Cancer cell CCR2 orchestrates suppression of the adaptive immune response

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Running title

CCR2 signaling in cancer cells causes immune evasion

Summary

C-C chemokine receptor type 2 (CCR2) expressed on monocytes facilitates their recruitment to tumors. Here, CCR2 signaling in cancer cells is shown to suppress immune control of tumors, in large part by enabling cancer cells to inhibit CD103+ dendritic cell recruitment.

ABSTRACT

C-C chemokine receptor type 2 (CCR2) is expressed on monocytes and facilitates their recruitment to tumors. Though breast cancer cells also express CCR2, its functions in these cells are unclear. We found that *Ccr2* deletion in cancer cells led to reduced tumor growth and ~2-fold longer survival in an orthotopic, isograft breast cancer mouse model. Deletion of *Ccr2* in cancer cells resulted in multiple alterations associated with better immune control: increased infiltration and activation of cytotoxic T lymphocytes (CTLs) and CD103+ cross-presenting dendritic cells (DCs), as well as upregulation of MHC class I and downregulation of checkpoint regulator PD-L1 on the cancer cells. Pharmacological inhibition of CCR2 increased cancer cell sensitivity to CTLs and enabled the cancer cells to induce DC maturation toward the CD103+ subtype. Consistently, *Ccr2*$^{-/-}$ cancer cells did not induce immune suppression in *Batf3*$^{-/-}$ mice lacking the CD103+ DC subtype. Our results establish that CCR2 signaling in cancer cells can orchestrate suppression of the immune response.

INTRODUCTION

Tumors escape immune control via multiple mechanisms (Dunn et al., 2002; Dunn et al., 2004). These mechanisms include cancer cell-intrinsic changes that alter how the cancer cell is recognized by the immune system and extrinsic changes that suppress immune cell activities. For example, cancer cells can intrinsically decrease the surface expression of major histocompatibility complex (MHC) class I, making them effectively invisible to cytotoxic T lymphocytes (CTLs) (Garrido et al., 2016). Moreover, cancer cells can increase expression of programmed cell death ligand 1 (PD-L1/B7-H1), which binds the PD-1 receptor on activated T cells, resulting in T cell anergy and thereby protecting against T cell-mediated killing (Chen et al., 2016). Extrinsic mechanisms include the downregulation of co-stimulatory molecules (*e.g.*, CD86) on antigen-presenting cells; the secretion of cytokines that directly inhibit CTLs; the promotion of regulatory T cell infiltration; and the recruitment of myeloid-derived suppressor cells (MDSCs) (Igney and Krammer, 2002). In contrast, infiltration of CD103+ dendritic cells (DCs) in mice, phenotypically similar to CD141+ DCs in humans (Hildner et al., 2008), has emerged as a mechanism by which tumors may be kept under immune control: CD103+ DCs are highly efficient at acquiring and processing exogenous antigens, which they directly present on MHC class I molecules to CD8+ CTLs. Even a modest accumulation of CD103+ DCs in tumors has been associated with improved immune-mediated tumor control (Broz et al., 2014; Roberts et al., 2016).

Chemokine receptors mediate the recruitment of immune cells to sites of inflammation and to tumors. C-C chemokine receptor type 2 (CCR2) is expressed by several bone marrow-derived cell types (including inflammatory monocytes, myeloid precursor cells, and immature DCs), as well as B and T lymphocytes (Lim et al., 2016). CCR2-expressing cells are recruited to sites of inflammation by C-C chemokine ligand 2 (CCL2) (Deshmane et al., 2009). Chemotherapy treatment promotes CCR2-dependent infiltration of tumor-promoting myeloid cells to murine mammary tumors (Nakasone et al., 2012), and

CCL2/CCR2-mediated recruitment of CCR2+ inflammatory monocytes to the lung has been shown to promote breast cancer extravasation and metastasis in mice models (Qian et al., 2011). Furthermore, elevated levels of CCL2 in tumors and in serum are associated with advanced disease and poor prognosis in breast carcinoma patients (Lebrecht et al., 2004; Lebrecht et al., 2001; Soria and Ben-Baruch, 2008). These findings have sparked interest in targeting the CCR2 pathway to modulate the innate immune response for therapeutic benefit in cancer. However, CCR2 is also expressed by breast cancer cells, and activation of CCR2 by CCL2 can induce cancer cell migration and survival through Smad3-, p42/44MAPK-, and Rho GTPase-mediated signaling (Fang et al., 2012). *In vivo*, the potential role(s) of CCR2 signaling in cancer cells have not been well investigated, largely because they were thought to be minor compared to the roles of CCR2 in myeloid cells.

In this study, we report that CCR2 signaling in cancer cells plays a surprisingly major role in regulating the immune response to murine breast tumors. We show that CCR2 in cancer cells supports immune escape by inhibiting CD103+ DC infiltration and maturation and by suppressing cytotoxic T cell activity. CCR2 expression on cancer cells represents a previously uncharacterized mechanism for immune suppression. Thus, our data support the notion that the CCL2/CCR2 axis is an important immune modulatory pathway in cancer, utilized by both immune cells and cancer cells to orchestrate immune suppression.

RESULTS

CCR2 in cancer cells promotes primary tumor growth in an orthotopic, isograft breast cancer mouse model

To investigate the potential effects of CCR2 on breast tumor growth and metastasis, we crossed *Ccr2*$^{-/-}$ mice (Boring et al., 1997) with mouse mammary tumor virus - polyoma middle T (MMTV-PyMT) mice, a model of luminal B breast cancer (Guy et al., 1992; Lin et al., 2003). The *Ccr2* genotype did not influence tumor onset (**Fig. 1a**); however, loss of even one allele of *Ccr2* significantly reduced tumor growth rates (**Fig. 1b**). Consistently, survival time was significantly longer for the MMTV-PyMT;*Ccr2*$^{-/-}$ and MMTV-PyMT;*Ccr2*$^{+/-}$ mice compared to that of the MMTV-PyMT;*Ccr2*$^{+/+}$ mice (**Fig. 1c**).

The importance of CCR2 signaling for monocyte recruitment is well understood, but CCR2 is also expressed by human breast cancer cells (Fang et al., 2012). In accordance with this report, we detected *Ccr2* mRNA and CCR2 protein in the cancer cells of the tumors developing in MMTV-PyMT;*Ccr2*$^{+/+}$ mice (**Fig. 1d**, **e**). To determine the relative contributions of cancer cell vs. host CCR2 to tumor growth, we isolated primary cancer cells (purity >90%; **Fig. S1a**) from MMTV-PyMT;*Ccr2*$^{+/+}$ and MMTV-PyMT;*Ccr2*$^{-/-}$ mice and transplanted them orthotopically to mammary fat pads of either *Ccr2*$^{+/+}$ or *Ccr2*$^{-/-}$ syngeneic host mice (**Fig. 1f**). Loss of *Ccr2* in the host did not alter tumor growth, consistent with our previous report (Nakasone et al., 2012). However, loss of *Ccr2* in cancer cells significantly reduced tumor growth rates, leading to ~2-fold longer survival (**Fig. 1g, h**). *Ccr2* mRNA levels of *Ccr2*$^{+/-}$ cancer cells were lower than those of *Ccr2*$^{+/+}$ cancer cells (**Fig. S1b**), and tumors derived from *Ccr2*$^{+/-}$ cancer cells exhibited the same slow growth as tumors derived from *Ccr2*$^{-/-}$ cancer cells (**Fig. S1c**). This result suggests that a threshold level of CCR2 expression in MMTV-PyMT cancer cells is required to promote tumor growth.

CCR2+ inflammatory monocytes promote breast cancer metastasis (Muller et al., 2001; Qian et al., 2011). The overall metastatic burden in the lung—the primary site of metastasis in the MMTV-PyMT model—was very variable in MMTV-PyMT;*Ccr2*$^{+/+}$mice and was not significantly different from that of MMTV-PyMT;*Ccr2*$^{-/-}$ mice (**Fig. S1d**). There were also no significant differences in the number of metastatic foci (representing seeding density) related to the *Ccr2* genotype (**Fig. S1e**). However, we found that the metastatic foci were larger in the lungs of MMTV-PyMT;*Ccr2*$^{+/+}$ mice than in the lungs of MMTV-PyMT;*Ccr2*$^{-/-}$ mice (**Fig. S1f**), suggesting that CCR2 promotes the growth of secondary lesions.

Ccr2 expression in cancer cells is associated with poor differentiation and reduced apoptosis sensitivity

The tumors from transplanted *Ccr2*$^{+/+}$ or *Ccr2*$^{-/-}$ cancer cells were markedly different at the histological level. The tumors originating from *Ccr2*$^{+/+}$ cancer cells consisted entirely of microlobules composed of sheets of large, neoplastic epithelial cells. The microlobules were surrounded by small amounts of fibrovascular stroma containing dense collagen, which is characteristic of poorly differentiated MMTV-PyMT tumors. The neoplastic cells had round to oval nuclei, prominent nucleoli, scant cytoplasm, and indistinct cell borders, typical of undifferentiated cells. In contrast, the tumors originating from *Ccr2*$^{-/-}$ cancer cells were more differentiated. In many areas, the cancer cells contained lipid vacuoles of various sizes. These tumors also had large cystic areas lined by a single or double layer of small, polarized epithelial cells, and the lumina were filled with proteinaceous secretions (**Fig. 2a**).

To understand why tumors from *Ccr2*$^{-/-}$ cancer cells grew slower than did tumors from *Ccr2*$^{+/+}$ cancer cells, we next examined the rates of proliferation and apoptosis in the tumors. There was no difference in proliferation as determined by nuclear Ki67 staining (**Fig. 2b**), and primary *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ cancer cells also grew similarly *in vitro* (**Fig. 2c**). However, we found a higher percentage of cells

undergoing apoptosis in tumors from *Ccr2*$^{-/-}$ cancer cells than from *Ccr2*$^{+/+}$ cancer cells (**Fig. 2d**). Furthermore, while the cell populations were equally viable under normal cell culture conditions, *Ccr2*$^{-/-}$ cancer cells were more sensitive to serum-free conditions (**Fig. 2e**), consistent with a previous study showing that CCR2 signaling can protect cancer cells from apoptosis induced by serum starvation (Fang et al., 2012). We found that the levels of CCL2, CCL12 (another CCR2 ligand), and most other cytokines were similar between MMTV-PyMT;*Ccr2*$^{+/+}$ and MMTV-PyMT;*Ccr2*$^{-/-}$ tumors (**Fig. S2a**). Only IL-16 and granulocyte colony-stimulating factor (G-CSF) were significantly reduced in the MMTV-PyMT;*Ccr2*$^{-/-}$ tumors. When secretions specifically from the cancer cells were analyzed, CCL2, CCL12, G-CSF, and IL-16 were all secreted to the same degree by *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ cancer cells (**Fig. S2b**).

Ccr2$^{-/-}$ cancer cells have increased expression of interferon (IFN) response genes and of genes involved in MHC class I antigen presentation

To determine which pathways in the *Ccr2*$^{-/-}$ cancer cells lead to reduced tumor growth and increased cell death, we isolated cancer cells from *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ isografted tumors grown in wild-type hosts for equal amounts of time and performed transcriptome profiling by RNA-seq. Overall, 520 genes were differentially expressed (adjusted p-value <0.05), ~40% of which were upregulated in *Ccr2*$^{-/-}$ cancer cells compared to *Ccr2*$^{+/+}$ cancer cells. Among the differentially expressed genes, we detected upregulation of MHC I (H2-K1) and Tap1, two genes required for antigen presentation, and IFI27, an IFN response gene, in the *Ccr2*$^{-/-}$ cancer cells (**Fig. 3a**). Vimentin, which is found in poorly differentiated epithelial cells and in mesenchymal cells, was downregulated in *Ccr2*$^{-/-}$ cancer cells. Consistently, gene set enrichment analysis (GSEA) showed robust induction of genes involved in antigen presentation and of IFN response genes in *Ccr2*$^{-/-}$ cancer cells compared to *Ccr2*$^{+/+}$ cancer cells (**Fig. 3b**). We also observed a pronounced luminal gene expression signature in the *Ccr2*$^{-/-}$ cancer cells (**Fig. 3b**), in agreement with the noted

histological differences (**Fig. 2a**). Consistently, gene ontology (GO) term analysis revealed altered expression of pathways involved in 1) regulation of the adaptive immune system and in 2) response to immune-mediated cell killing (**Fig. 3c**).

Cancer cell CCR2 promotes immune suppression

The increased expression of genes associated with IFN response and antigen presentation, together with the prolonged growth delay and increased apoptosis in tumors from *Ccr2*$^{-/-}$ cancer cells, are consistent with an ongoing adaptive immune response in the tumors. We therefore determined by flow cytometry whether there were any differences in the infiltrating immune cell populations (for gating strategy, see **Fig. S3**). There was a significant shift toward more CD8+ CTLs and fewer CD4+ helper T cells in tumors from *Ccr2*$^{-/-}$ cancer cells than in tumors from *Ccr2*$^{+/+}$ cancer cells (**Fig. 4a**), with no overall change in CD3+ T cell infiltration (**Fig. S4a**). PD-1 is a checkpoint surface receptor found on activated CD8+ T cells (Chen et al., 2016), and its expression was increased on CD8+ T cells from tumors from *Ccr2*$^{-/-}$ cancer cells (**Fig. 4b**). CTLs kill, in part, through the release of granzyme B, a process that requires lysosomal-associated membrane protein 1 (LAMP-1, also known as Cluster of Differentiation 107a, CD107a). In tumors from *Ccr2*$^{-/-}$ cancer cells, LAMP-1 was increased on the cell surface of CD8+ T cells (**Fig. 4c**), while granzyme B levels were reduced in CD8+ T cells (**Fig. 4d**), indicating increased levels of prior CD8+ degranulation, and therefore cytotoxic activity. Unexpectedly, regulatory T lymphocytes (CD4+FoxP3+ regulatory T cells), which are typically immunosuppressive, were present at elevated levels in tumors from *Ccr2*$^{-/-}$ cancer cells (**Fig. S4b**).

Tumors from *Ccr2*$^{-/-}$ cancer cells had multiple changes in the adaptive immune cell infiltrate, all consistent with an active immune response. Therefore, we tested whether an adaptive immune response contributed to the differences in growth rates between the *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ tumors by transplanting cancer cells in parallel into immunosuppressed athymic (nude) or fully immunocompetent mice (**Fig. 5a**).

In the immunocompetent hosts, tumors from *Ccr2*$^{-/-}$ cancer cells grew significantly slower than tumors from *Ccr2*$^{+/+}$ cancer cells (**Fig. 5b**), as previously observed (**Fig. 1g**); however, in the T cell-deficient athymic hosts, the *Ccr2*$^{-/-}$ tumors grew almost as fast as the *Ccr2*$^{+/+}$ tumors (**Fig. 5c**). Furthermore, in the athymic hosts, tumors from *Ccr2*$^{-/-}$ cancer cells no longer had more apoptotic cells than tumors from *Ccr2*$^{+/+}$ cancer cells (**Fig. 5d**). These data further suggest that CCR2 expressed by cancer cells enables the tumors to escape the adaptive immune response. The differentiated histological phenotype of the tumors from the *Ccr2*$^{-/-}$ cancer cells was maintained in the athymic mice (**Fig. 5e**). Thus, the histological differences were not the result of an altered adaptive immune response.

We next speculated that the reduced growth of tumors from *Ccr2*$^{-/-}$ cancer cells could be a result of increased sensitivity to CTL-induced cell death, given that the *Ccr2*$^{-/-}$ cancer cells showed increased cell death in serum-free conditions. To test this possibility, we used the MMTV-PyMT-chOVA model (Engelhardt et al., 2012), which is driven by the PyMT oncogene and co-expresses ovalbumin (OVA) as a model tumor antigen and mCherry for tracking. We generated MMTV-PyMT-chOVA;*Ccr2*$^{+/+}$ and MMTV-PyMT-chOVA;*Ccr2*$^{-/-}$ mice and isolated primary cancer cells from the resulting tumors. When these cancer cells were challenged with activated CTLs isolated from OT-1 mice (where T cells are engineered to recognize an OVA peptide presented by MHC class I), we found that *Ccr2*$^{-/-}$ cancer cells were approximately twice as sensitive to CTLs as *Ccr2*$^{+/+}$ cancer cells (**Fig. 6a**). This difference also persisted when exogenous OVA peptides were added to the cancer cells. Upon treatment with a pharmacological inhibitor against CCR2 (RS 102895 hydrochloride (Mitchell et al., 2013)), the MMTV-PyMT-chOVA;*Ccr2*$^{+/+}$ cancer cells became as sensitive to the OVA-specific CTLs as the MMTV-PyMT-chOVA;*Ccr2*$^{-/-}$ cancer cells (**Fig. 6b**). In contrast, the CCR2 inhibitor had no effect on the specific cytotoxicity of the CTLs to the MMTV-PyMT-chOVA;*Ccr2*$^{-/-}$ cancer cells.

To test if *Ccr2* expression in cancer cells also regulated sensitivity to CTLs *in vivo,* we measured the clonal expansion of T cells, an indicator of active proliferation of antigen-specific T cells, by sequencing the complementarity determining region 3 (CDR3) region of the T cell receptor (TCR) β chain. We found that clonality, a value corresponding to the extent of clonal expansion of T cells (**Fig. 6c**), was significantly higher in tumors from $Ccr2^{-/-}$ cancer cells than in tumors from $Ccr2^{+/+}$ cancer cells (**Fig. 6d**), while there was no difference in clonality in the spleens of these groups of mice (**Fig. 6e**). These results suggested that tumor-recognizing CTLs were actively expanding due to antigen recognition in tumors from $Ccr2^{-/-}$ cancer cells, but that this expansion did not alter the systemic T cell response. Both $Ccr2^{+/+}$ and $Ccr2^{-/-}$ cancer cells express the PyMT tumor antigen, and $Ccr2^{+/+}$ cancer cells grew at the same rate regardless of whether $Ccr2^{+/+}$ or $Ccr2^{-/-}$ cancer cells were transplanted to the contralateral mammary gland (**Fig. S4c**). Together, these results suggest that the expression of CCR2 by cancer cells enables the tumors to establish a locally immune-suppressive microenvironment.

Cancer cell CCR2 alters MHC class I and PD-L1 expression

CCR2 signaling can activate STAT transcription factors in cancer cells (Chen et al., 2015; Izumi et al., 2013), and altered STAT1 or STAT3 signaling in cancer cells can lead to a less immune-suppressive microenvironment (Ahn et al., 2017; Jones et al., 2016). Therefore, we tested whether STAT activation was altered in the $Ccr2^{-/-}$ cancer cells. We found no consistent differences between the activation of STAT1, STAT3, or p65/RELA when whole tumor lysate was assayed by Western blot (**Fig. 7a**). By immunohistochemistry, we found that the tumors contained patches of cancer cells with high expression of phopho-STAT1, with more of these patches in the tumors from $Ccr2^{-/-}$ than from $Ccr2^{+/+}$ cancer cells (**Fig. 7b, c**). No difference was observed for phospho-STAT3. These data are consistent with the possible involvement of STAT1 signaling as a regulator of an immune-suppressed microenvironment.

We next sought to understand how CCR2 signaling in cancer cells resulted in more efficient immune suppression. RNA-seq analysis of the cancer cells showed increased expression of genes involved in antigen presentation in the *Ccr2*$^{-/-}$ tumors (**Fig. 3c**). Consistently, we found high levels of MHC class I on *Ccr2*$^{-/-}$ cancer cells and almost undetectable levels on *Ccr2*$^{+/+}$ cancer cells in immunocompetent host mice by immunohistochemistry (**Fig. 8a, b**). However, in immune-deficient host mice, MHC class I levels were high on both *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ cancer cells (**Fig. 8a, b**), suggesting that MHC class I expression is not directly regulated by CCR2 signaling in the cancer cells, but rather is regulated by the different immune responses between these tumors.

PD-L1 is a potent suppressor of T cell activation and proliferation and is expressed on many types of cancer cells (Freeman et al., 2000). There was significantly higher expression of PD-L1 on *Ccr2*$^{+/+}$ cancer cells than on *Ccr2*$^{-/-}$ cancer cells (**Fig. 8c**). Since both MHC class I and PD-L1 are IFN-γ responsive genes, we compared IFN-γ levels between tumors from *Ccr2*$^{+/+}$ or *Ccr2*$^{-/-}$ cancer cells, but detected no differences in mRNA levels from whole tumor lysate (**Fig. S5a**) or in IFN-γ protein expression in infiltrating CD8+ T cells (a major source of IFN-γ) (**Fig. S5b**). *In vitro,* recombinant IFN-γ treatment resulted in similar upregulation of *Pd-l1* mRNA levels in *Ccr2*$^{+/+}$ *and Ccr2*$^{-/-}$ cancer cells (**Fig. 8d**). Thus, although we were unable to identify the means of PD-L1 regulation, *Ccr2*$^{-/-}$ cancer cells were still able to respond to IFN-γ by upregulating PD-L1. Interestingly, we found a similar strong positive correlation between *CCR2* and *PD-L1* expression in human breast cancer (**Fig. 8e**) when analyzing RNA-seq data from 1,245 invasive breast carcinoma samples (Cancer Genome Atlas, 2012). This correlation was found in all three major subtypes of breast cancer (**Fig. S5c**). Lastly, we tested if PD-L1 blockade would alleviate immune suppression of the MMTV-PyMT tumors *in vivo*. However, treatment with anti-PD-L1 therapy had no significant effect on the growth of *Ccr2*$^{+/+}$ tumors, and also, as expected, no effect on the low PD-L1-expressing *Ccr2*$^{-/-}$ tumors (**Fig. 8f**). Thus, although reduced PD-L1 likely contributed to an increased

sensitivity to CTLs in tumors from *Ccr2*$^{-/-}$ cancer cells, targeting PD-L1 expression was insufficient to reduce growth of the *Ccr2*$^{+/+}$ tumors.

Cancer cell CCR2 prevents the infiltration of cross-presenting CD103+ DCs

Since PD-L1 expression alone was not responsible for the immune suppression in *Ccr2*$^{+/+}$ tumors, we examined infiltration levels of immune populations that might be involved in immune suppression. There was no difference in CD11b+MHC class II+F4/80+ macrophages between tumors from *Ccr2*$^{+/+}$ and from *Ccr2*$^{-/-}$ cancer cells (**Fig. S5d**). There were, however, significantly fewer CD11b+MHC class II- myeloid cells in tumors from *Ccr2*$^{-/-}$ cancer cells (**Fig. S5e**). This population includes subsets of MDSCs, immature myeloid cells with immune-suppressive activity. Specifically, we found fewer granulocytes and granulocytic MDSCs (CD11b+CD11c-Ly6G+Ly6C^{low} cells) and more inflammatory monocytes and myeloid MDSCs (CD11b+CD11c-Ly6G-Ly6C^{high} cells) in tumors from *Ccr2*$^{-/-}$ cancer cells than from *Ccr2*$^{+/+}$ cancer cells (**Fig. S5f, g**).

DCs are critical for T cell activation, and we observed more than double the level of CD103+ DCs—a DC subtype that is very proficient in cross-presenting antigens to CD8+ T cells—in tumors from *Ccr2*$^{-/-}$ cancer cells than in those from *Ccr2*$^{+/+}$ cancer cells (**Fig. 9a**). The number of CD103+ DCs was also increased in the draining lymph nodes (dLNs) for these tumors (**Fig. 9b**). Furthermore, DCs in tumors from *Ccr2*$^{-/-}$ cancer cells displayed higher levels of CD86, a surface marker upregulated on activated DCs (**Fig. 9c**). A concern when using a transplant system to evaluate immune response is the potential for an artificial immune reaction toward antigens on the transplanted cells. However, there were also more CD103+ DCs in the spontaneously developing tumors in MMTV-PyMT;*Ccr2*$^{-/-}$ mice than in MMTV-PyMT;*Ccr2*$^{+/+}$ mice (**Fig. S5h**). The difference in CD103+ DC numbers was already apparent in the dLNs two days after injection of irradiated cancer cells into the mammary fat pad (**Fig. 9d**), suggesting a direct

signal from the cancer cells. However, infiltration of CD103+ DCs was not increased in tumors from *Ccr2*$^{-/-}$ cancer cells in immune-compromised mice (**Fig. 9e**). This finding suggests a feedback loop between T cells and DCs that is critical for CD103+ DC recruitment to the tumors.

We next asked whether a factor secreted by *Ccr2*$^{-/-}$ cancer cells was responsible for DC maturation toward the CD103+ DC subtype. Indeed, when bone marrow-derived cells were cultured with conditioned medium from *Ccr2*$^{-/-}$ rather than from *Ccr2*$^{+/+}$ cancer cells, the total number of DCs was not different, but a significantly higher percentage of CD11c+ cells were positive for CD103+ (**Fig. 10a**). When the conditioned medium was collected from *Ccr2*$^{+/+}$ cancer cells treated with the CCR2 inhibitor, the percentage of CD103+CD11c+ cells also increased (**Fig. 10b**), whereas adding the CCR2 inhibitor directly to the bone marrow-derived cultures did not by itself increase the percentage of CD103+ cells. Flt3 ligand and granulocyte-macrophage colony-stimulating factor (GM-CSF) can both stimulate DC maturation toward the CD103+ DC subtype (Broz et al., 2014). We found little flt3 ligand in the conditioned medium from the MMTV-PyMT-derived cancer cells, but a trend toward higher GM-CSF levels in the conditioned medium from *Ccr2*$^{-/-}$ cancer cells than in that from *Ccr2*$^{+/+}$ cancer cells was noted (**Fig. 10c**). Lastly, to test the importance of the CD103+ DCs in regulating an immune-controlling microenvironment, we transplanted *Ccr2*$^{-/-}$ and *Ccr2*$^{+/+}$ cancer cells into *Batf3*$^{-/-}$ mice, which lack the transcription factor basic leucine zipper transcription factor ATF-like 3 (BATF3) critical for CD103+ DC maturation. In these mice, the growth of *Ccr2*$^{-/-}$ cancer cell-derived tumors was restored to that of the *Ccr2*$^{+/+}$ cancer cell-derived tumors (**Fig. 10d**), and as expected, the percentage of infiltrating CD103+ DCs was significantly reduced (**Fig. 10e**).

DISCUSSION

The tumor-promoting effects of CCR2 are well-documented. However, they have mostly been attributed to CCR2-expressing immune and other host cells (Lim et al., 2016; Nakasone et al., 2012; Qian et al.,

2009; Qian et al., 2011; Wolf et al., 2012). Nevertheless, CCR2 is upregulated on breast cancer cells compared to normal breast epithelium, and cancer cell expression of CCR2 has been proposed to promote tumor growth through increased motility and invasion, cell proliferation, and cell survival (Fang et al., 2012; Lu et al., 2006). Here, we show that in the MMTV-PyMT mouse model, CCR2 signaling in cancer cells helps breast tumors establish a locally immune-suppressive microenvironment. Compared to tumors from *Ccr2*$^{+/+}$ cancer cells, the tumors from *Ccr2*$^{-/-}$ cancer cells have higher infiltration of CD103+ DCs and activated CD8+ T cells, as well as lower infiltration of granulocytic MDSCs. Furthermore, the *Ccr2*$^{-/-}$ cancer cells have higher surface levels of MHC class I and lower levels of PD-L1. It is likely the combination of all of these changes that results in more effective immune surveillance of the *Ccr2*$^{-/-}$ cancer cells and reduced growth of tumors from these cells (**Fig. 10f**). To our knowledge, the ability of cancer cell CCR2 signaling to orchestrate tumor immune suppression has not previously been recognized.

One of the most notable changes caused by targeting cancer cell CCR2 expression was the increased levels of CD103+ DCs in tumors from *Ccr2*$^{-/-}$ cancer cells. In *Batf3*$^{-/-}$ mice, which lack the transcription factor BATF3 required for CD103+ DC development, tumors from *Ccr2*$^{-/-}$ cancer cells grew as fast as tumors from *Ccr2*$^{+/+}$ cancer cells, supporting previous work showing that regulation of CD103+ DC infiltration is a critical step in tumor immune surveillance (Broz et al., 2014; Meyer et al., 2018; Roberts et al., 2016). Our data suggest that CCR2 signaling regulates the secretion of a factor(s) from the cancer cells that affects the polarization of DCs to the CD103+ subtype. Several factors have been proposed to affect the polarization of CD103+ DCs, including GM-CSF (Mayer et al., 2014), which tended to be secreted at higher levels by *Ccr2*$^{-/-}$ cancer cells than by *Ccr2*$^{+/+}$ cancer cells. However, it is unclear if GM-CSF could be solely responsible for the difference in CD103+ DC infiltration between tumors from *Ccr2*$^{+/+}$ or from *Ccr2*$^{-/-}$ cancer cells. A recent study reported that G-CSF secreted from MMTV-PyMT cancer cells can inhibit the differentiation of CD103+ DCs by downregulating interferon regulatory factor-

8 in DC progenitors (Meyer et al., 2018). Interestingly, G-CSF was one of only two cytokines found with reduced levels in MMTV-PyMT;*Ccr2*$^{-/-}$ tumors compared to MMTV-PyMT;*Ccr2*$^{+/+}$ tumors. However, secretion of G-CSF from isolated cancer cells was not reduced, suggesting that its altered secretion is not directly an effect of CCR2 signaling in cancer cells. Both G-CSF and GM-CSF are given as recombinant proteins to support the bone marrow with certain types of chemotherapy to reduce the risk of lethal febrile neutropenia (Mehta et al., 2015). However, preclinical data suggest that these cytokines play complex roles in regulating the immune response against tumors: they appear to have opposing effects on CD103+ DC differentiation, with GM-CSF stimulating CD103+ DC differentiation (Greter et al., 2012) and G-CSF inhibiting it (Meyer et al., 2018). However, although GM-CSF secretion in the tumor microenvironment potentially enhances immune responses against tumors by stimulating CD103+ DC differentiation, this cytokine can also mobilize immune-suppressive MDSCs, leading to suppression of an anti-tumor immune response (Bayne et al., 2012; Pylayeva-Gupta et al., 2012). Exploring therapeutic administration of blocking antibodies against G-CSF rather than recombinant GM-CSF with immune therapy could therefore be an important future direction.

Ccr2$^{-/-}$ cancer cells had multiple expression changes, including many that were consistent with an IFN response. Among these, increased surface expression of MHC class I might explain why *Ccr2*$^{-/-}$ cancer cells are more sensitive to T cell-mediated killing than *Ccr2*$^{+/+}$ cancer cells. However, since *Ccr2*$^{+/+}$ cancer cells did not downregulate MHC class I in nude mice, which lack T cells, we speculate that MHC class I expression is not directly regulated by CCR2 signaling in the cancer cells. Rather, the altered expression of IFN response genes, including MHC class I, may be an indirect result of an immune response in the tumors.

Results from an open-label, non-randomized phase I trial using a CCR2 inhibitor against locally invasive pancreatic cancer suggested that the inhibitor enhanced chemotherapy response, reduced

macrophage infiltration, and induced an anti-tumor adaptive immune response (Nywening et al., 2016). Our data suggest that the potential clinical benefits of CCR2 inhibitors might be caused by the combined inhibition of CCR2 signaling in both stromal and cancer cells. Interestingly, the loss of even one *Ccr2* allele was sufficient to reduce tumor growth in our study, suggesting that partial inhibition of CCR2 could result in clinical benefit. It is unclear why $Ccr2^{+/-}$ cancer cells phenocopy the $Ccr2^{-/-}$ cancer cells, but this phenomenon could be due to reduced dimerization of CCR2 in $Ccr2^{+/-}$ cancer cells, consistent with a prior report (Rodriguez-Frade et al., 1999), or because surpassing a threshold level of CCR2 signaling is required to induce immune suppression.

Despite the data suggesting that CCR2 inhibition has beneficial effects in cancer treatment, some studies have raised serious concerns about targeting the CCR2 ligand. Cessation of anti-CCL2 therapy resulted in increased pulmonary metastasis and decreased survival in a murine model of breast cancer, due to rapid mobilization of CCR2-expressing monocytes into circulation immediately upon cessation of therapy (Bonapace et al., 2014). Therefore, CCR2 inhibition likely will have to be used in combination with other therapies to maximize the eradication of existing cancer cells and to avoid complications of discontinuing the inhibition.

Our findings support the notion that the CCL2/CCR2 axis is an immune modulatory pathway in cancer, but with the added layer of complexity that cancer cells can hijack this chemokine receptor to orchestrate immune suppression. Preclinical experiments suggest that CCR2 targeting strategies can have detrimental effects, including the promotion of metastasis (Bonapace et al., 2014; Li et al., 2013). Therefore, future work that identifies the precise mechanisms by which CCR2 signaling in cancer cells induces immune suppression in breast cancer may set the stage for developing novel immune modulatory therapies. There are ongoing clinical trials using pharmacological inhibitors of CCR2 in cancer patients,

and our data suggest that the potential effects of inhibiting CCR2 signaling in cancer cells should be taken into account when interpreting the results from these trials.

MATERIALS AND METHODS

Mice

Ccr2$^{-/-}$, *Batf3*$^{-/-}$, and OT-1 mice were obtained from Jackson Laboratory (Bar Harbor, ME) and nude mice from Charles River Laboratory (Wilmington, MA). MMTV-PyMT mice (on C57BL/6 [BL/6 for short] background) were bred at Cold Spring Harbor Laboratory (CSHL), and MMTV-PyMT-chOVA mice (Engelhardt et al., 2012) were a kind gift from Dr. Mathew Krummel (University of California, San Francisco). MMTV-PyMT and PyMT-chOVA mice were intercrossed with *Ccr2*$^{-/-}$ mice. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at CSHL and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Measurement of *in vivo* tumor growth and lung metastasis

Tumor onset was determined by weekly palpation. Once a tumor was detected, its size was measured weekly by caliper, and tumor volume was calculated as (length x width^2)/2. Mice were sacrificed at either of two IACUC-approved endpoints: when tumors reached 25 mm or ulcerated. To determine metastatic burden, we adapted a previously published method (Nielsen et al., 2001). Briefly, lungs were placed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) in a vacuum desiccator for 1 h. The lungs remained in 4% PFA at 4°C for 48 h, followed by incubation in 20% sucrose in PBS at 4°C for 48 h. Lungs were embedded in Tissue-Tek O.C.T. compound embedding solution (Sakura, Torrance, CA). The blocks were then placed in a custom-made cutting chamber with razor blade inserts every 2 mm, and the block was cut into 2 mm sections. These sectioned lung pieces were then re-embedded in fresh O.C.T. to allow for cross-sectional cuts from the entire lung tissue. These cross-sections of the lungs were stained

with H&E and scanned by an Aperio ScanScope® CS System (Leica Biosystems, Buffalo Grove, IL). The metastatic burden was calculated as the percentage of metastasis/lung area and the number of foci/lung area, determined using Aperio eSlide Capture Devices software (Leica Biosystems).

Primary cancer cell isolation, culture, and transplantation

Aged-matched virgin females (C57BL/6, *Ccr2*$^{-/-}$, *Batf3*$^{-/-}$, or *Nu/Nu*), 6-12 weeks of age, were used as hosts for transplantation. Cancer cells were isolated from 2-3 tumors, each one 8-10 mm in diameter, from MMTV-PyMT;*Ccr2*$^{+/+}$ or MMTV-PyMT;*Ccr2*$^{-/-}$ mice. Tumors were mechanically dissociated and digested for 1 h with 1x collagenase/hyaluronidase (10X Solution, Stem Cell Technology, Cambridge, MA) containing DNase I (2 U/ml, Roche, Pleasanton, CA) in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 5% FBS (VWR Life Science Seradigm, Philadelphia, PA). Single cells and debris were removed from the resulting carcinoma organoid preparation by pulse centrifugation in HBSS supplemented with 5% FBS. Purified carcinoma organoids were dissociated into single cell suspension in 0.05% trypsin with 0.1% EDTA supplemented with 2 U/ml of DNase I (Roche) for 2-3 min. Single tumor cells were passed through a 100 μm cell strainer (BD Biosciences, San Jose, CA) and either plated in RPMI-1640 supplemented with 10% FBS for *in vitro* experiments or washed with PBS and immediately injected into the inguinal mammary glands of host mice (2.5×10^5 in 20 μl of 1:1 PBS/Matrigel; Corning, Corning, NY).

To transplant irradiated cancer cells, freshly isolated primary MMTV-PyMT;*Ccr2*$^{+/+}$ or MMTV-PyMT;*Ccr2*$^{-/-}$ cancer cells were plated in a 10 cm petri dish (2×10^6 per dish) in RPMI with 10% FBS, and received 80 Gy of irradiation in the Gammacell 40 Exactor (Best Theratronics, Ottawa, Ontario, Canada). After irradiation, cancer cells were washed with PBS and injected into the inguinal mammary glands of host mice (2.5×10^5 in 20 μl of PBS). To generate cancer cell conditioned medium, primary cancer cells

from either MMTV-PyMT;*Ccr2*$^{+/+}$ or MMTV-PyMT;*Ccr2*$^{-/-}$ tumors were plated in a 10 cm dish (2x10^6 per dish) in DMEM/F12 medium plus 10% FBS, 1% penicillin/streptomycin, with or without CCR2 antagonist (RS504393, 10 μM, Sigma-Aldrich, St. Louis, MO) for two days. The conditioned medium was collected and centrifuged at 300 x g for 10 min, and the supernatant was used in experiments.

To determine surface MHC class I expression, primary cancer cells from MMTV-PyMT;*Ccr2*$^{+/+}$ tumors were plated in a 6-well plate (1x10^6 per well) in DMEM/F12 medium plus 10% FBS, 1% penicillin/streptomycin, with or without CCR2 antagonist (RS504393, 10 μM, Sigma-Aldrich) or recombinant mouse CCL2 (479-JE, 10 ng/ml, R&D Systems, Minneapolis, MN) for 24 or 48 h as indicated in the legend.

In vivo anti-PD-L1 treatment

Tumor cells from MMTV-PyMT;*Ccr2*$^{+/+}$ or MMTV-PyMT;*Ccr2*$^{-/-}$ mice were transplanted as described above. On days 15, 18, 21, and 24 after transplantation, when tumors had formed, mice received 200 μg of anti-PD-L1 antibody by intraperitoneal injection (Clone 10F.9G2; Bio X Cell, West Lebanon, NH) or control rat IgG2b antibody (Bio X Cell) (Winograd et al., 2015). Tumor size was measured biweekly, and mice were sacrificed at IACUC-approved endpoint.

Analysis of human RNA-seq datasets

The invasive breast carcinoma patient RNA-seq dataset used for the analysis (n=1,215) was obtained from The Cancer Genome Atlas (TCGA). The dataset was loaded into a Jupyter notebook using pandas and processed using Python 3. The RSEM (RNA-Seq by Expectation Maximization) mean read counts for genes of interest were correlated in a pairwise manner, and corresponding statistical analysis was performed using NumPy. The correlation graphs and linear regression fits were plotted using matplotlib.

For subtype analysis, TCGA-designated subtype labels based on PAM50 gene set expression were used. Datasets without a designated subtype label were excluded from the analysis.

Flow cytometry

Tumors were harvested and mechanically dissociated for 30 min with Collagenase D (2 mg/ml; Sigma-Aldrich) and DNase I (4 U/ml; Roche) in RPMI. For flow cytometry of cells from lymph nodes, the lymph nodes were forced through a 100 μm cell strainer (BD Biosciences), and flow-through cells were collected for experiments. Cells were resuspended in 1x HBSS supplemented with 0.5% BSA and centrifuged at 300 x g. The cell suspension was filtered through a 70 μm cell strainer, and red blood cells were lysed using red blood cell lysing buffer (Sigma-Aldrich) for 1 min at room temperature.

For flow cytometry staining, cells (1×10^6) were incubated with mouse Fc Block (clone 2.4G2, BD Biosciences) for 10 min on ice, and then were stained with the appropriate antibodies to surface markers at 4°C for 30 min in the dark or were permeabilized and stained with intracellular antibodies overnight (see below for antibodies). Cell viability stain Zombie Red (BioLegend, San Diego, CA) was used to differentiate between dead and live cells. The stained populations were analyzed using an LSR II flow cytometer (BD, Franklin Lakes, NJ) (see **Fig. S2** for gating strategies) and FlowJo software (BD, Version 10). Antibodies: CD45-eFluor 450 (Clone 30-F11), CD11c-PE-Cy7 (Clone N418), MHC II (I-A/I-E)-APC-eFlour780 (Clone M5/114.15.2), CD103-FITC (Clone 2E7), CD274 (B7-H1, PD-L1)-PE-Cy7 (Clone MIH5), CD3-FITC (Clone 17A2), CD8-eFluor450 (Clone 53-6.7), CD107a (LAMP-1)-PE (Clone 1D4B), IFN-γ-PE (Clone XMG1.2), Granzyme B-FITC (Clone NGZB), γδ TCR-PE-Cy5 (Clone GL3), and FoxP3-PE (Clone NRRF-30) all from eBioscience (Waltham, MA); CD4-PerCP-Cy5.5 (Clone BM9), CD69-PE (Clone H1.2F3), F4/80-PerCP-Cy5.5 (Clone BM9), and CD86-BV510 (Clone GL-1) all from BioLegend; CD45-APC (Clone 30-F11), Ly6G-FITC (Clone 1A8), and CD11b-PE (Clone M1/70) from

BD Pharmingen; EpCAM-APC (Clone CI:A3-1, AbD Serotec [Hercules, CA]) and CCR2-flourescein (Clone 475301, R&D Systems). Prior to Granzyme B staining, cells were incubated for 2 h with Brefeldin A (Sigma-Aldrich, #B6542).

Cytokine array

Tumors (8-10 mm in diameter) were isolated from MMTV-PyMT;*Ccr2*$^{+/+}$ and MMTV-PyMT;*Ccr2*$^{-/-}$ mice and immediately snap-frozen in liquid nitrogen. They were then homogenized in PBS with the addition of protease inhibitors (Promega, Madison, WI). Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D Systems) was used according to the manufacturer's instructions. Films were scanned, and spots were analyzed using ImageJ software.

MTS assay

Proliferation of cancer cells isolated from MMTV-PyMT;*Ccr2*$^{+/+}$ and MMTV-PyMT;*Ccr2*$^{-/-}$ mice was measured using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS; Promega) according to the manufacturer's instructions. In this assay, the MTS tetrazolium compound (Owen's reagent) was bioreduced by metabolically active cells into a colored formazan product. The quantity of formazan product was measured in a 96-well plate by absorbance at 490 nm.

RNA purification and real-time quantitative PCR (RT-qPCR) analysis

Cancer cells, isolated as described above, were cultured for 48 h in RPMI supplemented with 10% FBS (Seradigm), followed by 24 h in serum-free RPMI. Total RNA was extracted using an RNAeasy kit (Qiagen, Germantown, MD), following the manufacturer's instructions. RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Reverse transcription was

performed with an ImProm-II Reverse Transcription System (Promega) with the following cycling conditions: 5 min at 70°C, 10 min at 4°C, 5 min at 25°C, 60 min at 42°C, and 15 sec at 70°C using 1 µg of total RNA extract for 20 µL of final volume. RT-qPCR analysis was performed using a TaqMan gene expression assay (Thermo Fisher Scientific) with *Ccr2*- and *Pd-l1*-specific primers (from (Boring et al., 1997) and Thermo Fisher Scientific (Mm00438270_m1 and Mm00452054_m1). RT-qPCR was performed on three independently isolated cancer cell populations and in triplicate for each sample. Relative quantitation was performed with the $2^{(-\Delta\Delta CT)}$ method using β-Actin expression for normalization and MMTV-PyMT;$Ccr2^{+/+}$ cancer cells as a reference sample.

RNA *in situ* hybridization

RNA *in situ* hybridization was performed on PFA-fixed paraffin-embedded tissue sections using an RNAscope Chromogenic 2.0 Detection Kit (Advanced Cell Diagnostics [ACD], Inc., Newark, CA). A *Ccr2* probe was custom-designed by ACD, Inc. (Catalog number 436261-C2) and used according to the manufacturer's instructions. We confirmed that no signal was found with the probe using $Ccr2^{-/-}$ tissues.

Hematoxylin and eosin staining

PFA-fixed paraffin-embedded tissue sections were deparaffinized and rehydrated following standard protocols, stained with Gill's Hematoxylin (Thermo Fisher Scientific, REF6765008) for 3 min, washed in tap water, rinsed with Nu-Clear II (Thermo Fisher Scientific, REF6769009), and then rinsed again first with tap water and then with Bluing Reagent (Thermo Fisher Scientific, REF 6769001). Slides were then stained with eosin (Sigma, HT110180), dehydrated following standard protocols, air-dried, and mounted with Cytoseal 60 (Thermo Fisher Scientific, Catalog number 831016).

Immunofluorescence staining

Frozen tissue sections were incubated with 1x blocking buffer (5% goat serum, 2.5% BSA in PBS) and Fc receptor blocker (Innovex Biosciences, Richmond, CA). Sections were incubated with Rabbit anti-CD3 polyclonal antibody (1:500 dilution; Abcam, Cambridge, MA) or Rat anti-MHC class I monoclonal antibody (Clone ER-HR52, 1:100 dilution; Abcam) in 0.5x blocking buffer overnight at 4°C. Secondary antibody, anti-Rabbit-Alexa568, or anti-Rat-Alexa568 (1:150 dilution; Life Technologies) was used for detection, and sections were counterstained with DAPI (1:200 dilution; Life Technologies). Images were collected at 40x magnification using an AX10 microscope and an AxioCam HRc camera (Zeiss, Thornwood, NY) and were analyzed using Volocity software (Version 6.3.0, PerkinElmer, Waltham, MA).

Immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Sections were deparaffinized and rehydrated for phospho-STAT3 and Ki67 staining. Antigen retrieval was carried out by boiling slides in 10 mM sodium citrate buffer (pH 6.0) for 6 min in a pressure cooker, while for phospho-STAT1 staining, antigen retrieval was done in Tris EDTA buffer (10 mM Tris Base, 1 mM EDTA, pH 9.0). The slides were blocked with 3% hydrogen peroxide, 1x blocking buffer (PBS containing 2.5% BSA and 5% goat serum), Fc receptor blocker (Innovex Biosciences), and finally avidin/biotin blocking buffer (Vector Laboratories, Burlingame, CA, SP-2001). Slides were then incubated with primary antibodies overnight at 4°C in 0.5x blocking buffer: rabbit anti-Ki67/MK167 (1:1,000 dilution; Novus International, Saint Charles, MO, NB110-89717), rabbit anti-phospho-STAT1 (1:200 dilution; Cell Signaling Technology, Danvers, MA), or rabbit anti-phospho-STAT3 (1:500 dilution; Cell Signaling Technology). After incubating with secondary biotinylated goat anti-rabbit IgG

antibody (1:500, Vector Laboratories, BA-1000) for 1 h at room temperature, slides were incubated with avidin-conjugated horseradish peroxidase (Vector Laboratories, PK-6100) for 30 min, and the signals were detected by a DAB (3,3'-Diaminobenzidine) substrate kit (Vector Laboratories, SK-4100). Lastly, sections were counter-stained with hematoxylin. TUNEL staining was performed on deparaffinized sections to detect late-stage apoptotic cells using the ApopTag peroxidase *in situ* apoptosis detection kit (Millipore, Burlington, MA), according to the manufacturer's instructions. Images were scanned by an Aperio ScanScope® CS System (Leica Biosystems), and positive nuclei were counted using Aperio eSlide Capture Devices software (Leica Biosystems).

ImmunoSEQ

Tumors from *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ transplants were isolated and immediately placed in liquid nitrogen for shipment. Amplification and sequencing were performed on the immunoSEQ platform (Adaptive Biotechnologies, Seattle, WA) using a multiplex PCR-based assay that exclusively targets rearranged T cell receptor genes. Sequencing data were analyzed using immunoSEQ Analyzer software. Shannon's Entropy (H), a measure of the richness and uniformity of the frequency of the T cell receptor repertoire distribution, was defined as:

$$H = -\sum_{i=1}^{N} P_i \log_2 P_i$$

Where N equals the number of unique clones and P_i the frequency of clones. To account for variation in sequencing depth, entropy was normalized by its maximum value (H_{max}):

$$H_N = H / H_{max}$$

Clonality is defined as C = 1 - H_N. (Sherwood et al., 2013). Sequencing files are available from the Adaptive Biotechnologies immunoSEQ web site.

Transcriptional profiling and bioinformatics analysis

Ccr2$^{+/+}$ or *Ccr2*$^{-/-}$ cancer cells were isolated from tumors and sorted as EpCAM+CD45-CD31- live cells (using 7-AAD Viability Staining Solution, Thermo Fisher Scientific) four weeks after orthotopic transplantation into the mammary glands of wild-type C57BL/6 mice. Total cancer cell RNA was extracted using a TRIzol extraction protocol. cDNA libraries were prepared using the Ovation® RNA-Seq System V2 (NuGEN Technologies, San Carlos, CA) according to the manufacturer's protocol and sequenced using a NextSeq 500 instrument (Illumina, San Diego, CA) to obtain 388 million 76 bp single-end reads. Reads were mapped to the reference mm9 mouse genome using Spliced Transcripts Alignment to a Reference (STAR) (Dobin et al., 2013) and were determined using HTSeq (Anders et al., 2015). Differential gene expression analysis was performed using DESeq2 (Love et al., 2014) with a false discovery rate cutoff of 5%. Hierarchical clustering was performed by sample and gene using normalized and log2-transformed gene expression values of differentially expressed genes. All of the analyses described above were performed on the Bioinformatics Shared Resource Galaxy server at CSHL. Gene set enrichment analysis (Subramanian et al., 2005) was performed on a pre-ranked gene list sorted by log2FoldChange value against v5.1 Hallmark gene sets using default parameters. Gene ontology term enrichment analysis was performed using GOrilla PANTHER (Eden et al., 2009).

Bone marrow-derived dendritic cell (BMDC) isolation, culture, and activation

BMDCs were Isolation and cultured as previously described (Inaba et al., 1992). Briefly, bone marrow was obtained from female C57BL/6 mice by flushing the femur and tibia with 2 ml of HBSS using a 1 ml insulin syringe with a 29G × ½ needle. The cells were washed with HBSS twice by centrifugation at 250 x g for 8 min and then suspended in BMDC culture medium (RPMI-1640 medium [Thermo Fisher

Scientific] containing 10% FBS, 20 ng/ml recombinant mouse GM-CSF [BioLegend], 50 units/ml penicillin, and 50 µg/ml streptomycin). The cells were then plated in 10 cm culture plates (2×10^6 cells per petri dish) and kept at 37°C in a 5% CO_2 incubator for three days, then split at day 4 into two plates. For activation of BMDCs with cancer cell conditioned medium, the non-attached BMDCs were harvested at day 6 by gently pipetting the cultures with medium. This BMDC population was then rinsed three times in HBSS and suspended in a) culture medium, b) conditioned medium from either MMTV-PyMT;*Ccr2*$^{-/-}$ or MMTV-PyMT;*Ccr2*$^{+/+}$ cancer cells, or c) conditioned medium from MMTV-PyMT;*Ccr2*$^{+/+}$ cancer cells cultured with CCR2 antagonist (RS504393, 10 µM, Sigma-Aldrich). After incubation with conditioned medium for 24 h, non-adherent BMDCs were harvested for flow cytometry.

Chromium release assay

Cytotoxic CD8+ T cells were isolated from spleens of OT-I transgenic mice by magnetic labeling and separation (Miltenyi Biotec, Cambridge, MA). Briefly, the spleen was forced through a 100 µm cell strainer (BD Biosciences), and flow-through cells were collected. After lysing red blood cells, the splenocytes were washed and incubated with CD8a+ T Cell Biotin-Antibody Cocktail, mouse (Miltenyi Biotec) at 4°C for 5 min, followed by incubating with anti-Biotin microbeads (Miltenyi Biotec) at 4°C for 10 min. The cell suspension was applied to an LS column (Miltenyi Biotec) in a magnetic field, and the flow-through (negatively selected) CD8+ T cells were collected. The isolated CD8+ T cells were cultured on plates coated with anti-mouse-CD3 (BioLegend) in RPMI-1640 containing 10% FBS, 50 µM of β-mercaptoethanol, 20 ng/ml mouse IL-2, 2 ng/ml mouse IL-7, and 1% penicillin/streptomycin for 6-9 days. Primary cancer cells were isolated as described above from MMTV-PyMT-chOVA;*Ccr2*$^{+/+}$ or MMTV-PyMT-chOVA;*Ccr2*$^{-/-}$ mice and cultured overnight with or without CCR2 antagonist (10 µM, Sigma RS504393) and OVA peptides (1 µg/ml) in DMEM/F12 medium with 10% FBS. For chromium (^{51}Cr)

release assay, cancer cells (1,000 per well) were incubated with 50 µl of ^{51}Cr solution (activity 1 mCi/ml, PerkinElmer) plus 50 µl of RPMI medium with 10% FBS for 2 h at 37°C. After rinsing the cells three times with RPMI plus 10% FBS, 50 µl of cancer cells (1,000) were plated into each well of a 96-well, V-bottom plate, followed by the addition of 50 µl of CD8+ T cells at a ratio of 10:1. After 4 h of incubation at 37°C, 50 µl of supernatant from each well was transferred to miniature vials, and radioactivity was determined by a Beckman scintillation counter (PerkinElmer). The percentage of specific lysis was calculated using the standard formula [(experimental - spontaneous release)/(total - spontaneous release) x 100] and expressed as the mean of triplicate samples.

Western blot

Snap-frozen tumor samples were homogenized in radio immunoprecipitation assay buffer with cOmplete™ Protease Inhibitor Cocktail Tablets (Roche) and phosphatase inhibitor cocktail (Thermo Fisher Scientific), and lysis was performed on ice for 30 min. Cell lysates were centrifuged for 15 min at 12,000 x g at 4°C. After centrifugation, the supernatant was mixed with 4x loading buffer plus 2.5% of β-mercaptoethanol and loaded on 8% SDS/PAGE gels. Protein bands were transferred to PVDF membranes (Bio-RAD) at 90 V for 2 h in a Bio-Rad Mini Trans-Blot system. The membrane was incubated sequentially with different primary antibodies: anti-p65, anti-p-p65, anti-p-STAT1, anti-p-STAT3, and anti-STAT3 (all from Cell Signaling Technology), and anti-STAT1 and anti-beta-Actin (from Santa Cruz Biotechnology, Dallas, Tx). Secondary antibodies were from LI-COR, (Lincoln, NE). Protein detection was performed using the Odyssey imaging system (LI-COR).

ELISA

Conditioned medium from *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ cancer cells was collected, and GM-CSF concentration was

evaluated by ELISA, following the manufacturer's instructions (R&D Systems). Briefly, 50 µl of cytokine standard or conditioned medium was added to the wells of the pre-coated ELISA plate and incubated at room temperature for 2 h. Plates were washed five times with wash buffer and incubated with a horseradish peroxidase-linked polyclonal antibody specific to mouse GM-CSF for 2 h, followed by incubation with substrate solution for 30 min. After adding 100 µL of stop solution, the optical density of each well was measured at 450 nm using a microplate reader. The concentrations of GM-CSF in the conditioned medium were calculated according to the standard curve.

Statistical analysis

All statistical analyses were performed using GraphPad Prism Version 6 software. Data were analyzed using Kaplan-Meier survival analysis, Student's t-tests, ANOVA, two-way ANOVA, chi-Square test, and log-rank (Mantel-Cox) test, as indicated in the figure legends, with an alpha of 0.05. The number of sampled units, N, is indicated in the figure legends.

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opinions, interpretations or policy of the State. **Competing interests:** D.T.F. is a co-founder of Myosotis, LLC, and a scientific advisory board member of iTeos Therapeutics, IFM Therapeutics, LLC, and Kymab. The other authors disclose no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

M.R.F. and M.E. designed the experiments. M.R.F. and X.Y.H. performed the *in vitro* experiments; M.R.F., C.O.d.S., and E.B. isolated RNA from cancer cells and performed the RNA-seq analysis. M.R.F. and X.Y.H. performed the animal experiments; A.S.A., M.R.F., A.P., and X.Y.H. performed flow cytometry; J.E.W. analyzed the histological sections; and M.R.F., X.Y.H, and A.E. performed immunohistochemistry and *in situ* hybridizations. X.Y.H., A.P., and M.R.F. designed and performed the chromium release experiments; and A.E., M.R.F., and X.Y.H. performed qPCR. D.T.F. provided experimental advice. M.R.F., X.Y.H., and M.E. wrote the manuscript.

Abbreviations

BMDC: bone marrow-derived dendritic cells

CCL2: C-C chemokine ligand 2

CCR2: C-C chemokine receptor type 2

chOVA: mCherry-ovalbumin

CTL: cytotoxic T lymphocyte

DC: dendritic cell

dLN: draining lymph node

G-CSF granulocyte colony-stimulating factor

GM-CSF: granulocyte-macrophage colony-stimulating factor

IACUC: Institutional Animal Care and Use Committee

IFN-γ: interferon gamma

LAMP-1: lysosomal-associated membrane protein 1

MDSC: myeloid-derived suppressor cell

MHC: major histocompatibility complex

MMTV: mouse mammary tumor virus

PD-L1: programmed cell death ligand 1

PFA: paraformaldehyde

PyMT: polyoma middle T

RPKM: reads per kilobase million

RPMI: Roswell Park Memorial Institute

TCGA: The Cancer Genome Atlas

TCR: T cell receptor

TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling

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FIGURE LEGENDS

Fig. 1: Cancer cell CCR2 promotes primary tumor growth, reducing overall survival

a. Tumor onset (tumor-free survival) is similar for MMTV-PyMT;*Ccr2*$^{+/+}$, MMTV-PyMT;*Ccr2*$^{+/-}$, and MMTV-PyMT;*Ccr2*$^{-/-}$ mice, as determined by weekly palpation and caliper measurement. N.S.=non-significant (Log-rank [Mantel-Cox] test; n=28 MMTV-PyMT;*Ccr2*$^{+/+}$, 20 MMTV-PyMT;*Ccr2*$^{+/-}$, and 18 MMTV-PyMT;*Ccr2*$^{-/-}$ mice).

b. Primary tumor growth is reduced in MMTV-PyMT;*Ccr2*$^{+/-}$ and MMTV-PyMT;*Ccr2*$^{-/-}$ mice compared to MMTV-PyMT;*Ccr2*$^{+/+}$ mice, as determined by weekly caliper measurement (mean +/- SEM, two-way ANOVA; n=21 MMTV-PyMT;*Ccr2*$^{+/+}$, 25 MMTV-PyMT;*Ccr2*$^{+/-}$, and 18 MMTV-PyMT;*Ccr2*$^{-/-}$ mice).

c. Time until IACUC-approved endpoint was increased for MMTV-PyMT;*Ccr2*$^{+/-}$ and MMTV-PyMT;*Ccr2*$^{-/-}$ mice compared to MMTV-PyMT;*Ccr2*$^{+/+}$ mice (Log-rank [Mantel-Cox] test; n=30 MMTV-PyMT;*Ccr2*$^{+/+}$, 35 MMTV-PyMT;*Ccr2*$^{+/-}$, and 29 MMTV-PyMT;*Ccr2*$^{-/-}$ mice).

d. Cancer cells express CCR2 (arrows) as determined by RNA *in situ* hybridization for *Ccr2* performed on paraffin-embedded tumor sections from MMTV-PyMT;*Ccr2*$^{+/+}$ mice. Image is representative of more than 10 mice. Scale bar=20 μm.

e. CCR2 is highly expressed on epithelial cells, as determined by flow cytometry performed on *Ccr2*$^{+/+}$ or *Ccr2*$^{-/-}$ tumors transplanted to C57BL/6 hosts. Anti-CCR2 primary antibody was gated on EpCAM+ cells and compared to background levels. Left panel shows one representative experiment and right panel depicts median fluorescence intensity for all experiments (mean +/- SEM, Student's t-test; n=5).

f. Schematic of transplantation experiments.

g. Primary tumor growth of *Ccr2*$^{-/-}$ cancer cells is reduced compared to that of *Ccr2*$^{+/+}$ cancer cells regardless of the genotype of the host, as determined by weekly caliper measurement (mean +/- SEM, two-way ANOVA; n=8 for all conditions).

h. Survival until IACUC-defined endpoint is increased in mice bearing *Ccr2*$^{-/-}$ cancer cells regardless of the genotype of the host (Log-rank [Mantel-Cox] test; n=8 for all conditions).

Fig. 2: Loss of CCR2 increases apoptosis *in vivo* with no effect on proliferation

a. Four representative photomicrographs of H&E-stained tumors from *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ cancer cells. Arrows denote cystic areas; also note lipid vacuoles in tumors from *Ccr2*$^{-/-}$ cancer cells (Scale bar=100 μm).

b. Proliferation is unchanged between *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ tumor transplants as determined by Ki67-positive nuclear stain. Left panels are representative photomicrographs (Scale bar=100 μm), and right panel shows quantification (mean +/- SEM, Student's t-test; n=10 and 9 for *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ transplants, respectively).

c. Proliferation is unchanged in *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ cancer cells *in vitro*, as determined by absorbance after 24 h using CellTiter 96 AQueous One Solution Cell Proliferation (MTS) Assay (mean +/- SEM, Student's t-test; n=4).

d. *Ccr2*$^{-/-}$ tumors have an increased apoptotic index, as determined by TUNEL stain. Left panels are representative photomicrographs, with arrows indicating apoptotic cells (Scale bar=100 μm), and right panel shows quantification (mean +/- SEM, Student's t-test; n=8 and 9 for *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ transplants, respectively).

e. *Ccr2*$^{-/-}$ cancer cells are less viable than *Ccr2*$^{+/+}$ cancer cells in serum-free conditions, as determined by absorbance in MTS assay after serum starvation for 24 h (mean +/- SEM, Student's t-test; n=3).

Fig. 3: *Ccr2*$^{-/-}$ cancer cells have increased expression of interferon (IFN) response genes and of genes involved in MHC class I antigen presentation

a. A hierarchically clustered heat map of *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ cancer cell samples based on expression levels of select genes associated with antigen processing and presentation, as well as epithelial-to-mesenchymal transition.

b. Gene set enrichment analysis plots showing enrichment of IFN response and luminal gene set expression in *Ccr2*$^{-/-}$ cancer cells (NES=normalized enrichment score)

c. Gene ontology (GO) term enrichment plots showing the most significantly overrepresented GO terms in *Ccr2*$^{-/-}$ cancer cells compared to *Ccr2*$^{+/+}$ cancer cells.

Fig. 4: Tumors derived from *Ccr2*$^{-/-}$ cancer cells have increased infiltration of activated CTLs

a. Tumors from *Ccr2*$^{-/-}$ cancer cells have fewer CD4+ T cells and more CD8+ T cells, as determined by flow cytometry gated on CD45+CD3+ cells. Left panels are representative examples of dot plots, and right panels show quantification of CD4+ and CD8+ cells (mean +/- SEM, Student's t-test; n=5).

b. PD-1 levels were increased in CD8+ T cells in tumors from *Ccr2*$^{-/-}$ cancer cells, as determined by flow cytometry gated on CD45+CD3+CD8+ cells (mean +/- SEM, Student's t-test; n=5).

c. LAMP-1 (CD107a) levels were increased in CD8+ T cells in *Ccr2*$^{-/-}$ tumors, as determined by flow cytometry gated on CD45+CD3+CD8+ cells (mean +/- SEM, Student's t-test; n=4 and 5 for *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ transplants, respectively).

d. Intracellular Granzyme B levels were decreased in CD8+ T cells in *Ccr2*$^{-/-}$ tumors, as determined by flow cytometry gated on CD45+CD3+CD8+ cells, after 2 h of incubation with Brefeldin A (mean +/- SEM, Student's t-test; n=5).

Fig. 5: Cancer cell CCR2 tumor growth requires functional adaptive immunity

a. Schematic of experimental design.

b. Tumors from *Ccr2*$^{+/+}$ cancer cells grow faster than tumors from *Ccr2*$^{-/-}$ cancer cells in mice with intact immune systems. Tumor burden was determined by weekly caliper measurement. Mice were sacrificed at IACUC-approved endpoint (mean +/- SEM, two-way ANOVA; n=20 per condition).

c. Tumors from *Ccr2*$^{+/+}$ cancer cells grow similar to tumors from *Ccr2*$^{-/-}$ cancer cells in athymic host mice. Tumor burden was determined by weekly caliper measurement. Mice were sacrificed at IACUC-approved endpoint (mean +/- SEM, two-way ANOVA; n=20 per condition).

d. Apoptosis was unchanged between *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ transplants to *Nu/Nu* nude hosts, as determined by TUNEL stain. Left panels are representative photomicrographs (Scale bar=100 μm), with arrows pointing to apoptotic cells; right panel shows quantification (mean +/- SEM, Student's t-test; n=8 and 6 for *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ transplants, respectively).

e. Representative H&E staining of transplanted tumors from *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ cancer cells to nude hosts. Arrows indicate highly necrotic areas (Scale bar=100 μm).

Fig. 6: Cancer cell *Ccr2* signaling protects against T cell-mediated killing

a. *Ccr2*$^{-/-}$ cancer cells are more sensitive to T cell cytotoxicity than *Ccr2*$^{+/+}$ cancer cells. Cytotoxicity of CD8+ OT-1 T cells toward MMTV-PyMT-chOVA;*Ccr2*$^{+/+}$ or MMTV-PyMT-chOVA;*Ccr2*$^{-/-}$ primary cancer cells, with or without OVA peptides, measured by chromium (Cr^{51}) release (mean +/- SEM, Student's t-test; n=3).

b. Inhibiting CCR2 increased T cell cytotoxicity against *Ccr2*$^{+/+}$ cancer cells. Cytotoxicity of CD8+ OT-1 T cells toward MMTV-PyMT-chOVA;*Ccr2*$^{+/+}$ or MMTV-PyMT-chOVA;*Ccr2*$^{-/-}$ cancer cells, with or without CCR2 inhibitor, measured by chromium (Cr^{51}) release (mean +/- SEM, Student's t-

test; n=4 and 3 for MMTV-PyMT-chOVA;*Ccr2*$^{+/+}$ and MMTV-PyMT-chOVA;*Ccr2*$^{-/-}$ cancer cells, respectively).

c. Model of clonality measured by T cell receptor (TCR) sequencing.

d. Productive clonality in tumors from *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ cancer cells (mean +/- SEM, Student's t-test; n=4).

e. Productive clonality in spleens was unchanged between *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ tumors (mean +/- SEM, Student's t-test; n=4).

Fig. 7: Localized STAT1 activation in *Ccr2*$^{-/-}$ tumors

a. Comparison of STAT1 activation in tumors from *Ccr2*$^{+/+}$ or *Ccr2*$^{-/-}$ cancer cells by Western blot analysis (left). Quantifications of protein expression normalized to β-actin levels (right) (mean +/- SEM).

b. Representative photomicrographs of p-STAT1 (top) and p-STAT3 (bottom) staining in sections of tumors from *Ccr2*$^{+/+}$ or *Ccr2*$^{-/-}$ cancer cells. Scale bar=100 μm.

c. Quantification of the immunohistochemical staining in (b), using H-score, shows higher STAT1 phosphorylation in tumors from *Ccr2*$^{-/-}$ than from *Ccr2*$^{+/+}$ cancer cells, but similar levels of STAT3 phosphorylation (mean +/- SEM, Student's t-test; n= 5).

Fig. 8: MHC class I expression is reduced and PD-L1 expression increased in *Ccr2*$^{+/+}$ cancer cells in immune competent mice

a. MHC class I is increased in *Ccr2*$^{-/-}$ compared to *Ccr2*$^{+/+}$ cancer cells after transplantation into C57BL/6 hosts but similar when transplanted to transplants to *Nu/Nu* nude hosts, as determined by immunofluorescence staining. Scale bar=50 μm.

b. Quantification of MHC class I immunofluorescence staining between *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ transplants to C57BL/6 and *Nu/Nu* nude hosts (mean +/- SEM, Student's t-test; n=4 and 5 for *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ transplants, respectively).

c. PD-L1 is decreased on *Ccr2*$^{-/-}$ cancer cells, as determined by flow cytometry gated on EpCAM+ cells (mean +/- SEM, Student's t-test; n=5).

d. PD-L1 mRNA levels are decreased on cultured *Ccr2*$^{-/-}$ cancer cells but respond equally well to IFN-γ treatment, as determined by qPCR. *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ cancer cells growing in culture were treated with either 0, 0.2, 2.0, or 20 ng/mL of recombinant IFN-γ for 48 h (mean +/- SEM, Student's t-test; n=3).

e. CCR2 and PD-L1 gene expression represented as reads per kilobase million (RPKM) value correlates in human breast cancer data (Pearson correlation coefficient=0.61, $p=1.8*10^{-124}$; n=1,242 patients).

f. Treatment with four intraperitoneal doses of anti-PD-L1 antibody (200 μg/injection) had no significant effect on growth of *Ccr2*$^{+/+}$ or *Ccr2*$^{-/-}$ tumors *in vivo*, compared to control rat IgG antibody (arrows indicate treatments; mean +/- SEM, two-way ANOVA; n=20 for all conditions).

Fig. 9: *Ccr2*$^{-/-}$ tumors promote infiltration and activation of cross-presenting CD103+ DCs

a. CD103+ DCs are increased in *Ccr2*$^{-/-}$ tumors compared to *Ccr2*$^{+/+}$ tumors, as determined by flow cytometry gated on CD45+CD11c+MHC class II+ cells. SSC=side scatter (mean +/- SEM, Student's t-test; n=5).

b. CD103+ DCs are increased in the draining lymph nodes (dLNs) of *Ccr2*$^{-/-}$ tumors compared to the dLNs of *Ccr2*$^{+/+}$ tumors, as determined by flow cytometry gated on CD45+CD11c+MHC class II+ cells (mean +/- SEM, Student's t-test; n=5).

c. DCs in *Ccr2*$^{-/-}$ tumors are more activated, as determined by flow cytometry for CD86+ gated on CD45+CD11c+MHC class II+ cells (mean +/- SEM, Student's t-test; n=5).

d. Flow cytometry on dLNs with or without injection of irradiated cancer cells. Note that CD103+ DCs are only increased in the dLNs of the glands after injection of *Ccr2*$^{-/-}$ cancer cells, not in non-dLNs from axillary glands (mean +/- SEM, Student's t-test; n=5).

e. CD103+ DCs are decreased in *Ccr2*$^{-/-}$ tumors compared to *Ccr2*$^{+/+}$ tumors in *Nu/Nu* nude hosts, as determined by flow cytometry gated on CD45+CD11c+ cells (mean +/- SEM, Student's t-test; n=5).

Fig. 10: Cross-presenting CD103+ DCs are critical for immune control of *Ccr2*$^{-/-}$ cancer cells

a. Conditioned medium from *Ccr2*$^{-/-}$ cancer cells induced similar numbers of CD11c DCs (left), but more CD103+ BMDCs (right) *in vitro*, compared to conditioned medium from *Ccr2*$^{+/+}$ cancer cells (mean +/- SEM, Student's t-test; n=3).

b. Conditioned medium from *Ccr2*$^{+/+}$ cancer cells cultured with CCR2 antagonist induced similar numbers of CD11c DCs (left), but more CD103+ BMDCs (right) *in vitro* than conditioned medium from control cells cultured in plain medium (mean +/- SEM, Student's t-test; n=3).

c. GM-CSF levels in conditioned medium from *Ccr2*$^{+/+}$ and *Ccr2*$^{-/-}$ cancer cells, as determined by ELISA (mean +/- SEM, unpaired t-test; n=5).

d. *Ccr2*$^{-/-}$ cancer cells showed accelerated growth in *Batf3*$^{-/-}$ hosts.

e. CD103+ DCs are increased in *Ccr2*$^{-/-}$ tumors compared to *Ccr2*$^{+/+}$ tumors in C57BL/6 hosts but are low in *Batf3*-/- hosts (mean +/- SEM, Student's t-tests; n=5).

f. Model of how cancer cell CCR2 orchestrates immune suppression.

Figure 1

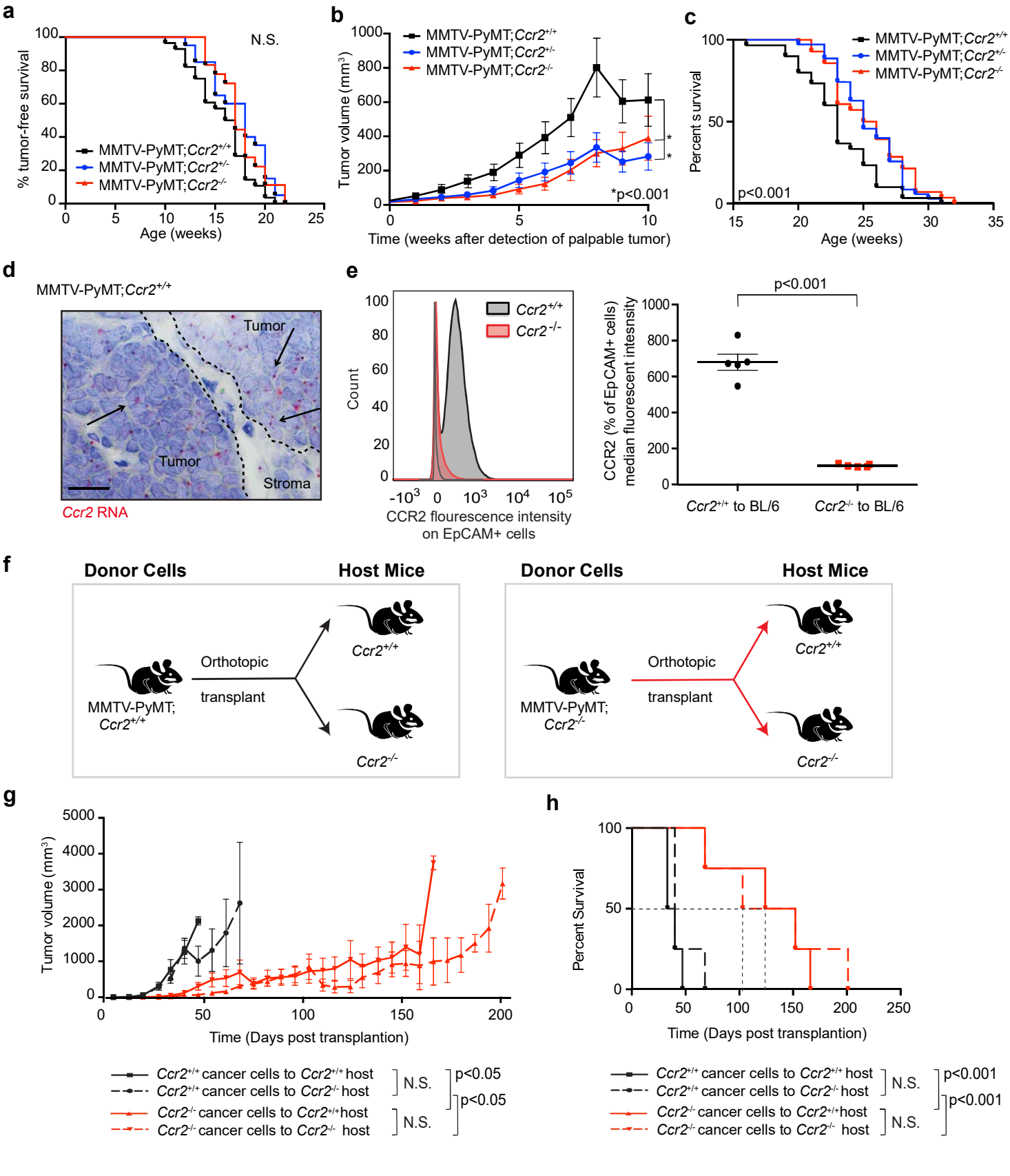

Figure 2

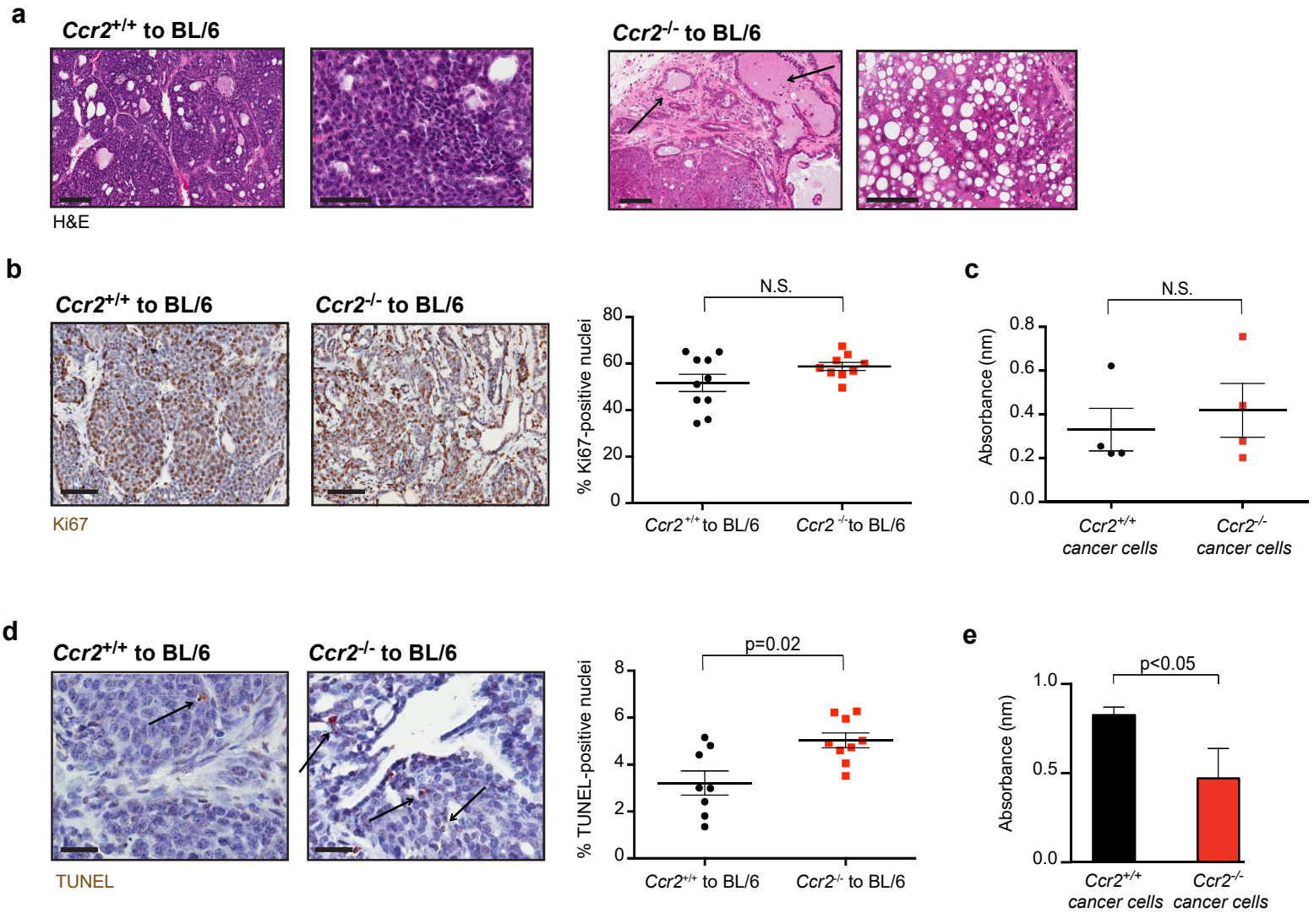

Figure 3

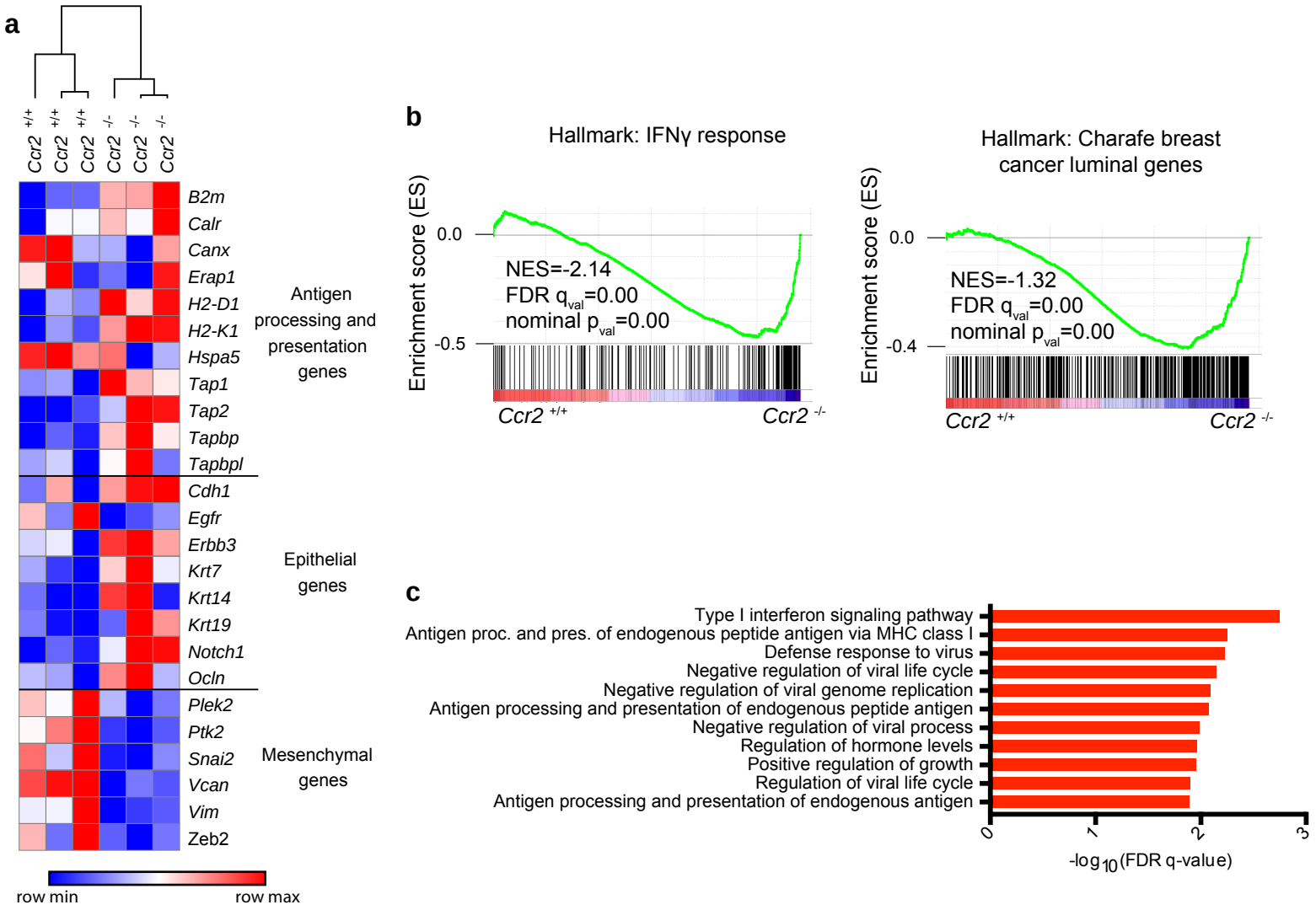

Figure 4

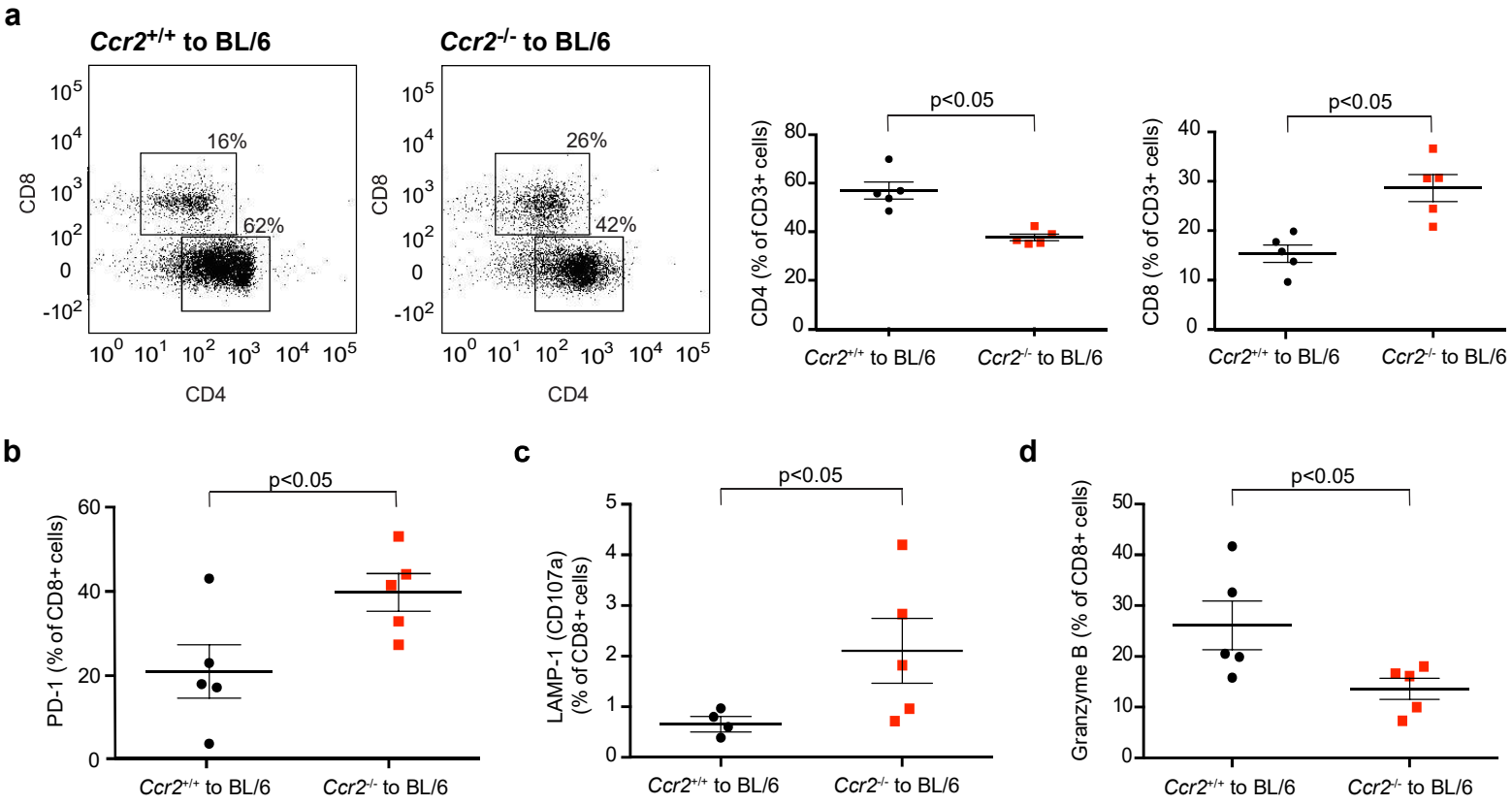

Figure 5

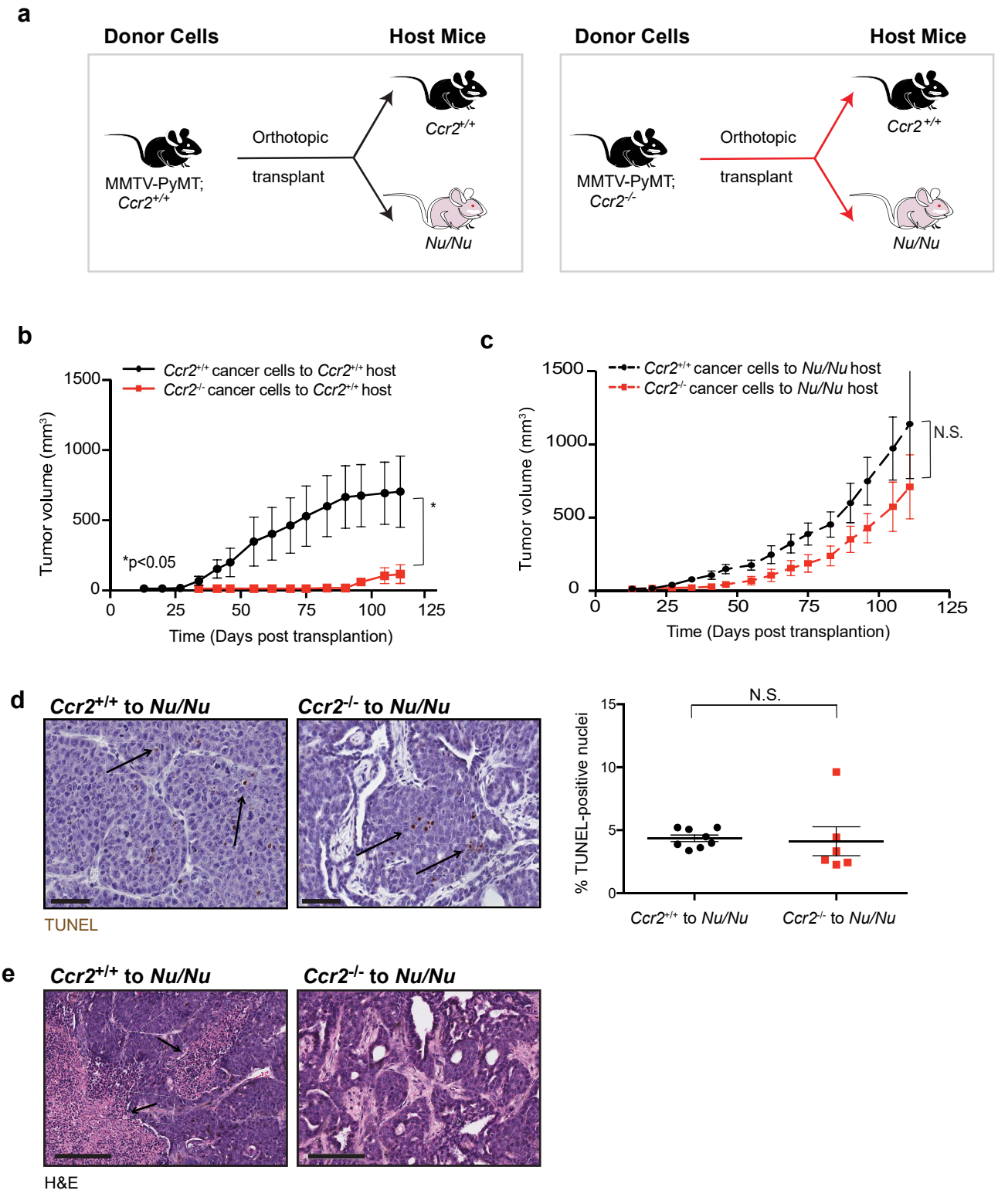

Figure 6

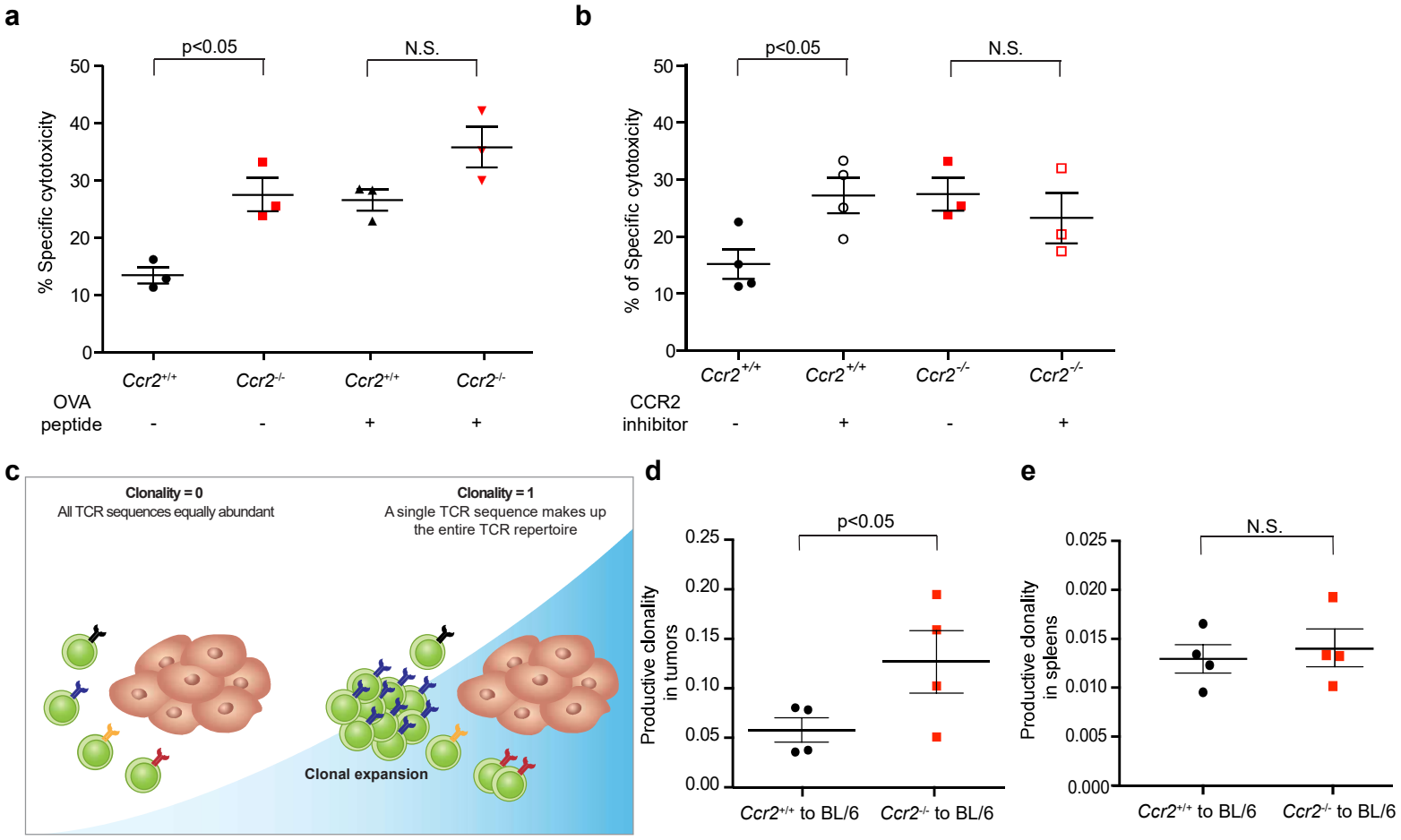

Figure 7

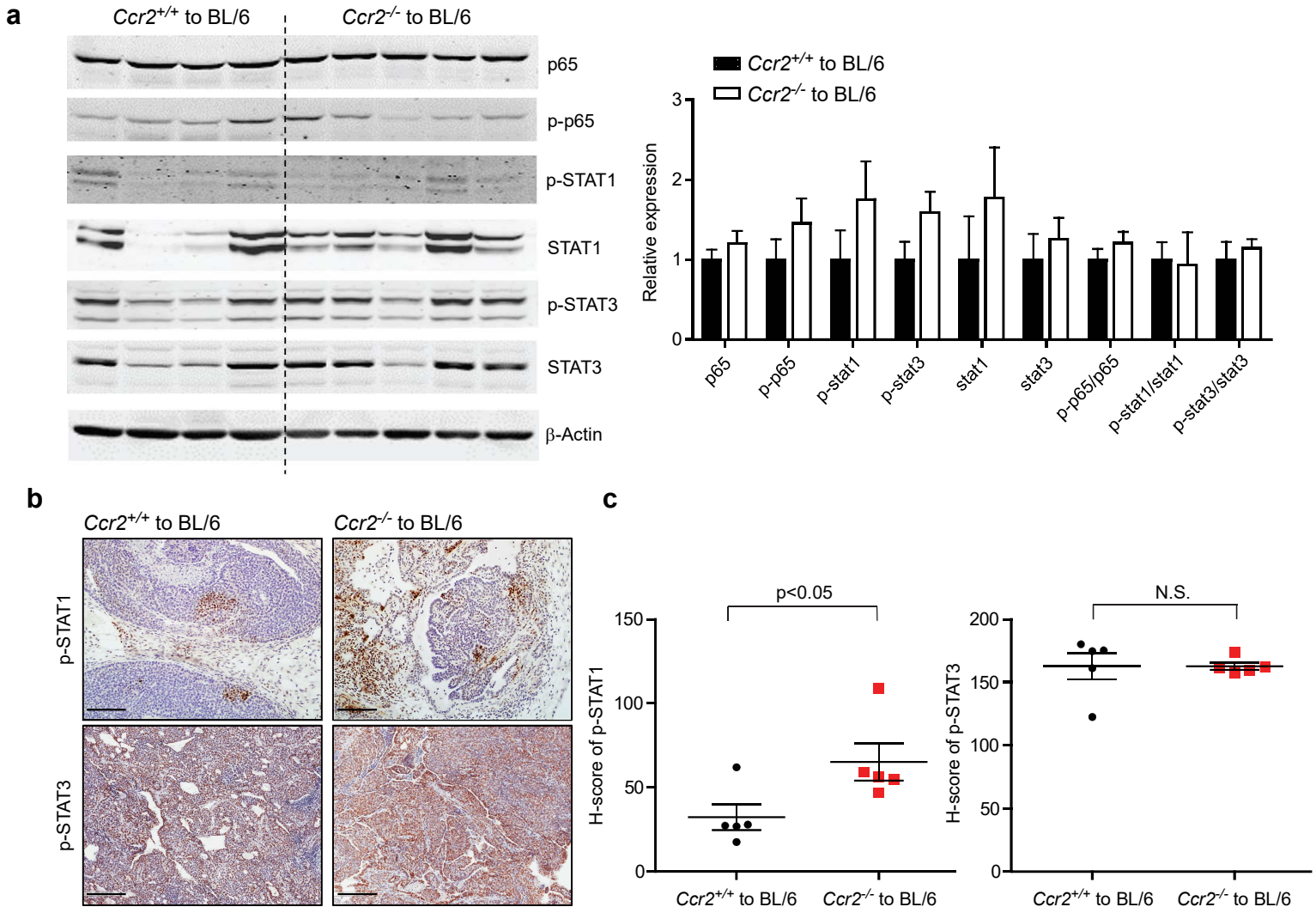

Figure 8

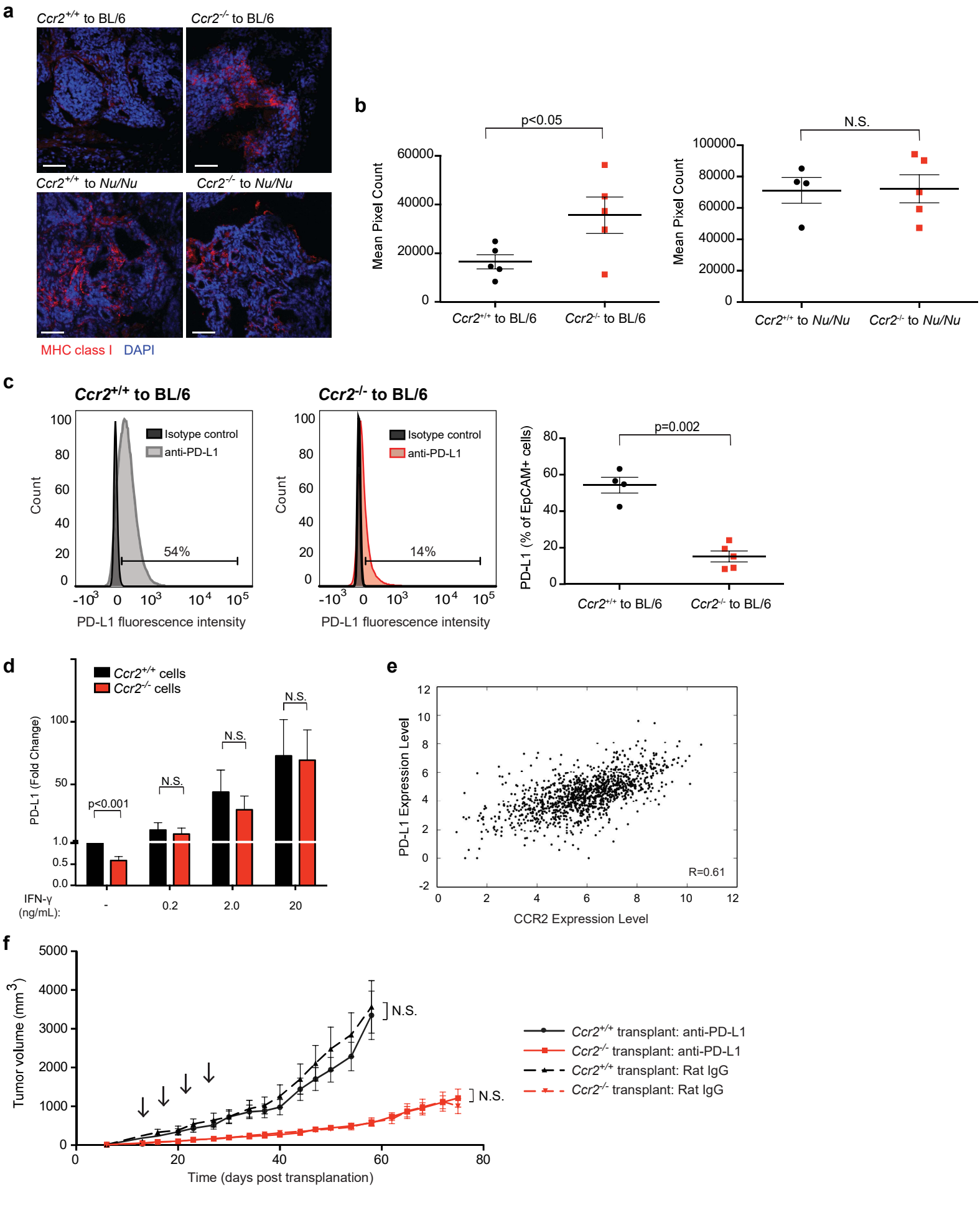

Figure 9

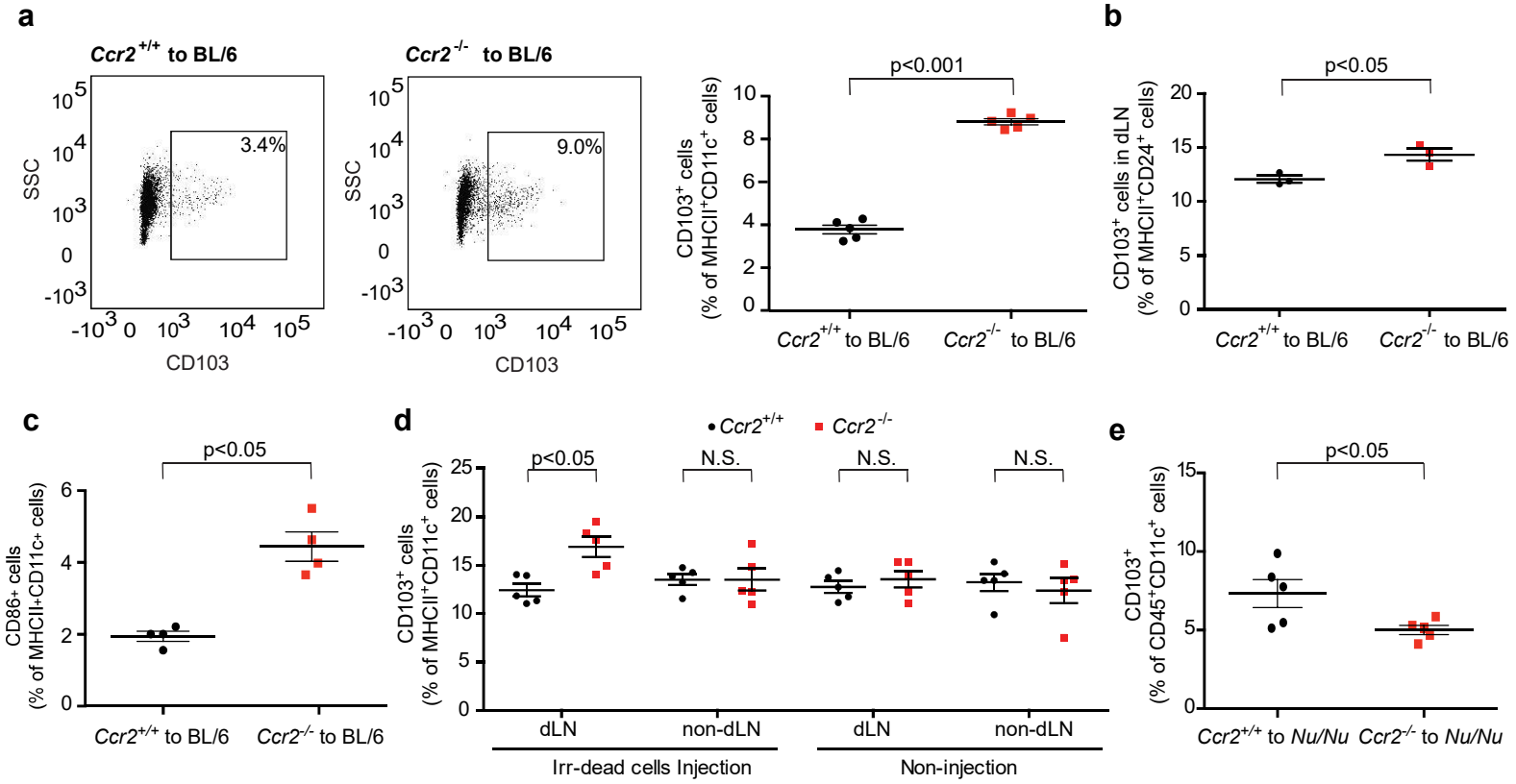

Figure 10