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ORIGINAL ARTICLE

TGFβ is responsible for skin tumour infiltration by macrophages enabling the tumours to escape immune destruction

Scott N Byrne, Matthew C Knox and Gary M Halliday

Infiltration of skin tumours by macrophages is an important step in tumour progression, although the mechanisms of macrophage recruitment to the tumour mass and the subsequent effects on tumour growth are poorly understood. Transfecting a murine regressing skin tumour with the gene for transforming growth factor (TGF)β enabled the tumours to grow progressively *in vivo* thus allowing us to study the role of this cytokine in tumour growth. Flow cytometry was used to show that TGFβ-mediated tumour progression was accompanied by an increase in tumour-associated macrophages (TAM) and a decrease in tumour-infiltrating dendritic cells (DCs). TAM in TGFβ-secreting tumours expressed lower levels of major histocompatibility complex II and CD86 compared to DC in control tumours and had a high phagocytic capacity as measured by uptake of latex beads *in vivo*. Indeed, TGFβ was directly responsible not only for the enhanced macrophage phagocytosis but also altering the ratio of antigen-presenting cells to favour macrophages over DC. Our results demonstrate that TGFβ recruitment and retention of macrophages at the tumour site enable effective tumour evasion of the host immune system and reinforces the need to target TGFβ in human cancer immunotherapy trials.
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In addition to the well-documented alteration to oncogenes and tumour suppressor genes, progression of cancer is also accompanied by the acquisition of abilities to evade detection and destruction by the host's immune system. An important immune-evasion mechanism that tumours acquire is the ability to secrete immunosuppressive cytokines. One cytokine, in particular, that has been associated consistently with an aggressive tumour phenotype is transforming growth factor β_1 (TGFβ). Torre-Amione *et al.*[1] showed that immunogenic skin tumours transfected with TGFβ evade the immune system via inhibition of cytotoxic T-cell activation. In support of a role of TGFβ in tumour immune evasion, we have shown previously that progressing but not regressing skin tumours constitutively secrete TGFβ that inhibits dendritic cell (DC) migration.[2] More recently, we showed that transfection of an ultraviolet radiation-induced regressor skin tumour with the gene for TGFβ enabled these tumours to grow progressively when transplanted into syngeneic mice. This was accompanied by an inhibition of DC migration from the tumour to the draining lymph nodes and a concurrent decrease in T-cell infiltration into the tumour.[3] These studies confirm that TGFβ has a profound affect on tumour growth, although the precise mechanisms by which TGFβ promotes tumour progression are still not completely understood.

In certain malignant diseases including the lung,[4] breast,[5] cervix,[6] bladder,[7] as well as squamous and renal cell carcinomas,[8,9] accumulation of macrophages within the tumour mass is associated with a poor prognostic outcome. Supporting this hypothesis, we reported previously that skin tumour progression was accompanied by an influx of phagocytic $CD11c^{-}CD11b^{+}MHC\ II^{+}$ macrophages.[10] More recently, Hagemann *et al.*[11] demonstrated that cancer cells chemically instruct infiltrating monocytes to differentiate into tumour-associated macrophages (TAM), that in turn secrete a wide variety of cytokines and chemokines. This ability of TAM to secrete immunosuppressive, angiogenic and cellular growth factors contributes to the ability of tumours to grow, evade detection by the immune system and metastasize.[12] There is also converse data demonstrating that TAM can migrate to lymph nodes[13] and activate T cells.[14]

In light of these conflicting reports, the aim of this study was to investigate the relationship between TGFβ secretion by tumours and the recruitment and retention of macrophages at the local site.

RESULTS

Regressing skin tumours undergo progression when transfected with the gene for TGFβ

The PGW skin tumour is a clone of the polyclonal UV13.1 cell line that has been transfected with an empty gene vector. This cell line

Dermatology Research Unit, Melanoma and Skin Cancer Research Institute, Sydney Cancer Centre, Royal Prince Alfred Hospital at University of Sydney, Sydney, New South Wales, Australia
Correspondence: Dr SN Byrne, Dermatology Research Unit, University of Sydney, Blackburn Building, D06, Sydney, NSW 2006, Australia.
E-mail: scottb@med.usyd.edu.au
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produces very little TGFβ (Figure 1a) and undergoes regression when injected subcutaneously into the backs of syngeneic C3H/HeN mice (Figures 1b and c). Transfection of this cell line with the gene for TGFβ (PGWTGF) results in the production of significantly increased amounts of TGFβ (Figure 1a), and the ability to evade immunological destruction and grow progressively (Figures 1b and c), as shown by the significantly increased tumour diameter and incidence.

TGFβ secretion decreases dendritic cells and increases macrophage infiltration into the tumour

Four-colour flow cytometry enabled the identification of tumour-infiltrating $CD45^+CD11c^+CD11b^+$MHC II^{high} DC and $CD45^+CD11c^-CD11b^+$MHC II^+ TAM (Figure 2a). $CD11c^+$ DC from both tumour groups expressed significantly higher levels of MHC II (over twofold higher mean fluorescence intensity) than $CD11c^-CD11b^+$ macrophages (Table 1). A detailed flow cytometric phenotypic analysis of these APC subsets showed no consistent difference in the expression of the chemokine receptors CCR6 or CCR7 nor in the expression of the costimulatory molecule CD80 (Table 1). CD86 analysis revealed that TAM infiltrating PGWTGF tumours expressed significantly lower levels of CD86 compared to DC infiltrating PGW tumours (Table 1; $P<0.05$). Analysis of the ratio of DC to TAM demonstrated that transfection with TGFβ not only decreased the percentage of tumour-infiltrating DC but increased the percentage of TAM (Figure 2b), however there was about fivefold greater proportion of DC than macrophages.

TGFβ alters the APC ratio and enhances macrophage phagocytosis *in vitro*

To investigate further the effect of TGFβ on APC number and function, we isolated and incubated whole spleen cells with various concentrations of recombinant TGFβ. Adding TGFβ to the culture media resulted in a greater than twofold reduction in the percentage of $CD11c^+CD11b^+$ DC and a concordant increase in the percentage of $CD11^-CD11b^+$ macrophages (Figure 3a). The TGFβ doses used in these experiments ($0.2–1.0\,ng\,ml^{-1}$) approximate the levels of TGFβ secretion by progressor PGWTGF tumours (as shown in Figure 1a). Tracking the phagocytic capacity of these APC using fluorescent latex beads showed that TGFβ enhanced macrophage but decreased DC phagocytosis (Figure 3b). Hence, TGFβ not only alters the ratio of DC to macrophages but also directly influences their ability to phagocytose particles.

Macrophages are the dominant phagocytic cells infiltrating progressing skin tumours *in vivo*

Previously we have used fluorescent beads to successfully create a snapshot in time of the phagocytic capacity of tumour infiltrating APC.[10] PGW- and PGWTGF tumour-bearing mice were injected intravenously with fluorescent beads 18 h prior to the removal and analysis of single cells by flow cytometry. A combination of anti-mouse CD11c, CD11b and MHC II was used to assess the phenotype of cells which had taken up the beads (Figure 4a). Similar to Figure 2a, DCs were identified as $CD11c^+CD11b^+$ while TAM as $CD11c^-CD11b^+$. Moreover, consistent with the data presented in Table 1, compared to DC, TAM had a significantly reduced expression of MHC II (Figure 4a). Total number of $bead^+$ cells was significantly greater in regressing PGW compared to progressing PGWTGF tumours (Figure 4b). More detailed analysis of the tumour infiltrating $bead^+$ APC subsets revealed that only 12 and 3% of the total DC-infiltrating PGW and PGWTGF tumours respectively had phagocytosed beads (Figure 4c). In contrast, almost half of the TAM within progressing PGWTGF tumours were phagocytic within the time frame of the experiment (Figure 4c). Hence, TAM have a much greater phagocytic capacity than DC, and in progressing skin tumours, despite their relatively small number compared to DC (Figure 2b), they are the dominant phagocytic cells.

DISCUSSION

The presence of phagocytic macrophages within TGFβ-secreting progressing but not vector-transfected regressing tumour clones demonstrates the importance of these cells in orchestrating tumour growth. Transfection of a regressor skin tumour cell line with the gene for TGFβ enabled evasion of immunological destruction resulting in tumour progression *in vivo*. Because we have found previously that progression can be associated with an accumulation of macrophages inside the tumour,[10] in a different model, we were interested in determining whether the TGFβ-mediated transformation of a regressor into a progressor tumour would also be associated with enhanced macrophage infiltration. Indeed, PGWTGF tumours were populated by significantly more macrophages and less DC than PGW regressor tumours indicating that TGFβ regulates the type of APC within skin tumours. Monocytes express high-affinity TGFβ type I/II receptors and TGFβ is a chemotactic factor for monocytes;[15,16] it is therefore possible that tumour-derived TGFβ acts as a chemokine recruiting peripheral

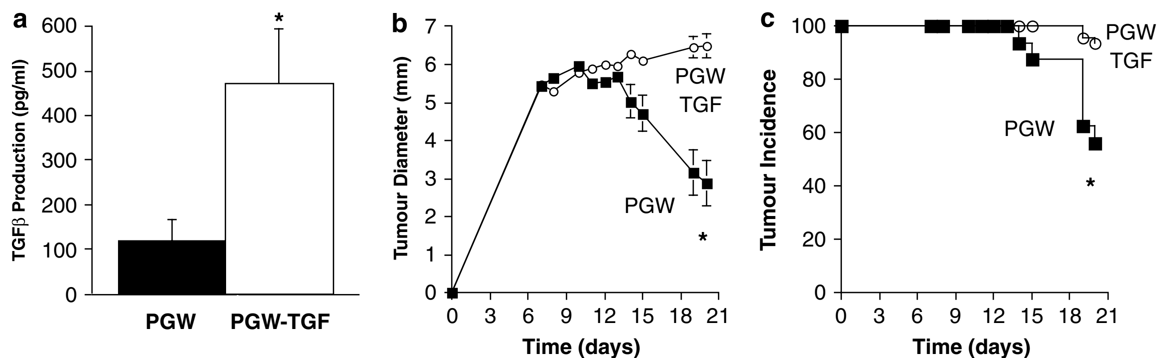

Figure 1 TGFβ-transfected tumour cells (PGWTGF) secrete elevated levels of TGFβ and undergo progressive tumour growth *in vivo*. (**a**) TGFβ production was assessed in supernatant collected from *in vitro*-cultured tumour cells using an enzyme-linked immunosorbent assay kit, mean+s.e.m. from four replicates shown. (**b**) Pooled tumour growth (mean diameter±s.e.m.) and (**c**) tumour incidence measurements from five independent experiments, PGW; n=16 mice and PGWTGF; n=48 mice (where s.e.m. is not obvious it is too small to be observed in this scale). Fully regressed tumours were included as zero for the calculation of mean diameter. *$P<0.05$. TGFβ, transforming growth factor-β_1.

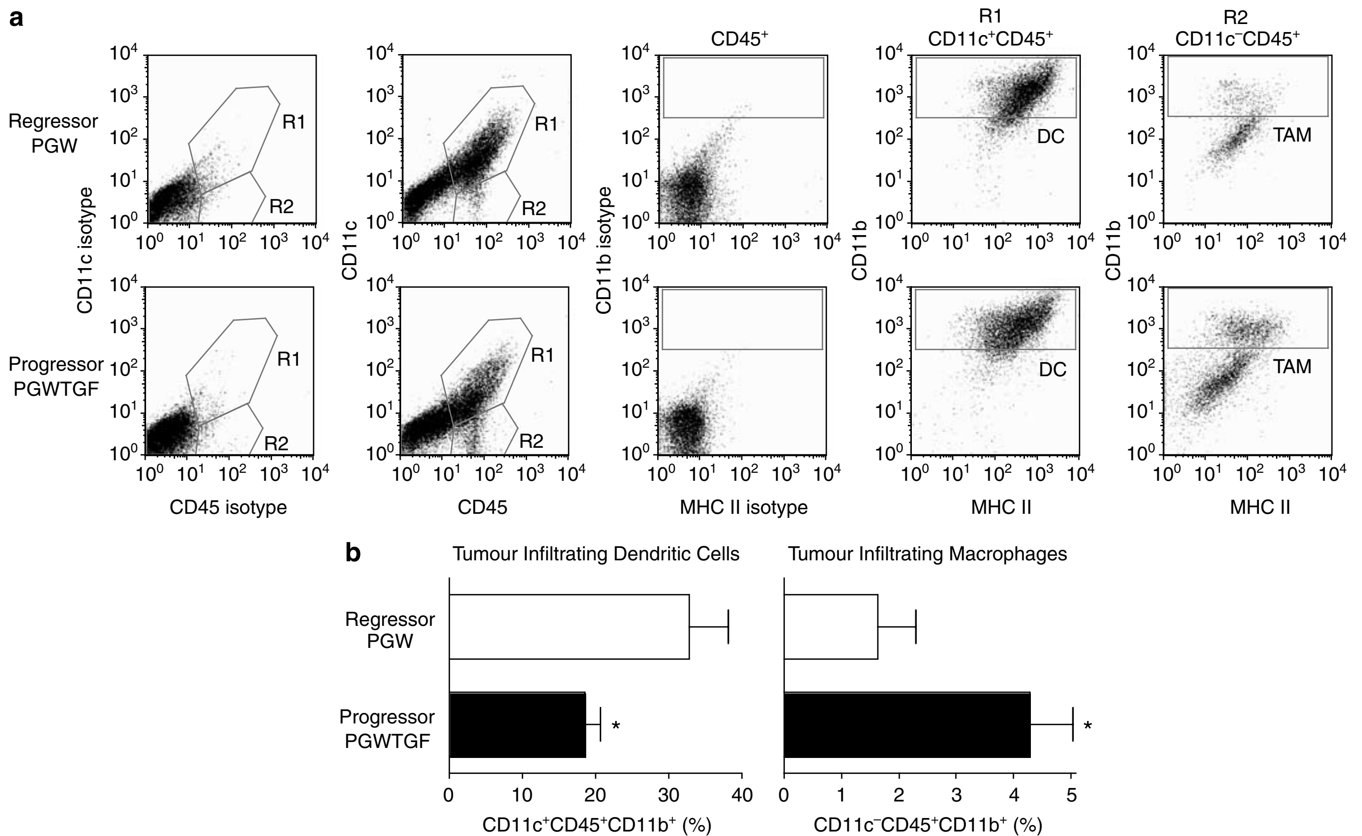

Figure 2 Progressor skin tumours are infiltrated by a significantly greater percentage of macrophages while regressor tumours are populated by dendritic cells. (**a**) The first column of dot plots show the level of isotype staining on whole tumour cell preparations compared to the level of CD45 and CD11c staining (second column). Four-colour flow cytometry identified CD11c$^+$CD45$^+$ DC (R1) and CD11c$^-$CD45$^+$ non-DC inflammatory cell infiltrates (R2). The third column of dot plots show the level of isotype staining for CD11b and MHC II on CD45$^+$ cells compared to the expression of CD11b and MHC II on DC (R1; fourth column) and non-DC inflammatory cells (R2; last column of dot plots). TAM were identified as CD11c$^-$CD45$^+$CD11b$^+$ (last column in (**a**)). Dot plots are representative of four separate experiments. (**b**) The percentage of CD11c$^+$CD45$^+$ CD11b$^+$ DC or CD11c$^-$CD45$^+$CD11b$^+$ TAM from four separate experiments were pooled. Mean+s.e.m. shown. $*P<0.05$. DC, dendritic cells; TAM, tumour-associated macrophages.

Table 1 Phenotype of tumour-infiltrating antigen-presenting cells

	Mean fluorescence index (MFI)±s.e. (n=*4*)		
	Regressor DC	*Progressor DC*	*Progressor TAM*
MHC II	32.4±14.7	36.5±13.5	16.7±4.0[a]
CD80	4.3±0.3	4.6±0.4	3.2±0.6
CD86	2.7±0.3	2.1±0.4	1.6±0.2[a]
CCR6	4.5±0.9	3.1±0.4	5.3±2.0
CCR7	40.0±19.4	29.4±0.8[ns]	22.7±0.8[ns]

[a]$P<0.05$; unpaired Student's *t*-test comparing regressor DC with progressor TAM.
Abbreviations: DC, dendritic cell; ns, non significant; TAM, tumour-associated macrophages.

blood monocytes to the tumour site where they differentiate into tissue-associated macrophages in preference to DC. The presence of high amounts of TGFβ would favour this macrophage pathway for two reasons, (1) monocytes that migrate through endothelium differentiate into DC, while those that remain in the subendothelial matrix become macrophages,[17] and (2) TGFβ can disrupt cell junctions in endothelial layers.[18] Hence, monocytes may be pushed to differentiate into macrophages rather than DC if TGFβ disruption of the endothelium allows monocytes to migrate into the tumour without crossing the endothelium. Our *in vitro* data showing that TGFβ alters the APC ratio by increasing the proportion of macrophages while at the same time decreasing DC percentages is novel and supports this hypothesis. Alternatively, the increase in TAM number following TGFβ transfection may be explained by local monocyte/macrophage proliferation,[19] although whether macrophages are recruited to the tumour site, or undergo local proliferation is unclear.

In certain malignant diseases, accumulation of TAM within a tumour mass is associated with a poor prognostic outcome. The precise reasons for this is not clear, though the consensus is that TAM facilitate tumour immune evasion, progression and metastasis via a coordinated release of immunosuppressive, angiogenic and cellular growth factors (reviewed in Elgert *et al.*[12]). In addition, TAM have a high phagocytic capacity that enables them to efficiently phagocytose apoptotic tumour cells.[20,21] This is potentially an important immune evasion mechanism as the more efficient phagocytic TAM compete and prevent DC from taking up tumour antigens. Indeed, DC access to apoptotic tumour cells is a key event in the initiation of strong and sustained T-cell-mediated antitumour immunity.[22] We used two approaches to address this issue. An *in vitro* assay of phagocytic capacity showed that consistent with previous reports,[23] TGFβ enhances macrophage uptake of fluorescent latex beads while concurrently inhibiting DC phagocytosis. This fluorescent latex bead-based assay was also used to measure the *in vivo* phagocytic capacity of APC within tumours and demonstrated that almost half of all the

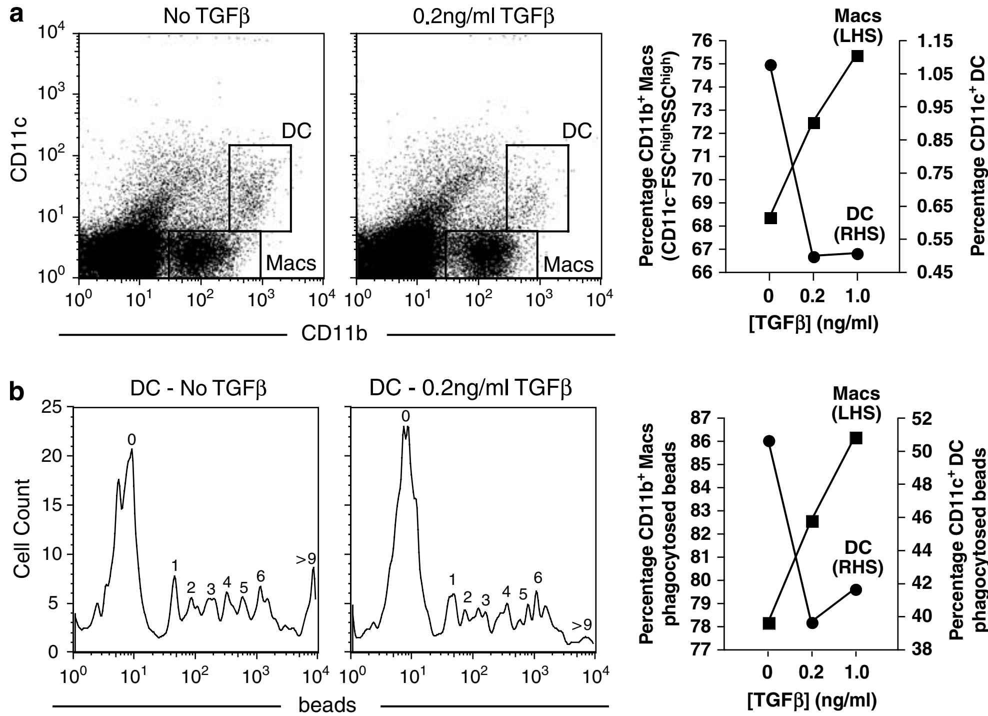

Figure 3 TGFβ alters the APC ratio and enhances macrophage phagocytosis *in vitro*. (**a**) A total of 2×10^6 whole spleen single-cell suspensions were incubated at various TGFβ concentrations for 24 h. CD11c+CD11b+ DC (●) and CD11c−CD11b+ Macs (■) were identified by flow cytometry and the percentage of cells at the various TGFβ concentrations was determined. (**b**) Fluorescently labelled latex beads were added for the final 3 h of incubation and the level of bead uptake was determined by flow cytometry. The numbers shown in the histograms refer to the number of beads that had been phagocytosed by each cell. The total number of phagocytic DC (●) and Macs (■) was analysed by flow cytometry and the change induced by TGFβ is shown in the right panel. APC, antigen-presenting cell; DC, dendritic cells; TGFβ, transforming growth factor-β_1.

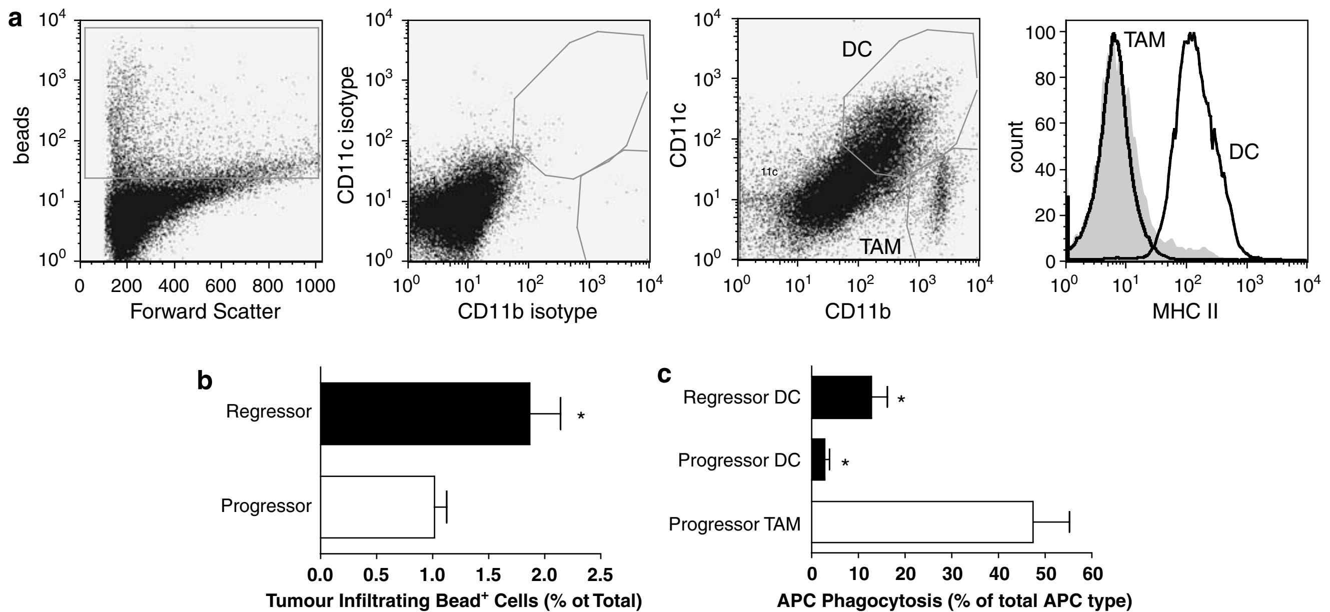

Figure 4 Macrophages are the dominant phagocytic cells infiltrating progressing skin tumours. (**a**) Injected intravenously 18 h prior to tumour removal and analysis by four-colour flow cytometry. The phenotype of the cells that had taken up beads were confirmed as either CD11c+CD11b+MHC II^{high} DC (bold histogram) or CD11c−CD11b+MHC II+ TAM (shaded histogram). Progressor tumour dot plots only are displayed as regressor dot plots looked similar. (**b**) Significantly more beads were taken up by any type of cell (percentage of total) within regressor (solid bar) compared to progressor (open bar) tumours ($n=7$). (**c**) Analysis of the percentage of each APC subset (DC in solid bars statistically compared to TAM in the open bar) that had taken up beads ($n=7$). In all cases mean+s.e.m. shown. *$P<0.05$. APC, antigen-presenting cell; DC, dendritic cells; TAM, tumour-associated macrophages.

macrophages within progressor tumours were highly phagocytic. The advantages of this assay is that phagocytic cells can be tracked by flow cytometry. Unfortunately, this only provides a snapshot of the level of phagocytic activity inside a tumour at a given point in time. Despite this limitation, even after a relatively short period of time (18 h after bead transfer), we could still identify numerous phagocytic cells within the tumours. Based on these results, we hypothesize that one-way TGFβ-secreting tumours evade the immune system is through preferential recruitment of macrophages which in turn remove dead and dying tumour cells thereby preventing DC access to immunogenic tumour antigens.

Peripheral uptake of antigen by DC and their subsequent maturation and migration to draining lymph nodes is a key event in the development of immunity. TGFβ may subvert this process at a number of levels. TGFβ-exposed monocytes develop into immature DC that express high levels of E-cadherin and CCR6,[24] both of which are molecules that anchor DC to peripheral skin sites. This may explain how TGFβ-secreting progressor tumours evade immune detection by trapping DC at the tumour site[2] and preventing their migration to draining lymph nodes.[3] However, even if DC from a TGFβ-secreting progressor tumour did successfully reach the draining lymph nodes, evidence from Kobie *et al.*[25] showing that TGFβ inhibits the antigen-presenting function of DC suggests that these APC may fail to activate tumour-specific T cells. Indeed, immature DC presenting antigen to naive T lymphocytes preferentially activates regulatory T-cell subsets.[26,27] Moreover, Sato *et al.*[28] showed that mature DC exposed to TGFβ failed to upregulate surface CCR7 and so were defective in their ability to migrate towards lymph node-derived CCL19. These multi-faceted, downregulating effects of TGFβ on the ability of DC to migrate and prime tumour-specific T cells may explain why TGFβ-secreting tumours grow progressively. However, based on our flow cytometric evidence, DC within both tumour groups had a similar phenotypic profile suggesting that DC in the tumour-draining lymph nodes dictate the outcome of the antitumour response. Based on this current study and our previous experiments showing a significant increase in skin-derived DC in regressor tumour-draining lymph nodes,[3] we hypothesize that mature DC migrate away from regressor (but not progressor) tumours where they activate effective antitumour immune responses. In contrast, TGFβ secretion by tumours pushes DC precursors to differentiate into macrophages. Therefore, inhibition of TGFβ by aggressive tumours in the clinic may facilitate not only the migration of mature, antigen-primed DC to draining lymph nodes, but prevent the differentiation of DC precursors into immune suppressive tumour-associated macrophages. Ultimately it is hoped this will enhance the overall antitumour immune response.

METHODS

Cell lines

Details of the tumour cell lines have been published by us previously.[3] Briefly, a clone of the UV13.1 cell line was transfected with either an empty vector (PGW) or a vector containing the gene for $TGF\beta_1$ (PGWTGF) by Transfectamine (Clontech, Palo Alto, CA, USA). The cell lines were maintained under routine tissue culture.

Tumour growth in mice

A total of 2×10^6 PGW or PGWTGF tumour cells in 50 μl phosphate-buffered saline were injected subcutaneously into the left and right flanks of female C3H/HeN (H-2^k) mice. All animals were aged 8–14 weeks and were used in accordance with animal ethics guidelines (University of Sydney, Australia). Tumour diameter was measured using an engineers' micrometer (Mitutoyo Corporation, Japan), taking the average of two diameter measurements. PGWTGF tumours grew progressively throughout the tumour monitoring, while PGW tumours grew progressively until around day 14 when they began to regress spontaneously. This was the time that the tumours were excised for analysis.

Measurement of TGFβ production by tumour cells *in vitro*

A total of 5×10^6 tumour cells ml^{-1} in a 24-well plate were cultured in Dulbecco's modified Eagle's medium with 2% fetal calf serum and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid for 24 h at 37 °C, 5% CO_2. Supernatant was collected and treated with 1 M HCl for 1 h at 4 °C and then neutralized with 1 M NaOH to activate any latent TGFβ. An enzyme-linked immunosorbent assay for TGFβ was subsequently performed on the cell culture supernatants with an OptEIA Set human TGF-β kit (Pharmingen, San Diego, CA, USA).

Flow cytometric analysis of tumour-infiltrating antigen-presenting cells

The method of making single-cell suspensions from *in vivo*-grown solid tumours and the subsequent preparation of those cells for analysis by flow cytometry has been described by us previously.[10] Single cells were incubated with antibodies against mouse CD45 (to identify cells of haematopoietic origin), CD11c (to identify DCs) and MHC Class II (to determine the maturation state of the antigen-presenting cells (APC)). Four-colour flow cytometry was then used to phenotype and enumerate the tumour-infiltrating leukocytes. CD45 (30-F11), CD11c (HL3), CD11b (Mac-1), I-A^k (11–5.2), CD80 (16–10A1) and CD86 (GL1) all from Pharmingen BD (Franklin Lakes, NJ, USA). Anti-mouse CCR6 (clone: 140706) was obtained from R&D Systems (Minneapolis, MN, USA). Murine CCR7 expression was detected using a fusion protein of human IgG fused to CCL19 (the specific chemokine for CCR7). The fusion protein was secreted by the cell line J558L (a kind gift from Jason G Cyster, Howard Hughes Medical Institute, University of California) and used directly from the culture supernatant generated in this laboratory as described previously.[29] Bound fusion protein was detected with phycoerytherin-conjugated anti-human IgG (Jackson Immunoresearch, West Grove, PA, USA). Isotype control antibody staining was used in parallel to ensure antibody specificity and to produce the electronic gates for analysis. Streptavidin allophycocyanate (Pharmingen) was routinely used to label biotinylated primary antibodies. Cellular events were acquired on a BD FACScalibur flow cytometer and analysed using CellQuest (Becton Dickinson, Franklin Lakes, NJ, USA) and FlowJo software (Treestar Inc., Ashland, OR, USA).

In vivo measurement of phagocytosis

Two hundred microliters of Fluoresbrite Carboxy YG or control non-fluorescent 2.6% solid latex microspheres with a diameter of 0.5 μm (Polysciences; Warrington, PA, USA) were injected intravenously into tumour-bearing mice. Tumours were removed 18 h later and single-cell suspensions were incubated with 1 ml of 20 mM ethylenediaminetetraacetic acid (Sigma, St Louis, MO, USA) in phosphate-buffered saline for 20 min at room temperature prior to labeling with antibodies to ensure beads were intracellular.

Effect of TGFβ on macrophage and dendritic cell number and phagocytosis *in vitro*

A single-cell suspension was prepared from the spleen of a C3H/HeN mouse and 2×10^6 cells at a concentration of 10^6 ml^{-1} were incubated in a 24-well plate with various indicated concentrations of recombinant hTGFβ (R&D Systems). Fluoresbrite Carboxy YG beads at a final concentration of 0.013% were added 21 h later and incubated for a further 3 h. At this time, suspended and adherent cells were recovered with an ethylenediaminetetraacetic acid/trypsin solution and washed extensively with ethylenediaminetetraacetic acid to remove surface beads. DC and macrophages were identified by flow cytometry using antibodies against CD11c and CD11b.

Statistical analysis

An unpaired two-tailed Student's *t*-test was used to analyse TGFβ production for each clone by enzyme-linked immunosorbent assay. *In vivo* tumour growth

was analysed using repeated measures of analysis of variance. Tumour incidence was analysed using Kaplan–Meier Survival test. Flow cytometry data and bead uptake experiments were all analysed using a *post hoc* Fisher's protected least significant difference (PLSD) analysis of variance test. For all analyses, a *P*-value of <0.05 was considered significant.

ABBREVIATIONS

APC, antigen-presenting cell; DC, dendritic cell; PGW, vector-transfected regressor clone; PGWTGF, TGFβ-transfected progressor clone; TAM, tumour-associated macrophages; TGFβ, transforming growth factor-β_1

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