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ORIGINAL ARTICLE

Bidirectional effect of CD200 on breast cancer development and metastasis, with ultimate outcome determined by tumor aggressiveness and a cancer-induced inflammatory response

N Erin[1], A Podnos[2], G Tanriover[3], Ö Duymuş[1], E Cote[2], I Khatri[2] and RM Gorczynski[2]

CD200 acts through its receptor (CD200R) to inhibit excessive inflammation. The role of CD200-CD200R1 interaction in tumor immunity is poorly understood. In this study, we examined the role of CD200-CD200R1 interaction in the progression and metastasis of highly aggressive 4THM murine-breast carcinoma using CD200 transgenic ($CD200^{tg}$) and CD200R1 knock-out ($CD200R1^{-/-}$) BALB/c mice. 4THM cells induce extensive visceral metastasis and neutrophil infiltration in affected tissues. CD200 overexpression in the host was associated with decreased primary tumor growth and metastasis, whereas lack of CD200R1 expression by host cells was associated with enhanced visceral metastasis. Absence of CD200R1 expression led to decreased tumor-infiltrating-cytotoxic T cells and increased the release of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6. In contrast, CD200 overexpression led to increased tumor-induced interferon-γ and IL-10 response and decreased TNF-α and IL-6 release. Neutrophil infiltration of tissues was markedly decreased in $CD200^{tg}$ animals and increased in $CD200R1^{-/-}$ mice. These findings are contradictory to what has been reported in the EMT6 mouse breast-cancer model. Other distinguishing features of tumor elicited by EMT6 and 4THM cell injections were also examined. Visceral tissues from mice bearing EMT6 tumors showed a lack of neutrophil infiltration and decreased IL-6 release in $CD200R1^{-/-}$ mice. EMT6 and 4THM cells also differed in vimentin expression and *in vitro* migration rate, which was markedly lower in EMT6 tumors. These results support the hypothesis that CD200 expression can alter immune responses, and can inhibit metastatic growth of tumor cells that induce systemic and local inflammatory response. Increasing CD200 activity/signaling might be an important therapeutic strategy for treatment of aggressive breast carcinomas.

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INTRODUCTION

CD200 is a widely expressed cell surface glycoprotein that belongs to the immunoglobulin family and regulates inflammation in autoimmunity, transplantation and viral infections.[1] CD200 functions through its receptor, CD200R, which is expressed mostly by myeloid cells,[2,3] and some subsets of lymphoid-derived cells.[2] There are five described CD200R isoforms in mice, CD200R1–R5; CD200R1 is reported to mediate the anti-inflammatory effects of CD200.[4] The role of CD200-CD200R1 interaction in tumor immunity is poorly understood. CD200 expression by leukemic tumor cells inhibits activation of tumor-specificT cells *in vitro*.[5] Treatment with anti-CD200 antibody can enhance tumor rejection by peripheral blood mononuclear cells (PBMC) in a hu-SCID adoptive transfer model.[6]

Although multiple studies have documented tumor-promoting effects of CD200 in leukemia,[7–9] there are conflicting results regarding its role in solid tumors. CD200 expression is associated with increased metastatic survival of squamous cell carcinoma,[10] and CD200 expression by human basal carcinoma is associated with tumor-initiating properties.[11] On the other hand, CD200 expression by melanocytes results in decreased lung metastasis, and CD200R activation by an agonistic monoclonal antibody (OX110) is associated with decreased CD200-negative melanoma tumor formation in the lungs.[12] These conflicting results regarding the role of CD200 in cancer progression and metastasis may be due to the bidirectional role of immune activation in cancer. Chronic activation of the immune system may induce carcinogenesis and metastasis by creating an inflammatory microenvironment, which increases DNA damage and malignant transformation.[13] Once malignant transformation occurs, cancer cells may also induce a chronic inflammatory response in the host, which enhances the metastatic process.[13]

In this study, we examined the role of CD200 in a murine model of breast cancer. The 4T1 breast carcinoma represents an inflammatory, aggressive, triple-negative breast cancer, originally obtained from a spontaneously formed breast cancer in a BALB/c mouse.[14] We previously reported that heart metastases of 4T1 cells, named 4THM, grow more aggressively and induce a systemic inflammatory response.[15–17] Recently, we have shown that more than 50% of 4THM cells express CD44 and are CD24 negative, under *in vitro* conditions, which is a feature of 'stemness'. Primary tumors formed by 4THM cells are positive for vimentin, another marker of cancer stem cells (CSCs).[18] The role of CD200 in breast cancer progression and metastasis has so far only been studied in an EMT6 breast carcinoma model, where CD200 overexpression resulted in increased lymph node metastasis.[19,20]

[1]Department of Medical Pharmacology, School of Medicine, Akdeniz University, Antalya City, Antalya, Turkey; [2]University Health Network, Toronto General Hospital, Toronto, ON, Canada and [3]Department of Histology and Embryology, School of Medicine, Akdeniz University, Antalya, Turkey. Correspondence: N Erin, Department of Medical Pharmacology, School of Medicine, Akdeniz University, B-blok kat 1 Immunoloji, Antalya 07070, Turkey.
Email: nerin@akdeniz.edu.tr
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To our knowledge, there have been no reports examining the effects of CD200 on visceral metastasis in an inflammatory, highly aggressive breast cancer model. Here, we examined the effects of CD200 on tumor growth, metastasis and anti-tumor immune responses to orthotopically-injected 4THM breast carcinoma cells using $CD200^{tg}$ and $CD200R1^{-/-}$ BALB/c female mice. We report that overexpression of CD200 by the host is associated with decreased metastatic growth of 4THM cells, the opposite of the results seen using EMT6 breast tumors.

RESULTS

Growth of primary tumors and lung/liver metastasis after injection of 4THM breast carcinoma cells is attenuated in hosts overexpressing CD200

Wild type (WT) and $CD200^{tg}$ mice were injected with 4THM cells (10^5 cells/mouse) orthotopically, with transgene induction using doxycycline (DOX)-water beginning 2 days before tumor injection. Primary tumor growth and visceral metastases were evaluated 24 days after the injection. As shown in Figure 1, 4THM primary tumor growth was attenuated in $CD200^{tg}$ mice, although all animals developed visible tumors at the site of injection by day 12.

Primary tumors regressed completely in 3/7 $CD200^{tg}$ mice with ~50% regression of the primary tumor observed in an additional animal (days 24 vs 12). Lung metastases were also reduced in $CD200^{tg}$ mice, consistent with the altered pattern of primary tumor growth (Figure 1). Since macroscopic nodules were clearly observed in lung tissues following fixation with Bouins solution and correlated with the microscopic results,[18] only the macroscopic findings are shown in Figure 1. However, microscopic analysis of lung tissues was performed in the three $CD200^{tg}$ mice in which primary tumors had completely regressed and no visible lung nodules were detected. No microscopic lesions were seen in lung tissues of these animals (Supplementary Figure 1: microscopic lung metastasis of WT and $CD200R1^{-/-}$ mice is shown).

Liver metastases were evaluated microscopically and analyzed to determine the extent of metastases as well as the frequency of metastatic lesions. As shown in Figure 1d, the degree of metastases was significantly decreased in $CD200^{tg}$ mice compared with WT mice. We have analyzed the data in the two groups of $CD200^{tg}$ mice with regression/nonregression of their primary tumors to determine whether liver metastases were detected in each. We observed occasional metastatic lesions in the liver tissues of mice with regression of primary tumors, but an overall decrease in metastasis was observed (data not shown). The levels of liver metastases in the $CD200^{tg}$ mice whose primary tumor persisted throughout the study were similar to those seen in WT mice, suggesting that CD200 over expression in the host resulted in the attenuation of growth of lung metastasis more effectively than liver metastasis.

Visceral metastasis of 4THM breast carcinoma was increased in $CD200R1^{-/-}$ mice

Lack of CD200R1 expression increased liver and lung metastasis in CD200RKO compared with both WT and $CD200^{tg}$ mice. The extent of metastasis and the frequency of lesions in the liver tissue were also increased in $CD200R1^{-/-}$ mice (Figure 1d). The rate and extent of primary tumor growth, on the other hand, was not significantly different among WT and $CD200R1^{-/-}$ animals (Figure 1b).

Flow cytometric analysis of CD45, CD200, CD3, CD8, CD4, Gr-1, CD11b and F4/80 expression by tumor infiltrating immune cells and draining lymph node cells

Lack of CD200 expression in $CD200^{-/-}$ mice has been reported to result in increased CD200R expression by myeloid cells.[21] Interestingly, 12 days after 4THM cell injection, the percentage of CD200-expressing tumor infiltrating $CD45^+$ cells was decreased in $CD200R1^{-/-}$ animals compared with WT, along with a fourfold decrease in the number of $CD3^+CD8^+$ tumor infiltrating T cells in the same mice (Figure 2a). However, the percentage of $CD4^+$ cells did not differ among the groups, and the percentage of $GR1^+$, $F4/80^+$ and $CD11b^+$ cells was also unchanged (data not shown). Levels of $CD3^+CD25^+$ cells in draining lymph nodes (DLNs) of $CD200R1^{-/-}$ animals injected with 4THM breast carcinoma cells (1.54%) were decreased ~twofold in comparison with levels in WT animals (3.95%; see Figure 2b). This was similar to data from primary tumors at 12 days following 4THM injections (0.61% vs 1.82%, respectively; see Figure 2b).

To verify that aberrant expression of leukocyte markers on 4THM tumor cells themselves did not interfere with the reported results, we examined CD45, CD3, CD200R and CD200 staining on 4THM cells grown *in vitro*. No cell-surface expression of CD3, CD45, CD200 or CD200R1 was detected on 4THM cells growing *in vitro*, nor was CD200 and CD200R expression detected by immunohistochemistry on 4THM and EMT6 primary tumors themselves grown in WT mice (Supplementary Figure 2). Data in Supplementary Figure 2 (Panels a, c, d and e) show, by comparison, similar staining using primary tissue from mice injected with a less aggressive EMT6 tumor.[19] Using liver tissue from WT mice bearing 4THM tumors as positive control, we observed, as expected, membranous staining of hepatocytes with CD200, with no expression on metastatic lesions within the liver tissue (Supplementary Figure 2b).

In the terminal stages of tumor growth (day 24) when animals started to die and primary tumors became necrotic, most of the cells obtained from tumor digestion were granulocytes, as determined by GR-1 staining, with 64.6 ± 6.6 and 60.5 ± 2.3 $GR\text{-}1^+$ cells in the tumor tissue of WT and $CD200R1^{-/-}$ animals, respectively. At this stage of primary tumor growth no significant differences for any other markers were observed between the different groups.

Increased tumor-induced IFN-γ responses in mixed leukocyte cultures using cells from tumor-bearing $CD200^{tg}$ mice

Mixed leukocyte cultures (MLCs) were prepared 12 and 24 days after 4THM injection, as described in Materials and methods, with control MLCs using cells from mice not injected with 4THM. Day 12 was chosen, because 4THM cells grow sufficiently to start metastasizing by this time, and the immune response to tumor cells would be evoked by day 12; day-24 MLCs were used to assess changes in the immune response in the presence of extensive disease. MLCs were stimulated either with lipopolysaccharide (LPS) or irradiated 4THM cells. IFN-γ responses were measured 40 h. after challenge.

The 4THM-induced IFN-γ response was significantly higher in MLCs of 4THM-injected $CD200^{tg}$ animals compared with WT and $CD200R1^{-/-}$ mice at day 12 (Figure 3). In contrast, LPS-induced IFN-γ secretion was higher in $CD200R1^{-/-}$ and $CD200^{tg}$ animals compared with WT. In control non-tumor-bearing mice, the levels of IFN-γ induced by LPS in $CD200^{tg}$, $CD200R1^{-/-}$ and WT animals were similar; challenge with irradiated 4THM cells did not induce IFN-γ secretion.

Irradiated 4THM cells induced IFN-γ responses only in $CD200^{tg}$ animals at day 24, although this response was attenuated compared with day 12. There was no LPS-induced IFN-γ response in WT and $CD200^{tg}$ animals at day 24, and again the LPS-induced IFN-γ response in $CD200R1^{-/-}$ animals at this time was diminished compared with day 12. All of the above are consistent with a generalized attenuation of IFN-γ production in all mice at day 24.

Attenuation of LPS-induced interleukin-10 secretion in MLCs of $CD200^{tg}$ mice 24 days after 4THM injection

Unstimulated MLCs of tumor-bearing animals secreted significant amounts of interleukin (IL)-10 (50 – 200 pg/ml), which was highest

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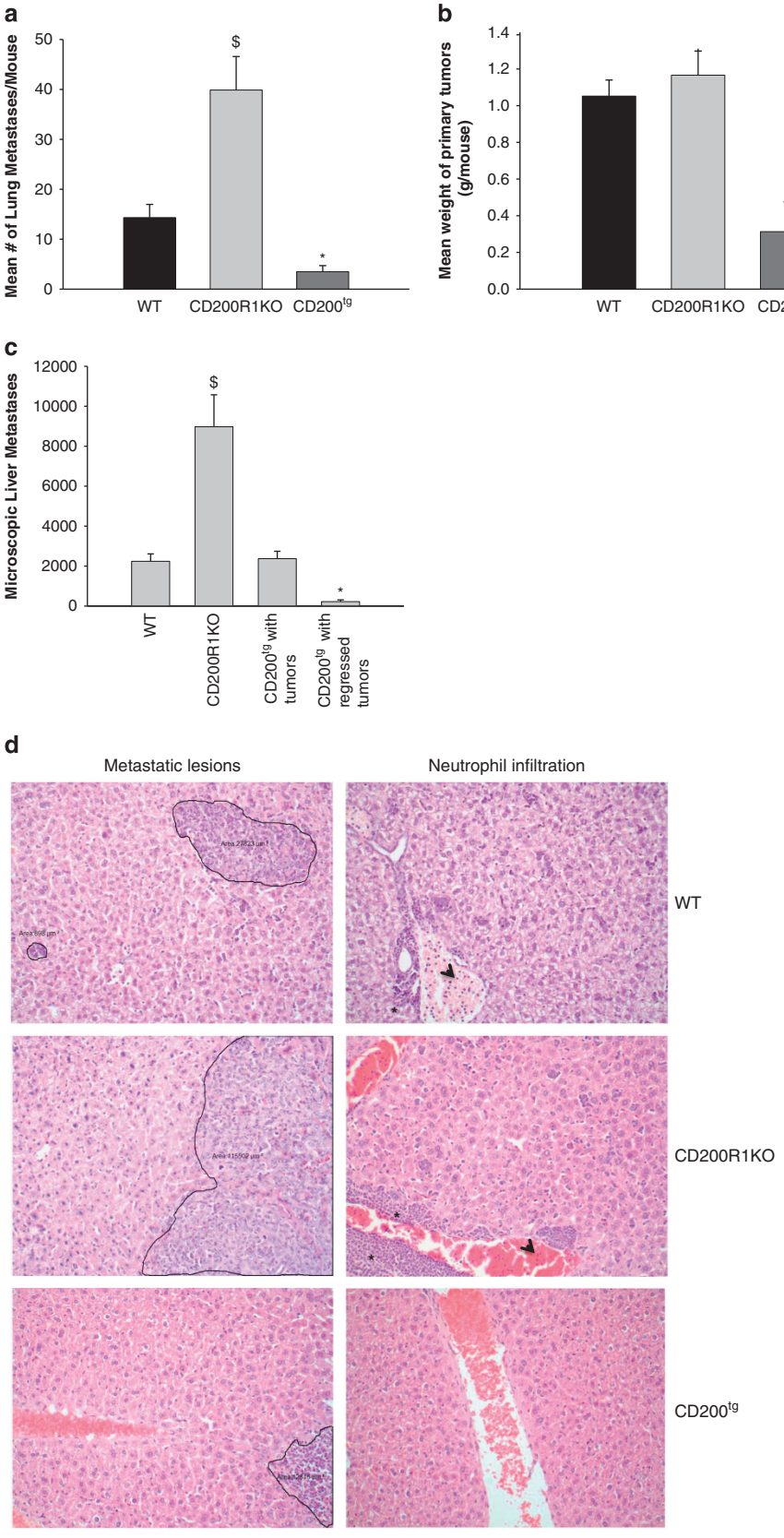


Figure 1. Changes in tumor growth and metastasis of 4THM cells in CD200^{tg} and CD200R1$^{-/-}$ mice. (**a**) changes in the number of lung nodules per mouse. $^{*,\$}P < 0.05$ significantly different from WT mice. (**b**) changes in primary tumor growth $^{*}P < 0.05$ significantly different from WT and CD200R1$^{-/-}$ mice. (**c**) changes in levels of microscopic liver metastases per mouse. $^{*,\$}P < 0.05$ significantly different from WT mice. (**d**) microscopic appearance of liver metastasis and neutrophil infiltration.

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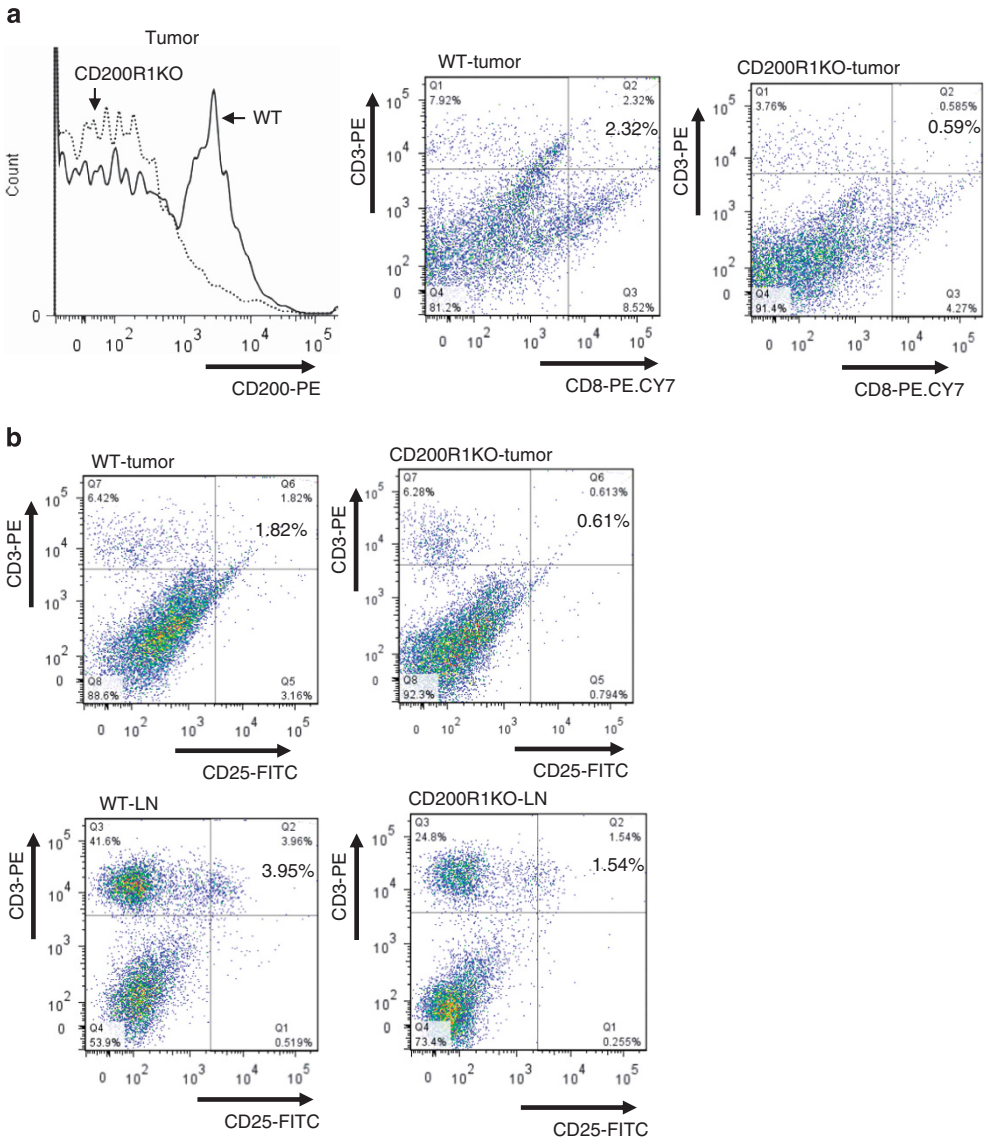


Figure 2. Flow cytometric analysis of immune cells within the primary tumor tissue and DLNs. (**a**) expression of CD200 on $CD45^+$ cells within tumor tissue; $CD3^+CD8^+$ cells infiltrating primary tumors from WT and $CD200R1^{-/-}$ mice. (**b**) $CD3^+CD25^+$ cells within 4THM primary tumors as well as DLNs of WT and $CD200R1^{-/-}$ mice.

in $CD200R^{-/-}$ mice (Figure 3c). In MLCs prepared 12 days after 4THM injection, IL-10 production following LPS challenge was significantly higher from cells of both $CD200R1^{-/-}$ and $CD200^{tg}$ animals compared with WT, whereas irradiated 4THM cells did not induce significant IL-10 secretion from any group (Figure 3c). However, by 24 days after tumor injection, only $CD200R1^{-/-}$ mice, but not WT or $CD200^{tg}$, produced IL-10 after LPS challenge.

LPS and irradiated 4THM cells induced IL-6 secretion by host immune cells, which was suppressed in MLCs of $CD200^{tg}$ mice 24 days after 4THM tumor cell injection

MLCs from all the tumor-bearing animals secreted significant amounts of IL-6 without challenge compared with control animals not bearing a tumor. Challenge with LPS or irradiated 4THM cells further enhanced IL-6 secretion in tumor-bearing animals 12 days after injection of tumor cells (Figure 4a). Challenge with irradiated 4THM cells induced significantly higher levels of IL-6 secretion in MLCs of $CD200^{tg}$ mice compared with WT or $CD200R1^{-/-}$ hosts at day 12. Interestingly, IL-6 responses induced by both 4THM or LPS challenge, as well as the basal cytokine secretion, were preserved/elevated in MLCs of WT and $CD200R1^{-/-}$ mice, respectively, 24 days post tumor injection, but were essentially abolished in $CD200^{tg}$ animals at this time (Figure 4b).

Attenuation of tumor necrosis factor-α secretion in cells from $CD200^{tg}$ compared with WT or CD200RKO mice

Basal tumor necrosis factor (TNF)-α secretion was below the limits of detection in all tumor-bearing animals and challenge with irradiated 4THM cells did not induce TNF-α secretion in MLCs of tumor-bearing or non-tumor-bearing mice. Challenge with LPS induced higher levels of TNF-α secretion in WT and $CD200R1^{-/-}$ tumor-bearing animals compared with non-tumor-bearing mice, or tumor-bearing $CD200^{tg}$ animals at day 12. By day 24 after 4THM injection MLCs of $CD200^{tg}$ animals failed to secrete TNF-α in response to LPS. MLCs of $CD200R1^{-/-}$ mice showed a similar TNF-α production at day 24 as compared with that observed at day 12, whereas MLCs of WT animals secreted less TNF-α at day 24 compared with day 12 (Figure 4c).

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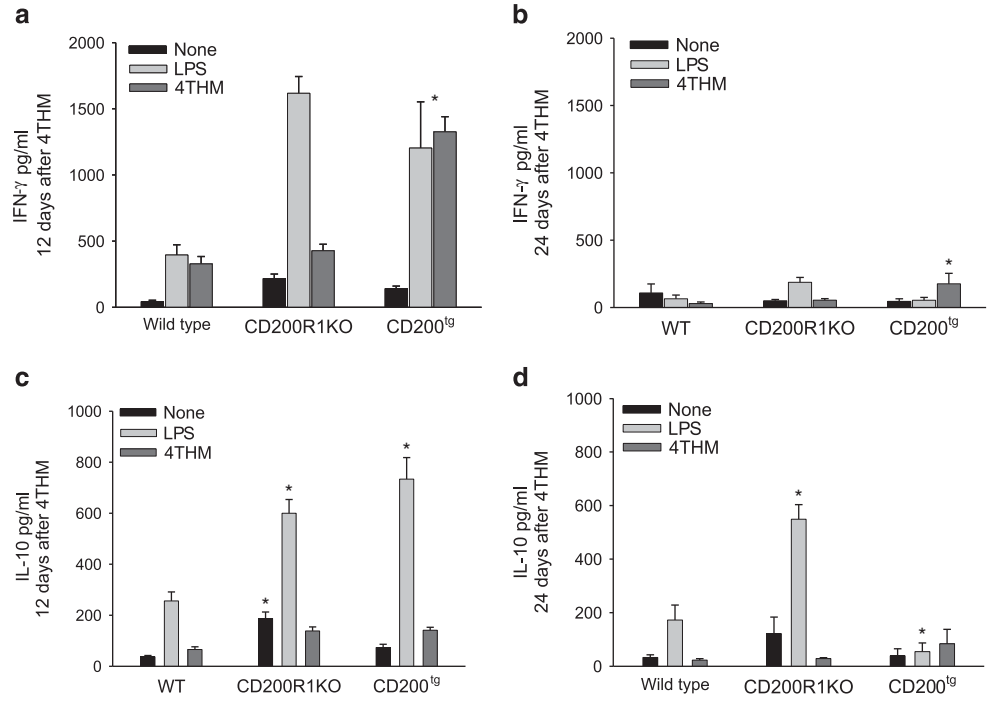


Figure 3. IFN-γ (**a**, **b**) and IL-10 (**c**, **d**) secretion from MLCs prepared from WT, CD200^{tg} and CD200R1$^{-/-}$ mice bearing 4THM tumors for 12 (**a**, **c**) and 24 (**b**, **d**) days. None indicates unstimulated MLCs; LPS-MLCs stimulated with LPS for 40 h; 4THM-MLCs stimulated with irradiated 4THM cells for 40 h. *$P < 0.05$ significantly different from corresponding WT MLCs.

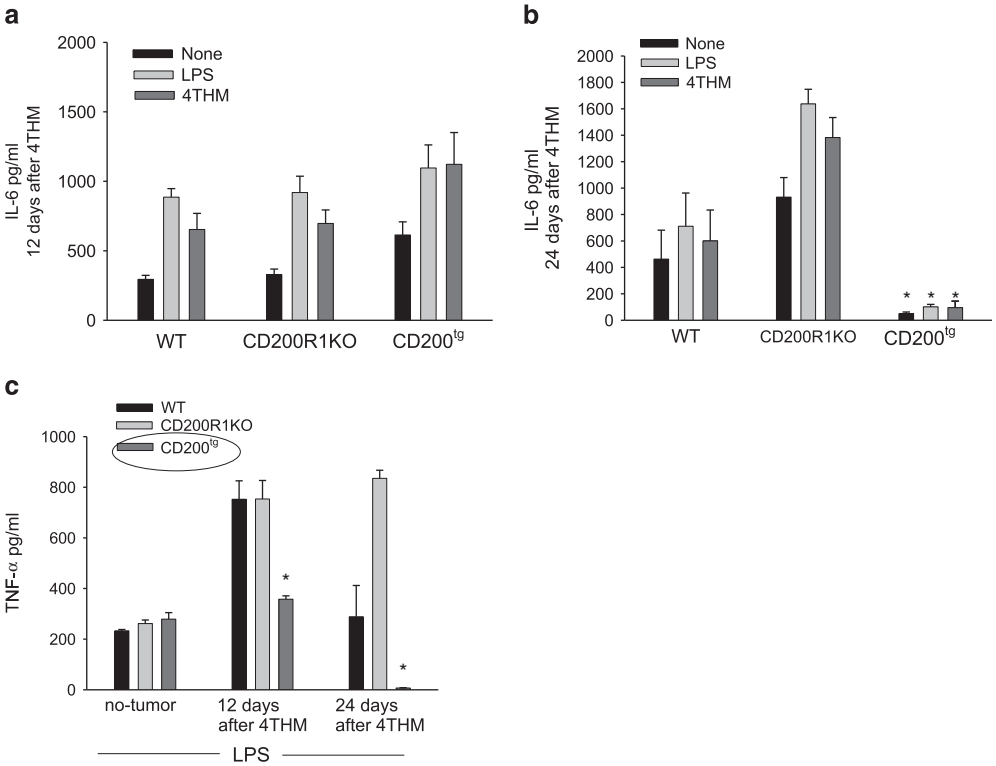


Figure 4. IL-6 (**a**, **b**) and TNF-α (**c**) secretion from MLCs prepared from WT, CD200^{tg} and CD200R1$^{-/-}$ mice bearing 4THM tumors for 12 and 24 days. **a**, **b** show IL-6 secretion at days 12/24, respectively. None indicates unstimulated MLC; LPS- MLC stimulated with LPS for 40 h; 4THM- MLC stimulated with irradiated 4THM cells for 40 h. **():** TNF-α secretion from MLCs stimulated with LPS for 20 h. * < 0.05 significantly different from corresponding WT MLCs.

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Table 1. Comparison of tumor growth/metastases and cytokine production in WT, CD200RKO and CD200^{tg} mice

	WT	*CD200RKO*	*CD200TG*
Lung metastasis (nodule/mouse)	14.33 ± 2.64	39.87 ± 6.7	3.5 ± 1.2
Liver metastasis (microscopic μm/area)	2241 ± 370	8972.46 ± 1599	2371.8 ± 370 (P(+)) 223 ± 85 (P(-))
Primary tumor growth (g/mouse)	1.05 ± 0.08	1.16 ± 0.14	0.31 ± 0.16
Neutrophil infiltration of metastatic tissue	++++	++++++	++
IFN-ɣ (pg/ml) 12 days	294 ± 77.5(LPS) 327 ± 55.8(T)	1617.08 ± 126.8(LPS) 427.01 ± 48.97(T)	1203.11 ± 349.39(LPS) 1325.6 ± 114.89(T)
IFN-ɣ (pg/ml) 24 days	65.85 ± 27.68(LPS) 29.05 ± 11.66(T)	86.96 ± 36(LPS) 54.63 ± 11.22(T)	54.1 ± 20.47(LPS) 176.07 ± 77.66(T)
IL-10 (pg/ml) 12 days	256.06 ± 35.26(LPS) 65.64 ± 10.74(T)	599.91 ± 54.03(LPS) 138.32 ± 15.9(T)	733.63 ± 84.47(LPS) 141.16 ± 11.69(T)
IL-10 (pg/ml) 24 days	172.61 ± 55.86(LPS) 22.97 ± 5.36(T)	548.96 ± 54.36(LPS) 28.66 ± 3.4(T)	54.52 ± 32.42(LPS) 84.02 ± 54.52(T)
TNF-α (pg/ml) 12 days	752.59 ± 72.91(LPS)	753.65 ± 73.11(LPS)	357.68 ± 13.32(LPS)
TNF-α (pg/ml) 24 days	288.61 ± 123.44(LPS)	835.30 ± 31.76(LPS)	< 7
IL-6 (pg/ml) 12 days	885.95 ± 61.8(LPS) 653.67 ± 115.52(T)	919.53 ± 117.07(LPS) 669.65 ± 97.56(T)	1095.73 ± 166.16(LPS) 1122.31 ± 228.55(T)
IL-6 (pg/ml) 24 days	711.29 ± 251.46(LPS) 600.20 ± 233.70(T)	1637.38 ± 110.29(LPS) 1382.47 ± 151.62(T)	101.56 ± 18.97(LPS) 96.73 ± 48.75(T)

P(+), mice with persistent primary tumors; P(-), mice with regression of primary tumors. Cytokines were measured in MLC 12 and 24 days after injections of 4THM cells; LPS, indicates levels in MLCs with LPS stimulation; T, indicates levels in MLCs after stimulation with irradiated 4THM. In the absence of challenge with LPS or tumor cells, cytokine levels were either low or below the limits of detection in all groups.

Table 1 shows a compilation of the various parameters discussed in Figures 1 – 5, indicating tumor growth, metastasis and cytokine production from the various mice used.

Significant differences in growth of, and host responses to, EMT6 and 4THM tumors

Examination of mice injected with 4THM or the less aggressive EMT6 breast cancer showed numerous differences in growth rate (Figures 5a and c) and host responses such as systemic leukocytosis (Figures 5d and f). Cells from tumor explants (Figures 5g and h) of 4THM injected mice spontaneously released 10 – 20 times more TNF-α and IL-6 into culture media than explants from EMT6 injected mice (Figure 5i), although interestingly WT, and not CD200R1$^{-/-}$ mice, released more IL-6 after challenge by EMT6 cells in MLC (Figure 5j) unlike the reverse situation with 4THM tumor bearers (Figures 4a and b). We also observed a ⩾twofold increase in the numbers of CD200$^+$ cells in DLN of WT mice bearing EMT6 tumors compared with mice bearing 4THM tumors (Figure 5k). Microscopic examination of lung and liver tissues from EMT6 tumor-bearing animals failed to demonstrate tumor metastasis (Supplementary Figure 3), unlike 4THM tumor bearers, where lung tissue was also found to stain strongly for S100A8 infiltrating cells (Supplementary Figure 4A). Interestingly, further comparison of primary tumor tissue of both 4THM and EMT6 tumor-injected mice showed an absence of S100A8 staining by either 4THM or EMT6 tumor cells themselves (Supplementary Figure 4B), but marked infiltration of 4THM tumors by cells staining for S100A8, whereas S100A8$^+$ immune cells were observed in the vascular structures of liver tissue of both 4THM and EMT6-injected animals (Supplementary Figure 4C), with more pronounced tissue infiltration by S100A8$^+$ cells in the 4THM-injected animals.

EMT6 and 4THM cells were also observed to differ in expression of vimentin, and in their *in vitro* migration rate. Higher vimentin expression was observed in 4THM primary tumors (Supplementary Figure 4D) correlating with their faster migration *in vitro* than EMT6 cells (Supplementary Figure 5). EMT6 cells were observed to migrate in clusters, which would make invasion through vascular structures less feasible, whereas 4THM cells migrated as single cells.

DISCUSSION

Cancer is a heterogeneous disease in which the outcome and response to treatment is determined by the tumor microenvironment, systemic and local host responses, and intrinsic features of the cancer cells. Given this complexity, it has proven difficult to define a single and a definitive role for any given molecule. CD200 seems to be one of a number of molecules with a bidirectional role in cancer development and metastasis. CD200 is known to exert both anti-inflammatory and immunosuppressive effects. We hypothesize that in the 4THM model described, CD200 exerts a potent anti-tumoral/antimetastatic effect on inflammation driven carcinogenesis and metastasis. Given that the degree of inflammation correlates with the degree of aggressiveness and treatment resistance in most carcinomas, CD200 agonists may thus exert potent anti-tumoral and anti-metastatic effects.

In support of the above hypotheses CD200 overexpression in CD200^{tg} mice was associated with decreased metastasis of the highly aggressive breast carcinoma cells (4THM), whereas lack of CD200R1 expression resulted in significantly increased lung and liver metastasis, consistent with the prediction that the protective effects of CD200 were, at least partly, mediated by CD200:CD200R1 interactions.[4] Lack of CD200R1 expression by the host was associated with decreased numbers of tumor infiltrating CD8$^+$ and CD3$^+$CD25$^+$ T cells, and increased production of inflammatory cytokines including TNF-α and IL-6. In contrast, CD200 overexpression by CD200^{tg} hosts resulted in increased tumor-induced IFN-γ and decreased TNF-α and IL-6 responses.

Cancer-related inflammation is a hallmark of cancer[22] and many cases is increased in parallel with tumor growth.[23] 4THM breast carcinoma cells induce extensive visceral metastasis and neutrophil infiltration of affected tissues, although neutrophil infiltration occurred independently of metastatic lesions. Neutrophil infiltration of tissues was markedly decreased in CD200^{tg} animals and increased in CD200R1$^{-/-}$ animals, in accordance with the proposed anti-inflammatory role of CD200.[1,2,4]

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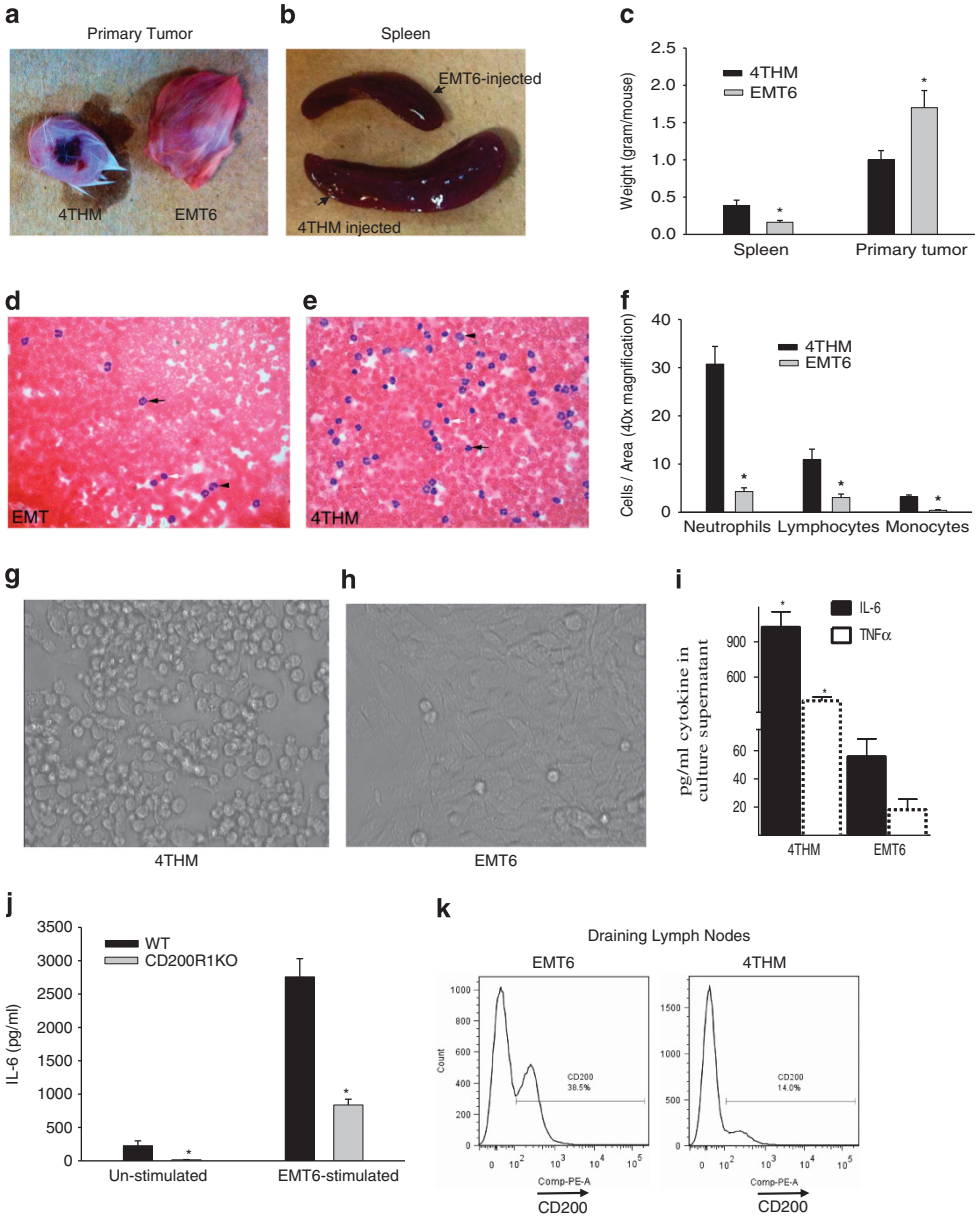


Figure 5. Growth and cytokine production from 4THM and EMT6 injected mice. (**a**–**c**) increased growth rate of EMT6 (**a** and **c**), and spleen weight in 4THM-injected animals (**b** and **c**). **d**–**f** greater numbers of neutrophils (black arrow), monocytes (black arrowhead) and lymphocyes (white arrow) in 4THM-injected animals (**d** and **e**; peripheral blood smears). (**f**) shows the mean number of immune cells per area ($n = 4$ mice). (**g** and **h**) appearance of 4THM and EMT6 tumor explants (harvested at 20d) 72 h after plating and immediately before harvesting CM for cytokine analysis. (**i**) cytokine production (24h cultures) of explants (see **g**, **h**) of primary 4THM or EMT6 tumors in WT mice. (**j**) IL-6 secretion from MLCs from mice with EMT6 tumors. MCLs were either unstimulated or stimulated with irradiated EMT6 cells for 40 h. *$P < 0{,}05$ significantly different from corresponding WT MLCs. (**k**) $CD200^+$ cells (Flow cytometry (FACS) staining) in DLNs of 4THM and EMT6-tumor bearing WT mice.

4THM cells are not only very aggressive and induce local and systemic inflammatory response, but they also include many properties of CSCs.[18] Recent studies demonstrated that CSCs might be responsible for the induction of local and systemic inflammation. CSCs are involved in tumor initiation and display increased metastatic potential.[24] Breast CSCs have been reported to express chemokines, including interleukin-8 (IL-8, as well as inflammatory cytokines, including TNF-α and IL-6. In our study, 4THM cells have been observed to secrete high levels of MIP-2 (the murine equivalent of IL-8: EN *et al.*, manuscript in preparation). Inflammatory mediators originating from CSCs can alter the tumor microenvironment and induce local inflammatory response, which can further potentiate the growth of CSCs.[25]

IL-6 is one of the main mediators of inflammation-induced stemness in breast cancer.[26,27] IL-6 has also been linked to metastasis through induction of EMT, increased cell invasion and migration, and recruitment of mesenchymal stem cells.[28,29] Interestingly, LPS and 4THM tumor-induced secretion of IL-6 was progressively increased in WT and $CD200R1^{-/-}$ mice and reached its greatest level in the late stages of disease (Figures 4b vs 4a). IL-6 secretion was observed from lymphocytes of tumor-bearing WT and $CD200R1^{-/-}$ animals even in the absence of additional challenge in MLCs. In contrast, IL-6 secretion was essentially abolished by 24 days after inoculation of tumor cells in $CD200^{tg}$ animals, although IL-6 secretion 12 days after inoculation of $CD200^{tg}$ mice with tumor cells were similar to levels observed in

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WT mice. This might imply that the 'early' inflammatory IL-6 response observed in WT, $CD200^{tg}$ and $CD200R1^{-/-}$ mice is responsible for fostering initial tumor growth, whereas further propagation and metastasis of tumor depends upon the absence of CD200-CD200R1 interactions which will otherwise contribute to an anti-inflammatory response providing effective immune surveillance.[30] Taken together, these findings are also in accordance with clinical studies that demonstrated increased serum concentrations of IL-6 in breast cancer patients are strongly associated with tumor stage and poor prognosis.[31]

TNF-α, a major pro-inflammatory cytokine, has been shown to induce stem cell-like features in breast cancer cells.[32] TNF-α mediates interactions between tumor and stromal cells, leading to an upregulation of genes implicated in tumor cell growth, survival, invasion, metastasis, inflammatory cell trafficking to the tumor site and neoangiogenesis.[33] Tumor-pomoting effects of TNF-α were demonstrated in triple negative breast carcinoma,[34] and clinical studies have also documented an important role for TNFα in advanced breast carcinoma resistant to conventional treatments.[35] As reported above, we found that WT animals bearing 4THM tumors produced TNF-α in response to LPS challenge, an effect which was even more pronounced in $CD200R1^{-/-}$ mice in the late stages of disease. In contrast, in $CD200^{tg}$ mice, negligible LPS-induced TNF-α production occurred by 24 days post-tumor injection. In all mice no detectable TNF-α was observed following 4THM challenge using cells harvested at either day 12 or 24 post tumor injection, although IL-6 production was observed under these circumstances (see above). This may reflect a different threshold for induction of these inflammatory cytokines by tumors growing *in situ*. In all cases however, the persistence of LPS-induced cytokine production in WT and $CD200R1^{-/-}$ mice at day 24 suggests the absence of a generalized 'tolerance' to an inflammatory stimulus within the tumor environment at this time period in the same animals.

IFN-γ is produced mainly by natural killer cells and specific T-cell subsets that play a critical role in an antitumor immune response.[36] IFN-γ induces activation of effector macrophages, which can lyse tumor cells, increase antigen presentation, and inhibit angiogenesis,[37] all of which are consistent with an anti-IFN-γ protective effect in the model described. Thus we observed that 4THM-induced IFN-γ production was greatest in MLCs of 4THM-injected $CD200^{tg}$ animals compared with WT and $CD200R1^{-/-}$ mice at day 12, and by day 24 irradiated 4THM cells were capable of inducing IFN-γ responses only in cells from $CD200^{tg}$ animals, consistent with increased growth in WT and $CD200R1^{-/-}$.

IL-10 can be secreted by both Th1 and Th2 cells and has a dual role on tumor development and carcinogenesis.[38] IL-10 has been reported to function as an anti-anti-inflammatory molecule preventing tumor development in animal models of chronic inflammation-induced carcinogenesis,[39] and inducing regression of established breast cancer metastases.[40] Conversely, IL-10 has been associated with tumor progression, and tumor IL-10 levels correlate with disease severity.[38] Interestingly, our data showed a biphasic effect of CD200 expression on IL-10 secretion during tumor growth. Thus in $CD200^{tg}$ mice IL-10 production induced by LPS/4THM was increased relative to unstimulated controls at 12 days post 4THM injection, but was essentially abolished by 24 days after injection, whereas persistence of LPS-induced IL-10 production was seen at 24 days in WT and $CD200R1^{-/-}$ mice. We speculate (see also above) that CD200 expression may be most important in later stages of tumor growth in this model (as IL-10 production declines) when it can later function as an alternative (to IL-10) modulator of anti-tumoral immune responses in carcinomas characterized by excessive inflammation.

Intra-tumoral CD8 T-cell infiltration is associated with delayed recurrence and extended survival in patients,[41] and infiltration of breast cancer with $CD8^+$ cytotoxic T lymphocytes is associated with a good response to chemotherapy.[42] Somewhat surprisingly, infiltration of $ER^{(-)}$ breast cancer with Treg cells has been reported to be associated with increased anti-tumor immunity and a more favorable prognosis.[43] In a similar vein, we observed increased metastasis in the absence of CD200-CD200R signaling, a putative immunosuppressive signaling interaction, in association with a decreased number of $CD8^+$ and $CD4^+CD25^+$ T cells within the tumor tissue. It is important to note, however, that we cannot exclude the possibility that the altered phenotype of T-cells within the tumor reflects a direct role for CD200:CD200R1 interactions in regulation of cell migration *per se*, rather than an indirect one on the environmental milieu (chemokines/cytokines and so on). Such an interpretation is amenable to experimental investigation using, for instance, adoptive transfer of WT ($CD200R1^+$ T cells) into $CD200R1^{-/-}$ tumor-bearing mice to investigate the effect of possible replenishment of $CD8^+$ tumor-infiltrating cells in such circumstances-such studies are currently underway.

The effects of CD200-CD200R interactions on development and metastasis of mouse 4THM breast carcinoma seemed to be the opposite of what was observed previously with EMT6 breast tumor cells.[19,44] Importantly, EMT6 tumors induce a systemic inflammatory response to a far lesser degree than 4THM tumors (see Figures 5d–f and l, comparing inflammatory cytokine production and systemic leukocytosis) and do not express vimentin (Supplementary Figure 4C) which is a marker for EMT and increased metastatic potential.[45] In agreement with these observations we observed (Supplementary Figure 5) that the migration ability of EMT6 cells was less than that of 4THM cells, and EMT6 cells migrated in clusters, which makes invasion through vascular structures less feasible, whereas 4THM cells migrated as single cells. Consistent with these differences, metastasis is more pronounced for 4THM than EMT6 tumors. These different features in turn might in part contribute to the differences observed in the host's response to the tumor. Thus the IL-6 response in MLCs for cells from EMT6 tumor bearers was greater in WT mice compared with $CD200R1^{-/-}$ animals, suggesting that despite a lack of CD200R1 signaling, an accelerated inflammatory response did not occur in EMT6 tumor bearers. This reduced IL-6 response might in turn explain the decreased EMT6 tumor growth and metastasis observed in $CD200R1^{-/-}$ animals.[19,44,46] Immunoediting of cancer cells has also been reported to lead to EMT and a more aggressive phenotype,[47] and thus we predict that EMT6 tumors are likely to be more immunogenic/less immunoedited. We suggest that CD200 expression increases the malignant potential of tumors that are highly immunogenic and poorly metastatic, whereas acting to inhibit the growth of highly aggressive carcinomas which present with extensive local and systemic inflammation.

Interestingly, S100A8, a calcium binding protein expressed by cells of myeloid lineage, has been reported to induce proinflammatory chemokines both intracellularly and extracellularly. Murine S100A8, also called CP-10, is a potent chemotactic factor for neutrophils, and induces sustained leukocyte recruitment *in vivo*.[48] More recently, evidence for S100A8 induction of chemokines in lung tissue was correlated with increased invasive and migratory abilities of tumor cells.[49] Carcinoma patients express higher serum S100A8 protein concentrations than adenoma patients,[50] and increased S100A8 expression was associated with lung metastasis.[51] In a recent study, Liu *et al.*[52] reported that bone marrow-derived $CD11b^+Gr1^+$ myeloid cells expressing S100A8 were increased in number in premetastatic brain lesions of mice bearing 4T1 tumors, and treatment of 4T1 tumor-bearing mice with the cyclooxygenase-2 inhibitor attenuated inflammatory chemokine expression, brain infiltration by $CD11b^+Gr1^+$ cells and formation of brain metastasis.

The data shown above and in supplementary figures are consistent with these observations, and show that premetastatic liver and lung tissue of mice injected with highly inflammatory

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4THM tumors are heavily infiltrated with S100A8$^+$ cells (Supplementary Figures 3 and 4).

In conclusion, comparison of the data above with those previously observed in the EMT6 breast cancer model, suggests that the effects of CD200-CD200R1 interaction on tumor progression in these models may reflect differences in immunogenicity and metastatic potential, and the capacity of the tumor cells themselves to induce chronic inflammation. The prognosis of triple negative breast cancers with a cancer stem cell phenotype is poor and effective treatments are lacking. 4THM cells originally derived from triple negative murine breast carcinoma cells (4T1).[14,17] The results reported in this manuscript indicate that systemic exposure to CD200 or CD200R1 agonists may prevent metastasis as well as local growth of this aggressive breast cancer. Inflammatory breast carcinoma is a highly aggressive, poorly differentiated malignancy that is associated with resistance to treatment and poor survival rates. The 4THM model mimics many aspects of inflammatory breast carcinoma, including the presence of breast CSCs, epithelial mesenchymal transition, extensive visceral metastases, lack of estrogen receptor expression and an undifferentiated state.[18] Our data are the first to suggest that CD200-CD200R1 interactions can decrease progression of metastatic inflammatory breast carcinoma, and taken together with our previous observations in an EMT6 tumor model, imply that modulating CD200 activity/signaling might be an important therapeutic strategy for the treatment of breast tumors, even in those characterized by increased systemic and local inflammatory response.

MATERIALS AND METHODS

Mice

Wild-type (WT) female BALB/c mice were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). Mice were housed five per cage under specific pathogen-free conditions and were allowed food and water *ad libitum*. All mice were used at 8–12 week of age. All animal experimentation was performed following the guidelines of an accredited animal care committee. The derivation of CD200R1$^{-/-}$ and CD200^{tg} mice, both on a BALB/c background was described previously.[19] Homozygous CD200^{tg} females received DOX (1 μg/ml) in their drinking water (DOX-water) to induce CD200 expression.[53]

Cell lines

4T1 breast cancer cells were previously derived from a spontaneously appearing breast tumor in a BALB/c female mouse. The 4THM cell line was derived from cardiac metastases of 4T1 cells by EN *et al.*[16,54] 4THM and EMT6 cells were grown in α-minimum essential medium (α-MEM) supplemented with penicillin and streptomycin and 10% fetal bovine serum.

Metastasis assay

4THM cells (1×10^5 cells/mouse) or EMT6 cells (5×10^5 cells/mouse) were injected orthotopically into the right upper mammary gland of 8–10 week female recipients. Necropsies were performed either 12 or 24 days after injection, with lung tissues stored in Bouin's fixative to visualize macroscopic nodules, as described before.[17] Liver tissue was originally fixed in 10% buffered formalin and sectioned after embedding in paraffin wax. Five sections from each tissue were stained with hematoxylin and eosin to determine the extent of metastases microscopically. For each animal, 20 photographs from 5 different sections were randomly taken at 20x magnification; areas of microscopic metastatic lesions were then selected and measured as mm^2 with Spot advanced 4.6 software (Diagnostic instruments, Inc 2006, Sterling Heights, MI, USA).

In some experiments (as noted in the text) equal numbers of EMT6 and 4THM cells ($1x10^5$/mouse) were injected into mammary fat pad of mice and animals were killed 20 days later. Blood samples were obtained before kiling to prepare peripheral blood smears stained with hematoxylin and eosin. Neutrophil, lymphocyte and monocyte numbers in the peripheral blood were enumerated using 10 slides at 40x magnification from each animal.

MLCs

Spleen and DLNs of animals injected with 4THM cells were removed aseptically and single-cell suspensions prepared for MLCs. Cells (4×10^6/well in 48-well tissue culture plate) were cultured alone (control for basal cytokine release) or stimulated with LPS 3 μg/ml or irradiated (20 Gray) 5×10^4 4THM cells. MLCs from control animals (not injected with 4THM cell) were also prepared. TNF-α levels were measured 20 h after challenge, whereas measurements of IFN-γ, IL-6 and IL-10 were performed in supernatants obtained 40 h after challenge. Standard ELISA kits were used for cytokine measurements (for IL-6, TNF-α and IL-10, from BD Biosciences (Mississauga, ON, Canada); for IFN-γ from eBioscience (San Diego, CA, USA)).

Analysis of cytokine expression in tumor tissue of tumor-bearing mice

Tumor tissue was minced into small pieces in medium (1 – 2 mm), passed through an 18-gauge needle to dissociate the tumor cells and plated in 24-well plates. Dulbecco's modified Eagle medium: nutrient mixture F-12 (DMEM-F12) medium containing 0.1% FBS was added to cultures for 24 h three days after plating to obtain CM. IL-6 and TNF-α levels were determined in CM using enxyme linked immunosorbant assay (ELISA).

Antibodies and cell staining

All anti-mouse monoclonal antibodies used for cell-surface phenotype characterization (CD200-PE, CD200R-FITC, CD45-PeCy.7, CD3-FITC, CD3-APC, CD8-PE, CD8-FITC, CD8-PeCy.7, CD4-FITC, CD4-PE, CD25-PeCy.7, GR-1-FITC, CD11b-PE and F4/80-PeCy.7) were purchased from BioLegend (San Diego, CA, USA). Multicolour flow cytometric analyses were conducted to characterize DLN cells, splenocytes, and tumor infiltrating cells. Tumors, DLNs and spleens were digested as described before.[44] The optimal concentration of antibody for staining was titrated individually for each antibody. Single colour controls were included in each experiment for compensation purposes, as well as fluorescence-minus-one (FMO) controls; all samples were analyzed in a CANTO-II flow cytometer, using FloJo software (Ashland, OR, USA). Samples from 2 – 3 mice were pooled per staining group and staining was performed in triplicate.

In vitro migration assay

Migration assays were performed using an Oris Cell Migration Assembly Kit (Platypus Technologies, Madison, WI, USA, Cat No: CMAUFL4). Cells were seeded in 5% FBS in the presence of stopper, which was removed 16 h later, and initial detection zones were photographed (time 0). 18 h later, photographs of the detection zone were taken. Areas of cell movement were marked as μm using Spot advanced 4.6 programme. Experiments were repeated twice with four replicates in each experiment.

Immunohistochemistry

Primary tumor tissue was fixed in 10% formalin and embedded in paraffin. 5 μm thick serial sections were collected. Staining with heterologous rabbit antibodies for mouse CD200 and CD200R (prepared after immunization in Freund's adjuvant with protein purified from supernatants of CHO cells transduced to express the respective cloned genes: Gorczynski *et al.* unpublished), S100A8 (GTX62287, Genetex, Irvine, CA, USA) and Vimentin antibody (1/100 dilution; Milipore Corporation, Billerica, MA, USA; Cat No. AB5733) was performed essentially as described elsewhere.[18]

Statistics

Student's *t*-test was used to compare metastatic indexes. When the variance expressed as s.d. between the groups differed, the Welch *t*-test or non-parametric (Mann–Whitney test) was used. Analysis of variance with Dunnett's post test was used for the analysis of cytokine results. *P*-values < 0.05 were considered biologically significant. Statistical analyses were performed using GraphPad InStat 3 software (San Diego, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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