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Interaction of Immune Cells and Soluble Factors Which Contribute to Local and Metastatic Tumor Control within the Tumor Microenvironment

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Abstract

Background and purpose: Transplantable immunogenic breast tumors (EMT6) in CD200RKO mice, but not wild-type (WT) animals, are cured of local and metastatic tumor growth following surgical resection and immunization with irradiated cells in adjuvant. By contrast tumor growth of the poorly immunogenic 4THM breast cancer was actually enhanced in CD200R1KO mice by comparison with WT mice. A novel two-phase culture system in which tumor invasion from a liquid phase occurs into a collagen gel containing bone marrow mesenchymal stromal cells (BMMSCs) was described which could recapitulate many of the differences observed *in vivo* between EMT6 and 4THM tumor cells. Invasion of tumor cells into the gel layer was monitored after collagenase digestion and culture of tumor cells at limiting dilution. Aliquots of the digest were assayed for cytokine levels in ELISA.

Results: Growth of both EMT6 and 4THM tumor cells into the collagen matrix was increased in collagen gels impregnated with BMMSCs. TGF β , IL-6 and IL-17 were detected in both gel and liquid phases following addition of DLN cells to the matrix. While inclusion of IL-6 and IL-17 alone in the gel matrix increased EMT6 tumor cell invasiveness, this was actually attenuated by inclusion of Draining Lymph Node (DLN) cells from EMT6 immune mice into the gel. This attenuation was abolished by anti-TGF β antibody, and re-established using recombinant TGF β . Anti-TGF β did not affect 4THM tumor invasion into collagen gels, though there was a trend to increased invasion with TGF β added to the gel matrix.

Conclusion: Micro-environmental stromal elements and the cytokines IL-6/IL-17/TGF β control local invasion of breast tumor cells. These effects are further differentially modulated in different tumors by the presence of additional inflammatory/immune responses.

Keywords: Tumor microenvironment • Invasion and metastasis • *In vitro* models • Cytokines • TGF β

Introduction

Immune and non-immune cells (stroma) within the tumor microenvironment (TME), along with factors produced by those cells, play important roles in tumorigenesis [1]. In early stages of tumor growth tumor-antagonizing immune cells/factors within TME seem to inhibit growth of cancer cells, while at later stages, escape from, or even inhibition of the effector function(s) of tumor-antagonizing immune cells, can occur [2,3]. Tumor-associated stromal cells (TASCs), novel stromal cells in the inflammatory TME which develop after long-term interaction with tumor cells, express higher levels of multiple proteins compared with their normal non-reactive counterpart [1]. In addition they secrete pro-tumorigenic factors, including IL-6, IL-8, Vascular Endothelial Growth Factor (VEGF), and matrix metalloproteinases, all of which contribute to augmented recruitment of tumor and pro-tumorigenic cells to the microenvironment [1]. The immune evasion capability of tumors (tumor checkpoints) has been studied leading to the development of clinical strategies which target molecules associated with checkpoint inhibition. Use of immune checkpoint inhibitors, as exemplified by the use of anti- CTLA4 and anti-PD antibodies, along with adoptive transfer of engineered immune cells (e.g. CAR-T) have revolutionized antitumor therapy for many solid tumors [4].

Identifying which targets may be suitable for further investigation in tumor growth immunomodulation, and their mechanism of action, represents a prime area of research in this field. *In silico* technology has been discussed in detail elsewhere as a methodology to identify the variety of cells and/or factors which contribute to the heterogeneity of the TME [3,5]. This is discussed in detail elsewhere making use of ESTIMATE methodology, followed by CIBERSORT and ultimately by direct gene expression analysis using tumor tissue [6]. However, in addition to identification of unique cell combinations, and differential gene expression data, research models, both *in vitro*, and ultimately *in vivo*, must be developed to improve understanding of the mechanism(s) by which various cells/factors interact to alter tumor growth, and how this may be manipulated to improve cancer survival.

We described a model 3D culture system to explore how cells/cytokines in the TME could control local growth and invasion of transplantable murine breast cancer cells [7-9]. A 2- phase 3-D culture system with bone marrow derived stromal cells (BMMSCs) from 12 day pre- cultures of T-depleted bone marrow cells embedded in a collagen matrix was overlaid with liquid cultures of tumor cells, and tumor cells infiltrating the collagen matrix investigated [7,10].

Tumor cell growth and survival involved cell–cell contact and cytokine production, including IL- 6 and IL-17 [11,12]. Inclusion of $CD4^+$ lymphocytes from tumor immunized mice along with BMMSCs in the collagen gel further regulated tumor invasion into the collagen gel phase [7].

Importantly, these data from preliminary *in vitro* studies mimicked data from earlier *in vivo* results studies, and the augmented tumor invasion associated with either exogenous IL-6 and IL-17 added to collagen gels containing stromal cells, or by added immune $CD4^+$cells, was (in the latter case) abolished by inclusion of anti-IL-6 or anti-IL-17 antibodies in the collagen matrix. Other studies also support an important role for members of the IL-17

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cytokine family in the TME in tumor invasion [13-15]. We have also shown that cell surface CD200:CD200R interactions can regulate breast cancer growth and metastasis *in vivo*, and established a similar influence of CD200:CD200R interactions for regulation of tumor invasion *in vitro* by utilizing BMMSCs and CD4+ cells from mice in which gene expression of CD200 and/or CD200R was disrupted [7,16-22]. We now report on the additional interactive role of TGFβ expression on local and distant breast cancer growth [23,24]. TGF-β is produced by cells in the tumor model described in Figure 3, with existing evidence from other groups that TGFβ is both a suppressor of tumor growth at early stages of tumor development, while at later stages may be implicated in promotion of invasion and metastasis [25,26].

Materials and Methods

Mice

8-week old wild type (WT) female BALB/c mice from Jax Labs, and CD200KO or CD200R1 knockout mice on a BALB/c background, are described elsewhere (20). All mice were housed 5/cage in an accredited facility at the University Health Network (UHN).

Ethics review

All studies were approved by a local institutional review board, certified by the Canadian Council on Animal Care (protocol AUP#1.15).

Monoclonal antibodies (mAbs)

All mAbs are described elsewhere [7,8,22].

EMT6 and 4THM tumor models and derivation of tumor-immune mice (22)

2x10^5 cells EMT6 and 4THM tumors carried *in vitro* (2 passages from frozen stock) were injected into the mammary fat pad of BALB/c female mice (WT or KO). Axillary lymph nodes draining the tumor (DLN) were harvested at the times indicated and single cell suspensions prepared after passing through a wire mesh. Where described, tumor bearing mice had tumors resected 14d after injection. Mice were immunized ip with homologous irradiated (2000Rads) tumors cells (5x10^6) mixed with MPLA (2μg/mouse) in incomplete Freund's adjuvant (IFA) 2 days after surgery. 5 mice/group) were used as a source of tumor-immune DLN 14d later.

3-D culture system to assess tumor invasion *in vitro*

The RAFT™ 3D cell culture system was used in accordance with the supplier's reagents [7]. In brief, 2.0 ml of 10 × MEM was added to 15.7 ml rat-tail collagen type I. After neutralization with 1.0 ml of a Neutralizing Solution 0.85 ml of a bone marrow stromal cell suspension (BMMSCs: 6 ×10^6 cells/ml) was added and mixed thoroughly. To produce BMMSCs bone marrow cells pooled from 3 control, non-tumor injected, donors of the indicated source were incubated α-Minimal Essential Medium containing 10% fetal calf serum (α F10) and 1mM dexamethasone for 12 d before trypsinization and washing.

Gels were cast in 24 well plates (750 μl/well), with 4 × 10^5 stromal cells/well, and incubated at 37°C for 15minutes. RAFT absorbers supplied by the manufacturer were used to remove interstitial fluid from the collagen gels, and 500 μl of fresh culture medium was added to each well before returning the plate to the incubator (37°C, 5% CO_2/air and 95% humidity). In some studies (see text) the stromal cell suspension also included 10 × 10^6/ml DLN from naïve or tumor immune mice-these wells contained 4 × 10^5 stromal cells and 7 × 10^5 DLN. 1d after initiation of culture the medium overlying the gel was replaced with medium containing tumor cells (1 × 10^2 of either EMT6 or 4THM origin). All groups included 6 replicate wells.

Assessment of tumor cells by limiting dilution culture analysis

2.5, 5 and 7 days post initiation of Lonza culture as above the liquid layer was removed for 2 wells/group, rinsed four times with 2ml PBS, and tumor cells adherent to the surface collagen were released by light trypsinization (300 μl trypsin/well at 37°C for 5 min) and enumerated by trypan blue dye exclusion were enumerated in the combined medium. The frequency of tumor cells in the collagen gel phase was assessed after digestion with collagenase (250 μl of 0.2% collagenase) for 30 min at 37°C, washing with 2 ml μF10/well, and analysis in a limiting dilution assay in microtitre plates (20 replicates: 6 0μl, 20 μl and 7 μ/well) with all wells containing 1 × 10^5 irradiated BALB/c splenocyte feeder cells to improved cloning efficiency at low numbers of tumor cells/well without having any effect on tumor cell viability/growth at non limiting tumor cell numbers [7]. An aliquot (280 μl) of the digested gel phase was retained for cytokine ELISA (below). Figure1 shows the kinetics of growth of EMT6/4THM tumor cells detected by limiting dilution using the various stromal cell feeder layers described.

ELISA assay for TNFβ, IL-6, IL-8 and IL-17 in 3D cultures

35 μl aliquots of the collagenase treated gel phase, or the medium phase of the 3D cultures described above, were assayed in duplicate for TNFβ, IL-6, IL-8 and IL-17 using commercial kits (BioLegend, USA). Similar ELISA analyses were performed with (2 μl and 6 μl) aliquots of serum from mice pooled within groups at sacrifice.

Statistics

Within experiments, comparison was made between groups using ANOVA, with subsequent paired Student's t-tests as indicated.

Results

Enhanced tumor invasion of 4THM and EMT6 tumor cells using BMMSCs

Growth of immunogenic EMT6 tumor cells is attenuated by inflammatory host reactions-in turn suppression of inflammation in CD200KO or CD200R1KO mice augments tumor growth *in vivo* [20]. In contrast, the reverse growth characteristics were seen for the poorly immunogenic 4THM, where enhanced tumor metastasis followed increased inflammation [22].

Similar differences in tumor invasion of 4THM and EMT6 tumors was seen in the 3D cultures discussed, with typical data shown in Figure 1, pooled from 3 studies of this type [7].

Invasion by 4THM tumor cells was ~5X greater than for EMT6 cells (compare Figure 1B *vs*. Figure 1A), regardless of the source of stromal cells used. Surprisingly, there was no difference in tumor invasion of EMT6 cells

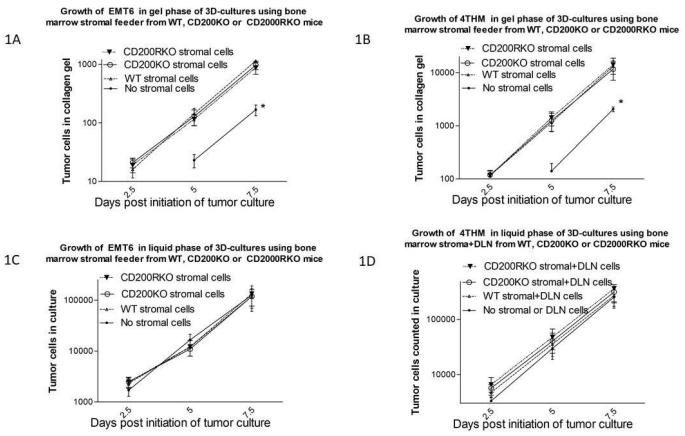

Figure 1. Comparison of tumor cells detectable in collagen gel phase **(Panels A/B)** or liquid phase **(Panels C/D)** of cultures. 4 × 10^5 BMMSCs from the mice indicated were included in the collagen gels. 100 EMT6 or 4THM tumor cells were seeded into the liquid culture overlying the collagen gels, with tumor cells enumerated at 2.5, 5 and 7 d post culture initiation by hemocytometer counting. Tumor cells in collagen gels were enumerated by limiting dilution culture of cells released following collagenase digestion of the gel. Data are pooled from 3 independent studies, each using 6 cultures per group, with 2 cultures harvested at each time point. **P* <0.05 compared with all groups with BMMSCs in collagen gel (Mann-Whitney U-test).

(or 4THM cells) seen in cultures with BMMSCs derived from WT/CD200KO or CD200RKO mice, despite the difference in metastasis by these tumors seen *in vivo* in such mice. This implies that stromal cell elements alone do not account for the differences seen *in vivo* [16,19]. 4THM cells grew ~2X faster than EMT6 cells in liquid cultures regardless of BMMSCs in the gel (panels C/D in Figure 1).

By inclusion in the gel matrix of DLN cells derived from tumor-bearer immunized mice there were now different invasion characteristics of EMT6 and 4THM *in vitro*, somewhat analogous to those seen *in vivo* (Figure 2). Thus when DLN cells from CD200RKO mice were used in collagen gels, a significant attenuation of invasion of EMT6 cells was seen, consistent with data *in vivo* and preliminary reports *in vitro* (Figure 2A) [7,22]. In this situation an enhanced inflammatory response is predicted in the absence of CD200:CD200R signaling, thus leading to inhibition of tumor invasion. By contrast, these same conditions actually enhanced invasion of 4THM cells, again consistent with previous *in vivo* data (Figure 2B) [7,22]. Regardless of the tumor cell studied (EMT6/4THM), or the DLN cells used, there was no noticeable effect on cells grown in the liquid culture phase (Figures 2C and 2D).

Importance of IL-6, IL-17 and TGFβ, but not TNFα, to tumor invasion *in vitro*

Both IL-6 and IL-17 (but not TNFα were reported to regulate metastasis/invasion of EMT6 cells *in vivo* [19]. In Lonza cultures in Figure 2, anti-6 and anti-IL-17 antibodies augmented tumor invasion while anti-TNFα had no observable effect [7,22]. Importantly, in the presence of non T depleted (but not T depleted) DLN cells from CD200RKO mice the augmented tumor invasion of EMT6 in vitro by recombinant IL-6/IL-17 was abolished, suggesting the existence of undocumented factor (s) which were further modulating tumor invasiveness [7]. To investigate whether TGFβ was one such factor was explored as follows.

First we analyzed culture medium from both the gel matrix and liquid culture phase in typical to those shown in Figure 2 for TNFα, IL-6, IL-17 and TGFβ using commercial ELISA kits. All cultures also included tumor-immune DLN cells from the mice shown, thus mimicking conditions *in vivo*. Panel A of Figure 3 shoes data using serum from WT or tumor-immunized mice as controls. No cytokines were detected above the level of sensitivity (5 pg/ml) for any sample assayed from the liquid culture phase, and these data are not shown to retain clarity.

Increased (~2.5 fold) TNFα, IL-6, IL-17 and TGFβ levels were seen in serum of 4THM tumor immune mice compared with EMT6 tumor immune mice, with levels in control mice ≤ 25 pg/ml for all cytokines (Figures 3A and 3B). Detectable cytokine levels were seen in collagen gels for both EMT6 and 4THM cultures when DLN cells from both CD200KO and CD200R1KO mice were added, compared with those cultures without DLN- this supports their potential importance, at least in part, in the increased tumor invasion seen in Figure 2A/B. However, it must be acknowledged that to date the source of cytokines, whether stromal cells (see (7)) or DLN cells, remains unexplored. Importantly, in EMT6 cultures, despite the increased levels of both IL-6 and IL-17 levels in cultures containing DLN in Figure 3A, tumor invasion into collagen gels was decreased (Figure 2A). This may reflect an effect of another functional activity, dependent upon DLN cells, which can attenuate the augmented invasion caused by IL- 6/IL-17. Furthermore, we have already argued that both *in vivo*/*in vitro*, TNFα does not seem to affect tumor invasion, and we speculated that TGFβ produced by tumor-immune DLN cells may be one of the undocumented contributors to the effects observed.

Anti-TGFβ antibody modifies immune DLN cell modulation of IL-6/IL-17 increased tumor invasion.

To assess whether TGFβ levels were independently and differentially affecting invasion of EMT6 and 4THM we initiated cultures as described for Figure 2 with immune DLN from CD200RKO mice, with further addition of recombinant IL-6/IL-17 (150 pg/ml) to both collagen gel and liquid phase cultures [7]. In addition, anti-TGFβ antibody (final concentration 10 μg/ml) was included in the gel matrix (see Materials and Methods). Data in Figure 4 are results pooled from two studies of this type.

Importantly, while again IL-6/IL-17 augmented invasion of EMT6 in Figure 4A, an effect attenuated by addition of immune DLN from CD200RKO mice in Figure 4A, addition of anti- TGFβ antibody relieved this suppression of invasion of EMT6 in cultures containing immune DLN cells from CD200RKO mice (Figure 4B *vs.* Figure 4A). Immune DLN cells from 4THM immune mice did not attenuate invasion of 4THM cells in the presence of IL-6/IL-17 (Figure 4C-trend towards enhanced invasion). Addition of anti-TGFβ did not further affect invasion in the presence of immune DLN and IL-6/IL-17 (Figure 4D).

Ability of TGFβ alone to modulate effect of IL-6+IL-17 increased tumor invasion

Data above show that immune CD4+ DLN cells from CD200RKO mice (but not T depleted CD4+ DLN cells) attenuated a presumed IL-6/IL-17 enhanced invasion of EMT6 but not 4THM cells *in vitro*, and that this effect of immune DLN cells on invasion of EMT6 was reversed by anti-TGFβ (Figure 4B). As additional evidence that TGFβ was a prominent mediator in the effect observed using immune DLN cells, we performed studies in which tumor invasion was measured using exogenous IL-6/IL-17 added to the gel layer, with additional recombinant TGFβ added in other groups. These data, shown in Figure 5, confirm the observations described in Figure 4, and show that indeed exogenous TGFβ (known also to be produced from immune DLN of

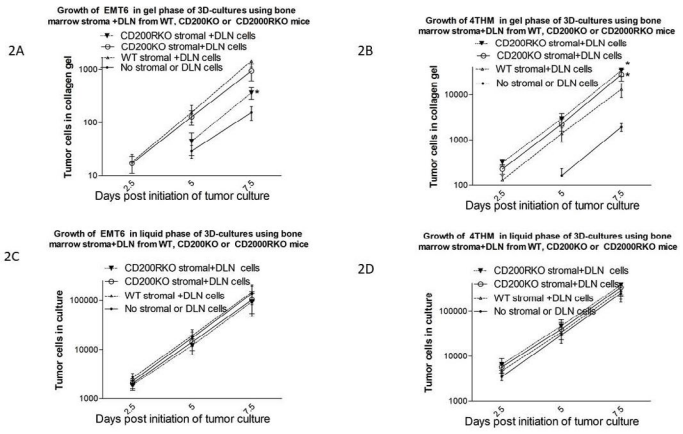

Figure 2. Comparison of tumor cells detectable in collagen gel phase **(Panels A/B)** or liquid phase **(Panels C/D)** of cultures with 4×10^5 BMMSCs and 7×10^5 DLN cells from the mice indicated added to the collagen gels. 100 EMT6 or 4THM tumor cells were seeded into the liquid culture overlying the collagen gels, with tumor cells in the liquid/gel phase enumerated as in Figure 1. Data are pooled from 2 independent studies; each using 6 cultures were used per group, with 2 harvested at each time point. *P <0.05 compared with group using stroma/DLN cells from WT mice in collagen gel (Mann-Whitney U-test).

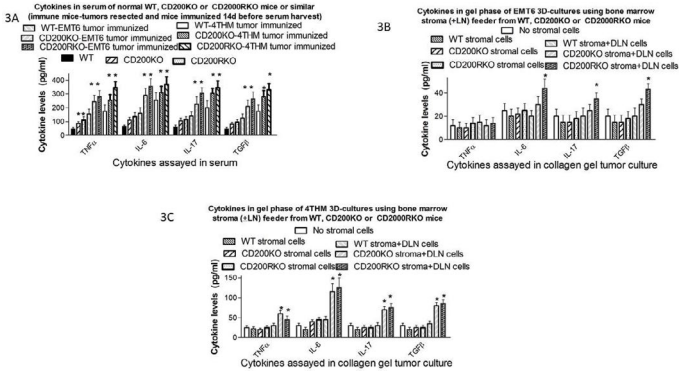

Figure 3. Panel A Mean Serum cytokine levels (assayed by ELISA) from 4/group control or tumor immune mice mice of the strain shown. *P <0.05 compared with corresponding WT control group B and C. Cytokine levels (pg/ml at 7.5 d of culture) in collagen gel phase of cultures shown in Figure 2, **Panel B** represents EMT6 cultures and **Panel C** represents 4THM cultures. Cytokine levels in serum of mice used to provide DLN cells for this study are shown in A. All values represent mean ± SD of 4 measures over 2 independent studies. *P <0.05 for culture groups shown compared with levels in equivalent groups with only stromal cells, or with stromal cells and WT DLN.

both EMT6 and 4THM immune mice-see Figure 3B), attenuated increased invasion of EMT6 tumor cells, but not 4THM tumor cells, *in vitro*. A trend to increased invasion of 4THM in the presence of additional TGFβ (see also effect of anti-TGFβ with 4THM cells in Figure 4) was seen.

Discussion

TGFβ is a multifunctional cytokine implicated in many cellular processes in both developing and adult organisms [23]. Its signaling role TGFβ in cancer is quite complex, with evidence for both early suppression (by inhibition of cell growth and apoptosis) and later enhancement of tumor formation [24]. Tumor promotion likely reflects the outcome of at least two different mechanisms. The one occurring through promotion of epithelial mesenchymal transition (EMT), leads to enhanced migration, invasion, infiltration, and extravasation of the tumor cells, while at least one of the others reflects action of a paracrine signaling pathway whereby TGFβ in the TME activates cancer-associated fibroblasts (CAF), promotes angiogenesis and may even inhibit anti-tumor immune responses causing promotion of tumor metastasis [25,26]. Such effects may help explain the recent evidence for a role for tissue resident macrophages in promotion of metastatic spread of ovarian cancer cells, and their adoption of a cancer stem cell phenotype [27]. TGFβ modulates activation/differentiation of multiple cell targets, including fibroblasts, mesenchymal stem cells, epithelial tumor cells, adipose tissue-derived stem cells, and endothelial cells and can also promote angiogenesis and migration in the TME, and modulate acquired immunity. Thus TGFβ inhibits T cell activation, proliferation, differentiation, and migration of ($CD8^+$) effector T cells (inhibition of IFNα and IL-2, and enhanced expression of PD-1), and both inhibits the differentiation of CD4+ T cells into various effector subtypes, and also promotes development of regulatory (immunosuppressive) CD4+ T cells [28-32]. Using a gene screening technique to assess correlation between gene expression frequencies and prognosis in human head and neck squamous cell carcinoma, CCR4 and CCR8 (chemokine receptor genes) and P2RY14 (involved in the G-protein pathway and regulation of the immune stem cell compartment) were identified as key genes important in the promotion of development of a TME which could stimulate anti-tumor immunity and inhibit tumor growth and invasion [33].

We have reported on an important role for inflammatory cytokines (IL-6/IL-17) on tumor growth and metastasis in immunogenic (EMT6) and non-immunogenic (4THM) mouse breast cancer lines *in vivo* and in a novel 3D culture cell model [7,17,19,22]. These studies are consistent with clinical reports showing an effect of IL-6/IL-17 on human tumor growth/metastasis [13,34-36]. Interactions between endogenous CD200:CD200R in the TME, an interaction thought to affect inflammatory and other immune pathways (20-22), are also important in regulation of growth and metastasis in these breast cancer models, though much remains to be understood concerning the mechanisms for these effects. The importance of understanding CD200:CD200R interactions in tumor growth control was emphasized by recent data showing that in 3D cultures, addition of exogenous IL-6 and IL-17 could enhance tumor invasion of both EMT6 and 4THM, while studies in which DLN cells from CD200RKO tumor immune mice were added to these cultures (Figures 2-4) highlighted a role for other factors which are implicated in regulation of growth/invasiveness of these two tumor lines [17,19,22].

Exploration of expression of prominent cytokines in tumor bearing and tumor-immunized mice which might affect tumor growth confirmed, as expected, an increased presence of IL-6 and IL-17 (Figure 3). While TNFα expression was similarly enhanced, we have been unable to modulate tumor invasion *in vitro* or growth in vivo by targeting TNFα [7]. Following growth of both tumors *in vivo* we also recorded enhanced expression of TGFβ in DLN (Figure 3). There are intriguing other research data showing the complexity of interactions which can modulate tumor cell growth/invasion-thus cancer cell escape from TGFβ-induced cell suppression can be modified by targeting another site of immunotherapy, namely simultaneous use of checkpoint inhibitors, such as PD-1/PD-L1 antibodies. The anti-tumor effect of the combination of PD-L1 antibody and TGF-β antibody is reported to be greater than that seen with single-drug treatment [37,38]. Data in Figures 4 and 5 support the hypothesis that TGFβ may be a crucial factor implicated in the enhanced EMT6 tumor invasion seen in the presence of IL-6/IL-17, which is attenuated by immune DLN (Figures 3 and 4). Thus addition of anti-TGFβ reduced the suppression by immune DLN cells of invasion of EMT6 induced by IL-6/IL-17. Furthermore, addition of recombinant TGFβ itself decreased invasion of EMT6 induced by IL-6/Il-17 (Figure 5). Note that for the highly inflammatory tumor 4THM, anti-TGFβ and recombinant TGFβ had, if anything, the opposite effects (Figures 4D and 5B). There was a trend to augmented tumor invasion induced by TGFβ, and towards attenuation of invasion in the presence of anti-TGFβ, which we suggest is most with a primary effect of TGFβ in this tumor on enhanced EMT and invasion [26,39]. This, in turn, is further consistent with our *in vivo* data indicating that attenuation of an inflammatory responseby the CD200:CD200R axis played no major role in control of growth/metastasis by 4THM, unlike EMT6.

Given that in the studies of Figure 5 no DLN cells were present in the cultures, the effect of TGFβ observed on either tumor must presumably be a direct one (on tumor cells themselves) or indirectly through the TME afforded by BMSCs. This in turn might imply activation of different TGFβ signaling pathways within the two different tumor cells, or that in combination with TME- associated factors, TGFβ results in activation of different pathways affecting tumor invasion -this we have attempted to portray in the schematic model in Figure 6). In the scenario shown in Figure 4, where immune cells from DLN were present, the differential effect of anti-TGFβ on invasion by the two tumors may be in part also explained by the role of TGFβ in altering multiple other pathways known to regulate tumor growth/invasion. As an example, it is known that 4THM tumor bearing mice have an increased number of myeloid-derived suppressor type cells, with enhanced splenomegaly and myelopoiesis present in mice, which can modulate tumor growth [17,40-42]. In comparison, in EMT6 tumor bearing

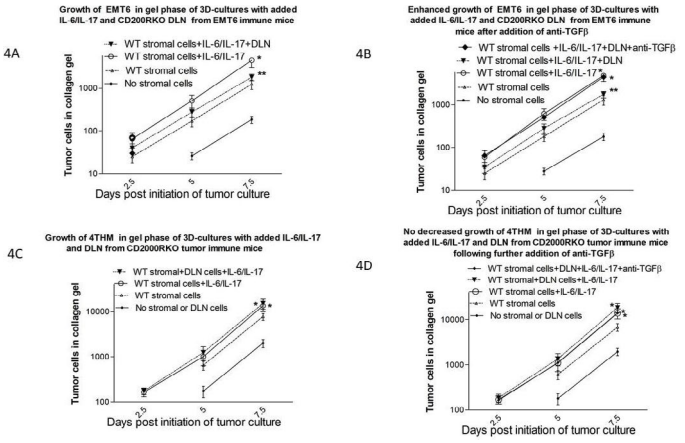

Figure 4. Comparison of EMT6 **(4A/4B)** or 4THM **(4C/4D)** tumor cells detectable in collagen gel phase of cultures including 4×10^5 BMMSCs. 150pg/ml recombinant IL-6 or IL-17, and in Figures 4B/4D anti-TGFβ antibody also, was added in both the collagen gel and liquid phase of cultures to the groups shown. Remainder of set-up as for Figures1-3. *P <0.05 compared with group with bone marrow stromal cells only (no cytokines) in collagen gel (Mann-Whitney U-test). ** P <0.05 compared with equivalent group without anti-TGFβ.

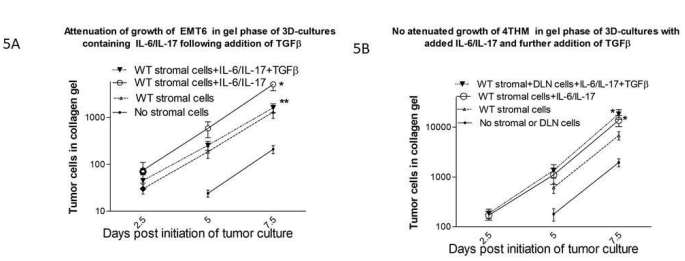

Figure 5. Effect of recombinant TGFβ (75pg/ml) added to gel and liquid phase of EMT6 **(5A)** or 4THM **(5B)** tumor cell cultures containing 4×10^5 BMMSCs and 150pg/ml recombinant IL-6 or IL-17 on migration of tumor cells into the gel phase of culture. Remainder of set-up as in Figures1-4.*P < 0.05 compared with other groups without cytokines (Mann-Whitney U-test); **P < 0.05 compared with equivalent group without TGFb.

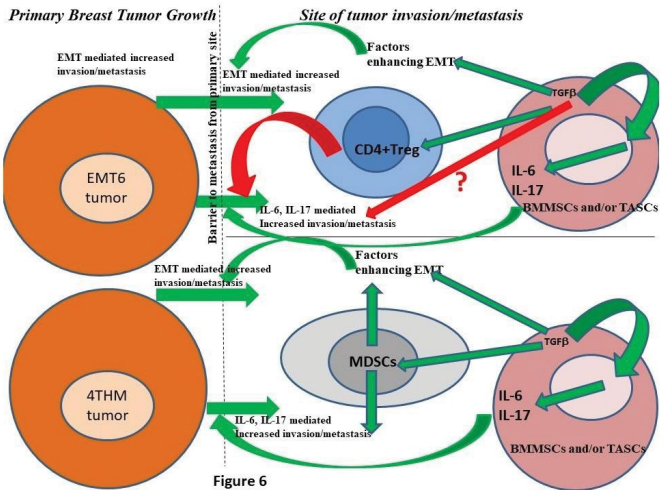

Figure 6. Schematic to help understand the differential effect of DLNs, MDSCs, $CD4^+$Tregs, IL-6, IL-17 and TGFβ on tumor invasion of EMT6 or 4THM tumors into the TME from a primary source. Note that EMT and both IL-6/IL-17 lead to enhanced tumor invasion for both EMT6 and 4THM tumors. However, differences are apparent in the effect of TGFβ, which for EMT6 may act on infiltrating $CD4^+$T cells resulting in their antagonism of IL-6/IL-17 mediated invasion, while for 4THM TGFβ may promote further production of IL-6/IL-17 from MDSCs, or may stimulate (from MDSCs) other factors enhancing EMT.

mice there is better evidence for a role for $CD25^+$Tregs in enhanced tumor growth [6,20,41,42]. TGFβ signaling is known to play a role in both MDSC and Treg development/function, consistent with the schematic model in Figure 6 [40-43]. However, as a further confounding issue to these interpretations, it should be noted that no studies have been performed to explore the effect of titration of the various cytokines (and antibodies) in the cultures described. Thus more detailed exploration of the mechanism(s) involved in the model system explored awaits more investigation.

Conclusion

Using an *in vitro* system to explore factors associated with increased invasiveness in two mouse breast cancer tumors, the non-immunogenic 4THM tumor, and the more immunogenic EMT6 tumor, the data shown indicate that the inflammatory cytokines IL-6 and IL-17 enhance invasiveness of tumors into a TME containing BMSCs. For EMT tumors, this enhancement is attenuated by EMT6 primed DLN cells, or by recombinant TGFβ. In contrast, immune DLN cells or TGFβ had no effect on attenuating invasiveness of 4THM. It is hypothesized that these differences may reflect a difference in previously reported MDSCs and/or $CD4^+$Tregs in these two models, and/or an intrinsic difference in TGFβ signaling or EMT in the tumors themselves.

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Author Contributions

The author is responsible for all aspects of the article.

Availability of Data and Materials

All data supporting the findings are reported in the enclosed. Individuals requesting access to original data can write the corresponding author.

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Conflict of Interest

The author declares that there are no conflicts of interest.

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