and lower (*CXCL2*^{low}) *CXCL2* expression showed that *CXCL2*^{hi} patients displayed
12 significantly more expression of *CD44* (**Fig. 5a**). These data suggest a positive correlation
13 between *CXCL2* and *CD44* expression in human tumours. Our data indicate that *CXCL2*-
14 mediated CD44 induction reflects the ability of tumour cells to educate TAMs. Therefore, the
15 correlation in expression between *CXCL2* and *CD44* should in turn have implications on
16 TAM education. Therefore, we performed GSEA comparing *CXCL2*^{hi} and *CXCL2*^{low} patients
17 using the abovementioned TAM signature. We observed that the TAM signature was
18 significantly enriched in *CXCL2*^{hi} patients (**Fig. 5b**), indicating that the increased CD44
19 expression in these patients consequently impacts TAM education.

20 When analysing difference between *CXCL2*^{hi} and *CXCL2*^{low} patients, we found an
21 expected upregulation of chemotactic response and myeloid cell recruitment, as shown by
22 gene ontology (GO) term analysis in *CXCL2*^{hi} patients (**Suppl. Fig. 5**), supporting the role of
23 *CXCL2* in controlling the myeloid cell compartment of tumours. Interestingly, we again
24 identified cholesterol metabolism as one of the most downregulated pathways in *CXCL2*^{low}
25 patients (**Fig. 5c**). Instead, *CXCL2*^{hi} patients showed induction of pathways such as lipid and
26 atherosclerosis, thus suggesting opposite profiles (**Fig. 5d**). Indeed, GSEA showed that the

1 same cholesterol metabolism gene set previously employed was significantly enriched in
2 CXCL2^{hi} cohort (**Fig. 5e**), indicating a similar cholesterol depletion as the one occurring in
3 peritoneal macrophages of our mouse model.

4

5 **TAM education mediated by endothelial *Rbpj* affects T cell cytotoxicity**

6 One of the genes downregulated in MN-derived macrophages from *Rbpj*^{ΔEC} tumour-
7 bearing mice is *Cd74* (**Fig. 4b**). CXCL2 is a ligand for CXCR2²¹, and CD44 is part of a
8 receptor complex that contains CD74 and CXCR2^{23,24}. Moreover, CD44 plays an important
9 role in CD74-mediated signal transduction²⁵. *In silico* analysis showed that CD74 interacts
10 closely with CD44 and CXCR2 (**Fig. 6a**). Besides, CD74 has been reported to be important
11 in TAMs in brain metastasis²⁶, and CD74 expression has been associated with worse
12 prognosis in metastatic ovarian cancer²⁷. Furthermore, CD74 was associated with an
13 immunosuppressive phenotype in macrophages²⁸. We decided to investigate whether the
14 downregulation of CD74 could have a consequence on TAM behaviour. For that, we
15 analysed publicly available data sets where IL4 responses in CD74-deficient macrophages
16 were analysed²⁹. GSEA showed that TAM signature was enriched in wild-type macrophages,
17 indicating that CD74 is not only regulated by IL4, but also necessary for TAM education (**Fig.**
18 **6b**). We extracted a signature with the 500 most enriched genes in control compared to
19 CD74-deficient macrophages stimulated with IL4 (CD74-mediated signature), representing a
20 group of genes induced by IL4 through CD74 activation. When comparing newly recruited
21 MN-derived macrophages from our EOC model by GSEA, we found that the CD74-mediated
22 signature was significantly enriched in macrophages isolated from control mice compared to
23 *Rbpj*^{ΔEC}. This suggests that newly recruited macrophages from *Rbpj*^{ΔEC} mice cannot induce
24 their immunosuppressive phenotype due to downregulation of CD74-mediated genes (**Fig.**
25 **6c**).

1 To verify the specific effect on the newly recruited macrophages, we repeated the
2 same analysis on resident macrophages (CD11b⁺/F4/80⁺/CCR2⁻), in which *Cd74* is not
3 differentially expressed between *Rbpj*^{ΔEC} tumour-bearing and control mice, and found no
4 enrichment of this gene set in any group, confirming that *Rbpj* deletion in ECs only affects
5 *Cd74* expression in newly recruited MN-derived macrophages (**Suppl. Fig. 6**). In summary,
6 only macrophages that have crossed the EC barrier as monocytes were affected by the lack
7 of *Rbpj* in the endothelium.

8 It has been previously reported that TME in metastatic ovarian cancer is highly
9 immunosuppressive and infiltration with immune effector cells has little impact in patients'
10 outcome³. This immunosuppressive microenvironment has been attributed to TAMs³. For
11 this reason, we wanted to test whether impaired TAM education could impact on the
12 phenotype of the TME. We isolated T cells from tumour-bearing *Rbpj*^{ΔEC} mice and their
13 littermate controls six weeks after intraperitoneal injection of ID8 cells (**Fig. 6d**). Results
14 demonstrated that T cells derived from *Rbpj*^{ΔEC} mice were more efficient in killing cultured
15 ID8 cells (**Fig. 6e**). This confirms that impairment in TAM education has a direct impact on T
16 cell cytotoxicity. In addition, the analysis of T cell populations in peritoneal cavity from
17 tumour-bearing mice revealed that, although the total frequency of T cells was not changed,
18 the cytotoxic CD8⁺ T cell population was significantly increased in *Rbpj*^{ΔEC} mice compared to
19 their littermate controls (**Fig. 6f**). In summary, our data reveal that *Rbpj* in ECs is necessary
20 for tumour cell-mediated education of MN-derived macrophages into TAMs (**Fig. 6g**).

21

22 Discussion

23 Collectively, this study provides evidence about a novel angiocrine axis influencing
24 the tumour immune microenvironment. We show how Notch/RBPJ-mediated transcription in
25 ECs, which is frequently hyperactive in tumours¹¹, is required for CXCL2-mediated monocyte

1 chemotaxis, induction of CD44 expression on monocytes, and the adoption of a TAM gene
2 signature in metastatic ovarian cancer.

3 Mice with EC-restricted *Rbpj* loss had impaired ovarian carcinoma growth in the
4 omentum and peritoneum and lower numbers of MN-derived macrophages in the peritoneal
5 fluid. These macrophages are essential for metastatic ovarian cancer development^{4,30}. As
6 such, the endothelium can influence tumour progression and metastasis by altering the
7 immune status of the TME. Specifically, we observed that monocyte recruitment is
8 potentiated, at least in part, through the release of *Rbpj*-mediated CXCL2 from ECs.
9 Interestingly, higher serum levels of CXCL2 in ovarian cancer patients are associated with
10 myeloid cell infiltration, poor prognosis and chemoresistance³¹. It should be noted that this
11 chemokine has been traditionally associated with the recruitment of neutrophils²¹. However,
12 there is evidence that CXCL2 also plays a role in the regulation of TAMs, especially those
13 derived from monocytes. For instance, the CXCL2 receptor CXCR2 on monocytes and
14 macrophages is important for the education of TAMs in prostate cancer³².

15 CXCR2 blockade has also been shown to re-sensitize ovarian cancer to cisplatin
16 treatment³³. Here we suggest that the CXCL2/CXCR2 axis might also have a role in the
17 recruitment and education of macrophages in ovarian cancer. By separating newly recruited
18 macrophages from macrophages that have reside in the peritoneal cavity for a longer period,
19 we observed that endothelial RBPJ is necessary for the education into TAMs by tumour
20 cells. Specifically, we report that MN-derived macrophages in contact with ECs lacking RBPJ
21 had a lower expression of the HA receptor CD44. This receptor gets stimulated by HMW-HA
22 produced by tumour cells to induce cholesterol depletion in macrophages, a crucial
23 mechanism by which tumour cells educate TAMs⁴. Therefore, we propose that through this
24 mechanism ECs can pre-condition MN-derived macrophages and contribute to their
25 immunosuppressive phenotype within the tumour microenvironment. Indeed, the data
26 showed that important genes involved in cholesterol depletion were reduced in MN-derived
27 macrophages from mice lacking RBPJ in their ECs. Consistently, T cells isolated from this

1 TME were more efficient at killing tumour cells, confirming the reduced immunosuppressive
2 potential of the MN-derived macrophages.

3 In conclusion, we demonstrate that peritoneal ECs are critically involved in the
4 recruitment and education of MN-derived macrophages in ovarian carcinoma.

5

6 **Material and methods**

7 **Animal models**

8 All animal procedures were approved by the local institutional animal care and use
9 committee (RP Karlsruhe, Germany and DKFZ) and performed according to the guidelines
10 of the local institution and the local government. Female C57BL/6 mice were group-housed
11 under specific pathogen-free barrier conditions.

12 Administration of tamoxifen diluted in sterile peanut (P2144, Sigma-Aldrich, St. Louis,
13 USA) in 8 to 12-week-old randomized mice was performed by oral gavage once with 100 μ l
14 (1 mg tamoxifen)³⁴. Control mice, which did not express Cre^{ERT2} were also treated with
15 tamoxifen.

16 Model of ovarian cancer: Three weeks after gene recombination, 5×10^6 ID8-luciferase
17 (ID8-luc) ovarian cancer cells were administered i.p. in PBS. For peritoneal lavage after
18 sacrificing the mice, 5 ml of ice-cold PBS (Gibco/Thermo Fisher Scientific, NY, USA) was
19 injected i.p., and after a careful massage to mobilize cells, peritoneal fluid was collected. For
20 analysis of ID8-luc tumour growth the cell suspension was centrifuged and supernatant was
21 collected. The cell pellet was suspended in 1 mL PBS and 100 μ L were used to determine
22 the luciferase activity. 100 μ L cell suspension were centrifuged and the cell pellet was
23 suspended in 100 μ L lysis buffer (Promega) and 20 μ L of lysed cells were pipetted into white
24 96-well plate in triplicates. 50 μ L of LAR substrate (Promega) was added to the lysed cell
25 suspension and luminescence signal was determined using the plate reader (ClarioStar,

1 BMG Labtech). For analysis of immune cell recruitment into the peritoneal cavity the
2 collected peritoneal cell suspension was centrifuged and red blood cells in the cell pellet
3 were lysed with 1 mL ACK (Thermo Fisher Scientific). After washing the cell suspension
4 was counted using Neubauer Counting chamber and 1×10^6 cells were used for flow
5 cytometry staining.

6 Model of peritoneal inflammation: To obtain newly recruited peritoneal macrophages,
7 1 mL thioglycolate (2 mg/mL in H₂O; B2551, Sigma-Aldrich) was injected into the peritoneum
8 three weeks after gene recombination. MN-derived macrophages were isolated 24 hours
9 after thioglycolate injection by their adherence to non-treated plastic petri dishes. Briefly,
10 after incubation of single cells in a petri dish for 30 min at 37°C, non-adherent cells washed
11 away with PBS.

12

13 **Flow cytometry**

14 For flow cytometry analysis, cells were suspended in 1 mL PBS with 2% FCS
15 (Biochrom). Cell suspension was incubated with the different fluorophore-coupled primary
16 antibodies for 20 min on ice. The following antibodies were used: CD45 (552848), CD11b
17 (552850) and CD4 (560468) and CD8 (557654) from BD Biosciences (Bedford, MA, USA);
18 CD3 (100203), F4/80 (123128), CCR2 (150608), CD44 (mouse and human 103007), CD74
19 (mouse (151005) and human (326811)) from BioLegend (St. Diego, CA, USA), MHCII (47-
20 5321-80, Life Technologies/Thermo Fisher Scientific, NY, USA) and Tim4 (12-5866-82, Life
21 Technologies/Thermo Fisher Scientific, NY, USA). Concentration of the different antibodies
22 was determined by titration. In the meanwhile, compensation beads (UltraComp eBeads,
23 Thermo Fisher) of the used primary antibodies were prepared. After staining, cells were
24 washed with PBS and stored on ice until acquisition. Acquisition was performed using BD
25 FACSCanto TM II, BD LSR for analysis or Aria for cell sorting (BD Biosciences).

1 Experiments were analysed using FlowJo Software. Flow cytometer results in percentage
2 were extrapolated to the total number of cells obtained from the cell counting.

3

4 **Whole mount staining**

5 The omentum was fixed for 1 hour in 1% PFA at room temperature and washed with
6 PBS. Tissues were washed three times for 5 min with permeabilization buffer (PBS, 0.1%
7 BSA and 0.2% Triton X-100) and treated with 0.5 mL blocking buffer (5% donkey serum
8 diluted PBS-T) for 1 hour at room temperature. Primary antibodies, CD45 (BD 553076) and
9 luciferase (ab185924), were diluted in PBS-T with 2% donkey serum. Next day, samples
10 were washed three times with PBS- 0.3% Tween. Afterwards secondary antibodies (1:200)
11 were incubated for 1 hour at room temperature, washed three times with PBS 0.3% Tween
12 and incubated with DAPI (1:10.000) for 15 min. Next, samples were incubated with clearing
13 solution (FUnGi: 60% glycerol (vol/vol), 2.5M fructose, 2.5M urea, 10mM Tris Base, 1.0mM
14 EDTA) at 4 °C overnight. Coverslips were mounted with FUnGi clearing solution and imaged
15 with a confocal microscope (LSM 710, Carl Zeiss). All images were processed with ZENblue
16 software (Carl Zeiss, Germany). Average mean intensities per image were counted with
17 ImageJ software (NIH, Bethesda, MD, USA).

18

19 **Immunohistochemistry**

20 Paraffin-embedded sections (3 µm) were de-paraffinized and re-hydrated in xylene
21 and step-wise reductions in alcohol concentrations. H&E staining was performed according
22 to standard protocols. Cytokeratin DAB and CD11b: antigen retrieval was performed at pH9
23 with citrate buffer. Primary antibodies CD11b (abcam, ab133357, 1:200), and Pan-
24 cytokeratin (undiluted, ZUC001-125), diluted in blocking solution, were incubated at 4°C
25 overnight. After washing, sections were incubated with secondary antibodies coupled with
26 ZytoChem Plus (HRP) Polymer anti-mouse, (ZYTOMED, ZYT-ZUC050-006) and ZytoChem

1 Plus (AP) Polymer anti-rabbit, (ZYTOMED, ZYT-ZUC031-006) diluted in antibody diluent
2 (Cell Signaling) for one hour at room temperature. Afterwards, slides were treated with DAB
3 Substrat Chromogen, Zytomed, ZYT-DAB057 and AP Red Kit, Zytomed, ZUC001-125.
4 Immunofluorescence staining: primary antibodies: CD31 (Abcam, ab28364, 1:50), VCAM
5 (Vcam, ab134047, 1:200) and α SMA (Sigma-Aldrich, A5228, 1:200) were diluted in blocking
6 solution. Fluorophore-conjugated secondary antibodies (Thermo Fisher Scientific) were
7 diluted in antibody diluent (Cell Signaling) together with isolectin-B4 (Thermo Fisher
8 Scientific, I32450). Images were obtained with slide scanner (Zeiss Axio Sacn.Z1, Carl
9 Zeiss) and a confocal microscope (LSM 710, Carl Zeiss). All images were processed with
10 ZENblue software (Carl Zeiss, Germany). Image quantification were proceeded with ImageJ
11 software (NIH, Bethesda, MD, USA).

12

13 **Cell culture**

14

15 Murine cardiac ECs (MCEC) were purchased from tebu-bio and cultured on gelatin-
16 coated surfaces in in DMEM containing 1 g/L D-glucose, 5 % FCS, 5 % HEPES, 100
17 units/ml penicillin and 100 μ g/ml streptomycin.

18 HUVEC were grown and maintained until passage 5 in Endopan-3 Growth Medium
19 containing 3% FCS and supplements (Pan-Biotech).

20 SK-OV-3 cells were kindly donated by Dr. Christiane Opitz (DKFZ, Heidelberg) and
21 cultured in DMEM containing 1 g/L D-glucose, 10 % FCS, 5 % HEPES, 100 units/ml
22 penicillin and 100 μ g/ml streptomycin.

23 ID8-luc cells were kindly donated by provided by Prof. Frances Balkwill (Barts Cancer
24 Institute, London, UK) and cultured in DMEM containing 1 g/L D-glucose, 10 % FCS, 5 %
25 HEPES, 100 units/ml penicillin and 100 μ g/ml streptomycin.

1 All cell culture experiments were performed in a laminar flow hood and cells cultured
2 at 37°C and 95% relative humidity and 5% CO₂. Cell culture were tested on a regularly basis
3 for mycoplasma contamination periodically and before injecting the tumour cells into the
4 mice.

5 To study the role of RBPJ in the regulation of Notch target genes, human umbilical
6 vein endothelial cells (HUVECs) were infected with lentivirus constructs for CRISPR/Cas9
7 (Addgene plasmid #52961, lentiCRISPR v2) induced knock-out for *Rbpj*³⁵. After 48 hours,
8 cells were infected with adenovirus (LifeTechnologies; pAD/CMV-V5-DEST) overexpressing
9 GFP as control or N1ICD.

10

11 **Isolation of peripheral blood nuclear cells (PBMC) from buffy coats**

12 Human buffy coats were purchased from blood donation service DRK Mannheim,
13 Germany. Peripheral blood nuclear cells (PBMC) were isolated by gradient centrifugation
14 using Biocoll density solution (L6715; Biochrom). Human buffy coat was diluted 1:1 with PBS
15 and added to the Biocoll density solution. This mixture was centrifuged at 430g for 20 min at
16 room temperature. After centrifugation, the white intermediate phase containing leukocytes
17 was collected and washed with PBS. To perform a positive isolation of monocytes, CD14
18 MACS beads were used with the LS column (130-042-402; Milentyi Biotec). The isolation of
19 CD14⁺ monocytes was performed following the manufacture's protocol.

20

21 **Transwell assay**

22 Human ovarian cancer cells were seeded at 100,000 cells/ml in 500 µl RPMI medium
23 without FCS for 48 hours. For the ECs monolayer, inserts were coated for 2 hours with 2
24 µg/ml fibronectin (1918-FN-02M; R&D Systems) in PBS. 50,000 human ECs (*RBPJ* knock-
25 out or shRNA for *CXCL2* (pLKO.1; Sigma Aldrich) and respective controls) were seeded on

1 top of the insert membrane for 48 hours. To analyse monocyte transmigration, 200,000
2 CD14⁺ cells were stained with carboxyfluorescein succinimidyl ester (CFSE; ThermoFischer)
3 and added onto the endothelial monolayer. The transwell plate was incubated for 2 hours.
4 For the chemotaxis assay with the recombinant proteins, transmigration of 50,000 CD14⁺
5 cells were analysed towards 60 ng/mL CXCL2 (PeproTech GmbH) in RPMI medium without
6 FCS for 30 min. After incubation, the remaining cell suspension in the upper well was
7 aspirated and the transwell was cleaned with a cotton swab. The migrated cells were fixed
8 with 4% PFA for 20 min at room temperature. Imaging of transwell was performed with Cell
9 Observer (Carl Zeiss). From each transwell five evenly spaced field picture were taken using
10 20x objective and analysis was performed with Image J software.

11

12 **Enzyme-linked Immunosorbent Assay (ELISA)**

13 Protein expression of CXCL2 (MIP-2) was quantified using an enzyme-linked
14 immunosorbent assay (ELISA; Abcam ab184862). Cell culture supernatant was collected
15 after 24 hours and ELISA was performed following the manufacture's protocol.

16

17 **Bone marrow-derived macrophages (BMDMs) differentiation**

18 Mouse macrophages were derived from the bone marrow of wild-type C57BL/6 mice.
19 Femurs and tibiae were flushed several times with DMEM and collected cells were
20 centrifuged. Bone marrow cells were suspended in media and seeded on 10 cm petri dishes
21 (Corning). To differentiate these cells into macrophages, 10 ng/ml M-CSF (PeproTech
22 GmbH) were added to Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher)
23 supplemented with 10% fetal calf serum (FCS) (Biochrom, UK). Differentiation occurred
24 within seven days. Cells were afterwards stimulated with indicated amount of recombinant
25 protein CXCL2 (250-15-20, PeproTech GmbH) in DMEM medium without FCS or
26 conditioned medium of endothelial cells.

1

2 **Immunostaining**

3 BMDMs were cultured in DMEM with 10% FCS (Biochrom, UK) and 250,000
4 cells/well were seeded into 24-well plates on top of coverslips. BMDMs were treated with 40
5 ng/mL CXCL2 (PeproTech GmbH) in DMEM without FCS for 72 hours. Cells were washed
6 with PBS and fixed with 4% PFA for 10 min. Then, the coverslips were washed three times
7 for 5 minutes with PBS, permeabilized with PBS with 0.1 % Triton X-100 for 10 minutes and
8 blocked for 30 min in blocking buffer (PBS in 5% FCS with 0.1% Tween 20 and 100 mM
9 glycine) for 1 hour at room temperature. The coverslips were incubated with antibody against
10 CD44 (1:1000) (Abcam, ab124515) and CD45 (1:500) (BD 553076) overnight at 4°C. The
11 coverslips were rinsed three times with blocking buffer and incubated with a secondary
12 antibody coupled to Alexa Fluor-488 and Alexa Fluor-546 (1:200) for 1 hour. The coverslips
13 were washed and incubated with a DAPI solution before they were washed again.
14 Coverslips were mounted and imaged with a confocal microscope (LSM 710, Carl Zeiss). All
15 images were processed with ZENblack software (Carl Zeiss, Germany).

16

17 **cDNA synthesis and qPCR**

18 RNA isolation from cell culture was performed using the InnuPrep Mini Kit (Analytik
19 Jena) according manufacture's protocol. RNA isolation from tissue was performed using
20 PicoPure RNA Isolation Kit (Acturus, Life Technology). 1 mL Trizol was added to the tissue
21 and homogenized for 1 min and a frequency of 30/sec. After disruption of the tissue, 200 µL
22 chloroform were added and mixed by inverting several times followed by a centrifugation
23 step for 15 minutes 12.000g at 4°C. Further RNA isolation steps were performed using the
24 manufacture's protocol. RNA concentration was measured using a Nanodrop 100 (Thermo
25 Fisher Scientific). Reverse transcription of isolated RNA into complementary DNA was
26 performed using High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific).

1 Quantitative real-time PCR (qPCR) was performed with SYBR Green PCR mix (Applied
2 Biosystems) on a QuantStudio3 Real-time PCR system (Applied Biosystems). Resulting fold
3 changes were calculated using the $2^{\Delta\Delta CT}$ method and mRNA expression was normalized to
4 the housekeeping gene (*Cph* for murine and *HPRT* for human samples).

5 Primers used for mouse genes

Gene Name	Forward Primer	Reverse Primer
<i>Cph</i>	ATGGTCAACCCACCGTG	TTCTTGCTGTCTTTGGAACTTTGTC
<i>Ccl1</i>	CTGCTGCTTGAACACCTTGA	GGTGATTTTGAACCCACGTT
<i>Ccl21</i>	ACCCAAGGCAGTGATGGA	CAGGGTTTGACATAGCTCA
<i>Cxcl2</i>	CCAACCACAGGCTACAGG	GCGTCACACTCAAGCTCTG
<i>Cxcl12</i>	GTCCTCTTGCTGTCCAGCTC	TAATTTGGGTCAATGCACA
<i>Cd44</i>	TCGATTTGAATGTAACCTGCCG	CAGTCCGGGAGATACTGTAGC
<i>Mmp9</i>	CTGGACAGCCAGACACTAAAG	CTCGCGGCAAGTCTTCAGAG

6 Primers used for human genes

Gene Name	Forward Primer	Reverse Primer
<i>HPRT</i>	TGTTGTAGGATATGCCCTTGACT	CTAAGCAGARGGCCACAGAAC
<i>CCL1</i>	CATTTGCGGAGCAAGAGATT	TGCCTCAGCATTTTTCTGTG
<i>CCL2</i>	CAGCCAGATGCAATCAATGCC	TGGAATCCTGAACCCACTTCT
<i>CCL21</i>	CCCAGCTATCCTGTTCTTGC	TCAGTCCTCTTGACGCCCTTT
<i>CXCL2</i>	GGCAGAAAGCTTGCTCAACCC	CTCCTTCAGGAACAGCCACCAA
<i>CXCL5</i>	AGCTGCGTTGCGTTTGTAC	TGGCGAACACTTGACAGATTAC
<i>CXCL8</i>	AAGAAACCACCGGAAGGAAC	AAATTTGGGGTGAAAGGTT
<i>CXCL12</i>	ATTCTCAACACTCCAACTGTGC	ACTTTAGCTTCGGGTCAATGC

7

8 Cytotoxicity assay

9 To analyse the T cell killing potential by the lactate dehydrogenase (LDH)-
10 Cytotoxicity Assay Kit (Ab65393, Abcam), ID8 cells (7,500 ID8 cells in 100 μ L DMEM
11 medium) were seeded in a 96-well plate one day before T cell sorting. T cells were sorted
12 from tumour bearing *Rbpj* ^{Δ EC} and control mice after six weeks of tumour growth and 10,000
13 CD3⁺ cells were co-cultured with ID8 cells in technical triplicates including untreated control
14 and blank. After overnight incubation, cytotoxicity and killing potential was measured by LDH
15 amount in the cell supernatant using the LDH-Cytotoxicity Assay Kit (Ab65393, Abcam)
16 following the manufacturer's protocol.

1

2 ***In silico* analysis of promotor region**

3 To determine RBPJ binding sites³⁶ (5'-GTGGGAA-3') in the promotor region of the
4 murine (NM_009140; chr5+:90902580-90903927) and human (NM_002089; chr4-
5 :74100502-74099123) CXCL2-encoding gene, we used ApE plasmid Editor by M. Wayne
6 Davis (<https://jorgensen.biology.utah.edu/wayned/apel/>).

7

8 ***In silico* protein-protein interaction**

9 We used the Search Tool of Interacting Genes/Proteins database (STRING v11.5) to
10 perform *in silico* protein-protein interaction analysis³⁷. Given CXCR2 protein as input,
11 STRING can search for their neighbour interactors, the proteins that have direct interactions
12 with the inputted proteins; then STRING can generate the PPI network consisting of all these
13 proteins and all the interactions between them. All the interactions between them were
14 derived from high-throughput lab experiments and previous knowledge in curated databases
15 at medium level of confidence (score ≥ 0.40).

16

17 **Gene set enrichment analysis (GSEA)**

18 GSEA (Broad Institute) was used to determine if a list of genes (gene signature) was
19 enriched between different groups. A defined list of genes exhibits a statistically significant
20 bias in their distribution (false discovery rate (FDR)) within a ranked gene list using the
21 software GSEA³⁸ resulting to an enrichment in one of the compared groups (normalized
22 enrichment score (NES)) obtained from microarray or public available data sets as indicated
23 in the figure legend.

24

25 **Ingenuity Pathway Analysis (IPA)**

1 IPA software (Qiagen) was used to identify predicated upstream regulators and
2 differentially regulated pathways in newly recruited macrophages (based on microarray
3 data). For the analysis of data, fold-changes were uploaded to the software. Differentially
4 regulated pathways and upstream regulator analysis was performed from obtained
5 microarray data.

6

7 **Pathway analysis**

8 Pathway analysis were obtained from public external databases (EnrichR) and
9 analysed as -2log fold changes.

10

11 **Human ovarian cancer patient RNAseq data analysis**

12 Human ovarian cancer patient bulk tumour RNA-sequencing data was obtained from
13 the Cancer Genome Atlas (TCGA) database. Stratification in $CXCL2^{high}$ and $CXCL2^{low}$
14 patients was performed using R Studio software. Patients were assigned to the different
15 groups using $CXCL2$ expression below the first or higher than the third quartile. Normalised
16 raw counts of CD44 were plotted comparing the two different groups.

17

18 **Statistical analysis**

19 Normality was tested when sample size allowed it. Those samples with normal
20 distribution where compared using Students' t-test (with Welch's correction when groups
21 had different sizes). When normality was rejected, Mann-Whitney U-test was used.
22 Comparison analysis was performed with analysis of variance (ANOVA) with Tukey post-test
23 when more than two groups were analysed. Statistical analysis and the generation of the
24 graphs were performed using GraphPad Prism 9 (GraphPad Software, Inc.; La Jolla, CA,
25 USA).

1

2 **Schematic Figures**

3 Schematics were created using BioRender.com.

4

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23

24 **Author Contributions**

1 R.M., E.A-S, I.M., S.B., L.W., L.J., T.Z., J.T., F.A.R., A.S., B.D.G., J.R-V. performed
2 experiments. A.C., T.B., A.F. and J.R-V. contributed to analysis of results. A.F. and J.R-V.
3 conceived the original hypothesis. R.M., E.A-S, A.F. and J.R-V. planned experiments. R.M.,
4 E.A-S, J.R-V. and A.F. wrote the manuscript. J.R-V. directed and supervised the work.

5

6 **Competing Interests statement**

7 Authors declare that they have no competing interests.

8

9 **References**

- 10 1 Ferlay, J. *et al.* Cancer statistics for the year 2020: An overview. *Int J Cancer*,
11 doi:10.1002/ijc.33588 (2021).
- 12 2 Kipps, E., Tan, D. S. & Kaye, S. B. Meeting the challenge of ascites in ovarian cancer: new
13 avenues for therapy and research. *Nat Rev Cancer* **13**, 273-282, doi:10.1038/nrc3432 (2013).
- 14 3 Hensler, M. *et al.* M2-like macrophages dictate clinically relevant immunosuppression in
15 metastatic ovarian cancer. *J Immunother Cancer* **8**, doi:10.1136/jitc-2020-000979 (2020).
- 16 4 Goossens, P. *et al.* Membrane Cholesterol Efflux Drives Tumor-Associated Macrophage
17 Reprogramming and Tumor Progression. *Cell Metab* **29**, 1376-1389 e1374,
18 doi:10.1016/j.cmet.2019.02.016 (2019).
- 19 5 Noy, R. & Pollard, J. W. Tumor-associated macrophages: from mechanisms to therapy.
20 *Immunity* **41**, 49-61, doi:10.1016/j.immuni.2014.06.010 (2014).
- 21 6 Rafii, S., Butler, J. M. & Ding, B. S. Angiocrine functions of organ-specific endothelial cells.
22 *Nature* **529**, 316-325, doi:10.1038/nature17040 (2016).
- 23 7 Augustin, H. G. & Koh, G. Y. Organotypic vasculature: From descriptive heterogeneity to
24 functional pathophysiology. *Science* **357**, doi:10.1126/science.aal2379 (2017).
- 25 8 Alsina-Sanchis, E., Mulfarth, R. & Fischer, A. Control of Tumor Progression by Angiocrine
26 Factors. *Cancers (Basel)* **13**, doi:10.3390/cancers13112610 (2021).
- 27 9 Zhao, Q. *et al.* Single-Cell Transcriptome Analyses Reveal Endothelial Cell Heterogeneity in
28 Tumors and Changes following Antiangiogenic Treatment. *Cancer Res* **78**, 2370-2382,
29 doi:10.1158/0008-5472.CAN-17-2728 (2018).
- 30 10 Zhao, Q. *et al.* Heterogeneity and chimerism of endothelial cells revealed by single-cell
31 transcriptome in orthotopic liver tumors. *Angiogenesis* **23**, 581-597, doi:10.1007/s10456-
32 020-09727-9 (2020).
- 33 11 Wieland, E. *et al.* Endothelial Notch1 Activity Facilitates Metastasis. *Cancer Cell* **31**, 355-367,
34 doi:10.1016/j.ccell.2017.01.007 (2017).
- 35 12 Bray, S. J. Notch signalling in context. *Nat Rev Mol Cell Biol* **17**, 722-735,
36 doi:10.1038/nrm.2016.94 (2016).
- 37 13 Limbourg, F. P. *et al.* Essential role of endothelial Notch1 in angiogenesis. *Circulation* **111**,
38 1826-1832, doi:10.1161/01.CIR.0000160870.93058.DD (2005).
- 39 14 Hasan, S. S. *et al.* Endothelial Notch signaling controls insulin transport in muscle. *EMBO Mol*
40 *Med* **12**, e09271, doi:10.15252/emmm.201809271 (2020).

- 1 15 Ramasamy, S. K., Kusumbe, A. P., Wang, L. & Adams, R. H. Endothelial Notch activity
2 promotes angiogenesis and osteogenesis in bone. *Nature* **507**, 376-380,
3 doi:10.1038/nature13146 (2014).
- 4 16 Sorensen, I., Adams, R. H. & Gossler, A. DLL1-mediated Notch activation regulates
5 endothelial identity in mouse fetal arteries. *Blood* **113**, 5680-5688, doi:10.1182/blood-2008-
6 08-174508 (2009).
- 7 17 Jabs, M. *et al.* Inhibition of Endothelial Notch Signaling Impairs Fatty Acid Transport and
8 Leads to Metabolic and Vascular Remodeling of the Adult Heart. *Circulation* **137**, 2592-2608,
9 doi:10.1161/CIRCULATIONAHA.117.029733 (2018).
- 10 18 Zhu, T. S. *et al.* Endothelial cells create a stem cell niche in glioblastoma by providing NOTCH
11 ligands that nurture self-renewal of cancer stem-like cells. *Cancer Res* **71**, 6061-6072,
12 doi:10.1158/0008-5472.CAN-10-4269 (2011).
- 13 19 Etzerodt, A. *et al.* Tissue-resident macrophages in omentum promote metastatic spread of
14 ovarian cancer. *J Exp Med* **217**, doi:10.1084/jem.20191869 (2020).
- 15 20 Verginelli, F. *et al.* Activation of an endothelial Notch1-Jagged1 circuit induces VCAM1
16 expression, an effect amplified by interleukin-1beta. *Oncotarget* **6**, 43216-43229,
17 doi:10.18632/oncotarget.6456 (2015).
- 18 21 Jablonska, J., Wu, C. F., Andzinski, L., Leschner, S. & Weiss, S. CXCR2-mediated tumor-
19 associated neutrophil recruitment is regulated by IFN-beta. *Int J Cancer* **134**, 1346-1358,
20 doi:10.1002/ijc.28551 (2014).
- 21 22 Taki, M. *et al.* Snail promotes ovarian cancer progression by recruiting myeloid-derived
22 suppressor cells via CXCR2 ligand upregulation. *Nat Commun* **9**, 1685, doi:10.1038/s41467-
23 018-03966-7 (2018).
- 24 23 Gilliver, S. C., Emmerson, E., Bernhagen, J. & Hardman, M. J. MIF: a key player in cutaneous
25 biology and wound healing. *Exp Dermatol* **20**, 1-6, doi:10.1111/j.1600-0625.2010.01194.x
26 (2011).
- 27 24 Alban, T. J. *et al.* Glioblastoma Myeloid-Derived Suppressor Cell Subsets Express Differential
28 Macrophage Migration Inhibitory Factor Receptor Profiles That Can Be Targeted to Reduce
29 Immune Suppression. *Front Immunol* **11**, 1191, doi:10.3389/fimmu.2020.01191 (2020).
- 30 25 Shi, X. *et al.* CD44 is the signaling component of the macrophage migration inhibitory factor-
31 CD74 receptor complex. *Immunity* **25**, 595-606, doi:10.1016/j.immuni.2006.08.020 (2006).
- 32 26 Priego, N. *et al.* STAT3 labels a subpopulation of reactive astrocytes required for brain
33 metastasis. *Nat Med* **24**, 1024-1035, doi:10.1038/s41591-018-0044-4 (2018).
- 34 27 Cortes, M. *et al.* Tumor-associated macrophages (TAMs) depend on ZEB1 for their cancer-
35 promoting roles. *EMBO J* **36**, 3336-3355, doi:10.15252/embj.201797345 (2017).
- 36 28 Figueiredo, C. R. *et al.* Blockade of MIF-CD74 Signalling on Macrophages and Dendritic Cells
37 Restores the Antitumour Immune Response Against Metastatic Melanoma. *Front Immunol* **9**,
38 1132, doi:10.3389/fimmu.2018.01132 (2018).
- 39 29 Przybyl, L. *et al.* CD74-Downregulation of Placental Macrophage-Trophoblastic Interactions
40 in Preeclampsia. *Circ Res* **119**, 55-68, doi:10.1161/CIRCRESAHA.116.308304 (2016).
- 41 30 Moughon, D. L. *et al.* Macrophage Blockade Using CSF1R Inhibitors Reverses the Vascular
42 Leakage Underlying Malignant Ascites in Late-Stage Epithelial Ovarian Cancer. *Cancer Res* **75**,
43 4742-4752, doi:10.1158/0008-5472.CAN-14-3373 (2015).
- 44 31 Zhang, F., Jiang, J., Xu, B., Xu, Y. & Wu, C. Over-expression of CXCL2 is associated with poor
45 prognosis in patients with ovarian cancer. *Medicine (Baltimore)* **100**, e24125,
46 doi:10.1097/MD.00000000000024125 (2021).
- 47 32 Di Mitri, D. *et al.* Re-education of Tumor-Associated Macrophages by CXCR2 Blockade Drives
48 Senescence and Tumor Inhibition in Advanced Prostate Cancer. *Cell Rep* **28**, 2156-2168
49 e2155, doi:10.1016/j.celrep.2019.07.068 (2019).

1 33 Henriques, T. B. *et al.* Inhibition of CXCR2 plays a pivotal role in re-sensitizing ovarian cancer
2 to cisplatin treatment. *Aging (Albany NY)* **13**, 13405-13420, doi:10.18632/aging.203074
3 (2021).

4 34 Alsina-Sanchis, E. *et al.* Intraperitoneal Oil Application Causes Local Inflammation with
5 Depletion of Resident Peritoneal Macrophages. *Mol Cancer Res* **19**, 288-300,
6 doi:10.1158/1541-7786.MCR-20-0650 (2021).

7 35 Sanjana, N. E., Shalem, O. & Zhang, F. Improved vectors and genome-wide libraries for
8 CRISPR screening. *Nat Methods* **11**, 783-784, doi:10.1038/nmeth.3047 (2014).

9 36 Lake, R. J., Tsai, P. F., Choi, I., Won, K. J. & Fan, H. Y. RBPJ, the major transcriptional effector
10 of Notch signaling, remains associated with chromatin throughout mitosis, suggesting a role
11 in mitotic bookmarking. *PLoS Genet* **10**, e1004204, doi:10.1371/journal.pgen.1004204
12 (2014).

13 37 Szklarczyk, D. *et al.* The STRING database in 2021: customizable protein-protein networks,
14 and functional characterization of user-uploaded gene/measurement sets. *Nucleic Acids Res*
15 **49**, D605-D612, doi:10.1093/nar/gkaa1074 (2021).

16 38 Subramanian, A. *et al.* Gene set enrichment analysis: a knowledge-based approach for
17 interpreting genome-wide expression profiles. *Proc Natl Acad Sci U S A* **102**, 15545-15550,
18 doi:10.1073/pnas.0506580102 (2005).

19 39 de Vos van Steenwijk, P. J. *et al.* Tumor-infiltrating CD14-positive myeloid cells and CD8-
20 positive T-cells prolong survival in patients with cervical carcinoma. *Int J Cancer* **133**, 2884-
21 2894, doi:10.1002/ijc.28309 (2013).

22

23

24

1 Figure Legends

2 **Figure 1. Reduced tumour burden in omentum and peritoneum of *Rbpj*^{ΔEC} mice.**

3 **a**, Model for the spread and proliferation of epithelial ovarian cancer (EOC) cells in the
 4 omentum. **b**, Schematic illustration of oral tamoxifen administration and metastatic EOC
 5 protocol. **c**, Representative pictures of ID8 tumour-bearing mouse omentum and microscopic
 6 images of omentum stained with H&E. Asterisk indicates pancreas. Scale bar, 2 mm. **d**,
 7 Representative images of immunohistochemistry staining for CD31 (white) and DAPI (blue)
 8 in omentum of ID8 tumour-bearing *Rbpj*^{ΔEC} and control mice. At four weeks after tumour
 9 injection. Scale bar, 50 μm. Quantification of vessel density. Bar graphs show mean±SD;
 10 two-tailed, Welch's corrected t-test. **e**, Representative images of immunohistochemistry DAB
 11 cytokeratin (brown) staining. Scale bar, 20 μm. Tumour burden quantification of control
 12 (n=12) and *Rbpj*^{ΔEC} mice (n=9). Bar graphs show mean±SD; two-tailed, Welch's corrected t-
 13 test **f**, Luciferase activity in peritoneal cavity of tumour-bearing *Rbpj*^{ΔEC} compared to control
 14 mice four weeks after tumour injection. Quantification of luciferase levels in control (n=12)
 15 and *Rbpj*^{ΔEC} (n=10). Bar graphs show mean±SD; two-tailed, Welch's corrected t-test.

16

17 **Figure 2: Reduced MN-derived macrophage recruitment in metastatic EOC of *Rbpj*^{ΔEC}** 18 **mice.**

19 **a**, Representative images of whole mount staining of tumour nodules of the omentum for
 20 luciferase (red), CD45 (green) and DAPI (blue) four weeks after tumour injection in *Rbpj*^{ΔEC}
 21 and control mice. Scale bar, 20μm. Quantification of infiltrating immune cells (CD45⁺) into
 22 tumour nodules (luciferase⁺). Bar graphs show mean±SD; two-tailed, unpaired Mann-
 23 Whitney U-test. **b**, Representative images of tumour infiltrating myeloid cells, CD11b⁺ (pink)
 24 in tumour areas, cytokeratin (brown) from *Rbpj*^{ΔEC} and control mice four weeks after tumour
 25 injection. Scale bar, 50 μm. Quantification of CD11b⁺ infiltrating cells area normalized by
 26 tumour area. Bar graphs show mean±SD; two-tailed, Welch's corrected t-test. Analysis of

1 myeloid cells within the peritoneal cavity after four weeks of tumour growth of *Rbpj*^{iΔEC} and
2 control mice by flow cytometry. Percentage of **c**, myeloid cells (CD45⁺, CD11b^{high}) and **d**,
3 macrophages (CD45⁺, CD11b^{high}, F4/80⁺) relative to alive cells. Bar graphs show mean±SD;
4 two-tailed, unpaired Mann-Whitney U-test. **e**, Representative flow cytometer plot of
5 monocyte-derived macrophages characterization by F4/80 and MHCII expression into LPM,
6 IntPM and SPM in *Rbpj*^{iΔEC} mice compared to controls and their quantification. Bar graphs
7 show mean±SD; two-tailed, unpaired Mann-Whitney U-test. **f**, Quantification of newly
8 recruited monocyte-derived macrophages (Tim4⁺ CCR2⁺). Bar graphs show mean±SD; two-
9 tailed, unpaired Mann-Whitney U-test. **g**, Scheme of transwell assay with human CD14⁺
10 monocyte through a monolayer of human umbilical vein endothelial cells (HUVECs) towards
11 human ovarian cancer cells (SK-OV-3). **h**, Analysis of cell tracer carboxyfluorescein
12 succinimidyl ester (CFSE) stained migrated CD14⁺ monocyte through monolayer of HUVECs
13 with *RBPJ* KO and their representative images. Bar graphs show mean±SD; two-tailed, T-
14 student's t-test.

15

16 **Figure 3. Endothelial Notch-mediated recruitment via CXCL2.**

17 **a**, Quantification of mRNA expression of CCL1, CCL21, CXCL12, and CXCL2 upon N1ICD
18 overexpression, knock-out of *RBPJ* or their combination in HUVECs. Bar graphs show n-fold
19 vs GFP transduced as mean±SD; two-tailed, unpaired Mann-Whitney U-test. **b**,
20 Quantification of mRNA expression in whole peritoneal fat tissue from *Rbpj*^{iΔEC} and control
21 mice. Bar graphs show mean±SD; two-tailed, unpaired Mann-Whitney U-test. **c**,
22 Quantification of mRNA expression in whole peritoneal fat tissue from ecN1ICD and control
23 mice. Bar graphs show mean±SD; two-tailed, unpaired Mann-Whitney U-test. **d**, Scheme of
24 RBPJ binding in CXCL2 promotor region in murine and human genes. **e**, Enzyme-linked
25 immunosorbent assay (ELISA) of CXCL2 of cell culture supernatant from HUVECs infected
26 with N1ICD and GFP as control. Bar graphs show mean ±SD; two-tailed, unpaired Mann-
27 Whitney U-test. **f**, Analysis of cell tracer carboxyfluorescein succinimidyl ester (CFSE)

1 stained migrated CD14⁺ monocyte towards CXCL2 cytokine. Bar graphs show mean±SD;
 2 two-tailed, unpaired Mann-Whitney U-test. **g** ELISA of CXCL2 of HUVECs with knock down
 3 of CXCL2. Bar graphs show mean±SD; two-tailed, unpaired Mann-Whitney U-test. **h**
 4 Scheme of transwell assay with human CD14⁺ monocyte through a monolayer of human
 5 umbilical vein endothelial cells (HUVECs) towards human ovarian cancer cells (SK-OV-3).
 6 Analysis of cell tracer carboxyfluorescein succinimidyl ester (CFSE) stained migrated CD14⁺
 7 monocyte through monolayer of HUVECs with knock down of CXCL2. Bar graphs show
 8 mean±SD; two-tailed, T-student's t-test.

9

10 **Figure 4. Essential role of endothelial *rbpj* for tumour-mediated TAM education.**

11 **a**, Schematic illustration of sorting of newly recruited monocyte-derived macrophages for
 12 microarray analysis after four weeks of tumour growth in *Rbpj*^{ΔEC} and control mice. **b**, Gene
 13 set enrichment analysis (GSEA) of the tumour associated-macrophages (TAM) signature in
 14 recruited macrophages from *Rbpj*^{ΔEC} vs. control mice compared with 20 most differentially
 15 regulated genes. **c**, Ingenuity pathways analysis (IPA) for upstream regulator in recruited
 16 macrophages from *Rbpj*^{ΔEC} and control mice. Analysis of significant differentially regulated
 17 pathways by **d**, ingenuity pathways (IPA) and **e**, pathway analysis. **f**, GSEA of cholesterol
 18 homeostasis in recruited macrophages from *Rbpj*^{ΔEC} vs. control mice with 20 most
 19 differentially regulated genes. **g**, Mean fluorescence intensity (MFI) of CD44 expression of
 20 human monocytes co-cultured with human ECs carrying an *RBPJ* knock-out. Bar graphs
 21 show mean ±SD; two-tailed, unpaired Mann-Whitney U-test. Quantification relative to control
 22 from Bone marrow-derived macrophages (BMDMs) after stimulation with conditioned
 23 medium from immortalized mouse cardiac ECs (MCECs) with *Rbpj* knock-out and control of
 24 **h**, *Cd44* and **i**, *Mmp9* mRNA expression. Bar graphs show mean±SD; two-tailed, paired
 25 students T-test. **j**, Quantification of mRNA expression of *Cd44* from macrophages isolated
 26 from peritoneal lavage of *Rbpj*^{ΔEC} mice after 24 hours of thioglycollate i.p. injection relative to
 27 control. Bar graphs show mean±SD; two-tailed, unpaired students T-test. **k**, BMDMs control

1 and stimulated with CXCL2 (40ng/ml) for 72 hours. Quantification of mRNA expression of
2 *CD44* relative to control. Bar graphs show mean±SD; two-tailed, paired students T-test. **I**,
3 Representative images of BMDMs stained with CD44 (white), CD45 (red) and DAPI (blue) of
4 control and stimulated with CXCL2 (40ng/ml) for 72 hours.

5

6 **Figure 5. CXCL2 expression in ovarian cancer patients correlates with CD44**
7 **expression and TAM education.**

8 **a**, Analysis of *CD44* raw counts in CXCL2^{hi} (n=97) vs CXCL2^{low} (n=100) patient stratification
9 of publicly available bulk tumour of human ovarian cancer from the Cancer Genome Atlas
10 (TCGA) database using R Studio software. Bar graphs show median; two-tailed, unpaired T-
11 student's t-test. **b**, GSEA of the tumour associated-macrophage (TAM) signature in
12 CXCL2^{low} vs. CXCL2^{high} ovarian cancer patients from the TCGA database. Analysis of KEGG
13 pathways of **c**, CXCL2^{low} and **d**, CXCL2^{high} patients fold changes and shown as Log2-ratio
14 from extracted p-values. **e**, GSEA of cholesterol homeostasis in CXCL2^{low} vs. CXCL2^{high}
15 ovarian cancer patients from the TCGA database.

16

17 **Figure 6. Loss of endothelial *Rbpj* increases cytotoxic potential and proportion of**
18 **cytotoxic T cells in peritoneal lavage of tumour-bearing mice.**

19 **a**, *In silico* protein-protein interaction analysis shows the association between CXCR2, CD44
20 and CD74. Results were mapped with CXCR2 as query protein using the STRING database.
21 Protein-protein results are obtained using medium confidence interaction score (0.400). Line
22 thickness of network edges indicates the strength of data support. **b**, GSEA of publicly
23 available data (E-MTAB-3309) of TAM signature in WT vs. CD74 knock-out (KO) IL-4-
24 treated BMDMs with 10 most differentially regulated gene extracted from. **c**, GSEA of CD74-
25 mediated gene signature in recruited macrophages from *Rbpj*^{ΔEC} vs. control mice with 10
26 most differentially regulated genes. **d**, Schematic illustration of T cell sorting for cytotoxicity

1 assay after six weeks of tumour growth. **e**, Lactate dehydrogenase (LDH)-cytotoxicity assay
2 of sorted CD3⁺ T cells and incubation with *in vitro* cultivated murine ovarian cancer cells
3 (ID8) measured by absorbance at 450nm including blank correction. Bar graphs show
4 mean±SD; two-tailed, unpaired Mann-Whitney U-test. **f**, Percentage of CD3⁺ cells (relative to
5 CD45⁺ cells) and their proportion in CD4⁺ and CD8⁺ T cells (relative to CD3⁺ cells) in
6 peritoneal lavage of *Rbpj*^{ΔEC} and control mice 4 weeks after tumour injection. Bar graphs
7 show mean±SD; two-tailed, unpaired Mann-Whitney U-test. **g**, Model of endothelial Notch1-
8 dependent recruitment and education of MN-derived macrophages (TAM) into the tumour
9 microenvironment. Tumour cells (TC) activate Notch1 on the tumour endothelium. Activation
10 of endothelial Notch signalling leads to a secretion of angiocrine factors, especially CXCL2,
11 which leads to an increased infiltration of monocyte-derived macrophages (by its receptor
12 CXCR2) into the tumour microenvironment. Loss of endothelial Notch signalling inhibits TC-
13 induced education of TAMs by priming of monocytes (MN) leading to a downregulation of
14 hyaluronan receptor, CD44 as well as pro-tumorigenic receptor CD74 on TAMs. Model
15 created with BioRender.com.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.