Nuanced role for dendritic cell intrinsic IRE1 RNase in the regulation of antitumor adaptive immunity

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82 **ABSTRACT**

83 The IRE1/XBP1s axis of the unfolded protein response (UPR) plays divergent roles in

84 dendritic cell (DC) biology in steady state versus tumor contexts. Whereas tumor

85 associated DCs show dysfunctional IRE1/XBP1s activation that curtails their function, the

86 homeostasis of conventional type 1 DCs (cDC1) in tissues requires intact IRE1 RNase

87 activity. Considering that cDC1s are key orchestrators of antitumor immunity, it is

88 relevant to understand the functional versus dysfunctional roles of IRE1/XBP1s in tumor

89 DC subtypes. Here, we show that cDC1s constitutively activate IRE1 RNase within

90 subcutaneous B16 melanoma and MC38 adenocarcinoma tumor models. Mice lacking

91 XBP1s in DCs display increased melanoma tumor growth, reduced T cell effector

92 responses and accumulation of terminal exhausted CD8$^+$ T cells. Transcriptomic studies

93 revealed that XBP1 deficiency in tumor cDC1s decreased expression of mRNAs encoding

94 XBP1s and regulated IRE1 dependent decay (RIDD) targets. Finally, we find that the

95 dysregulated melanoma growth and impaired T cell immunity noticed in XBP1 deficient

96 mice are attributed to RIDD induction in DCs. This work indicates that IRE1 RNase activity

97 in melanoma/MC38-associated DCs fine tunes aspects of antitumor immunity

98 independently of XBP1s, revealing a differential role for the UPR axis that depends on the

99 DC subtype and cancer model.

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101 **Keywords:** Dendritic cells, immunity, IRE1, melanoma, unfolded protein response.

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ABBREVIATIONS

BM: bone marrow

cDC: conventional DC

cDC1: type 1conventional DC (XCR1$^+$ DC)

cDC2: type 2conventional DC (CD11b$^+$ DC)

DC: dendritic cell

DEG: Differentially expressed gene

ER: endoplasmic reticulum

ERAI: ER stress-activated indicator

Flt3L: FMS-related tyrosine kinase 3 ligand

FP: fluorescent protein

GSEA: Gene set enrichment analysis

IRE1: inositol-requiring enzyme 1 alpha

KO: Knock-out

MdC: Myeloid derived Cell

RIDD: regulated IRE1-dependent decay

ROS: reactive oxygen species

TAM: tumor-associated macrophages

TCR: T cell receptor

TdLN: Tumor draining lymph node

UPR: unfolded protein response

XBP1s: spliced XBP1

XBP1u: unspliced XBP1

INTRODUCTION

A crucial arm of antitumor immunity relies on effective activation of tumor specific cytotoxic CD8^{+} T cells endowed with the ability to eliminate cancer cells. This process is critically dependent on type 1 conventional dendritic cells (cDC1), which excel in cross-presentation of tumor-associated antigens (1–3), secrete soluble factors that potentiate CD8^{+} T cell function (2, 4–7), and prevent the generation of terminal exhausted CD8^{+} T cells committed to irreversible dysfunctional phenotypes in tumors (8). Tumor cDC1 infiltration correlates with improved prognosis (2, 5, 9) and better response to immune checkpoint blockade (ICB) therapy (9–12). Besides, therapeutic strategies expanding/activating tumor cDC1s have shown promising results in clinical trials (11, 13, 14). Additional DC subsets such as type 2 DCs (cDC2s), and a novel DC activation state termed 'mregDCs' (mature DCs enriched in immunoregulatory molecules) can also boost antitumor CD4^{+} and CD8^{+} T cell responses (7, 15–17), indicating that cDCs are interesting candidates in immunotherapy. However, the molecular mechanisms safeguarding the function of these cells in tumors have not been fully elucidated.

An emerging intracellular pathway regulating DC biology is the inositol-requiring enzyme 1 alpha (IRE1) branch of the unfolded protein response (UPR), which is an adaptive cellular response maintaining the fidelity of the cellular proteome (18). Upon endoplasmic reticulum (ER) stress, the endoribonuclease (RNase) domain of IRE1 splices *Xbp1* mRNA, generating the transcription factor XBP1 spliced (XBP1s), master regulator of protein folding and ER biogenesis (18–20). The IRE1 RNase domain can also promote the degradation of a subset of mRNAs/miRNAs in a process known as 'regulated IRE1-dependent decay' (RIDD) (21), which is a mechanism beginning to be understood in pathological settings including metabolism, inflammation and cancer (22–24).

161　In steady state, IRE1 regulates cDC homeostasis via constitutive activation of its RNase

162　domain, a feature noticed in cDC1s across several tissues (25, 26). Furthermore, cDC1s

163　are markedly sensitive to perturbations in the IRE1/XBP1s axis, as genetic loss of the

164　transcription factor XBP1 alters proteostatic programs and counter activates the RIDD

165　branch, which mediates the decay of various mRNAs involved in integrin expression, ER

166　to golgi transport and antigen presentation, among others (25, 26). The selectivity of the

167　IRE1/XBP1s axis in cDC1s is underscored in microarray studies of XBP1 deficient cells,

168　which change the transcriptomic landscape of cDC1s but not cDC2s (26). As such, cDC1s

169　opt the IRE1/XBP1s axis for proper function in steady state, but it is unclear if the pathway

170　displays similar roles in cDC1s infiltrating tumors. This is a relevant question, as reported

171　work shows that metabolically stressed tumors elicit maladaptive UPR activation in

172　certain tumor immune cells (including DCs), which reprograms their phenotype towards

173　dysfunctional states that promote tumor growth (27, 28). For instance, DCs infiltrating

174　ovarian cancer (typified by expression of the cDC2/monocyte marker CD11b^{+} (29))

175　display persistent IRE1/XBP1s activation that triggers aberrant intracellular lipid

176　accumulation, resulting in impaired immunostimulatory functions and leading to tumor

177　progression (30). Thus, these data suggest that IRE1 may play different, or even opposite

178　roles in DC biology depending on the subtype or the inflammatory context. As such, a

179　correct delineation of the role of the enzyme in tumor cDCs is required to understand if

180　intervention of this UPR branch can be targeted for potential cancer therapies.

181　In this work, we study the role of IRE1/XBP1s in DCs by focusing on two

182　immunoresponsive tumor models: subcutaneous mouse B16/B78 melanoma and MC38

183　colon adenocarcinoma (2, 10). We identified that cDC1s display constitutive IRE1 RNase

184　activity in tumors, which follows a lineage-intrinsic trait not influenced by the tumor

185　microenvironment. In contrast to previous reports (30), deletion of XBP1 in DCs did not

186   decrease tumor burden. Rather, XBP1 deficiency in DCs resulted in a moderate increase

187   of tumor growth, lower frequencies of intratumoral effector T cells, and accumulation of

188   terminal exhausted TIM-3$^+$CD8$^+$ cells in the melanoma model. Transcriptomic studies

189   revealed that XBP1 deficient tumor cDC1s downregulate proteostatic processes and

190   decrease the expression of mRNAs encoding XBP1s and regulated IRE1 dependent decay

191   (RIDD) targets. Importantly, animals bearing double deletion of IRE1 RNase and XBP1 in

192   DCs display normal tumor growth and adaptive immunity in the melanoma model,

193   highlighting a role for IRE1 RNase hyperactivation in fine tuning aspects of antitumor

194   immunity via DCs.

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RESULTS

cDC1s constitutively activate IRE1 RNase in subcutaneous melanoma and MC38 colon carcinoma tumors

The tumor microenvironment contain activators of the IRE1/XBP1s axis that are detected by immune cells (27, 30–32). To identify relevant cell types activating IRE1 RNase in tumors, we analyzed the immune composition of B16 tumors of ERAI mice, a mouse strain that reports IRE1 RNase activity through expression of Venus Fluorescent Protein (VenusFP) fused with the sequence of *Xbp1s* mRNA (33) (validated in (25, 26, 31)). To allow unsupervised identification of IRE1 RNase cellular targets, we devised a 17-color flow cytometry panel and data was visualized on a *t*-distributed stochastic neighbor embedding (*t*-SNE) map. Cells were grouped into populations by DBScan-guided automated clustering (Fig. 1A-B, Supp. Fig. 1A), which identified 15 cell clusters that included CD8$^+$ T cells, CD4$^+$ T cells, monocyte-derived cells (MdCs), MHC-II-expressing MdCs, NK cells, NKT cells, B cells, neutrophils and cDC1s. As expected (17), cDC2s and tumor associated macrophages (TAMs) (clusters 4 and 6) showed a degree of heterogeneity and convergence. Also, our analysis revealed two undefined clusters based on surface marker expression; Cluster 11: CD4$^+$ CD11c$^+$ CD26$^+$, and Cluster 14: CD3$^+$ CD4$^+$ CD11b^{int} F4/80$^+$ MHC-II^{high} CD11c^{int} CD26^{high}.

Next, IRE1 RNase activity from the ERAI reporter mice line was determined in the clusters of the t-SNE plot (Fig. 1C-D). Data indicated that among CD45$^+$ hematopoietic cells, cDC1s represented the population with highest fluorescence intensity of VenusFP (Fig 1C-D, cluster 15). Additional immune cells including cDC2s, MdC, MHC-II$^+$MdC, TAM, neutrophils, NK cells and cells from cluster 11 also showed noticeable VenusFP induction, albeit at lower levels than cDC1s; whereas CD4$^+$ T cells, CD8$^+$ T cells and B cells showed little or no induction of VenusFP compared to cells from control animals (Fig 1D). Manual

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236　gating analysis (Fig. 1E-F, Supp. Fig. 2A) confirmed that the mean fluorescence intensity

237　(MFI) of VenusFP in melanoma-associated cDC1s was higher than additional myeloid and

238　lymphoid cells (Fig. 1E-F). Notably, similar results were observed in cDC1s infiltrating

239　MC38 tumors (Supp. Fig. 1B). Finally, these data was confirmed by PCR for endogenous

240　*Xbp1* spliced and unspliced forms from tumor cDC1s isolated from wild type (WT)

241　animals implanted with the B16-FLT3L melanoma cell line (which expresses the DC-

242　differentiation factor FMS-like tyrosine kinase 3 ligand (FLT3L) (11, 34)). Data in Fig. 1G-

243　H showed that tumor cDC1s expressed marked levels of *Xbp1s*, which was even superior

244　to the levels noticed in bone-marrow derived DCs (BMDC) stimulated with the

245　pharmacological UPR inducer tunicamycin. Altogether, these data indicate that cDC1s

246　display prominent activation of the IRE1/XBP1s axis in tumors.

247　We next interrogated whether the augmented IRE1 RNase activity observed in tumor

248　cDC1s is a lineage-intrinsic signature or if it is a feature imposed by the tumor

249　microenvironment. We quantified the VenusFP MFI of cDC1s directly exposed to the

250　tumor (tumor cDC1 and migratory cDC1s of the tumor draining lymph node (TdLN)),

251　versus TdLN resident cDC1s, which do not access to the tumor site (gating strategy in

252　Supp. Fig. 2B) (35). Data depicted in Fig. 1I indicates that tumor cDC1s express lower

253　levels of VenusFP than resident cDC1s, indicating that tumor exposure does not increase

254　IRE1 RNase activity in these cells. Similar observations were made for cDC2s (Fig 1I). In

255　fact, cDC1s infiltrating MC38 tumors express markedly lower levels of VenusFP than

256　spleen cDC1s (Supp. Fig. 1C). Of note, this observation was not replicated in monocytes,

257　which express higher VenusFP levels in the tumor compared to the spleen (Fig 1J).

258　Altogether, these data suggest that IRE1 RNase activation in tumor cDCs is a stable lineage

259　intrinsic trait not driven by the microenvironment.

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261 **XBP1 deletion in CD11c-expressing cells results in increased melanoma tumor**

262 **growth**

263 To gain insights on the role of XBP1 in DCs during melanoma tumor growth, we studied

264 the *Itgax*-Cre x *Xbp1*$^{fl/fl}$ mice (36, 37), referred to as 'XBP1$^{\Delta DC}$' mice, in which exon 2 of

265 *Xbp1* is excised in CD11c-expressing cells, resulting in absence of the transcription factor

266 in DCs (26). These animals were compared to control littermates (*Xbp1*$^{fl/fl}$ animals with

267 no expression of Cre), referred to as 'XBP1^{WT}' mice. XBP1^{WT} and XBP1$^{\Delta DC}$ mice were

268 implanted with the B78-ChOVA melanoma line, a B16 variant that expresses the

269 ovalbumin (OVA) antigen and mCherry fluorescent protein (2). Data in Figure 2A-B

270 indicate that XBP1$^{\Delta DC}$ mice showed moderate but noticeable acceleration of tumor growth

271 and significantly larger tumor size than tumors from XBP1^{WT} mice on day 12 post

272 implantation (80.44 ± 8.927 vs 56.56 ± 6.174 mm^3, p = 0.0312, mean ± s.e.m.) (Fig. 2B).

273 As a second tumor model, we also analyzed growth of subcutaneous MC38 murine colon

274 adenocarcinoma tumors, which also showed a trend towards increased tumor growth in

275 XBP1$^{\Delta DC}$ mice, but without reaching statistical significance (Supp. Fig. 3A-C).

276 To understand if XBP1 deficiency in the CD11c compartment resulted in altered cell

277 recruitment, we quantified the immune cell composition at the melanoma tumor site.

278 XBP1^{WT} and XBP1$^{\Delta DC}$ mice show similar numbers of CD45$^+$ cells (Fig. 2C), and comparable

279 composition of tumor cDC1/cDC2 and resident and migratory cDC1/cDC2s in the TdLN

280 (Fig. 2D-E). Furthermore, conditional *knock-out* and control animals also showed

281 comparable frequencies of myeloid and lymphoid cells (Fig. 2F-H). These data indicate

282 that XBP1s expression by DCs infiltrating melanoma and MC38 tumor does not promote

283 tumor progression. Rather, loss of XBP1s in DCs leads to increased melanoma tumor

284 growth by mechanisms that are independent of immune cell recruitment.

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286 **XBP1 deletion in CD11c-expressing cells results in impaired antitumor T cell**

287 **responses and disbalanced precursor/terminal exhausted T cell ratio**

288 We next focused our analysis on DC function. It was recently identified a conserved

289 immunoregulatory transcriptional program activated by tumor DCs based on the co-

290 expression of the molecules CD40 and PDL-1 plus the cytokine IL-12 (15). Our analysis

291 revealed that tumor and migratory cDC1/cDC2s from XBP1$^{\Delta DC}$ mice express normal levels

292 of these molecules (Fig. 3A, Supp. Fig. 4A). We also studied bone marrow cDC1s generated

293 upon culture with OP9-DL1 stromal cell line plus FLT3L (reported in (38, 39)). We found

294 that bone marrow cDC1s from XBP1$^{\Delta DC}$ mice produce lower levels of IL-12 in

295 unstimulated conditions (Fig 3B, Supp. Fig. 4B). However, upon tumor exposure,

296 expression of IL-12 was comparable between XBP1 sufficient and deficient cDC1s. We

297 conclude that XBP1s regulate certain parameters of DC activation in steady state that are

298 restored upon tumor encounter.

299 Considering previous findings showing that XBP1s deficient cDC1s have impaired cross-

300 presentation abilities in steady state (26), we interrogated if the mice line was able to

301 cross-present melanoma-associated antigens. We implanted B78ChOVA tumors in

302 XBP1^{WT} and XBP1$^{\Delta DC}$ mice and quantified the presence of endogenous OVA-specific CD8$^+$

303 T cells using H-2K^b-OVA$_{257\text{-}264}$ tetramers. XBP1^{WT} and XBP1$^{\Delta DC}$ contained similar

304 frequencies of OVA-specific CD8$^+$ T cells in tumors and TdLN (Fig. 3C-D, Supp. Fig 4C). A

305 similar response was obtained when tracking proliferation/early activation of CD8$^+$ T

306 cells isolated from pmel mice, which possess transgenic CD8$^+$ T cells bearing a TCR

307 selective for the melanoma-associated antigen gp100 (40) (Supp. Fig. 4D-E). Thus, we

308 conclude that XBP1 deletion in tumor-associated CD11c$^+$ cells does not prevent cross-

309 presentation of melanoma-associated antigens.

310 We next investigated the quality of the antitumor T cell response evoked in XBP1$^{\Delta DC}$ mice.

311 As a measure of T cell quality, we analyzed cytokine producing T cells from tumors of

312 XBP1^{WT} and XBP1$^{\Delta DC}$ mice. Tumors from XBP1$^{\Delta DC}$ mice contained lower frequencies of

313 IFN-γ-producing and TNF-producing CD8$^+$ T cells, which also resulted in decreased

314 frequencies of double producers IFN-γ$^+$TNF$^+$ CD8$^+$ T cells and triple producers IFN-

315 γ$^+$TNF$^+$IL-2$^+$ CD8$^+$ T cells (Fig. 3E-F). These observations were also noticed in the CD4$^+$ T

316 cell compartment, as reduced frequencies of IFN-γ$^+$ CD4$^+$ T cells and IFN-γ$^+$ TNF$^+$ CD4$^+$ T

317 cells were found in tumors from XBP1$^{\Delta DC}$ mice (Fig. 3G). As such, absence of XBP1 in

318 CD11c-expressing cells results in decreased CD8$^+$ and CD4$^+$ T cell effector function in

319 melanoma tumors. Analysis of the MC38 model showed that whereas the CD8$^+$ T cell

320 response was not affected, there was a significant reduction in the frequencies of IFN-γ-

321 producing and IFN-γ/ TNF-producing CD4$^+$ T cells (Supp. Fig. 4F-G).

322 Impaired cytokine production is a hallmark of CD8$^+$ T cell exhaustion in cancer (41, 42),

323 a process characterized by a progressive loss of function that culminates with the

324 generation of terminal exhausted TIM-3$^+$CD8$^+$ T cells unable to control tumor growth

325 (42). TIM-3$^+$CD8$^+$ T cells do not proliferate, are unresponsive to anti-PD1 therapy (41, 43)

326 and originate from 'precursor exhausted' CD8$^+$ T cells, a T cell state characterized by the

327 expression of the transcription factor TCF-1 (termed 'TCF1$^+$CD8$^+$ T cells'), which retain

328 proliferative potential and can be reinvigorated through anti-PD1 therapy (41, 43, 44).

329 We determined the presence of intratumoral TCF-1$^+$CD8$^+$ T cells and TIM-3$^+$CD8$^+$ T cells

330 in melanoma tumors from XBP1$^{\Delta DC}$ mice and control animals. Tumors from XBP1$^{\Delta DC}$ mice

331 show decreased infiltration of TCF-1$^+$CD8$^+$ T cells and increased proportions of TIM-

332 3$^+$CD8$^+$ T cells compared to tumors from control animals (Figure 3H-I). Additionally,

333 TIM3$^+$ CD8$^+$ T cells from XBP1^{WT} and XBP1$^{\Delta DC}$ mice display a *bona-fide* terminal exhausted

334 phenotype, with elevated levels of CD39, TOX and granzyme B (Supp. Fig. 4H). These

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335 findings are consistent with data depicted in figure 3E-F showing lower frequencies of

336 polyfunctional cytokine-producing CD8$^+$ T cells in tumors from XBP1$^{\Delta DC}$ mice, which is an

337 attribute of precursor exhausted TCF-1$^+$CD8$^+$ T cells (41). Altogether, our data shows that

338 XBP1s in the CD11c$^+$ compartment coordinates the balance of CD8$^+$ T cell profiles in

339 melanoma.

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341 **Tumor cDC1s from XBP1$^{\Delta DC}$ mice display signs of RIDD**

342 Thus far, our data indicate that XBP1s expression in DCs modulates melanoma tumor

343 growth and the balance of effector and exhausted T cell subsets. However, it is unclear if

344 these processes depend on XBP1s-transcriptional activity, as previous work

345 demonstrated that XBP1s deficiency leads to hyperactivation of IRE1 RNase and RIDD in

346 steady state cDC1s (25, 26). Thus, we interrogated if XBP1 deficiency also resulted in

347 increased IRE1 RNase activity in tumor cDC1s (Fig 4A). We measured expression of *Xbp1*

348 spliced/unspliced mRNA in tumor cDC1s from control and XBP1$^{\Delta DC}$ mice. Although

349 XBP1$^{\Delta DC}$ cDC1s are unable to synthesize XBP1s protein, these cells still generate *Xbp1*

350 mRNA bearing the IRE1 cleavage sites, which serves as an assay to monitor IRE1 RNase

351 activity (26). Data in Figure 4B show that tumor cDC1s isolated from XBP1$^{\Delta DC}$ mice

352 express marked levels of *Xbp1s* mRNA compared to control counterparts, which is an

353 indicative sign of IRE1 RNase hyperactivation.

354 To determine RIDD on protein level in tumor DCs, we determined surface expression of

355 the integrin CD11c, a dimeric partner of *Itgb2* (coding the integrin CD18), which is a

356 reported mRNA substrate of IRE1 RNase (26). CD11c surface expression depends on

357 RIDD-mediated degradation of *Itgb2* mRNA and therefore, it can be used as a surrogate

358 marker for RIDD activity. Data in Fig. 4C show that tumor cDC1s from XBP1$^{\Delta DC}$ express

359 lower surface levels of CD11c than control counterparts, confirming RIDD induction on

360   protein level. Similar effect was observed in cDC1 subsets from the TdLN (Fig 4D). These

361   data show that XBP1-deficient cDC1s display signs of RIDD in melanoma tumors.

362   Interestingly, additional APCs such as tumor cDC2s and TAM from XBP1$^{\Delta DC}$ mice showed

363   a modest but noticeable reduction in CD11c expression (Fig. 4C), suggesting that these

364   cells may also induce RIDD upon XBP1 loss, albeit at lower extent than cDC1s.

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366   **Gene expression profiles of tumor cDC1s deficient for IRE1 RNase and XBP1**

367   Given that XBP1 deficient cDC1s show signs of RIDD at the tumor site, we analyzed the

368   transcriptomic signature downstream of IRE1 RNase in melanoma-infiltrating cDC1s. To

369   identify specific XBP1-dependent and RIDD-dependent targets, we carried out a parallel

370   analysis of the transcriptome of tumor cDC1s deficient for XBP1, or double deficient for

371   the RNase domain of IRE1 and XBP1. To generate double *knock-out* animals for the IRE1

372   RNase and XBP1 in CD11c-expressing cells, we crossed XBP1$^{\Delta DC}$ mice with *Ern1*$^{fl/fl}$ mice,

373   which bear loxP sites flanking exon 20 and 21 of the *Ern1* gene and generates a truncated

374   IRE1 isoform lacking the RNase domain (45) (referred to as "XBP1$^{\Delta DC}$/IRE1^{trunc}DC mice").

375   As such, XBP1$^{\Delta DC}$ mice lack the transcription factor and activate RIDD, whereas double

376   deficient XBP1$^{\Delta DC}$/IRE1^{trunc}DC mice lack both XBP1s and RIDD (Supp. Fig 5A-C). With this

377   strategy, XBP1s target genes are identified as transcripts that are downregulated in tumor

378   cDC1s from both XBP1$^{\Delta DC}$ and XBP1$^{\Delta DC}$/IRE1^{trunc}DC mice. In contrast, RIDD-dependent

379   transcripts are recognized as mRNAs that decrease their expression in XBP1-deficient

380   tumor cDC1s, but which expression is restored in XBP1$^{\Delta DC}$/IRE1^{trunc}DC animals.

381   Tumor cDC1s were isolated by cell sorting from B16 melanoma tumors of control,

382   XBP1$^{\Delta DC}$ or XBP1$^{\Delta DC}$/IRE1^{trunc}DC mice and the transcriptome was analyzed by bulk RNA

383   sequencing (RNA-seq). 51 differentially expressed genes (DEG) were identified among

384   XBP1$^{\Delta DC}$ or XBP1$^{\Delta DC}$/IRE1^{trunc}DC cDC1s (adjusted p-value < 0.05 and |Fold Change| > 1.5)

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385   (Fig. 5A). Biological pathway enrichment analysis using Gene Ontology (GO)

386   knowledgebase revealed that a large proportion of DEGs are constituents of the response

387   to misfolded proteins and transport and localization of ER proteins (see Biological

388   Process, Fig. 5B). There was also an overrepresentation of protein disulfide-isomerases

389   (see Molecular Function, Fig. 5B) and on intracellular localization level, most deregulated

390   genes encoded ER proteins (see Cellular Component, Fig. 5B). Next, we analyzed the DEGs

391   per mice line, which clustered these genes into three main groups: (1) genes upregulated

392   in XBP1-deficient and IRE1 RNase/XBP1-deficient tumor cDC1s; (2) genes

393   downregulated in both XBP1-deficient and IRE1 RNase/XBP1-deficient tumor cDC1s

394   (XBP1s targets); and (3) genes downregulated exclusively in XBP1-deficient tumor cDC1s

395   (potential RIDD targets) (Fig. 5A). In group (1), we identified five transcripts including

396   *Hspa5* which encodes BiP, a chaperone induced upon UPR activation (46). This finding

397   indicates that XBP1-deficient and IRE1 RNase/XBP1-double deficient tumor cDC1s show

398   signs of ER stress (Fig. 5A). We also identified *Cox6a2* (subunit of cytochrome C oxidase),

399   which was previously identified as an upregulated gene in XBP1-deficient cDC1s (26).

400   Transcripts in group (2) include protein disulfide isomerases (*Erp44, Txndx11, Txndc5,*

401   *P4hb*) chaperones (*Dnajb9/Hyou1*); glycosylation proteins (*Rpn1, Alg2, Serp1*), proteins

402   involved in transport to the ER (*Sec61a1, Sec61b, Spcs2, Spcs3, Ssr3*) and from the ER to

403   Golgi (*Bet1, Surf4*) (Fig. 5A). Additional canonical XBP1s targets (*Stt3a* and *Edem2)* were

404   identified when the cut off value was set below 1.5-fold (Supp. Fig 6A). Gene Set

405   Enrichment Analysis (GSEA) revealed that the transcriptome of both XBP1$^{\Delta DC}$ and

406   XBP1$^{\Delta DC}$/IRE1^{trunc}DC tumor cDC1s were depleted of targets related to protein

407   glycosylation, ER to Golgi transport, protein localization to the ER and lipid biosynthesis

408   (Fig 5C-D).

409   Finally, group (3) includes the canonical RIDD substrates *Bloc1s1*, *St3gal5* (21), *Itgb2* (26),

410   plus a subset of transcripts with heterogeneous functions (Fig. 5A) that range from lipid

411   synthesis and metabolism members (*St3gal5, Gm2a, Abca2*), Ca^{+2} homeostasis (*Stim2,*

412   *Pkd1*), protein folding (*Qsox1, Mlec*), a steroid binding receptor (*Paqr7*), an amino acid

413   transporter (*Slc1a5*) , a member of the nuclear pore complex (*Nup210*), an E3-ubiquitin

414   ligase (*Rnf130*), signaling receptors in immunity and development (*Fcrla*, *Notch4*,

415   respectively) to *Eif2ak3,* which encodes the UPR transducer PERK. Consistent with the

416   functional heterogeneity of RIDD targets, GSEA did not reveal differences at the level of

417   biological processes between XBP1$^{\Delta DC}$ and XBP1$^{\Delta DC}$/IRE1^{trunc}DC cDC1s (Fig 5C-D). Even

418   though some processes such as antigen processing and presentation and cell-redox

419   homeostasis are downregulated exclusively in tumor cDC1s from XBP1$^{\Delta DC}$ mice (thereby

420   suggesting RIDD dependency), these processes display low enrichment scores. To sum

421   up, these data indicate that tumor cDC1s from XBP1$^{\Delta DC}$ mice display an altered XBP1s

422   transcriptional program related to protein homeostasis and folding, and counter activate

423   RIDD. Using GSEA and reported XBP1s-target and RIDD-target gene datasets (31, 47) we

424   confirmed downregulation of the canonical XBP1s transcription program in both XBP1$^{\Delta DC}$

425   cDC1s and double deficient XBP1$^{\Delta DC}$/IRE1^{trunc}DC cDC1s (Fig 5E-F, Supp. Fig. S6B),

426   whereas RIDD-dependent targets are predominantly downregulated in XBP1$^{\Delta DC}$ cDC1s

427   (Fig 5E-F, Supp Fig. S6B). Finally, our findings reveal that melanoma-infiltrating cDC1s do

428   not show signs of dysfunctional XBP1s activity, as genes related to triglyceride

429   biosynthesis that are associated with diminished DC function in other cancer settings are

430   not downregulated upon XBP1s or IRE1 RNase loss (Supp. Fig. 6C-D) (30). Taken together,

431   these results demonstrate that XBP1 deficiency in tumor cDC1s impairs transcriptomic

432   programs associated with the maintenance of proteostasis and induces RIDD.

433

434 **RIDD activation in DCs accounts for the changes related to tumor growth and**

435 **dysregulated antitumor T cell immunity noticed in XBP1$^{\Delta DC}$ mice**

436 Our observations raise the question as to whether the increased tumor growth and T cell

437 dysregulation noticed in XBP1$^{\Delta DC}$ mice is due to XBP1s- or RIDD-dependent outputs in

438 DCs. To address this issue, we implanted the B78ChOVA cell line in control and

439 XBP1$^{\Delta DC}$/IRE1^{trunc}DC animals (Fig. 6A-B), in which RIDD is abolished (Supp. Fig. 5A-C).

440 Remarkably, in contrast to the observations noticed in XBP1$^{\Delta DC}$ mice (Fig. 2A), the tumor

441 size of animals lacking both XBP1 and IRE1 RNase in DCs was comparable to that

442 observed in control animals (41.78 ± 9.033 vs 53.90 ± 12.36 mm^3, p=0.42, mean ± s.e.m.)

443 (Fig. 6B), indicating that RIDD activation in CD11c-expressing cells accounts for the

444 increased tumor growth. Furthermore, we examined whether the reduced frequencies of

445 cytokine producing CD8$^+$ T cells and the disbalance between TCF-1$^+$/TIM-3$^+$ CD8$^+$ T cells

446 noticed in XBP1$^{\Delta DC}$ mice are also dependent on RIDD. Analysis of tumor-infiltrating CD8$^+$

447 T cells revealed no differences in the frequencies of IFN-γ producing CD8$^+$T cells, TNF

448 producing CD8$^+$T cells, IL-2 producing CD8$^+$T cells, nor in the proportion of double or

449 triple cytokine producers between XBP1$^{\Delta DC}$/IRE1^{trunc}DC mice and control animals (Fig.

450 6C-D). Similar results were observed for IFN-γ producing or double IFN-γ/ TNF-α

451 producing CD4$^+$ T cells (Fig. 6E). In addition, analysis of the composition of precursor-

452 exhausted/terminal exhausted T cells revealed similar infiltration of TCF-1$^+$/TIM-3$^+$ CD8$^+$

453 T cells in melanoma tumors from XBP1$^{\Delta DC}$/IRE1^{trunc}DC mice versus control animals (Fig.

454 6F-G), indicating that IRE1 RNase activity in DCs accounted for the accumulation of

455 dysfunctional CD8$^+$ T cells in melanoma. Altogether, these data indicates that

456 hyperactivation of the RNase domain of IRE1 in DCs fine tunes melanoma tumor growth

457 and antitumor T cell immunity.

458

DISCUSSION

The IRE1/XBP1s axis has emerged as a critical regulator of immunity and cancer (27, 48, 49). The differential mechanisms by which IRE1 signaling integrates the intensity and duration of ER stress to regulate cell fate is particularly noticed in the immune system, with cells such as cDC1s, B cells, NK cells and eosinophils that opt for an intact IRE1/XBP1s axis to maintain cellular health (25, 31, 50, 51), or cells including TAM/MdCs or intratumoral T cells, which acquire dysfunctional phenotypes upon enforced activation of the UPR sensor (27, 32, 52).

Here, we report that loss of XBP1 in CD11c-expressing cells results in increased melanoma tumor growth, decreased frequencies of cytokine-producing T cells and accumulation of terminal exhausted TIM-3$^+$CD8$^+$ T cells. Notably, this effect is abrogated in XBP1$^{\Delta DC}$/IRE1^{trunc}DC mice, demonstrating that IRE1 RNase-dependent, XBP1s-independent outputs account for the dysregulated antitumor immunity in melanoma. We also observe a milder phenotype in the MC38 model, suggesting that different tumor models differentially regulate the IRE1/XBP1s axis in DCs.

The results presented in this work contrast with previous studies demonstrating that persistent IRE1/XBP1s activation in tumor DCs curtails their antitumor function. In ovarian cancer, the same XBP1$^{\Delta DC}$ mice line show marked inhibition of tumor progression and improved antitumor immunity (30). One possibility accounting for these differences may be related to the different composition of DCs infiltrating these cancer models. In ovarian cancer models, tumor-associated DCs are spontaneously immunosuppressive (53) and in fact, whereas depletion of CD11c$^+$ cells delays ovarian cancer progression in later stages (54), the same process curtails CD8$^+$ T cell priming in melanoma (55). Furthermore, XBP1 deficiency in ovarian cancer DCs does not lead to RIDD activation (30), which contrasts to the evidence presented in this work. Thus, as result of these

484   combined data, we must consider that the outcome of the IRE1 outputs in tumor DCs may

485   drastically differ depending on the DC subset and the cancer type. In fact, data presented

486   here show that animals lacking both XBP1 and IRE1 RNase in DCs display normal

487   melanoma tumor growth and T cell immunity, indicating that the deletion of an entire

488   branch of the UPR in DCs does not have a predetermined role across different tumor types.

489   These results complement existing literature and may be relevant for building a

490   comprehensive understanding on future manipulation of the IRE1/XBP1s axis in cancer.

491   In addition, even though we use a genetic model of XBP1 deletion to reveal the scope of

492   RIDD in tumor DCs which may not recapitulate physiological responses, our work alludes

493   to cDC1s as regulators of anti-melanoma T cell immunity in XBP1$^{\Delta DC}$ mice. First, cDC1s are

494   the subset with the highest IRE1 RNase activity within melanoma and MC38 tumor niches,

495   and T cell parameters altered by XBP1 loss in CD11c^{+} cells, such as the induction of

496   intratumoral IFN-γ producing CD8^{+} T cells and the maintenance of precursor exhausted

497   TCF1^{+}CD8^{+} T cells are attributed to cDC1 function (8, 11). Furthermore, these processes

498   are also dependent on RIDD activation by DCs, which is more strongly induced in cDC1s

499   from XBP1$^{\Delta DC}$ mice (25, 26). Nevertheless, as we also observe reduced frequencies of

500   tumor infiltrating CD4^{+} IFNγ^{+} T cells in XBP1$^{\Delta DC}$ mice in melanoma and MC38 models, a

501   contribution of IRE1 RNase in CD4^{+} T cell priming by tumor cDC1s (56) or also by cDC2s

502   (17) cannot be excluded.

503   On a mechanistic level, we do not find a role for IRE1 RNase in cross-presentation of tumor

504   antigens, contrasting with previous findings in steady state cDC1s (26). These data

505   suggest that tumors may shape the spectra of XBP1s/RIDD targets in infiltrating cDC1s or

506   that additional mechanisms (or DC subtypes) may compensate for the process. In fact,

507   *tapbp* mRNA, a previously identified RIDD target in XBP1-deficient splenic DCs

508   contributing to antigen cross-presentation is not found as DEG in the transcriptomic

509 analysis of this study. In addition, growing evidence demonstrating the capacity of tumor

510 DCs to carry out cross-dressing of MHC-I/peptide complexes from tumor cells adds a new

511 layer of complexity that remains to be addressed (57, 58). However, one novel candidate

512 identified in this analysis as a potential RIDD substrate, the ER-resident FC receptor Like

513 A (*Fcrla*) (Fig. 5A), has been previously identified as part of a BATF3/IRF8 transcriptional

514 program that confers tumor immunogenicity in cDC1s independently of cross-

515 presentation (59). As such, data presented here delineates for first time the XBP1s-

516 dependent and RIDD-dependent targets in tumor cDC1s, which may serve as basis for

517 future studies focused on addressing the role of selective IRE1 RNase targets involved in

518 the regulation of antitumor immunity.

519 Multiple efforts are currently focused on the development of pharmacological compounds

520 targeting the IRE1 RNase active site and XBP1s *in vivo*, many of which have shown

521 translational potential in cancer (60). A recent study revealed that RIDD regulates

522 expression of the MHC-I heavy chain mRNAs in DCs and that inhibition of IRE1 RNase

523 through systemic administration of small molecules greatly attenuates tumor growth in

524 4T1 and CT26 models, by a mechanism proposed to be dependent on DC cross-

525 presentation (61). Even though we do not find MHC-I heavy chain mRNAs as DEGs in the

526 transcriptomic analysis of tumor cDC1s from XBP1$^{\Delta DC}$/IRE1^{trunc}DC mice, and we do not

527 find an improved melanoma tumor response in XBP1$^{\Delta DC}$/IRE1^{trunc}DC animals, future

528 studies are required to integrate these findings through experiments that include kinetics

529 of comparable tumor models. Finally, the work presented here serves as a proof-of-

530 concept study demonstrating that IRE1 RNase dependent, XBP1s-independent outputs in

531 DCs may also contribute to fine-tuning antitumor immunity.

532

533

MATERIAL AND METHODS

RESOURCE AVAILABILITY

Further information and requests for resources and reagents should be directed to

Fabiola Osorio (fabiolaosorio@med.uchile.cl).

Materials availability

This study did not generate new unique reagents.

Data and code availability

RNA-seq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in supplementary resources table. This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

ERAI (33), XBP1^{WT}(XBP1fl/fl (36)), XBP1$^{\Delta DC}$ (XBP1fl/fl x CD11c-Cre (37)), XBP1^{WT}/IRE1^{WT} (XBP1fl/fl x IRE1fl/fl (45)), XBP1$^{\Delta DC}$ IRE1^{trunc}DC (XBP1fl/fl x IRE1fl/fl x CD11c-Cre) mice were bred at Universidad de Chile and Fundación Ciencia y Vida in specific pathogen-free conditions. Also, for RNA seq studies XBP1^{WT}, XBP1$^{\Delta DC}$, XBP1^{WT}/IRE1^{WT} and XBP1$^{\Delta DC}$ IRE1^{trunc}DC mice were bred at the animal facility at VIB institute. pmel-1 mice (40) were kindly donated by Dr F. Salazar-Onfray. All mice were kept on a C57BL/6 background. Litters with mice of both sexes at 6–14 weeks of age were used for experiments.

558

Cell Lines

560   B78-ChOVA cells were kindly provided by Dr. Matthew Krummel (UCSF) (2). B16-F10

561   cells were obtained from ATCG (#CRL-6475). B16-FLT3L cell line (62) was provided by

562   Dr. Maria Rosa Bono (University of Chile). MC-38 cell line was provided by Dr. Álvaro

563   Lladser (Universidad San Sebastian). OP9 cells expressing Notch ligand DL1 (OP9-DL1)

564   (63) were kindly provided by Dr. Juan Carlos Zuñiga-Pflucker (Sunnybrook Research

565   Institute, Canada). Cells were cultured under standard conditions prior to injection into

566   mice. Briefly, cells were cultured in DMEM (B78-ChOVA) or RPMI-1640 (B16-F10/B16-

567   FLT3L/MC-38) supplemented with 10% v/v inactivated fetal bovine serum (FBS, Gibco),

568   100 U/mL penicillin (Corning), 100 µg/mL streptomycin (Corning) and 0.55 mM 2-

569   Mercaptoethanol (Gibco). For MC-38 culture, media was supplemented additionally with

570   non-essential amino acids (ThermoFisher Scientific) and 1 mM sodium pyruvate

571   (ThermoFisher Scientific). Cells were cultured on T75 tissue-culture treated plastic flasks

572   at 37°C, 5% CO_2. Cells were split every other day. OP-DL1 cells were cultured in MEM-

573   alpha medium supplemented with 20% FBS (Gibco), 100 U/mL penicillin (Corning), 100

574   µg/mL streptomycin (Corning), 1mM sodium pyruvate (brand) and 0.55 mM 2-

575   Mercaptoethanol (Gibco).

576

METHOD DETAILS

Tumor Model

579   Tumor cell lines were harvested, washed with PBS, and resuspended in a final injection

580   volume of 50 µl PBS. 5×10^5 (B16/B78-ChOVA) or 1×10^6 (MC-38) tumor cells were injected

581   in the right flank of shaved mice intradermally and allowed to grow for 10-15 days. For

582   tumor growth curves, tumor size was determined by two orthogonal measurements with

583   a caliper and the volume was estimated as (width^2 x length)/2.

584

585   ***Preparation of Cell Suspensions***

586   Tumors were minced and digested with Collagenase D (1 mg/mL, Roche) and DNAse I (50

587   µg/mL, Roche) for 30 minutes at 37°C in a water bath. Digested tissue was then passed

588   through a 70 µm cell strainer, followed by red blood cell lysis with RBC lysis buffer

589   (Biolegend). Single cells were kept on ice.

590   For whole intratumoral immune cell profiling and DC stainings, CD45-biotin magnetic

591   positive selection (MACS, Miltenyi) was performed to enrich for total tumor immune

592   infiltrate.

593   For intratumoral T cell stainings, hematopoietic cells were enriched by density gradient

594   centrifugation with 40/70 Percoll (GE Healthcare) for 20 min at 700xg.

595   Tumor draining lymph nodes (tdLNs) were minced and digested with Collagenase D (1

596   mg/mL, Roche) and DNAse I (50 µg/mL, Roche) for 45 minutes at 37°C in a water bath.

597   Digested tissue was then passed through a 70 µm cell strainer and single cells were kept

598   on ice.

599

600   ***Bone marrow derived DCs generation***

601   Bone marrow cells from femurs and tibias were cultured in presence of 20 ng/ml mouse

602   recombinant GM-CSF (Biolegend) for 8 days. Fresh culture medium with cytokine was

603   added on day 3, and on day 6. After harvesting and when indicated, cells were stimulated

604   with 1 ug/mL Tunicamycin (Sigma) for 8h followed by total RNA extraction with Trizol

605   (Invitrogen).

606

607 ***Bone marrow derived cDC1s generation and tumor lysate stimulation***

608 Bone marrow cells from femurs and tibias were cultured in presence of 100 ng/ml

609 recombinant human FLT3-L (Peprotech). After three days of differentiation, cells were

610 plated onto a monolayer of OP9-DL1 stromal cells and co-cultured for additional 6 days

611 in P24 plates as previously reported (38, 39).

612

613 For tumor lysate preparation B78-ChOVA cells were washed twice with PBS, resuspended

614 at 8×10^6 cells/mL in RPMI supplemented with 10% FBS and aliquoted in cryotubes. Cell

615 suspensions were subjected to heat-shock (42°C for 60 min) followed by three cycles of

616 freeze/thaw (liquid nitrogen/waterbath at 37°C). Tumor lysates were stored at -80°C

617 until use.

618 BM-derived cDC1s were harvested and plated with B78-ChOVA lysates (50 uL/mL) in

619 round-bottom p96 plates. After 14h, Brefeldin A (GolgiPlug, BD) was added and four

620 hours later, cells were harvested and stained for intracellular IL-12p40 expression by

621 flow cytometry.

622

623 ***Xbp1s splicing assay***

624 Total RNA was isolated either by Trizol (Invitrogen) or RNAeasy plus Micro Kit (Qiagen).

625 cDNA was prepared using M-MLV reverse transcriptase (Invitrogen). The following

626 primers were used for conventional PCR amplification of total Xbp1: Fwd: 5'-

627 ACACGCTTGGGAATGGACAC-3' and Rev: 5'-CCATGGGAAGATGTTCTGGG-3' (26); and for

628 beta actin (*Actb*): Fwd: 5'-CTAAGGCCAACCGTGAAAAG-3' and Rev: 5'-

629 TTGCTGATCCACATCTGCTG-3' or alternatively for beta actin (*Actb*): Fwd 5'-

630 GTGACGTTGACATCCGTAAAGA-3' and Rev: 5'-GCCGGACTCATCGTACTCC-3'. PCR

631 products were analyzed on agarose gels.

632

Flow Cytometry and Cell Sorting

For surface staining, cells were incubated with anti-Fc receptor antibody (anti-CD16/32, Biolegend) and then stained with fluorochrome-conjugated antibodies in FACS buffer (PBS + 1% FBS + 2mM EDTA) for 30 min at 4°C. Viability was assessed by staining with fixable viability Zombie (BioLegend) or LIVE/DEAD fixable (Invitrogen). A biotinylated antibody was used for F4/80 staining, followed by a second staining step with Streptoavidin-APC (Biolegend) for 30 min at 4°C. Flow cytometry was performed on BD Fortessa LSR instrument. Analysis of flow cytometry data was done using FlowJo software. Cell sorting was performed using a BD FACS Aria III.

642

Transcription Factors and Granzyme B intracellular staining

After surface staining, cells were fixed and permeabilized using Foxp3 transcription factor staining set (eBioscience) followed by intracellular staining of transcription factors (Foxp3, Tcf1, Tox) and/or granzyme B as indicated by the manufacturer protocol.

647

T cell stimulation and intracellular cytokine staining

Tumor and TdLN cell suspensions were stimulated *ex-vivo* prior to staining with 0.25 μM phorbol 12- myristate 13-acetate (PMA; Sigma) and 1 μg/mL Ionomycin (Sigma) at 37°C and 5% CO_2 for 3.5 hr in the presence of Brefeldin A (BD GolgiPlug). After stimulation, cells were surface stained as mentioned above. Then, cells were fixed and permeabilized using BD Cytofix/Cytoperm fixation/permeabilization kit (BD) followed by intracellular staining of cytokines (IFN-γ, IL-2 and TNF-α) as indicated by the manufacturer protocol.

655

Tetramer staining

25

657   For OVA-specific CD8+ T cell quantification cells were incubated with PE H2-K^b-OVA

658   (SIINFEKL) tetramers (MBL) at room temperature for 30 min protected from light,

659   followed by surface staining and FACS analysis.

660

661   ***t-SNE and clustering***

662   For tSNE visualization of tumor immune infiltrate a multicolor flow cytometry panel was

663   used including 19 parameters (FSC, SSC, Viability, CD45, VenusFP, XCR1, CD4, NK1.1,

664   CD26, F4/80, Ly6G, MHCII, CD24, CD3e, Ly6C, CD8a, CD11c, CD11b, CD19). Cells were

665   compensated for spillover between channels and pre-gated on CD45+ Live singlets using

666   FlowJo. Flowjo workspace was imported into the R environment using CytoML v2.4.0 ,

667   FlowWokspace v4.4.0 and FlowCore v2.4.0 packages (64–66). The intensity values of

668   marker expression were then biexp-transformed via the flowjo_biexp_trans function of

669   FlowWorkspace using parameters ChannelRange=4096, maxValue=262144, pos=4.5,

670   neg=0 and widthBasis=-10. Subsequently 5.000 cell events from each mouse (4 WT and 4

671   ERAI) were randomly sampled and combined for a total of 40.000 single cells. Sampled

672   data was min-max normalized, and subjected to dimensionality reduction by Barnes-

673   Hutts implementation of t-Distributed Stochastic Neighbor Embedding (tSNE) using

674   RtSNE v0.15 package (67). Thirteen parameters were used for tSNE construction (XCR1,

675   CD4, NK1.1, CD26, F4/80, Ly6G, MHCII, CD24, CD3e, Ly6C, CD8a, CD11c, CD11b and CD19)

676   and the parameters were set to iterations=1000 and perplexity =30. After dimensionality

677   reduction, automatic clustering was performed using density based spatial clustering

678   (DBSCAN) using DBSCAN v1.1.8 package (68). Dotplot for marker expression among

679   clusters and Violin plots for VenusFP were then generated using ggplot2 v3.3.5 package

680   (69).

681

682 ***In vivo T cell proliferation assay***

683 LN cells from pmel-1 TCR transgenic mice were isolated and enriched for CD8+ T cells by

684 magnetic negative selection using CD8+ T cell isolation kit (MACS, Miltenyi). Enriched

685 CD8+ T cells were surface stained and naïve CD8+ T cells were purified by cell sorting

686 (CD8a+, CD62L high, CD44low, CD25 neg). After sorting, naïve CD8+ T cells were labeled

687 with Cell Trace Violet (CTV, Invitrogen). $1x10^6$ naïve CD8+ T cells were adoptively

688 transferred into B16-F10 tumor-bearing mice at day 7 after tumor challenge. *In vivo*

689 proliferation and CD44/CD25 expression of transferred T cells was analyzed by FACS in

690 tumor draining lymph nodes 4 days after adoptive transfer.

691

692 ***RNA-seq***

693 Cell suspensions from tumor tissue pooled from 2-4 B16 bearing mice were enriched in

694 immune cells by positive selection with CD45+ biotin magnetic beads (MACS, Miltenyi).

695 Enriched cells were surface stained and 5-20 $x10^3$ intratumoral cDC1s were sorted

696 directly in RLT lysis buffer (Qiagen) containing 2-mercaptoethanol. Immediately after

697 sorting, collected cells were homogenized through vortex and frozen on dry ice before

698 storage at -80°C. Total RNA was extracted with RNAeasy Plus Micro kit (Qiagen). RNA

699 sequencing was performed at VIB Nucleomics Core using SMART-seq v4 pre-

700 amplification followed by single-end sequencing on Illumina NextSeq500. Preprocessing

701 of the RNA-seq data was performed by Trimmomatic v0.39 and quality control by FastQC

702 v0.11.8. Mapping to the reference mouse genome was performed by STAR v2.7.3a and

703 HTSeqCount v0.11.2 was used for counting. Limma v3.42.2 (70) was used to normalize

704 the data. Genes which did not meet the requirement of a count per million (cpm) value

705 larger than 1 in at least 4 samples were filtered. This resulted in an expression table

706 containing 11066 genes. EdgeR v3.28.0 (71) was utilized to perform differential

707   expression analysis. Benjamini-Hochberg correction was used to adjust the p-values for

708   multiple testing. Differentially expressed genes were filtered as genes with a |FC| > 1.5

709   and adjusted p-value < 0.05. Heatmaps were created using pheatmap v1.0.12 package

710   (72) on log2 normalized and mean centered gene expression data.

711

712   ***Gene Set Enrichment Analysis***

713   Over-Representation Analysis (ORA) and Gene Set Enrichment Analysis (GSEA) were

714   performed using *ClusterProfiler* v4.0.5 package (73) in R and Gene Ontology (GO)

715   knowledgebase gene sets. ORA results were considered significant when the q-value was

716   below 0.01. GSEA was performed on pre-ranked mode using as rank metric the signed

717   log10 transformed p-values derived from the differential expression analysis. GSEA was

718   run using the GO:BP database or literature lists of Xbp1-targets and RIDD-targets (47).

719   Results were considered significant when the adjusted p-value was below 0.05.

720

721   **QUANTIFICATION AND STATISTICAL ANALYSIS**

722   No statistical methods were used to predetermine sample size. The experiments were

723   not randomized, and the investigators were not blinded to allocation during

724   experiments and outcome assessment. Statistical analysis was conducted using

725   GraphPad Prism software (v9.1.2). Results are presented as mean ± SEM. Two groups

726   were compared using two tailed t-test for normal distributed data (Shapiro-Wilk test) or

727   using a non-parametric two-tailed Mann-Whitney test as indicated in figure legends.

728   Multiple groups were compared using one-way ANOVA with Tukey post-test. A p-value

729   < 0.05 was considered statistically significant.

730

731   **STUDY APPROVAL**

732   All animal procedures were performed in accordance with institutional guidelines for

733   animal care of the Fundación Ciencia y Vida, the Faculty of Medicine, University of Chile

734   and the VIB, Belgium, and were approved by the local ethics committee.

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Author contributions

751   F.F, MR.B, S.J and F.O designed the research; F.F, S.R, S.G, C.F, D.F did the experiments. F.F,

753   S.R, S.J and F.O analyzed the results; C.DN and C.M helped with RNA-seq data analysis;

754   D.Fe provided technical assistance and experimental expertise, T.I and A.L provided

755   critical reagents. F.F. and F.O wrote the manuscript

756

Acknowledgements

770

Declaration of interest

772   The authors declare no competing interests.

773

774

FIGURE LEGENDS

Figure 1. cDC1s are predominant cellular targets of IRE1 activation in melanoma tumors.

(A-F) B16-F10 melanoma cells were implanted intradermally on ERAI or control mice and 11 days after implantation, tumor tissue was analyzed by multicolor flow cytometry. n=4 mice per group, representative of two independent experiments. **(A)** t-SNE of 40.000 immune (CD45+) infiltrating cells from melanoma of ERAI or control mice. Colors indicate unsupervised clustering by DBSCAN. **(B)** Marker expression across the different cell clusters identified in (A). See also Supp. Fig 1A. **(C)** t-SNE map colored by VenusFP signal intensity from control or ERAI mice. cDC1 cluster is highlighted in a red circle. **(D)** VenusFP signal quantification across the different cell clusters identified in (A). Median fluorescence intensity for VenusFP is depicted with a "+" inside each violin plot. **(E-F)** Histograms (E) and quantification (F) of VenusFP signal from manually gated immune populations from B16-F10-bearing WT and ERAI mice (see gating strategy in Supp. Fig.2A). Statistical significance is depicted as compact letter display, ANOVA and Tukey post-test between ERAI mice. **(G-H)** Xbp1 splicing assay (G) of tumor cDC1s isolated by cell sorting from B16-FLT3L-bearing WT mice compared to BMDCs treated with 1 µg/mL of the ER-stressor Tunicamycin (Tun) or medium for 8h. (H) Bars show the image quantification of the ratio between Xbp1 spliced (Xbp1s) and total Xbp1 (Xbp1s + Xbp1u). n= 3 samples per group (representative of three independent experiments), mean ± s.e.m, ANOVA and Tukey post-test, ** $p<0.01$, *** $p<0.001$. **(I)** Quantification of VenusFP signal from tumor and tumor draining lymph node migratory (mig) and resident (res) cDCs (see gating strategy in Supp. Fig.2B). ** $p<0.01$, ANOVA and Tukey post-test between ERAI mice. n=4 mice per group, representative of two independent experiments, mean ± s.e.m. **(J)** Quantification of VenusFP signal from

800   intratumoral and spleen monocytes (CD11b^{hi} Ly6C^{hi} cells). *** p<0.001, t-test between

801   ERAI mice. n= 3 mice (ERAI) or 1 mouse (WT), representative of two independent

802   experiments, mean ± s.e.m.

803

804   **Figure 2. XBP1 deletion in CD11c-expressing cells results in increased melanoma**

805   **tumor growth**

806   XBP1^{WT} and XBP1$^{\Delta DC}$ mice were implanted with B78ChOVA tumors. **(A)** Tumor growth

807   curves monitored over a period of 12 days. n=19 mice per group. Pooled data from 4

808   independent experiments. **(B)** Tumor size at day 12 post implantation. * p<0.05, two-

809   tailed t-test. n= 51 mice (XBP1^{WT}) or 53 mice (XBP1$^{\Delta DC}$) from animals used throughout

810   this study (pooled data from 12 independent experiments), boxplot. **(C)** Cell counts for

811   tumor immune infiltrate (CD45+). n=31 mice (XBP1^{WT}) or 29 mice (XBP1$^{\Delta DC}$), pooled

812   data from 8 independent experiments, mean ± s.e.m. **(D)** Frequencies of intratumoral

813   cDC subsets. n=5-10, pooled data from two (cDC2) or three (cDC1) independent

814   experiments, mean ± s.e.m. (E) Frequencies of cDC subsets in TdLN. n=4 mice per group,

815   representative of two independent experiments, mean ± s.e.m. **(F-G)** Frequencies of

816   tumor lymphoid and myeloid populations. n=3-12 mice per group, pooled data from two

817   (neutrophils, TAMs, NK) or three (MdCs, T) independent experiments, mean ± s.e.m. **(H)**

818   Tumor Treg (CD3$^+$CD4$^+$Foxp3$^+$), Tconv (CD3$^+$CD4$^+$Foxp3$^-$) and CD8$^+$ T cells (CD3$^+$CD8$^+$)

819   frequencies. n=8 mice per group, pooled data from two independent experiments, mean

820   ± s.e.m.

821

822   **Figure 3. XBP1 deletion in CD11c-expressing cells results in impaired antitumor T**

823   **cell responses and dysbalanced precursor/terminal exhausted T cell ratio.**

824 **(A)** CD40 and PD-L1 expression by tumor cDCs from B78-ChOVA bearing XBP1^{WT} or

825 XBP1$^{\Delta DC}$ mice. n=7 mice per group, pooled data from two independent experiments,

826 mean ± s.e.m. See also TdLN data in Supp. Fig 4A. **(B)** Intracellular IL-12 expression of *in*

827 *vitro* generated cDC1s (FLT3-L/OP9-DL1) upon stimulation with B78-ChOVA lysates.

828 Gated on cDC1s (MHC-II$^+$ CD11c$^+$ CD8$^+$ CD11b$^-$). *p < 0.05, two-tailed Mann-Whitney test.

829 Each dot represents a biological replicate, n=5, data pooled from three independent

830 experiments, mean ± s.e.m. **(C-D)** Tetramer$^+$ CD8+ T cell frequencies in tumor and TdLN

831 from B78-ChOVA bearing XBP1^{WT} or XBP1$^{\Delta DC}$ mice. Gated on CD3$^+$CD8$^+$ T cells. n=8 mice

832 per group, pooled data from two independent experiments, mean ± s.e.m. **(E-G)**

833 Cytokines expression by tumor CD8$^+$ T cells (E-F) or CD4$^+$ T cells (G) from B78-ChOVA

834 bearing XBP1^{WT} or XBP1$^{\Delta DC}$ mice after ex vivo stimulation with PMA/ION in the

835 presence of BFA. *p < 0.05, two-tailed Mann-Whitney test. n=18 mice (XBP1^{WT}) or 17

836 mice (XBP1$^{\Delta DC}$), pooled data from 4 independent experiments, mean ± s.e.m. **(H-I)**

837 Precursor exhausted (PD1$^+$TCF1$^+$ TIM3^{neg}) and terminal exhausted (PD1$^+$TCF1^{neg} TIM3$^+$)

838 CD8$^+$ T cell tumor frequencies from B78-ChOVA bearing XBP1^{WT} or XBP1$^{\Delta DC}$ mice. Gated

839 on CD3$^+$CD8$^+$ T cells. * p<0.05, **p<0.01, two-tailed t-test. N=8 mice per group, pooled

840 data from two independent experiments, mean ± s.e.m.

841

842 **Figure 4. Tumor cDC1s from XBP1ΔDC mice display signs of RIDD.**

843 (A) Upon Cre mediated recombination in XBP1$^{fl/fl}$ mice, a premature stop codon is

844 introduced in the *Xbp1* mRNA sequence, preventing the translation of a functional XBP1s

845 protein. XBP1s absence is reported to trigger IRE1 RNase hyperactivation and induce

846 RIDD in certain cell types (26, 36). However, IRE1 RNase activity can still be monitored

847 by determining Xbp1 mRNA splicing ratio. (B) Scheme depicting LoxP sites and IRE1

848 splicing sites at *Xbp1* locus (top) and PCR analysis of Xbp1 splicing in intratumoral

849   cDC1s isolated from B16-bearing XBP1^{WT} and XBP1$^{\Delta DC}$ mice (bottom). Each lane

850   represents different mice. Xbp1u: Xbp1 unspliced; Xbp1s: Xbp1 spliced; Actb: beta actin.

851   (C) CD11c expression by intratumoral cDC1, cDC2 and TAMs from B16-bearing XBP1^{WT}

852   and XBP1$^{\Delta DC}$ mice. Gray histogram depicts unstained control. * p<0.05, ** p<0.01, ****

853   p<0.0001, two-tailed t-test. n=4 mice per group, representative of two independent

854   experiments, mean ± s.e.m. (D) CD11c expression by cDC subsets in the TdLN from B16-

855   bearing XBP1^{WT} and XBP1$^{\Delta DC}$ mice. Gray histogram depicts unstained control. *p<0.05,

856   two-tailed Mann-Whitney test. n=4 mice per group, representative of two independent

857   experiments, mean ± s.e.m.

858

859   **Figure 5. Gene expression profiles of tumor cDC1s deficient for IRE1 RNase and**

860   **XBP1.**

861   WT, XBP1$^{\Delta DC}$ and XBP1$^{\Delta DC}$/IRE1^{trunc}DC mice were implanted with B16 tumors. After 12

862   days, tumor cDC1s were isolated by cell sorting and total RNA was sequenced by RNA-

863   seq. **(A)** Heatmap of differentially expressed genes (DEGs). Three groups of genes were

864   identified by the pattern of expression among the three genotypes. **(B)** Over

865   representation analysis of DEGs over the Gene Ontology (GO) database. **(C)** Gene Set

866   Enrichment Analysis (GSEA) of WT vs XBP1$^{\Delta DC}$ cDC1s using GO:Biological Process

867   database. **(D)** GSEA of WT vs XBP1$^{\Delta DC}$/IRE1^{trunc}DC cDC1s using GO:Biological Process

868   database. **(E-F)** GSEA using XBP1s- and RIDD-target gene sets from literature (So et al,

869   2012, Cell Metabolism).

870

871   **Figure 6. RIDD activation in DCs accounts for the changes related to tumor growth**

872   **and dysregulated antitumor T cell immunity noticed in XBP1ΔDC mice.**

34

873   XBP1^{WT}/IRE1^{WT} and XBP1$^{\Delta DC}$/IRE1^{trunc}DC mice were implanted with B78ChOVA cells.

874   **(A)** Tumor growth curves monitored over a period of 12 days. n=20 mice

875   (XBP1^{WT}/IRE1^{WT}) or 24 mice (XBP1$^{\Delta DC}$/IRE1^{trunc}DC), data pooled from 4 independent

876   experiments. **(B)** Tumor size at day 12 post implantation. n=24 mice (XBP1^{WT}/IRE1^{WT})

877   or 28 mice (XBP1$^{\Delta DC}$/IRE1^{trunc}DC), data pooled from 5 independent experiments, mean ±

878   s.e.m. **(C-D)** Cytokines expression by tumor CD8$^+$ T cells after ex vivo stimulation with

879   PMA/Ionomycin in the presence of BFA. Gated on CD3$^+$CD8$^+$ T cells. N=9 mice

880   (XBP1^{WT}/IRE1^{WT}) or 11 mice (XBP1$^{\Delta DC}$/IRE1^{trunc}DC), data pooled from three

881   independent experiments, mean ± s.e.m. **(E-F)** Precursor exhausted (PD1$^+$TCF1$^+$

882   TIM3^{neg}) and terminal exhausted (PD1$^+$TCF1^{neg} TIM3$^+$) CD8$^+$ T cell tumor frequencies.

883   Gated on CD3$^+$CD8$^+$ T cells. n=9 mice (XBP1^{WT}/IRE1^{WT}) or 11 mice

884   (XBP1$^{\Delta DC}$/IRE1^{trunc}DC), data pooled from three independent experiments, mean ± s.e.m.

885

SUPPLEMENTARY FIGURE LEGENDS

887   **Supplementary Figure 1. Immune analysis of melanoma tumors derived from**

888   **ERAI mice.**

889   **(A)** t-SNE map as in figure 1a. Color gradient shows the expression of the indicated

890   marker**. (B)** Quantification of VenusFP signal from manually gated immune populations

891   from MC-38 bearing WT and ERAI mice. **** p < 0.0001, ANOVA and Tukey post-test

892   between ERAI mice. n=3 (WT) or 5 (ERAI) mice per group, representative of two

893   independent experiments.(C) Quantification of VenusFP signal of tumor- and spleen-

894   cDC1s from MC-38 bearing WT and ERAI mice. **** p<0.0001, t-test between ERAI mice.

895   n=3 (WT) or 5 (ERAI), representative of two independent experiments.

896

897 **Supplementary Figure 2. Gating strategy for tumor associated cDCs.**

898 **(A)** Gating strategy for identification of immune infiltrated populations in tumors.

899 Representative plots from B16 melanoma tumor. **(B)** Gating strategy for identification of

900 migratory (mig) and resident (res) cDC1s and cDC2s in tumor draining lymph node.

901 Representative plots from B16 melanoma TdLN.

902

903 **Supplementary Figure 3. Tumor growth and T cell infiltration in MC-38 bearing**

904 **XBP1$^{\Delta DC}$ mice.**

905 XBP1^{WT} and XBP1$^{\Delta DC}$ mice were implanted with MC-38 tumors. **(A-C)** Tumor volume

906 curves (A), tumor volumes (B) at end point and tumor weight (C) at end point. n=12-13

907 mice per group. Pooled data from two independent experiments.

908

909 **Supplementary Figure 4. Tumor immune cell analysis of XBP1$^{\Delta DC}$ mice.**

910 **(A)** Related to figure 3A. CD40 and PD-L1 expression by TdLN cDCs. n=7 mice per group,

911 pooled data from two independent experiments, mean ± s.e.m. **(B)** Related to figure 3B.

912 Dot plots of IL-12 intracellular expression by *in vitro* generated cDC1s stimulated with

913 B78-ChOVA lysates. **(C)** Related to figure 3C-D. Fluorescence minus one (FMO) and

914 tumor-free WT mouse as negative controls for tetramer staining. **(D-E)** XBP1^{WT} and

915 XBP1$^{\Delta DC}$ mice were implanted with B16-F10 tumors. Seven days after implantation,

916 CD8$^+$ naïve T cells isolated from pmel1 transgenic mice were labeled with Cell Trace

917 Violet and were adoptively transferred into tumor-bearing mice. Four days later,

918 transferred cell proliferation and CD44/CD25 expression was quantified by FACS. n=8

919 mice (XBP1^{WT}) or 15 mice (XBP1$^{\Delta DC}$), data pooled from two independent experiments,

920 mean ± s.e.m. **(F-G)** CD8+ T cell (F) and CD4+ T cell (G) frequencies and profiles in MC-

921 38 bearing XBP1^{WT} and XBP1$^{\Delta DC}$ mice. n=11-13, pooled data from two independent

922   experiments, mean ± s.e.m. **(H)** Related to figure 3H-I. Representative histograms of

923   different markers associated with terminal exhausted CD8$^+$ T cells. Gated on

924   CD3$^+$CD8$^+$PD1$^+$TCF1$^+$ or CD3$^+$CD8$^+$PD1$^+$TIM3$^+$ as shown in figure 3H.

925

926   **Supplementary Figure 5. IRE1/XBP1s double deficient cDC1s are unable to**

927   **activate RIDD.**

928   **(A)** PCR analysis of Xbp1 splicing in intratumoral cDC1s isolated from B16-bearing

929   XBP1^{WT}/IRE1^{WT} vs XBP1^{ΔDC}/IRE1^{trunc}DC mice. Each lane represents different mice.

930   Xbp1u: Xbp1 unspliced; Xbp1s: Xbp1 spliced; Actb: beta actin. **(B)** CD11c expression by

931   intratumoral cDC1, cDC2 and TAMs from B16-bearing XBP1^{WT}/IRE1^{WT} vs

932   XBP1^{ΔDC}/IRE1^{trunc}DC mice. n=4 mice per group, mean ± s.e.m. **(C)** CD11c expression by

933   cDC subsets in the TdLN from B16-bearing XBP1^{WT}/IRE1^{WT} vs XBP1^{ΔDC}/IRE1^{trunc}DC mice.

934   *p<0.05, two-tailed Mann-Whitney test. n=4 mice per group, mean ± s.e.m.

935

936   **Supplementary Figure 6. RNAseq analysis of WT, XBP1 deficient WT vs IRE1/XBP1**

937   **double deficient cDC1s.**

938   **(A)** Heatmap of genes with an adjusted p-value < 0.05 but with a fold change (FC) below

939   the 1.5 threshold. Table summarize log2FC and adj p-values for WT vs XBP1 and WT vs

940   IRE1/XBP1 deficient cDC1s. **(B)** Related to figure 5F-G. GSEA using XBP1s- and RIDD-

941   target gene sets from literature (So et al, 2012, Cell Metabolism). **(C)** GSEA of the

942   "triglyceride biosynthetic process" gene set (GO: 0019432) in WT vs XBP1^{ΔDC} cDC1s

943   (left) or WT vs XBP1^{ΔDC}/IRE1^{trunc}DC cDC1s (right) showing not statistically significant

944   enrichment (q-value > 0.05). **(D)** Normalized expression (z-scores) for genes of the

945   "triglyceride biosynthetic process" gene set (GO: 0019432) in WT, XBP1^{ΔDC}, or

946   XBP1^{ΔDC}/IRE1^{trunc}DC cDC1s.

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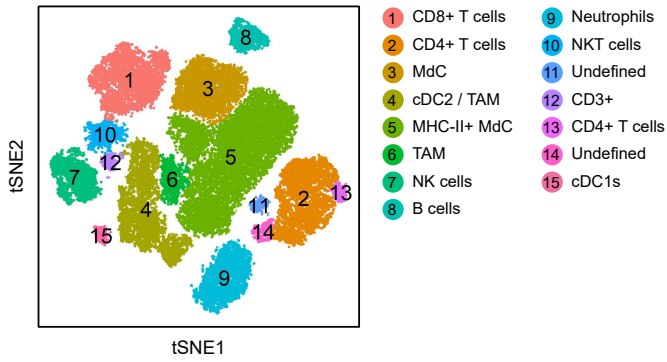1030　Cells. *Cancer Cell* 2020;37(6):786-799.e5.

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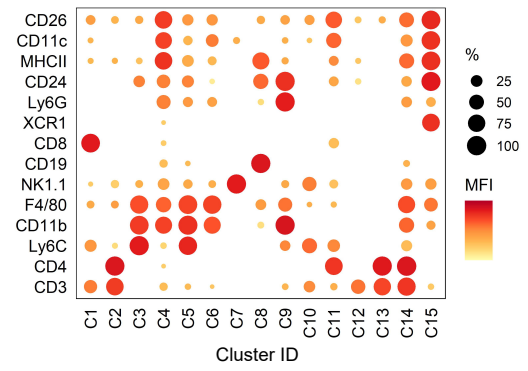1072   boosting antitumor immunity. *Cancer Res.* 2008;68(18):7684–7691.

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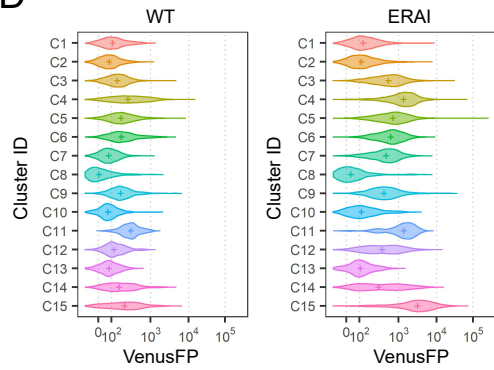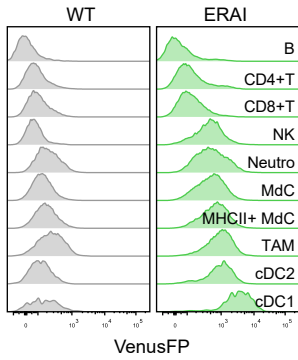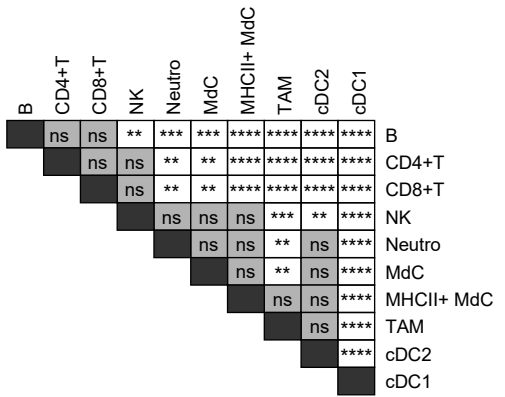1110 data. *Innov.* 2021;2(3):100141.

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Figure 1
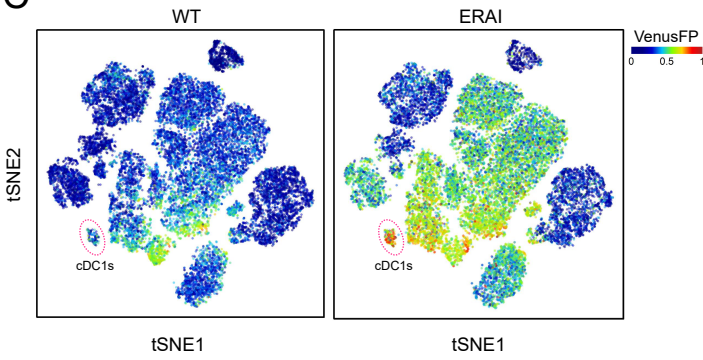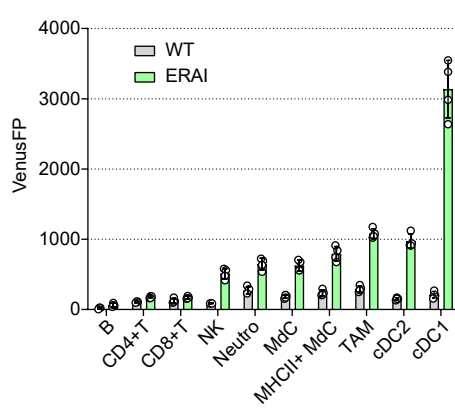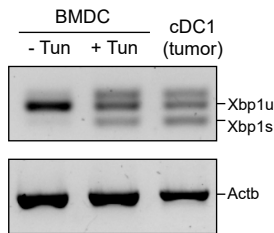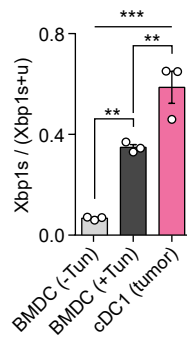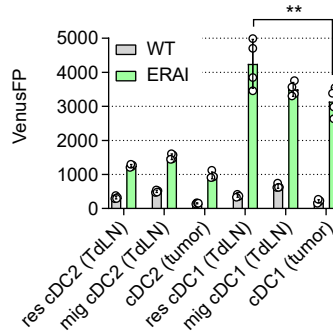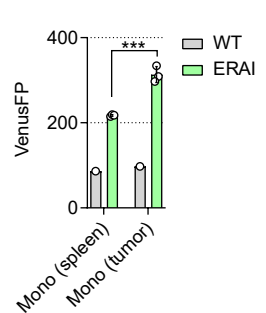
Figure 2

Figure 3

Figure 4

A

IRE1

e.g. *Itgb2*

RIDD

Xbp1 (s) mRNA

XBP1s

B

IRE1 splicing sites

XBP1^{WT} → XBP1s

XBP1$^{\Delta DC}$ → Cre → No protein

XBP1^{WT} XBP1$^{\Delta DC}$

Xbp1u
Xbp1s

Actb

Xbp1s / (Xbp1s+u)

■ XBP1^{WT}
■ XBP1$^{\Delta DC}$

**

C

cDC1

cDC2

TAM

% of mode

CD11c

gMFI CD11c

■ XBP1^{WT} ■ XBP1$^{\Delta DC}$

****   **   *

cDC1   cDC2   TAM

D

mig cDC1

mig cDC2

% of mode

CD11c

gMFI CD11c

■ XBP1^{WT} ■ XBP1$^{\Delta DC}$

*   *

mig cDC1   mig cDC2

Figure 5

Figure 6