Original Article

Alteration of early dendritic cell activation by cancer cell lines predispose immunosuppression, which cannot be reversed by TLR4 stimulation.

Ying Ying Kong[1], Martina Fuchsberger[1], Magdalena Plebanski[1,†]*, and Vasso Apostolopoulos[2,†,*]

[1] Department of Immunology, Monash University AMREP Precinct, Melbourne, Victoria 3004, Australia
[2] Centre for Chronic Disease, College of Health and Biomedicine, Victoria University, Melbourne, Victoria 3004, Australia

[†] These authors contributed equally to this work.

* Correspondence address. Tel: +61 3 9919 2025 (V.A.), fax: +61 3 9919 2465 (V.A.); Tel: +61 3 9903 0667 (M.P.) E-mail: Vasso.Apostolopoulos@vu.edu.au (V.A.)/ Magdalena.Plebanski@monash.edu (M.P.)

Abstract

Dendritic cells (DCs) have shown promise for use in cancer vaccine and cancer immunotherapy studies. However, we demonstrate that cancer cell lines can negatively interfere with DC generation in GM-CSF derived cultures, although cancer cells are able to enhance CD80 cell surface activation marker and cytokine secretion. Furthermore, in the presence of cancer cells, GM-CSF derived DCs are unable to stimulate T cells. Additional stimulation with LPS can not fully reverse the suppressive effect of cancer cells or supernatant. Hence, it is imperative to understand the immunosuppressive effects of cancer on DCs in order for DC-based cancer immunotherapy to be successful.

Keywords: dendritic cells, early dendritic cells, GM-CSF, cytokines

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Introduction

Cancer cells, being genetically less stable than normal cells, mutate and transform under the constant surveillance of the immune system. Through this selection pressure, they modify the expressions or alter the expression levels of normal proteins, which render them capable of evading apoptosis and express immunosuppressive cytokines [1, 2]. This allows them to resist being removed, prevent stem cells from differentiating into mature dendritic cells (DCs) and suppress Th1 immune response [3].

Over the last decade, many studies have attempted to establish the critical role of DCs in the maintenance of immunologic integrity, and given their unique characteristics, they have been promising targets for developing new cancer immunotherapies. However, the strongly immunosuppressive nature of tumour cells prevents an effective immune response from being activated. To date, both human and murine cancer immunological studies have used various sources of cancer antigens, such as, whole tumour cells, dead tumour cells, cancer-associated proteins/peptides and DNA, and have achieved limited protection against tumours [4, 5]. Unfortunately, these results only extended patients' lifespan by a few months at best [6]. Recent cancer immunotherapeutic studies have also concentrated on eradicating cancer immunosuppression on T-cell activation. These studies focused on the blockade of the T-cell inhibitory receptors cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death-1 (PD-1) and its ligand, PD-L1 [7-12]. While these studies have shown an improvement in patients' overall response rate and overall survival, they only extended patients' lifespan by close to a year. Retrospectively, the ideal focus of cancer immunotherapies is probably to generate DCs that are stable upon activation and able to initiate lasting interaction with T-cells to eliminate the tumour cells, and then use them in conjunction with anti-CTLA-4 and/or anti-PD-1 therapy to boost T-cell response.

DCs express co-stimulatory markers such as CD80 and CD86 which are important in mediating T-cell functions [13-16]. Engagement of CD28 on T-cell by CD80 and CD86 induces T-cell activation, proliferation and secretion of immunostimulatory cytokines such as IL-1, IL-6 and IL-12 [17]. However, studies have also shown that CD80 and CD86 can bind to CTLA-4, which induces T-cell inhibition through the production of indoleamine 2,3-dioxygenase [4, 18].

We have shown that the early GM-CSF derived DCs are fully functional in terms of endocytosis, up-regulating activation markers upon TLR stimulation [14, 16]. They are

comparable, if not better, to the GM-CSF + IL-4 equivalents. These early GM-CSF-derived DCs express lower levels of activation markers than early GM-CSF + IL-4-derived DCs, however as we demonstrate herein, these early DCs derived from GM-CSF cultures rapidly increased the expression levels of CD80, CD86 and MHCII upon LPS and CpG stimulation [14, 16], and are capable of secreting cytokines and chemokines after stimulation. In addition, these early GM-CSF-derived DCs, like the early GM-CSF + IL-4-derived DCs, can stimulate T-cell proliferation after exposure to LPS. Using these early GM-CSF-derived DCs, we determined the effects of cancer cells (with the use of the *in vitro* thymoma cell line, RMA) on cell surface co-stimulatory markers, ability to activate T-cell proliferation and cytokine secretion. These early GM-CSF-derived DCs have the potential to be used in cancer immunotherapy studies.

Materials and Methods

Mice

C57BL/6 and BALB/c mice (aged 6-8 weeks) used throughout this study were purchased from the Precinct Animal Centre in Alfred Medical Research and Education Precinct and Monash Animal Services (Melbourne, Australia).

Generation of early DCs from GM-CSF-derived cultures

Bone marrow (BM) cells from femurs and tibias of C57BL/6 mice were collected and treated with ACK lysis buffer (155 mM $NHCl_4$, 0.1 mM Na-EDTA, 10 mM $KHCO_3$, pH 7.2) to lyse erythrocytes. Cells were washed and cultured for 3 or 4 days at 5 x 10^5 cells/ml with complete media consisting of RPMI 1640, 2 mM L-glutamine (Sigma-Aldrich, St Louis, USA), 20 mM HEPES (Sigma-Aldrich), 0.1 mM 2-mercaptoethanol (2-ME) (Sigma-Aldrich), 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma-Aldrich) and 10 % heat-inactivated foetal calf serum (FCS; Sigma-Aldrich) at 37^{o}C and 5% CO_2. GM-CSF (10 ng/ml; PeproTech, Rocky Hill, USA) with or without IL-4 (5 ng/ml; PeproTech) was added to induce DC generation. Cells were harvested on day 3 or 4 unless otherwise stated by gentle re-suspension.

Co-incubation of cancer cells and supernatant with DC cultures

Cancer cell lines (B16-OVA and RMA) were cultured at 37ºC and 5% CO_2, and at ~80% confluence they were removed from the culture flasks. The harvested cells were centrifuged at 300 *g*, 4ºC for 5 min and the supernatant was collected. The remaining cell pellet was re-suspended in 10 ml of fresh cold culture media. Cancer cells, at 1.25 x 10^4 cells/ml, 2.5 x 10^4 cells/ml or 5 x 10^4 cells/ml, were added to 5 x 10^5 DCs derived from a GM-CSF BM culture. Furthermore, to examine the effect of soluble factors of such cancer cells, 1 ml of cancer supernatant (SN) was added to 5 x 10^5 DCs in 1 ml of culture medium. One milliliter of fresh culture media was added to wells containing only DCs as negative control. Cultures were incubated at 37ºC and 5% CO_2 for 24 h.

Induction of DC activation with LPS

DCs (5 x 10^5 cells) from day 3 or 4 cultures were cultured with or without RMA cells (5 x 10^4 cells) or RMA supernatant (1 ml) in 24-well plates in 1 ml of complete media for 24 h. LPS (1 µg/ml, derived from *Escherichia coli*) (0111:B4; Sigma-Aldrich) was added to the cultures, which were incubated a further 24 h at 37ºC and 5% CO_2. LPS was added to cultured DCs without cancer cells for a positive control; and wells of DC cultures with no LPS acted as a negative control. After 24 h, the plates were spun, supernatants were collected, and cells were harvested and stained with fluorescent surface markers (CD11c, CD80, CD86, and MHCII) for analysis by flow cytometry.

Flow cytometry and data analysis

Antibodies were purchased from BD Biosciences (Franklin Lakes, USA) unless otherwise stated. Culture cells were harvested and labelled with anti-CD11c V450 (HL3) to identify DCs. To study the phenotype and maturation status of DCs, cells were also labelled with anti-B7H1 PE (MIH5), biotin-conjugated anti-B7H3 (M3.2D7; eBioscience, San Diego, USA), anti-B7H4 Alexa Fluor 488 (297219; R&D Systems, McKinley Place NE, USA), anti-CD11b PECy7 (M1/70), biotin-conjugated anti-CD80 (16-10A1), anti-CD86 PE (GL1), Gr-1 PerCP Cy5.5 (RB6-8C5) and anti-MHCII APCCy7 (M5/114.15.2) where appropriate. Either streptavidin FITC or streptavidin APC was used as secondary antibodies. Dead cells were discriminated by staining harvested cells with live/dead Fixable Aqua Dead Cell Stain Kit (Invitrogen, Life Technologies, Australia Pty Ltd, Victoria, Australia). T-cells were labelled with anti-CD3 FITC (17A2), anti-CD4 PerCP (RM4-5) and anti-CD8α APC (53-6.7) to scrutinise T-cell purity. Antibodies were prepared in flow cytometry staining buffer (PBS containing 2% FCS) and cells were stained for 20 min on ice in the dark. Following

incubation, cells were washed with staining buffer and centrifuged at 300 *g* at 4 ºC for 5 min. The supernatant was removed and the cells were re-suspended in 100 µl of PBS containing 1% paraformaldehyde. Samples were acquired with LSRII (BD Biosciences) at AMREP Flow-Cytometry Core Facility (Melbourne, Victoria, Australia). Data was analysed with FlowJo Flow Cytometry Analysis Software (TreeStar Inc, Ashland, USA).

Cytokine detection

To detect and quantify various cytokines secreted within the culture, early GM-CSF-derived BM cultures were either left un-stimulated, stimulated with LPS, or co-cultured with RMA cells or RMA SN. Supernatant was collected at the end of each experiment and analysed by cytometric bead array (CBA) (BD Biosciences), following manufacturer's instructions. Briefly, for CBA inflammation kit, 50 µl of culture supernatant or standard cytokine solutions (0-5000 pg/ml) was incubated with 50 µl of cytokine capture beads and PE detection reagent for 2 h. For TGF-β flex-set, latent TGF-β in the 100 µl of culture supernatant was activated with 20 µl of 1M HCl for 10 min, then neutralised by 20 µl of 1.2 M NaOH/0.5 M HEPES. Then, 50 µl of neutralized culture supernatant or standard TGF-β cytokine solution (0-10 000 pg/ml) was incubated with 50 µl of cytokine capture beads and PE detection reagent for 2 h. Samples were acquired with LSRII. Acquisition setup involved scattering of beads and fluorescence compensation was standardised with cytometric setup beads prior to sample acquisition. Estimated concentrations of cytokines were calculated using FCAP Array software (Soft Flow Inc, St. Louis Park, USA).

T-cell purification

Splenocytes from BALB/c mice were collected and incubated with ACK lysis buffer for 3 min at room temperature to remove erythrocytes. T-cells from splenocytes were isolated based on the manufacturer's instructions using the MACS Pan-T cells isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, remaining cells were washed and incubated with a cocktail of biotin-conjugated antibodies against CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, MHCII, and Ter-119 for 10 min on ice and further incubated with anti-biotin magnetic beads for 15 min in the 4 ºC. The isolation of untouched T cells was achieved by depletion of magnetically labelled cells. A small aliquot of the sorted T-cells was labelled with anti-CD3 FITC (17A2), anti-CD4 PerCP (RM4-5) and anti-CD8α APC (53-6.7) to scrutinize T-cells purity (purity ≥ 95%). The antibodies were prepared in flow cytometry staining buffer (PBS + 2% FCS) and the cells

were stained for 20 minutes on ice in the dark. The samples were then acquired on the LSRII (BD Biosciences) at the AMREP Flow-Cytometry Core Facility. Data were analysed using FlowJo Flow Cytometry Analysis Software.

Mixed lymphocyte reaction and T-cell proliferation

Cells from day 3 or 4 GM-CSF BM cultures from C57BL/6 mice that were either left un-stimulated, stimulated with LPS, or co-cultured with RMA cells or RMA SN were harvested, washed, and sorted for $CD11b^+$ cells where necessary to remove RMA cells with BD Influx (BD Biosciences). Then, 3×10^3, 10^4 or 3×10^4 cells from sorted cultures were incubated with 10^5 allogenic BALB/c T-cells in 150 µl of complete media (quadruplicates, 96-well U-bottom plates) for 72 h at 37°C and 5% CO_2. Next, 1 µCi ^{3}[H]-thymidine was added and cultures were incubated for a further 18 h at 37°C and 5% CO_2. To assess T-cell proliferation, radioactivity was measured in counts per minute (CPM) using the TopCount NXT Microplate Scintillation and Luminescence Counter (PerkinElmer Inc, Waltham, USA).

Statistical analysis

The data generated are shown in the mean ± SD. All values are graphed and analysed for statistical significance using Prism (GraphPad Software Inc, La Jolla, USA). Statistical significance was indicated by *P*-value < 0.05.

Results

RMA and RMA SN decreased DC generation in BM cultures but induced differential expression of activation markers on GM-CSF derived early DCs

To test the effect of the murine thymoma cancer cell line RMA on DCs generated from GM-CSF-derived bone marrow cultures at day 3 or 4, cells were either left unperturbed, or exposed to LPS (1 µg/ml), RMA cells (5×10^4 cells) or supernatant (SN) from RMA culture (RMA SN) for 24 h. In response to the exposure to RMA or RMA SN, DC generation (% $CD11c^+$ cells) was significantly decreased on day 3 (from 40.38% ± 1.84% to 22.96% ± 4.93% when exposed to RMA cells, and to 25.9% ± 5.99% when exposed to RMA SN) and day 4 cultures (from 41.58% ± 4.98% to 31.08% ± 2.06% when exposed to RMA, and to 34.41% ± 3.23% when exposed to RMA SN) compared with untreated cultures (**Fig. 1A**). In contrast, a trend to an increase of DC numbers in response to LPS activation was observed (day 3, 45.13% ± 2.46%; day 4, 47.53% ± 3.92%) (**Fig. 1A**). The

cell surface marker expression of CD80 and CD86 to LPS, RMA and RMA SN were determined (**Fig. 1B**). Both LPS and RMA cells significantly increased surface expression of CD80 (from 5.38% ± 1.38% to 13.39% ± 0.39% when exposed to LPS, and to 20.18% ± 2.21% when exposed to RMA) and CD86 (from 20.97% ± 2.86% to 34.3% ± 2.89% when exposed to LPS, and to 31.7% ± 5.43% when exposed to RMA) on day 3. Surprisingly, RMA SN only increased surface expression of CD80 (23.78% ± 0.73%) but not CD86 (17.5% ± 3.06%) on day 3. We further analysed the ratio of CD80:CD86 expressions within each condition, and noted that RMA (0.64 ± 0.10) and RMA SN (1.38 ± 0.29) significantly increased the ratio on day 3 DCs, in contrast to LPS (0.40 ± 0.03), which retained a similar ratio to untreated DCs (0.26 ± 0.09) (**Fig. 1C**). MHCII expression was significantly up-regulated when DCs were exposed to LPS, RMA or RMA SN. However, no differences were detected between the three stimulants (data not shown). Similar changes in expression of co-stimulatory molecules were noted when stimulating GM-CSF cultures on day 4, despite a lower overall expression of the activation markers on DCs. Titration studies using a different cancer cell line (B16-OVA) confirmed this result (**Fig. 2**), suggesting that this observation is not restricted only to RMA, and it is effective to other cancer cell lines.

RMA and RMA SN prevent T-cell proliferation when incubated with DCs

To investigate the ability of the early DCs to stimulate T-cell proliferation after exposure to LPS, RMA and RMA SN, stimulants were added to the cultures on day 3 and cells were incubated for a further 24 h at 37^{o}C and 5% CO_2 before they were harvested and sorted for $CD11b^+$ cells. Then 3 x 10^3, 1 x 10^4 or 3 x 10^4 sorted cells were incubated with 10^5 allogenic BALB/c T-cells ($CD3^+$ cells enriched from the spleen with pan T-cell isolation kit) in 150 µl of complete media in 96 well U-bottom plates for a further 3 days at 37^{o}C and 5% CO_2. To measure T-cell proliferation, 1 µCi of ^{3}[H]-thymidine was added and incubated for an additional 18 h. Early DCs co-cultured with RMA or RMA SN inhibited T-cell proliferation (on both day 3 and day 4 of GM-CSF cultures) compared with DC cultures without RMA or RMA SN (**Fig. 3**). Early DCs that were co-cultured with control stimulant, LPS, promoted T-cell proliferation. These data indicated that RMA and RMA SN altered the activation status of DCs, which prevented these cells from inducing T-cell proliferation.

Cytokine secretion in GM-CSF derived bone marrow cultures in the presence of RMA

Cytokine profile from day 3 and day 4 GM-CSF cultures after stimulation with LPS, RMA or RMA SN for 24 hours at 37ºC and 5% CO_2 was determined (**Fig. 4**). To harvest the supernatants, plates were spun at 300 *g*, 4 ºC for 5 min and the supernatants were carefully taken off and analysed for the presence of several cytokines and chemokines (IL-6, IL-10, IL-12p70, IFN-γ, CCL2, TNF-α, TGF-β) using CBA detection kit (BD Biosciences). IL-6 level was increased from 487.23 ± 36.76 pg/ml to 15478.23 ± 2794.99 pg/ml, IL-10 level was increased from 0 pg/ml to 463.00 ± 7.67 pg/ml, CCL2 level was increased from 310.99 ± 21.05 pg/ml to 1058.78 ± 140.66 pg/ml, and TNF-α levels from 656.81 ± 25.77 pg/ml to 9823.2 ± 180 pg/ml in response to stimulation with LPS (**Fig. 4**). Cells incubated with RMA cells or RMA SN also showed increased cytokine secretion, although at much lower levels than those noted with LPS stimulation. It was interesting to note that in day 4 GM-CSF cultures, LPS induced higher IL-6 level (from 362.54 ± 160.94 pg/ml to 28042.37± 2587.13 pg/ml), but lower levels of IL-10 (from 0 pg/ml to 250.91± 8.77 pg/ml), CCL2 (from 126.69 ± 0.47 pg/ml to 180.47 ± 12.58 pg/ml) and TNF-α (from 369.19 ± 8.28 pg/ml to 7373.1 ± 219.16 pg/ml) when compared with day 3 cultures. However, cells incubated with RMA and RMA SN had no significant alterations in the cytokine secretions. In contrast, no significant difference in the amount of TGF-β in the culture had been detected in supernatant of day 3 or day 4 GM-CSF cultures regardless of the stimulants. No IFN-γ or IL-12p70 was detected in either day 3 or day 4 GM-CSF cultures, regardless of the stimulant (data not shown).

Further incubation with LPS did not significantly increase early DC numbers or co-stimulatory marker expression on DCs that have been pre-exposed to RMA cells or RMA SN

In order to determine whether an additional inflammatory stimulus could reverse the RMA-induced effect on early DC cultures by stimulating the cultures that have been exposed to RMA cells or RMA SN with LPS (1 µg/ml) for another 24 h at 37ºC and 5% CO_2. We observed a small, but insignificant increase in DC generation in cultures stimulated with RMA + LPS when compared to cultures stimulated with RMA only in in both day 3 and day 4 cultures (**Fig. 5A**). The same trend could be seen in day 3 and day 4 DCs + RMA cultures. Apart from increased DC generation, on day 3, the $CD11c^+$ cells

also showed elevated expression of CD80 (from DCs + RMA 20.18% ± 2.21% to DCs + RMA + LPS 23.63% ± 3%; from DCs + RMA SN 23.78% ± 0.73% to DCs RMA SN + LPS 26.68% ± 2%). Interestingly, the additional LPS stimulation significantly increased CD86 expression (from DCs + RMA 31.7% ± 5.43% to DCs + RMA + LPS 48.1% ± 6.38%) (**Fig. 5B**). Similar effects were noted in cultures stimulated with RMA cells or RMA SN on day 4, although the effect was less prominent (**Fig. 5B**). When the ratio of CD80:CD86 was measured, it was clear that LPS decreased the ratio in cultures exposed to RMA cells (from 0.65 ± 0.1 to 0.5 ± 0.08) and RMA SN (from 1.38 ± 0.29 to 1.04 ± 0.1) on day 3, albeit not significant (**Fig. 5C**). However, the decrease diminished in day 4 cultures exposed to RMA cells and RMA SN. It was also noted that the ratio of CD80:CD86 was still significantly higher in both day 3 and day 4 cultures exposed to RMA SN further stimulated with LPS (**Fig. 5C**) when compared with cultures stimulated only with LPS (0.40 ± 0.03). There was also upregulation of CD40 expression on day 3 DCs after treatment with RMA cells or RMA SN, but not comparable to DCs stimulated with LPS (data not shown); no differences were noted in CD40 expression with day 4 DCs stimulated with LPS, RMA or non-stimulated (data not shown).

Additional incubation with LPS increased cytokine secretions by GM-CSF-derived cells that have been pre-exposed to RMA cells or RMA SN

Additionally, we examined the SNs of the GM-CSF-derived cells that had been pre-exposed to RMA cells or RMA SN and stimulated again with LPS (1 μg/ml) for 24 h at 37^{o}C and 5% CO_2 for changes in cytokine secretions compared with cultures without the addition of LPS stimulation. We noted a drastic increase of IL-6 from 1316.51± 266.95 pg/ml to 31983.5± 0.433 pg/ml, IL-10 from 25.25 ± 3.1 pg/ml to 484.69 ± 77.77 pg/ml, TNF-α from 1123.26 ± 186.08 pg/ml to 8418.98 ± 576.82 pg/ml and the chemokine CCL2 from 805.88 ± 161.73 pg/ml to 2647.51 ± 65.06 pg/ml in day 3 GM-CSF derived cell cultures that had been pre-exposed to RMA cells after additional LPS stimulation when compared to cultures without additional LPS stimulation (**Fig. 6**). Similar observations were made for day 4 GM-CSF derived cells, pre-exposed to RMA cells and stimulated with LPS for 24 h, although overall cytokine secretion was lower when compared with day 3 cultures, except for IL-6. Cultures pre-exposed to RMA SN showed a slightly different pattern. While IL-6 secretion in day 3 cultures after LPS stimulation was comparable to that in cultures pre-exposed to RMA cells, it was significantly lower in supernatants from day 4 GM-CSF-derived cells pre-exposed to RMA SN + LPS (RMA + LPS 13983.5 ± 0.43

pg/ml; DCs + RMA SN + LPS 3905.43 ± 1280.48 pg/ml). IL-10 levels were increased in supernatant from day 3 cultures, though not as much as that seen in the day 4 cultures pre-exposed with RMA cells (from DCs + RMA SN 27.45± 14.98 pg/ml to DCs + RMA SN + LPS 42.74 ± 9.42 pg/ml). Interestingly, negligible levels of IL-10 were detected in day 4 GM-CSF cultures pre-exposed to RMA SN, and further stimulation with LPS did not change that. Similarly, a small increase of CCL2 was detected in day 3 cultures pre-exposed to RMA SN and stimulated with LPS, but no change was seen under the same conditions in day 4 GM-CSF-derived cultures. We also tested TGF-β secretion. However, further stimulation of day 3 or day 4 GM-CSF-derived cultures pre-exposed to either RMA cells or RMA SN with LPS did not yield any detectable differences (data not shown). No IFN-γ or IL-12p70 could be detected in the cultures.

Discussion

We have demonstrated that early GM-CSF-derived DCs are highly functional when compared to the early GM-CSF + IL-4 DCs (**Fig. 7**). In addition, we previously demonstrated that reactive oxygen species (ROS) induction in GM-CSF-derived DCs correlated with inflammatory DC functionality and expansion. Day 4 GM-CSF DC cultures expressed very high levels of ROS and day 3 ROS(lo) DCs were highly responsive to toll-like receptor stimuli (LPS) by rapid upregulation of CD80, CD86, and MHC class II, in contrast to the low response of day 6 ROS(hi) DCs [16]. Herein, we further explored the effects of the *in vitro* cancer cell line RMA on early GM-CSF bone marrow-derived DCs. We hypothesised that exposure to cancer cell lines such as RMA may activate the early GM-CSF-derived DCs, increasing the expression of co-stimulatory molecules, secretion of pro-inflammatory cytokines and stimulation of T-cell proliferation. First, we analysed the effects on DC (identified as $CD11c^+$ cells) generation and expression of activation markers upon exposure to RMA cells and RMA SN. We noted a significant decrease in the percentage of DCs within the culture after exposure to RMA cells or RMA SN but not after LPS stimulation for both, cultures stimulated on day 3 as well as day 4. This effect was also seen when using another cancer cell line, B16-OVA, for stimulation, suggesting that the effect is not limited to the RMA cell line. It was interesting that RMA cells appear to skew the expression of co-stimulatory markers towards CD86, when in contrast, RMA SN induced a higher percentage of cells to express CD80, as is shown in the higher ratio of CD80:CD86 expression when compared with RMA cells. CD80 and CD86 are

important and well-studied activation markers that bind to the T-cell co-stimulatory markers CD28 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, CD152) [19-21]. CTLA-4 is known to act as a strong negative regulator for T-cell activation [22] and several studies have shown that CD80 has a higher affinity to binding CTLA-4 than CD86 [23, 24]. Driving the expression of the co-stimulatory molecules on the DCs towards CD80 over CD86 will ensure a stronger CTLA-4 response, thus weakening the T-cell response to any foreign antigen in the periphery. These results suggest that early DCs that were exposed to RMA SN are unable to stimulate T-cell proliferation.

Indeed, no T-cell proliferation was induced by DCs which had been pre-exposed to RMA SN. Interestingly, DCs exposed to RMA cells before incubation with T-cells were not able to induce T-cell proliferation either, which suggests additional mechanisms are being engaged by the cancer cell line. It has been demonstrated in many studies that DCs can express inhibitory markers to regulate T-cell function [25-28]. Earlier results from our lab showed that GM-CSF-derived DCs express moderate levels of inhibitory marker, programmed death ligand 1 (PD-L1 or B7H1) but not B7H3 and B7H4 in their resting state (**Fig. 8**). While it has not been tested if RMA cells or RMA SN increase any of the inhibitory marker expression, these GM-CSF-derived early DCs induce robust T-cell proliferation after LPS stimulation in spite of the pre-existing PD-L1 expression, suggesting that these DCs can induce a strong immune response when given a proper stimulation. This was in line with a study done by Bhattacharya *et al.* [29] where they demonstrated that BM-derived DCs selectively expand regulatory T-cells in the presence of OX40L but not PD-L1. Furthermore, blockade of CD80 on BM-derived DCs has also been shown to discourage regulatory T-cell expansion. The lack of T-cell proliferation was not only triggered by the binding to co-stimulatory markers expressed on DCs, but also triggered by cytokines found in the immediate cellular environment. IL-6 has been shown to trigger a strong T-cell response [30]. IL-6 acts as both a pro-inflammatory and an anti-inflammatory cytokine. In cancer patients, IL-6 is highly elevated, leading to poor prognosis [31], and is abundant at the tumour microenvironment where it plays a role in cancer metastasis via down-regulation of E-cadherin [32]. In mice, CT-26 colorectal cancer cells expressing IL-6 exert tumour-promoting activities by activating growth and survival [33]. TNF-α, a pro-inflammatory cytokine, is involved in pathological process of chronic inflammation, autoimmune diseases and cancer growth [34]. Furthermore, the combination of pro-inflammatory cytokines, TNF-α and IL-6, has been shown to synergistically induce strong signal for T-cell proliferation [35] and cancer cell growth [36].

We demonstrated that LPS induced expression of co-stimulatory markers and high levels of cytokine secretion, both of which can provide strong signals to promote T-cell proliferation. On the other hand, RMA cells and RMA SN did induce much lower levels of cytokine secretion and differential expression of co-stimulatory markers on DCs. The cytokine profile together with the altered CD80 and CD86 expression may contribute to the absence of T-cell proliferation by the early DCs that have been pre-exposed to RMA cells or RMA SN. In addition, further studies need to be conducted to measure the effects of RMA and RMA SN on the expression of B7H1 on these DCs.

In an attempt to reverse the negative effects from the exposure of the DCs to the cancer cells or SN, GM-CSF DC cultures that had been co-incubated with RMA cells or RMA SN were stimulated with LPS. The additional stimulation with LPS increased cytokine secretion in the cultures pre-exposed to RMA cells and RMA SN compared with the same cultures without additional LPS stimulation. Also, generation of DCs in the GM-CSF-derived cultures could be rescued to a small extent by addition of LPS post exposure to RMA cells or RMA SN. We furthermore observed a trend in LPS-stimulated cultures that showed a reduction of the ratio of CD80:CD86 expression. However, these changes were not significant. Although we did not achieve significance in our experiments, the results are in line with studies that reported positive therapeutic effects from administering LPS or synthetic lipid A molecule into animal cancer models [37, 38]. The inhibition of tumour growth was associated with a dose-dependent effect, and animals received antigenic memory from LPS-eradicated tumours [39, 40]. These results are promising, but more research is required to determine the ideal dose of LPS to obtain a tumouricidal effect while, at the same time, avoiding the toxic side effects of LPS.

Human DCs, particularly the classic myeloid DCs, express activation markers such as CD80, CD86 and HLA-DR (equivalent to MHCII), which is similar to mice [41]. Due to the similarities in activation pathway in these DCs, we speculate that human cancer cell lines may interact with culture DCs in similar ways. However, one major difference between human and mice DCs is the differential expression of TLRs. For example, human DCs only express low levels of TLR4 when compared with their mouse counterparts [42, 43]. While lower TLR4 expression corresponds to a weaker response to LPS [44], a higher dose of LPS can still initiate DC activation [45]. The differences in TLR expressions may also affect DCs' response against cancer cells. Therefore, there is still much testing to be conducted to test the possible use of this DC model in future cancer immunotherapies.

In summary, we show that RMA cells and RMA SN can induce differential co-stimulatory marker expressions and cytokine secretion in early DCs derived from GM-CSF cultures and that further stimulation with LPS can not fully reverse the suppressive effect of cancer cells or supernatant. Although these early DCs were previously shown to be potent activators of T-cell stimulation, this is inhibited in the presence of cancer, and further studies are required to determine the function and use of GM-CSF-derived DCs and their ability to be used in cancer immunotherapy studies.

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Figure Legends

Figure 1. RMA and RMA SN decrease DC generation in bone marrow cultures but induce differential expression of activation markers on these GM-CSF derived DCs
Day 3 and day 4 DCs generated from GM-CSF bone marrow cultures either not stimulated (NS; black), stimulated with LPS (dark grey), RMA (5 x 10^4 cells/ml) (light grey) or RMA SN (white) for 24 h. (A) The percentage of DCs ($CD11c^+$ cells) generated and (B) surface expression of CD80 and CD86 on DCs were determined by flow cytometry. (C) Ratios of CD80:CD86 expression on early DCs were calculated. Results shown are the average of 3 mice, Two-way ANOVA was used used to calculate statistical significance. Results are shown as the mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 2. Cancer cell lines decrease DC generation in bone marrow cultures and increase expression of co-stimulatory markers Day 3 and day 4 DCs generated from GM-CSF bone marrow cultures either not stimulated (NS; black), stimulated with B16-OVA at 2.5 x 10^4 cells/ml (dark grey), 5 x 10^4 cells/ml (light grey) or 10^5 cells/ml (white) for 24 h. (A) Percentage of DCs ($CD11c^+$ cells) generated; and (B) the expression of co-stimulatory markers (CD80, CD86) was measured by flow cytometry. Results shown are the average of 3 mice, two-way ANOVA was used used to calculate statistical significance. Results are shown as the mean ± SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3. DCs pre-exposed to RMA cells and RMA SN can not induce T-cell proliferation T-cells were incubated with day 3 or day 4 DCs which were either not stimulated (black), stimulated with LPS (dark grey), stimulated with RMA (light grey) or stimulated with RMA SN (white) for 24 hours beforehand. Culture cells were harvested and incubated with T-cells at ratios of 1:30, 1:10 and 1:3 for 3 days. Proliferation of T-cells was measured by $^3[H]$-thymidine incorporation. Results shown are the average of 3 mice. Two-way ANOVA was used used to calculate statistical significance. Results are shown as the mean ± SD. ** $P < 0.01$, *** $P< 0.001$.

Figure 4. Cytokine and chemokine secretion in GM-CSF-derived bone marrow cultures in the presence of RMA Culture supernatant from the day 3 and day 4 cultures either not stimulated or stimulated with LPS, RMA or RMA supernatant (RMA SN) was harvested and cytokines (IL-6, IL-10, CCL2, TNF-α and TGF-β) secreted within the culture were analysed using a cytometric bead array (CBA). Results shown are the average of 3 mice. Two-way ANOVA was used used to calculate statistical significance. Results are shown as the mean ± SD. * $P < 0.05$, ** $P < 0.01$, ***$P < 0.001$.

Figure 5. Additional incubation with LPS does not rescue early DC generation from GM-CSF cultures pre-exposed to RMA cells or RMA SN or change expression of co-stimulatory markers Day 3 or day 4 cultures were either left unstimulated (NS), stimulated with RMA cells or SN for 24 hours before being further stimulated with LPS for 24 hours, at 37C, 5% CO2. Cultures were harvested and (A) percentage of DCs ($CD11c^+$ cells) and (B) expression of co-stimulatory markers (CD80 and CD86) on these DCs were analysed by flow cytometry. (C) Ratios of CD80:CD86 expression on early DCs were

calculated by dividing the percentage of CD80 with percentage of CD86. Results shown are the average of 3 mice. Two-way ANOVA was used used to calculate statistical significance. Each condition was compared with NS condition (black) unless stated otherwise. Results are shown as the mean ± SD. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

Figure 6. Further incubation with LPS increases cytokines and chemokine secretion within the culture (A) Day 3 or day 4 cultures were stimulated with RMA cells or SN for 24 hours before being further stimulated with LPS for 24 hours at 37C, 5% CO2 both times. Culture supernatants from each culture conditions were collected and cytokine secretion was analysed with cytometric bead array (CBA). (B) Ratios of CD80 expression: cytokine secretion by early DCs were calculated by dividing the percentage of CD80 expression with the amount of cytokine secreted in pg/ml. The same calculation was done for the ratios of CD86 expression:cytokine secretion by early DCs. Results shown are the average of 3 mice. Two-way ANOVA was used used to calculate statistical significance. Results are shown as the mean ± SD. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

Figure 7. Comparison between GM-CSF and GM-CSF + IL-4-derived DCs (A) The endocytosis of bone marrow-derived DCs were analysed by incubating the cells with 40 nm or 500 nm nanoparticles for 1 hour before these cells were harvested and analysed using flow cytometry. (B) Bone marrow derived DCs were cultured for 3 to 5 days before they were stimulated with LPS for 24 hours; NS depicts non-stimulated. The expression levels of activation markers, CD80, CD86 and MHCII were measured using flow cytometry. Each histogram shown represents 3 different experiments, bar graphs were results from 3 mice. Two-way ANOVA was used to compare both experimental conditions at each time-point. (C) Supernatants from bone marrow-derived GM-CSF DC and GM-CSF+IL-4 DC cultures generated on day 3, day 4 and day 5 were collected and cytokines were measured with cytometric bead array (CBA). Bar graphs were results from 2 mice. Results are shown as the the mean ± SD. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$.

Figure 8. Expression of co-inhibitory molecules on GM-CSF derived early DCs Day 3 DCs were generated from GM-CSF bone marrow cultures and the expression of B7H1 (PD-L1), B7H3 and B7H4 were analysed using flow cytometry. Each dot plot is representative of 3 different mice.